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SPECIAL REPORT

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4-DAMP analogues reveal heterogeneity of M_1 muscarinic receptors

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The muscarinic receptors responsible for two effects elicited by McN-A-343, i.e. the relaxation of the rat duodenum and the inhibition of the twitch contraction of rabbit vas deferens, were investigated by use of derivatives of 4-diphenyl acetoxy-N-methyl piperidine methobromide (4-DAMP). Both receptors had been previously identified as M_1 on the basis of the high affinity shown towards pirenzepine. Schild analysis of antagonism revealed that the affinities of 4-DAMP and three of its analogues in the rat duodenum were significantly different from those estimated in rabbit vas deferens. These data indicate that distinct receptor subtypes mediate duodenal relaxation and vas deferens inhibition of twitch contraction and suggest that receptors classified as M_1 by means of pirenzepine affinity constitute a heterogeneous population.

Introduction The classification of muscarinic receptors has long relied on the discriminative properties of the antagonist pirenzepine (Hirschowitz *et al.*, 1984). While receptors with low affinity for pirenzepine, originally defined as M_2, have subsequently proved to be heterogeneous and have been further characterized by means of newly developed selective antagonists (Levine *et al.*, 1988), the identification of the M_1 subtype is still based on pirenzepine recognition of receptor sites occurring with high affinity. Thus, in accordance with the high sensitivity shown towards pirenzepine blockade, two recently described neuronal responses have been attributed to stimulation of M_1 receptors. These are the relaxation of the isolated small intestine of the rat, due to muscarinic-mediated activation of a neural inhibitory pathway (Micheletti *et al.*, 1988) and the inhibition of the electrically-induced twitch contraction of the rabbit isolated vas deferens, occurring through prejunctional inhibitory muscarinic receptors (Eltze, 1988). Both responses are elicited by McN-A-343 and antagonized by pirenzepine with an affinity in the nanomolar range, consistent with interaction at M_1 sites.

To characterize further the receptor responsible for the above described effects, we studied the antagonism by 4-diphenyl acetoxy-N-methyl piperidine methobromide (4-DAMP) and some related compounds. Although commonly employed for its ability to discriminate between smooth muscle (M_3) and cardiac (M_2) receptors, 4-DAMP appears also endowed with some M_1 selectivity, in that it was shown to bind to M_1 receptors with high affinity (Berrie *et al.*, 1983) and to antagonize the M_1-mediated depolarization of the rat superior cervical ganglion with a pA_2 close to that found in the ileum (Brown *et al.*, 1980).

We now show that 4-DAMP and three of its derivatives antagonize the duodenal relaxation and the inhibition of twitch in the vas deferens with different affinities, indicating that a hitherto undescribed subtype is responsible for either of the two effects studied.

Methods Rats (male, Sprague Dawley, 200 g) were killed by cervical dislocation. Duodenum preparations were set up as described by Micheletti *et al.* (1988). Briefly, segments of duodenum of about 2 cm length were mounted in Tyrode solution (mM: NaCl 137, KCl 2.68, $CaCl_2$ 1.82, $NaHCO_3$ 5.9, $MgCl_2$ 1, NaH_2PO_4 0.42 and glucose 5.6) maintained at 37°C, under 1.5 g tension. Relaxation was induced by administering sequential concentrations of McN-A-343 at 15 min intervals.

Rabbits (male, New Zealand, 2.5–3.0 kg) were killed by cervical dislocation and exsanguinated. The epididymal portion of the vas deferens was mounted under 500 mg tension in the following solution (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, containing yohimbine 1 μM, and maintained at 32°C (Eltze, 1988). Preparations were continuously stimulated through platinum electrodes at 30 V, 0.5 ms, 0.05 Hz. McN-A-343 was added in cumulative concentrations.

In either preparation, isotonic contractions were recorded (Basile 7050 transducer). Antagonists were allowed 60 min equilibration period; each concentration was tested on a separate preparation. Affinity (expressed as $-\log K_B$) was estimated by Schild analysis, fitting linear regressions of individual experiments by the least squares method and verifying parallelism before calculating dose-ratios. Statistical comparison of Schild regressions was performed by parallel line bioassay (Finney, 1964), calculating potency ratios and confidence limits for $P < 0.05$ and $P < 0.01$. Significance was accepted at the $P < 0.05$ level.

All drugs used were synthesized in our laboratories: McN-A-343 (4-chlorophenylcarbamoyloxy-2-butynyltrimethylammonium chloride); pirenzepine; 4-DAMP (4-diphenylacetyoxy-N-methyl piperidine methobromide); α-methyl 4-DAMP (4-diphenyl-α-methylacetoxy-N-methylpiperidine methobromide); DA 6206 (1-iminoethyl)-4-piperidinyl α-methyl-α- phenylbenzeneacetate hydrochloride); DA 6264 (1-guanyl-4-piperidinyl α-methyl-α- phenylbenzeneacetate hydrochloride); DA 6265 (1-iminomethyl-4-piperidinyl α-methyl-α-phenylbenzeneacetate hydrochloride) (Eur. Pat. Appl. 309424).

Results McN-A-343 produced a relaxation of the rat duodenum and an inhibition of the twitch contraction of rabbit vas deferens with potencies comparable to those previously described (Micheletti *et al.*, 1988; Eltze, 1988): $-\log EC_{50}$ (M), 6.30 ± 0.05 and 5.86 ± 0.07, ($n = 15$, each), respectively.

The compounds investigated inhibited the effects of McN-A-343 in both preparations. The substances acted as competitive antagonists, producing parallel rightward displacements of McN-A-343 concentration-response curves and yielding slopes of Schild plots not significantly different from unity. The values found are reported in Table 1 and compared with those obtained with pirenzepine.

The potencies of 4-DAMP and its three amidino derivatives, DA 6206, DA 6264 and DA 6265, in inhibiting duodenal relaxation differed from those found in antagonizing the reduction of twitch contraction of rabbit vas deferens. In the case of 4-DAMP, affinity was higher for the vas deferens ($P < 0.05$), while its analogues displayed a tenfold greater affinity ($P < 0.01$) for the duodenal response. The compound α-methyl 4-DAMP did not distinguish between the two preparations. Affinity values in duodenum and vas deferens were

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Table 1 Affinity estimates and Schild slopes for 4-DAMP and its derivatives as antagonists at muscarinic receptors mediating rat duodenum relaxation and inhibition of rabbit vas deferens twitch contraction

	Rat duodenum		*Rabbit vas deferens*	
Compound	$-\log K_B$	Slope	$-\log K_B$	Slope
α-Methyl 4-DAMP	9.65 (0.14)	1.04 (0.18)	9.75 (0.30)	1.09 (0.24)
DA 6206	8.01 (0.19)	1.07 (0.19)	6.95 (0.09)**	1.14 (0.17)
DA 6264	8.40 (0.21)	0.91 (0.15)	7.22 (0.09)**	1.21 (0.12)
DA 6265	9.04 (0.10)	0.99 (0.09)	8.21 (0.16)**	0.83 (0.16)
4-DAMP	8.13 (0.26)	0.96 (0.21)	8.71 (0.10)*	1.23 (0.18)
Pirenzepine	8.09 (0.09)	1.14 (0.08)[a]	8.12 (0.05)	1.10 (0.12)

[a] Data from Micheletti *et al.* (1988).
* $P < 0.05$ and ** $P < 0.01$ in comparison with duodenum (parallel line bioassay).
Results from 9–15 replicates. In parentheses, s.e.mean.

very close and nearly identical to those reported for ileal and cardiac receptors (Barlow *et al.*, 1986, Table 1). Finally, estimates of pirenzepine affinity in either preparation were strictly comparable.

Discussion The present observations show that two muscarinic receptors, previously classified as belonging to the M_1 subtype, are in fact heterogeneous. These receptors mediate the relaxation of the rat isolated duodenum and the inhibition of the twitch contraction in rabbit vas deferens, the two effects being elicited by McN-A-343. While being antagonized by pirenzepine with high affinity, they display different sensitivities towards 4-DAMP and some related antagonists. As 4-DAMP and its amidino-derivatives show opposite selectivities, it seems unlikely that affinity measurements in the present studies were affected by the species used. The significantly different affinities found for these compounds thus indicate that distinct receptor subtypes sustain relaxation of duodenum and inhibition of rabbit vas deferens contraction.

A corollary to the present results is therefore that the selectivity of pirenzepine appears inadequate to define M_1 receptors, since the antagonist shows high affinity also for a non-M_1 subtype. This conclusion warrants some comment.

Of the five muscarinic receptor genes (m1–m5) described (Bonner *et al.*, 1987; 1988), the first three seem to correspond to M_1–M_3 receptors (Levine & Birdsall, 1989). The affinity pattern of pirenzepine for these subtypes is well-known. On the other hand, M_4 and M_5 receptors have not yet been distinctly identified and the knowledge available relates mainly to the localization and binding properties of the expressed m4 and m5 species. The present knowledge can be summarized as follows: (1) m1, m2, m3 and m4 receptors are coexpressed in the forebrain, including the cerebral cortex, a region widely employed for studies on M_1 receptors (Buckley *et al.*, 1989). (2) There are no known antagonists capable of specifically interacting with m4 and m5 receptors (Buckley *et al.*, 1989). (3) Pirenzepine is able to recognize m4 receptors with high affinity (Bonner *et al.*, 1987; Peralta *et al.*, 1987). This latter evidence was obtained during investigation of the properties of the individual subtypes expressed in transfected mammalian cells. Pirenzepine showed strikingly close affinities in displacing [^{3}H]-quinuclidinyl benzilate from m1 and m4 receptors in COS-7 cells (35 *vs.* 36 nM, Bonner *et al.*, 1987), and human embryonic kidney cells (less than 3 fold difference, Peralta *et al.*, 1987). A rather discrepant finding has been recently reported by Buckley *et al.* (1989), who showed that in CHO-K1 cells, pirenzepine was 6 fold less potent at m4 than m1 receptors; notably, affinity was not estimated because of the shallow Hill slope at m4 receptors. Plausible explanations for the low Hill number include negative cooperativity or post-translational modifications. Actually, the affinities of muscarinic receptor subtypes are known to be largely affected by membrane environment, since pirenzepine fails to discriminate between M_1 and M_2 subtypes following receptor solubilization and purification (Wang *et al.*, 1983; Berstein *et al.*, 1989), and the purified M_1 subtype recovers the original affinity upon interaction with factors present in membrane preparations (Berstein *et al.*, 1989).

In light of the above, the possibility cannot be excluded that previous studies may have classified m4 receptors as M_1 (Buckley *et al.*, 1989). It is then conceivable that an M_4 receptor subserves either of the functional responses studied here. Studies on cells that express the cloned individual subtypes should help to define the nature of the receptors present in duodenum and vas deferens and clarify the selectivity pattern of 4-DAMP and its analogues. These latter agents contain an amidine moiety of the formamidine (DA 6265), acetamidine (DA 6206) and guanidine (DA 6264) type. We have recently shown that the same amidine substitution affected the discriminative properties of hexocyclium derivatives, enhancing their selectivity for smooth muscle (M_3) receptors (Micheletti *et al.*, 1990). The improved selectivity of those antagonists seems to depend largely on a decrease of the M_2 affinity. In contrast, the M_2 and M_3 affinities of the 4-DAMP derivatives, DA 6265, DA 6206, DA 6264, are unchanged (data not shown).

It is hoped that the use of antagonists containing the amidine moiety will improve our knowledge of muscarinic receptor subtypes.

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SHORT COMMUNICATION

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Pretreatment with rh-GMCSF, but not rh-IL3, enhances PAF-induced eosinophil accumulation in guinea-pig airways

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Intraperitoneal injections of recombinant human granulocyte-macrophage colony stimulating factor (rh-GMCSF, 50 $\mu g\,kg^{-1}$ daily) or interleukin-3 (rh-IL3, 50 $\mu g\,kg^{-1}$ daily) for two days, induced an increase in the percentage of bone marrow and pulmonary airway eosinophils in the guinea-pig. In addition, rh-IL3-treated animals exhibited an increase (21%) in blood neutrophils. Exposure of guinea-pigs to an aerosol of platelet activating factor (PAF) gives rise to a selective pulmonary eosinophil accumulation, maximal at 48 h. The eosinophilic response to PAF was significantly enhanced in rh-GMCSF-treated guinea-pigs but was suppressed in rh-IL3-treated animals.

Introduction Eosinophils are closely associated with allergies and asthma, and the inflammatory nature of these cells is well established, since toxic proteins secreted by eosinophils have the capacity to cause tissue damage (Frigas & Gleich, 1987; Tai *et al.*, 1987). In the guinea-pig, platelet activating factor (PAF) induces a selective accumulation of eosinophils within the airways (Lellouch-Tubiana *et al.*, 1988; Sanjar *et al.*, 1990). However, the eosinophilic response to PAF does not approach the intensity of an allergic reaction (Hutson *et al.*, 1988), which indicates an involvement of other mediators. Since the cellular response of sensitized animals (Samter, 1949; Sanjar *et al.*, 1989) or man (Kline *et al.*, 1932; Henocq & Vargaftig, 1988), to stimuli other than antigen, is qualitatively and quantitatively different from that of normal individuals, it is likely that sensitization in some way alters the response pattern. Recent studies may provide a molecular basis for such changed behaviour, since exposure of leucocytes to recombinant cytokines *in vitro* can induce a heightened responsiveness to activation stimuli (Owen *et al.*, 1987; Dahinden *et al.*, 1988). In the present study, the ability of recombinant human granulocyte-macrophage colony stimulating factor (rh-GMCSF) or interleukin-3 (rh-IL3) to enhance the eosinophilic response to an aerosol of PAF has been investigated in the guinea-pig.

Methods Dunkin-Hartley guinea-pigs (450–650 g) were given intraperitoneal injections of saline, rh-GMCSF (50 $\mu g\,kg^{-1}$, daily) or rh-IL3 (50 $\mu g\,kg^{-1}$, daily) for 2 days. Treated animals were divided into two groups; one group was exposed to PAF and the other not; in either case lungs were lavaged 48 h later. Guinea-pigs were exposed individually to an aerosol of PAF (10 ml of a 10 $\mu g\,ml^{-1}$ solution in sterile saline containing bovine serum albumin (BSA) 0.25%), generated in a Devilbiss nebulizer (flow rate of 7 l min^{-1}) for 1 h (Sanjar *et al.*, 1990). Blood (5 ml) was collected by cardiac puncture in sodium citrate (2.8%) from ether anaesthetized guinea-pigs prior to death. Bronchoalveolar lavage (BAL) was performed with 4 × 10 ml aliquots of Hank's balanced salt solution (HBSS) containing disodium edetate (EDTA, 10 mM), HEPES (10 mM) and BSA (0.3%). Bone marrow was collected from femurs of guinea-pigs not exposed to PAF, by inserting an 18-gauge needle into the bone lumen and flushing with 10 ml of HBSS. Blood samples were layered over an equal volume of a mixture (10/16, v/v) of Hypaque (30% w/v)/methylcellulose (1% w/v) in order to sediment erythrocytes. The leucocyte-rich layer was washed and resuspended (1 ml) in HBSS. Cytospin preparations of BAL, blood and bone marrow cells were stained by Leishman's stain for differential cell counting (light microscopy × 1000) and total BAL cells were counted in a micro cell counter (model cc-180, Sysmex, Switzerland). Results are presented either as cells ($\times 10^4$) ml^{-1} BAL fluid or as percentage of cell populations. Student's *t* test (two tailed) for groups of unequal size was used for statistical analyses.

Results No change in eosinophil numbers was observed in BAL with rh-GMCSF ($n = 14$) or rh-IL3 ($n = 9$) treatment as compared with saline ($n = 10$) treatment (Figure 1). However, the percentage of eosinophils was higher in rh-GMCSF or rh-IL3 groups (saline, 5.8 ± 0.8%; rh-GMCSF, 13.4 ± 1.2% $P < 0.0005$; rh-IL3, 10.1 ± 1.6% NS). Similarly there was a small increase in the number of bone marrow eosinophils (saline, 6.2 ± 1.6% $n = 3$; rh-GMCSF, 10.5 ± 0.9% $n = 6$; rh-IL3, 10.1 ± 0.9% $n = 3$). No changes in blood eosinophils were observed in the different treatment groups; however, rh-IL3-treated guinea-pigs exhibited a significantly higher neutrophil population (saline, 44.3 ± 3.6%, rh-GMCSF, 45.9 ± 2.0%; rhIL3, 53.0 ± 3.9% $n = 9$, $P < 0.04$). Exposure of saline- or rh-GMCSF-treated guinea-pigs to an aerosol of PAF resulted in an increase of both the total eosinophil numbers (Figure 1) and in the percentage of these cells (saline, 21.1 ± 2.5% $P < 0.005$; rh-GMCSF, 31.5 ± 4.9% $P < 0.005$).

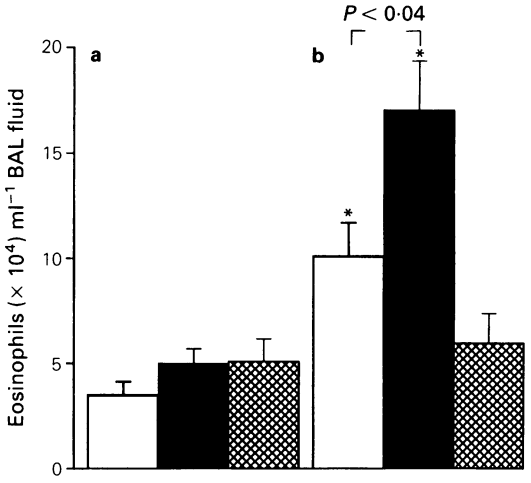

Figure 1 Number of eosinophils recovered by bronchoalveolar lavage (BAL) from saline (open columns), rh-GMCSF (solid columns) or rh-IL3 (cross-hatched columns)-treated guinea-pigs either with (a) no PAF challenge or (b) 48 h after exposure to an aerosol of PAF (10 $\mu g\,ml^{-1}$). * indicates statistical significance (*t* test) of $P < 0.001$ when comparing PAF-exposed groups with guinea-pigs not exposed to PAF.

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Furthermore, the response of rh-GMCSF-treated animals was significantly greater ($P < 0.04$) than saline-treated controls (Figure 1). No increases in the percentage or numbers of eosinophils was observed in rh-IL3-treated animals following exposure to PAF (Figure 1).

Discussion This study demonstrates that administration of recombinant human cytokines (rh-GMCSF or rh-IL3) to guinea-pigs can lead to changes in leucocyte populations in blood, bone marrow or airways, when doses higher than those normally used to treat patients with leukopenia are given. However, the capacity of rh-GMCSF to increase the eosinophilic response of the guinea-pig to aerosolised PAF is of greater importance, since such an enhanced reactivity to a variety of stimuli is a feature of sensitized guinea-pigs (Samter, 1949; Sanjar *et al.*, 1989) or man (Kline *et al.*, 1932; Henocq & Vargaftig, 1988). These observations imply that exposure to cytokines, such as GMCSF, during sensitization may be responsible for the exaggerated eosinophil response. *In vitro* experiments reinforce such a proposal (Owen *et al.*, 1987). Under similar experimental conditions, and despite a similar increase in the percentage of airway eosinophils, rh-IL3-treated guinea-pigs did not respond to PAF. An increase in blood neutrophils may explain this phenomenon, since infected animals which have high blood or BAL neutrophils, do not give an eosinophilic response either to PAF or to antigen (unpublished observations). The study of allergic mediators in primed cells or animals may lead to greater understanding of cellular responses in disease processes.

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Atropine-resistant relaxation induced by high K^+ in iris dilator muscle of the rat and pig

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1 The effects of high K^+ ion concentration on the isometric tension in dilator muscle strips of the rat and porcine iris were examined. A high K^+ solution, prepared by the replacement of Na^+ in the medium with equimolar K^+, was applied in the presence of 1 μM phentolamine, 1 μM propranolol and 1 or 10 μM atropine. High K^+ (>20 mM) induced a biphasic response; an initial phasic contraction followed by relaxation rather than tonic contraction.

2 An additional application of a Ca^{2+} antagonist, 1 μM nifedipine or nicardipine, almost completely blocked the K^+-induced initial contraction and enhanced the following relaxation. The effect of K^+ under these conditions was concentration-dependent in the range 20 to 80 mM. The maximum amplitude of the atropine-resistant relaxation induced by high K^+ corresponds to 50–75% of that produced by acetylcholine in the absence of atropine. A similar K^+-induced relaxation was observed in the porcine iris dilator.

3 The atropine-resistant relaxation in the rat iris dilator was not affected by pretreatment with 10 μM ouabain. The relaxation induced by 40 or 80 mM K^+ in the porcine dilator was slightly enhanced or not affected, respectively, in the presence of 1 μM ouabain. Application of 10 μM ouabain *per se* induced relaxation in the porcine iris dilator.

4 The low Na^+ ion concentration present in high K^+ solutions was not responsible for the K^+-induced relaxation since the complete replacement of Na in the medium with Tris did not affect significantly the relaxation produced by high K^+-containing solutions.

5 Neither 1 μM tetrodotoxin, 10 μM indomethacin, 10 μM nordihydroguaiaretic acid nor hypoxic conditions affected the high K^+-induced relaxation.

6 The inherent tone of the rat iris dilator was not affected by either 8-bromo cyclic GMP, dibutyryl cyclic GMP (0.1–0.3 mM) or nitroprusside (1–100 μM).

7 These results may suggest that the atropine-resistant relaxation induced by high K^+ is not due to either activation of the Na-K pump or release of a relaxing factor produced by oxidative metabolism. Although the relaxation mechanism has not been elucidated, it is probably not mediated by an increase in cellular cyclic GMP levels.

Introduction

Several substances including acetylcholine (ACh), 5-hydroxytryptamine histamine, adenosine di- or triphosphate, A23187 and bradykinin, induce vasodilatation via the release of a relaxing factor (endothelium-derived relaxing factor, EDRF) from endothelial cells (for reviews see: Furchgott, 1983; Vanhoutte *et al.*, 1986). It has been strongly suggested that nitric oxide (NO) is the major EDRF responsible for acetylcholine-induced relaxation (Palmer *et al.*, 1987; Ignarro *et al.*, 1987). The mechanisms of EDRF-induced relaxation of vascular smooth muscles are attributable to an activation of guanylate cyclase and subsequent increase in guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Rapoport & Murad, 1983; Rapoport *et al.*, 1983). It has been also demonstrated that high K^+ solutions induce EDRF release (Rubanyi & Vanhoutte, 1988).

Iris dilator is another smooth muscle tissue which shows marked relaxation in response to ACh (cat; Schaeppi & Koella, 1964, rat; Narita & Watanabe, 1981, and man; Yoshitomi *et al.*, 1985). Although the mechanisms of ACh-induced relaxation in iris dilator have not been clarified yet, a release of relaxing factor has been suggested (Hasegawa *et al.*, 1988). In the investigation of substances which induce relaxation in a similar manner to ACh in iris dilator muscle, we found that high K^+ solution induces a substantial relaxation in the presence of atropine, phentolamine, propranolol and nifedipine or nicardipine.

The present study was undertaken to examine whether the relaxation is a direct or an indirect (cf. EDRF in vasculature) effect of high K^+.

Methods

Preparation

The preparation of the rat iris dilator and tension measurement from it were carried out by methods described in detail previously (Narita & Watanabe, 1981; 1982a). Briefly, about one sixth of whole iris (about 3 mm width at the outer edge of the iris) was dissected from the isolated eye. This muscle strip was mounted horizontally in a 4 ml organ bath and was perfused with bathing solution. The pupillary end of the preparation was pinned on a sheet of silicone rubber which formed the bottom of the bath. The other end was fixed to an isometric transducer lever. Porcine dilator strips of a similar width (3 mm) were prepared and mounted in a similar manner. At the start of an experiment, resting tone was adjusted to between 10 and 15 mg or 30 and 35 mg in the rat or porcine dilator, respectively.

Recording

Isometric tension was measured by a force displacement transducer which was made in the laboratory (Imaizumi & Watanabe, 1983) for small tension measurement. The electrical signal was amplified by an operational amplifier (μA 725,

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Fairchild), and recorded on a pen-recorder (Toa Denpa, Tokyo). Nerve stimulation was performed by field stimulation via a pair of platinum wire electrodes which were placed at both sides of the strip. A train of 60 pulses (0.1 ms in duration, at 30 Hz in frequency and supramaximal current) were applied once every 2 or 3 min. The stimulator used was made in the laboratory and had high output current up to 250 mA (Imaizumi & Watanabe, 1983).

Solutions

The bathing solution was a modified Krebs solution of the following composition (mM): NaCl 120, KCl 4.7, $CaCl_2$ 2.2, $MgCl_2$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 14. The solution was bubbled with 95% O_2 and 5% CO_2. The Tris-buffered solution had the following composition (mM): NaCl 112, KCl 5.9, $CaCl_2$ 2.2, $MgCl_2$ 1.2, Tris (hydroxymethyl) aminomethane (Tris) 25.0, glucose 14. When Na-free solution was prepared, Na in the Tris-buffered solution was replaced with equimolar Tris. The pH of the Tris-buffered solution was adjusted to 7.4 with HCl. This solution was gassed by 100% O_2. To do experiments under hypoxic conditions in the normal Krebs solution, a gas mixture of 95% N_2 and 5% CO_2 was used. When the normoxic gas mixture was changed to an anoxic one, the Po_2 in the modified Krebs solution decreased from over 600 mmHg to below 5 mmHg within 5 min. The pH of the solutions was kept at 7.4 at 35–37°C. High K^+ solutions were prepared by replacing Na^+ in the modified Krebs or Tris buffered solution with equimolar K^+.

Drugs and statistics

The following drugs were used: acetylcholine chloride, atropine sulphate, ethylene glycol-bis(aminoethylether) N,N,N′,N′-tetraacetic acid (Wako Pure Chemical Industries, Ltd., Osaka), phentolamine mesylate (Ciba-Geigy), ouabain (Tokyo Kasei Industry Co. Ltd., Tokyo), 8-bromoguanosine 3′,5′-cyclic monophosphate and N^2,2-O-dibutyryl guanosine 3′,5′-cyclic monophosphate (Sigma, MO, U.S.A.), nordihydroguaiaretic acid (Sigma). Propranolol and nifedipine were synthesized and kindly given by Kyowa Hakko Ltd. (Tokyo, Japan). Tetrodotoxin, indomethacin and nicardipine were kindly given by Dr S. Okuda (the University of Tokyo), Sumitomo Chemical and Yamanouchi Pharmaceutical Company, respectively.

Summarized data are shown as mean values with s.e.mean. The statistical differences were tested by Student's t test and indicated by * $P < 0.05$ or ** $P < 0.01$ in the figures.

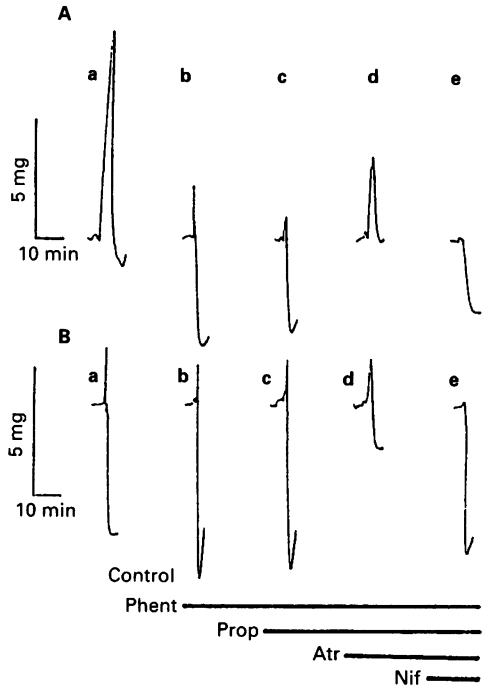

Figure 1 (A) Typical responses to 80 mM K^+ solution in the rat iris dilator. (a) Control; (b) in the presence of 1 μM phentolamine (Phent); (c), (b) plus 1 μM propranolol (Prop); (d), (c) plus 1 μM atropine (Atr); (e), (d) plus 1 μM nifedipine (Nif). (B) Responses in a preparation which showed marked relaxation rather than contraction in 80 mM K^+ solution. The order of drug-application (a–e) was the same as in (A). The initial fast upward deflection observed in (A,b), (B,a,b and c) includes artifacts of drug application.

Results

Effects of high K^+ in rat iris dilator

The dilator muscle of the rat iris had an inherent tone of about 10–15 mg as has been described previously (Narita & Watanabe, 1982a; Hasegawa *et al.*, 1988). Figure 1A shows the typical response of rat iris dilator to 80 mM K^+ solution. In the absence of antagonist drugs, Krebs solution containing 80 mM K^+ induced a phasic contraction which was never followed by a tonic contraction. The tension returned to the resting or a slightly lower level during the application of 80 mM K^+ (Figure 1Aa). The contraction was greatly reduced and became more transient after the addition of 1 μM phentolamine (Figure 1Ab). Moreover, a substantial relaxation followed the initial transient contraction. The amplitude of the relaxation was not affected or slightly decreased by a subsequent application of 1 μM propranolol (Figure 1Ac). A further addition of 1 μM atropine significantly decreased the relaxation (Figure 1Ad). This is the high K^+-induced contraction described in a previous study (Narita & Watanabe, 1982b). The contraction was phasic and the tension decreased to the resting level in the presence of 80 mM K^+. An additional application of 1 μM nifedipine abolished the contraction and, surprisingly, unmasked a further substantial relaxation. Figure 1B illustrates an example of the marked relaxation observed instead of contraction in response to high K^+. The existence of such preparations led us to identify an atropine-resistant relaxation induced by high K^+. Although responses similar to those shown in Figure 1A were apparently predominant, the extent of the relaxation varied widely from preparation to preparation unless a Ca^{2+} channel antagonist was applied in addition to the other antagonists.

Atropine-sensitive and -resistant components in high K^+ -induced relaxation

The atropine-resistant component of the high K^+-induced relaxation was determined in the presence of 1 μM phentolamine, propranolol and nicardipine. When the relaxant response to 40 or 80 mM K^+ (obtained before the application of 10 μM atropine) was taken as 100%, the atropine-resistant component of the response was 46.5 ± 5.4% ($n = 5$, $P < 0.01$ vs 100%) or 74.3 ± 7.1% ($n = 5$, $P < 0.01$ vs 100%), respectively. This might indicate that the relative contribution of endogenous ACh to the total relaxation induced by high K^+ is larger in 40 mM K^+ than in 80 mM K^+-containing solutions. However, the relaxation induced by 40 and 80 mM K^+ in the absence of atropine was 10.9 ± 0.4 and 10.8 ± 0.4 mg ($n = 5$) respectively, and was almost the whole inherent tone of the muscle. Therefore, relaxation induced by 40 and 80 mM K^+ in the absence of atropine was maximal. To confirm that 10 μM atropine was sufficient to block completely the effect of ACh released from nerve endings in high K^+ solutions, the tissues were treated with 0.3 μM physostigmine, which significantly enhances the relaxant response to exogenously applied ACh (Narita & Watanabe, 1982a). Treatment with 0.3 μM physostigmine for 20 min did not change the component resistant to 10 μM atropine (40 mM K^+: 50.5 ± 5.4%; 80 mM K^+:

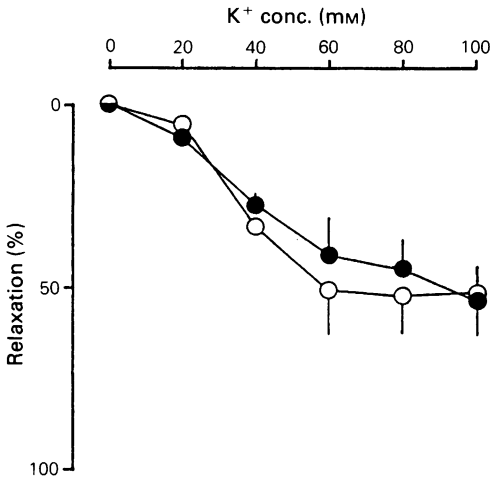

Figure 2 The concentration-response relationship for high K^+-induced relaxation. The response to 10 μM acetylcholine (ACh) was obtained before application of 1 μM phentolamine, nifedipine and 1 (●) or 10 (○) μM atropine and its amplitude was taken as 100%. Values represent mean and vertical lines show s.e.mean; $n = 6$.

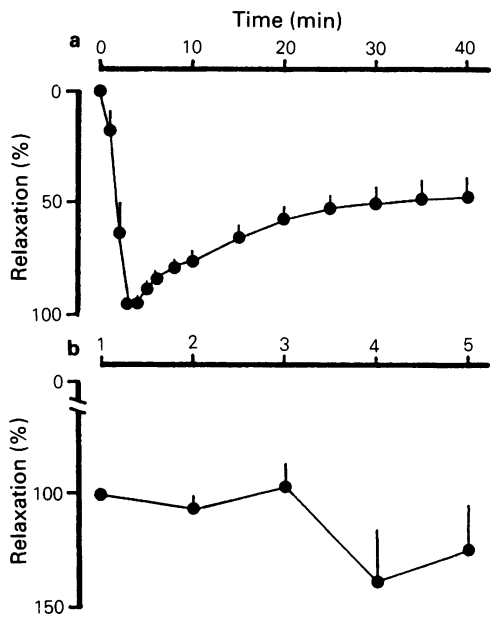

Figure 3 (a) The time course of 80 mM K^+-induced relaxation in the presence of 10 μM atropine, 1 μM phentolamine, propranolol and nicardipine. The normal Krebs solution was changed to 80 mM K^+ solution at time 0. The maximum relaxation in each preparation was taken as 100%. (b) The change in the peak amplitude of relaxation during repetitive applications of 80 mM K^+ solution for 5 min. The numbers on the horizontal axis represent the number of applications. The interval between applications was 30 min. The amplitude obtained at the first application was taken as 100%. Values represent mean and vertical lines shown s.e.mean; $n = 4$.

$75.3 \pm 6.0\%$, $n = 5$, $P > 0.05$ vs in the absence of physostigmine, respectively).

Figure 2 illustrates the relationships between the K^+ ion concentration and the relaxation obtained in the presence of 1 μM or 10 μM atropine. Although the relaxation appeared to be slightly larger at 60 and 80 mM K^+ in the presence of 10 μM atropine, the difference was not statistically significant ($P > 0.05$). It is apparent that the contribution of released ACh to the high K^+-induced relaxation can be ignored in the presence of atropine at concentrations over 1 μM. All the following experiments were performed in the presence of 10 μM atropine, 1 μM phentolamine, propranolol and nicardipine.

Characteristics of the atropine-resistant relaxation induced by high K^+

Figure 3a illustrates an averaged time course of relaxation induced by 80 mM K^+. The relaxation reached a maximum after 3 min and, thereafter, decreased gradually. Although the rate of decrease in amplitude of relaxation varied quite widely from preparation to preparation, it was much slower than the time of onset. The averaged amplitude at 30 min (Figure 3a) was approximately 50% of the maximum. When the solution was changed back to the normal Krebs solution, the muscle tone returned to the original level. Therefore, 80 mM K^+ solution induced a rather sustained relaxation. Similar results were obtained when 40 mM K^+ was applied. Figure 3b shows the rather constant responses to 80 mM K^+ solution applied five times at intervals of 30 min. Changes in the amplitude of relaxation for the five challenges were not statistically significant (Figure 3b; $P > 0.05$ vs 100%). The time course of the relaxation of each response did not change during repetitive applications (not shown). These two observations indicate that the high K^+-induced relaxation does not show apparent 'desensitization' or 'exhaustion'.

It has been shown that activation of the Na-K pump by slight elevation of K^+ ion concentration results in vasodilatation in some arteries (Toda, 1976). For this reason, the effects of high K^+ solution were examined in the presence of ouabain. It is, however, known that Na-K ATPase in the rat has a low sensitivity to ouabain (Allen & Schwartz, 1969; Schwartz *et al.*, 1975). For this reason the dilator muscle of the porcine iris was also used for this part of the study. In porcine iris dilator, high K^+ (> 20 mM) induced a small phasic contraction and a large relaxation in the absence of any blockers (not shown). Exogenously applied ACh elicited a dose-dependent relaxation but not contraction in the range 0.1 μM and 1 mM in the porcine dilator (not shown). Figure 4a shows that 10 μM ouabain did not affect the amplitude of 40 or 80 mM K^+-induced relaxation in the rat dilator. In porcine dilator, slow relaxation was elicited by treatment with 10 μM ouabain *per se* but not apparently with 1 μM ouabain (Figure 4c). This was not observed in the rat dilator (not shown). Treatment with 1 and 10 μM ouabain significantly enhanced the relaxation induced by 40 mM K^+ but not by 80 mM K^+ in porcine dilator.

The effect of 1 μM tetrodotoxin on 40 and 80 mM K^+-induced relaxation was also examined. The responses to 40 and 80 mM K^+ in the presence of tetrodotoxin (TTX) were 104.4 ± 3.3 ($n = 3$) and $101.6 \pm 2.7\%$ ($n = 3$) of the responses obtained before the application of TTX and not significantly different from controls, respectively. Muscle tone was not significantly affected by a 30 min treatment with either indomethacin (10 μM) or nordihydroguaiaretic acid (NDGA) (10 μM), an inhibitor of both lipoxygenase and cyclo-oxygenase (Tappel *et al.*, 1953; Morris *et al.*, 1980) and neither agent changed significantly the relaxation induced by 40 and 80 mM K^+.

The relaxation in high K^+ solution might be induced not by high K^+ itself but by low Na^+. To exclude this possibility, the high K^+-induced relaxation was examined in Na-free Tris-buffered solutions. A change of the solution from the normal Krebs solution to the Na-rich Tris-buffered solution did not affect the muscle tone. When the solution was changed to the Na-free solution, the tone of the muscle increased quickly by about 1.9 ± 0.8 mg ($n = 6$) and, thereafter, decreased gradually. The relaxation induced by 20 and 40 mM K^+ in Na-free solution appeared to be slightly slower than in Na^+-rich solutions. Moreover, the muscle tone only recovered incompletely from the high K^+-induced relaxation even after the K^+ concentration had been changed to the normal level (5.9 mM) under Na^+-free conditions. The amount of relaxation induced by high K^+ *per se* was, however, not significantly affected in Na-free solutions (Figure 5).

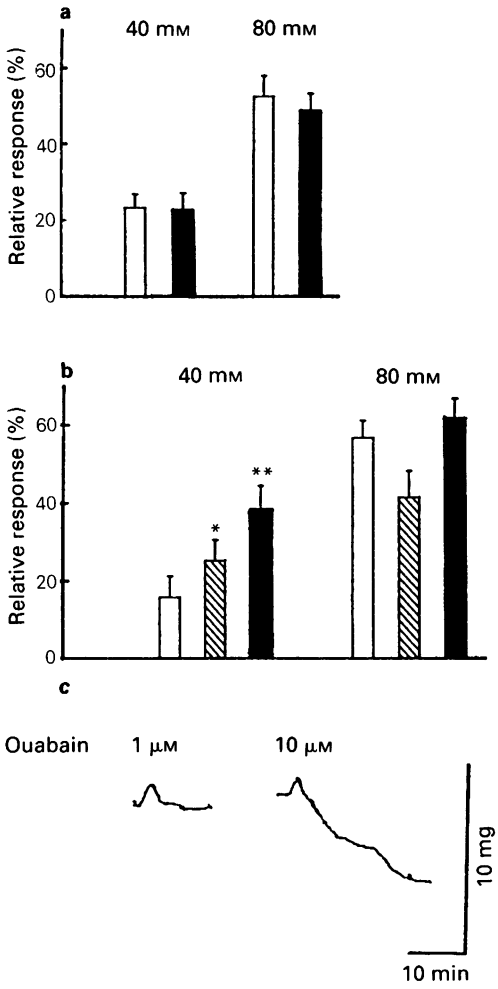

Figure 4 Effects of 1 or 10 μM ouabain on the relaxation induced by 40 or 80 mM K^+ in the rat (a) and porcine (b) dilators. The response to 10 μM (a) or 1 mM (b) acetylcholine (ACh) in the absence of blockers was taken as 100%, respectively. In the rat (a), open and solid columns illustrate the relative amplitude of high K^+-induced relaxation in the absence and presence of 10 μM ouabain, respectively ($n = 4$). In porcine dilator (b), open, hatched and solid columns denote the responses in the absence and presence of 1 or 10 μM ouabain, respectively ($n = 5$). (c) In porcine dilator, the application of 10 μM ouabain *per se* induced slow relaxation, while 1 μM ouabain did not change the tone significantly. The high K^+-induced relaxation was evaluated from the steady level obtained about 30 min after the application of 10 μM ouabain. Solutions always contained 10 μM atropine, 1 μM phentolamine and 1 μM nicardipine after the initial application of ACh. Values represent mean and bars show s.e.mean.

A comparison with EDRF-related relaxation in vascular tissues

In order to examine the possibility that EDRF might contribute to the high K^+-induced relaxation, effects of hypoxia and membrane-permeable cyclic GMP derivatives on the relaxation were tested. After incubation for 15 min under hypoxic conditions, the relaxation induced by 60 mM K^+ was $105.7 \pm 6.2\%$ ($n = 4$; $P > 0.05$ vs 100%) of the control obtained under preceding normoxic conditions. After the tissue had been returned to normoxic conditions, the relaxation was $86.7 \pm 5.5\%$ of the control ($n = 4$, $P > 0.05$ vs 100%). An application of 8-bromo cyclic GMP up to 3 mM did not change the resting tone in the rat dilator ($n = 4$, not shown), nor did dibutyryl cyclic GMP affect the tone at any concentration examined (0.01–3 mM, $n = 3$, not shown). Moreover, nitroprusside (1–100 μM) which activates cytosomal guanylate cyclase and increases cyclic GMP levels (Katsuki & Murad, 1977; Schultz *et al.*, 1977) did not elicit any changes in the tone ($n = 4$, not shown). However, dibutyryl cyclic AMP and 8-bromo cyclic AMP, dose-dependently (10 μM–3 mM) reduced the tone (not shown).

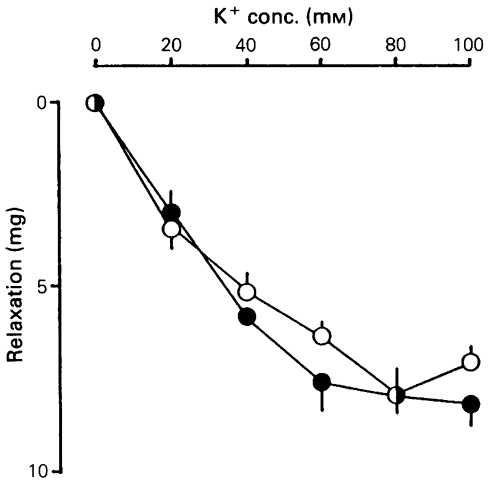

Figure 5 Effect of extracellular Na on high K^+-induced relaxation. Absolute values of the responses to several concentrations of K^+ are illustrated. Responses in 112 (●) and 0 (○) mM Na^+ solutions are shown. The concentration-response curve for high K^+ was not affected significantly by the absence of extracellular Na^+. Values represent mean and vertical lines show s.e.mean; $n = 6$.

Discussion

In general, when a smooth muscle preparation is treated with high K^+ solution, depolarization of cells and a sustained influx of Ca^{2+}, probably through voltage-dependent Ca channels (Imaizumi *et al.*, 1989), occurs and results in a phasic contraction followed by a tonic contraction (for reviews see, Weiss, 1975; Bolton, 1979). The high K^+-induced contraction is effectively blocked by Ca^{2+} channel antagonists such as verapamil, diltiazem and nifedipine (Godfraind *et al.*, 1986). From the start of our study with the rat iris dilator preparation, we recognized that the high K^+-induced contraction is transient and usually followed by a small relaxation rather than a tonic contraction. Since the relaxation was significantly reduced by atropine, this relaxation appeared to be due to release of endogenous ACh from parasympathetic nerve endings. It was considered that the lack of a tonic contraction might be due to a characteristic of the muscle. Our expectation was, therefore, that no response would be induced by high K^+ solution in the simultaneous presence of phentolamine, propranolol, nifedipine and a high concentration of atropine. In the present study, however, it was found that high K^+ elicited a large relaxation under these conditions. A possibility that the relaxation in dilator is an artifact caused by the contraction of sphincter muscle, which cannot be removed completely when a dilator strip is prepared for tension measurement, can be ruled out completely because of the following: firstly, the artifact due to sphincter muscle was minimized because the sphincter region was pinned to the rubber on the bottom of the bath. Secondly, the contraction of sphincter muscle induced by high K^+ was markedly decreased by treatment with 10 μM atropine and 1 μM nicardipine. Even when the tension was measured from a ring preparation of whole sphincter (Narita & Watanabe, 1981), a transient contraction of 1–5 mg lasted for only ~5 min in a high K^+ solution containing these drugs (cf. Figure 3a).

It has been pointed out that the relaxation induced by isoprenaline in dog iris is effectively blocked by timolol but not by propranolol (Yoshitomi & Ito, 1986). It may, therefore, be possible that the high K^+-induced relaxation was due to the release of endogenous noradrenaline and was not blocked by 1 μM propranolol. However, this possibility can be discarded because of the following: firstly, 100 μM noradrenaline did not

relax the dilator muscle even in the presence of 1 μM phentolamine (data not shown). Secondly, the relaxation induced by field stimulation was completely blocked by 1 or 10 μM atropine and, therefore, an adrenergic component was not present.

In some smooth muscle preparations, a small elevation of extracellular K^+ concentration elicits a significant relaxation (Toda, 1976; Webb & Bohr, 1978; Baysal *et al.*, 1979). Some of these relaxations have been explained by the contribution of the Na^+-K^+ pump to muscular tone since ouabain, a specific inhibitor of Na^+-K^+ ATPase, blocks the relaxation (Toda, 1976; Webb *et al.*, 1981; Souhrada & Souhrada, 1981). The high K^+-induced relaxation in the rat iris dilator was not affected by ouabain. The response in the porcine dilator, however, was enhanced by ouabain. The relaxing mechanism in porcine dilator therefore appears to be distinct from the activation of Na-K pump by high K^+. It is, however, known that the Na^+-K^+ ATPase of the rat has a low sensitivity to cardiac glycosides (Allen & Schwartz, 1969; Schwartz *et al.*, 1975). The contribution of Na^+-K^+ ATPase can therefore not be excluded in the rat iris dilator. It appears to be unlikely, however, that the mechanisms of high K^+-induced relaxation are different in the rat and pig. Of interest is the finding that 10 μM ouabain *per se* induced a sustained relaxation and enhanced the high K^+-induced relaxation in porcine dilator. A similar observation has been demonstrated in bullfrog hemilungs (Downes *et al.*, 1986). Further experiments were not done to elucidate this mechanism of ouabain-induced relaxation in the present study.

It is rather unlikely that the decrease in extracellular Na^+ rather than the increase in K^+ was responsible for the relaxation in high K^+ solution. In cardiac (Niedergerke, 1963) and smooth muscle tissues (Judah & Willoughby, 1964; Osa, 1971; Van Breemen *et al.*, 1979), a decrease in extracellular Na^+ induces an increase rather than a decrease in muscle tone or contracture. This is due to a decrease in Ca^{2+}-extrusion by Na^+-Ca^{2+} exchange or to an increase in Ca^{2+}-uptake by reversed Na^+-Ca^{2+} exchange (Reuter *et al.*, 1973; Blaustein *et al.*, 1986). The contracture which occurs in Na-free solution is removed by the addition of Na in some smooth muscle preparations (Van Breemen *et al.*, 1979). In the rat iris dilator, the complete replacement of Na in the bathing solution with Tris did not induce a contracture but a small increase in muscle tone. Moreover, the high K^+-induced relaxation was not affected significantly by the replacement and could be elicited repetitively in the Na-free solution. Therefore, the interaction between Na and Ca in the rat iris dilator might be different from that in other smooth muscles. It is clear, however, that the high K^+-induced relaxation is not dependent upon a decrease in Na concentration. The gradual decrease in muscle tone in Na-free solution after the initial small increase might be due to metabolic depression, which has been observed in other smooth muscle tissue (Karaki *et al.*, 1982).

It has been postulated that high K^+-induced relaxation in guinea-pig trachea is secondary to release of a non-adrenergic, non-cholinergic inhibitory transmitter(s) (Chideckel *et al.*, 1986). The release by high K^+ is sensitive to TTX in that preparation. In the rat iris dilator, no relaxation occurred in response to nerve stimulation in the presence of 1 μM or 10 μM atropine, 1 μM phentolamine, propranolol and nifedipine. Moreover, the high K^+-induced relaxation was not affected by TTX. Therefore, it is rather unlikely that the release of a non-adrenergic, non-cholinergic transmitter(s) contributes to high K^+ relaxation in iris dilator. The finding that indomethacin and NDGA did not affect the high K^+-induced relaxation indicates that products of cyclo-oxygenase and lipoxygenase are not included in the relaxation mechanisms.

Recently, it has been shown that high K^+ solution stimulated the release of EDRF from endothelial cells in a blood vessel and relaxes the smooth muscle in the presence of a Ca^{2+} antagonist (Rubanyi & Vanhoutte, 1988). The possibility of a similar mechanism occurring in iris dilator is worth studying for two reasons: firstly, the vascular system is well developed in the iris dilator region and thus EDRF released from vascular endothelium might have an effect on dilator smooth muscle. Secondly, the dilator tissue includes posterior epithelial cells which might release a relaxing factor similar to EDRF or related substances. The high K^+-induced relaxation was, however, not affected by hypoxic conditions (cf. Furchgott & Zawadzki, 1980). Moreover, two stable analogues of cyclic GMP, which mediate EDRF-induced relaxation in vasculature (Rapoport & Murad, 1983: Rapoport *et al.*, 1983), (dibutyryl cyclic GMP and 8-Br cyclic GMP) did not relax the dilator muscle. Therefore, the possibility that the release of EDRF (NO) is included in the high K^+-induced relaxation was completely ruled out.

In conclusion, a high K^+ solution relaxes iris dilator muscle of the rat and pig. The relaxation includes an atropine-resistant component in addition to the component due to the release of endogenous ACh. Although the possibility of the atropine resistant component being a direct or indirect effect of high K^+ is an open question, the involvement of the Na^+-K^+ pump, a non-adrenergic non-cholinergic transmitter, a product of the arachidonic acid cascade or EDRF is quite unlikely.

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Modification of the ischaemic-induced contraction in the sheep circumflex coronary artery by various pharmacological antagonists

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1 Sheep isolated circumflex coronary artery rings were exposed to simulated ischaemia (increased K^+ reduced pH, hypoxia, reduced glucose and addition of lactate). Simulated ischaemia caused a transient relaxation lasting approximately 5 min followed by a sustained contraction that was reversible on washing with oxygenated Krebs solution.

2 Haemolysate caused a rise in baseline tension and augmented the ischaemic contraction.

3 The ischaemic contraction was abolished by BW 755C 5 μM and reduced by indomethacin 1 μM, quinacrine 50 μM or the thromboxane A_2 antagonist BM 13177 10 μM. The leukotriene D_4 antagonist, ICI 198615, had no effect.

4 The ischaemic-induced contraction was enhanced by propranolol 1 μM, methiothepin 1 μM and reduced by ketanserin 1 μM.

5 The ischaemic contraction was markedly inhibited by trypsin (1 u ml^{-1}) and by verapamil, cromakalim or sodium nitroprusside.

6 The following had no or little effect on the ischaemic contraction: Sar^1-Thr^8-angiotensin II, mepyramine, atropine, the spin trapping agent PBN or the free radical scavenger dimethylsulphoxide. Superoxide dismutase caused a slight enhancement.

7 The ischaemic-induced contraction was abolished by the simultaneous administration, at subthreshold concentrations, either of two vasodilators (iloprost and adenosine) or of a vasodilator and a vasoconstrictor (iloprost and U46619).

8 The ischaemic relaxation phase was reduced by propranolol and indomethacin, abolished by haemolysate and enhanced by quinacrine or cromakalim.

9 It is concluded that the ischaemic-induced contraction is caused by mediators released from the endothelium including a product of the lipoxygenase pathway. There is also evidence that simulated ischaemia causes the release of noradrenaline and 5-hydroxytryptamine.

Introduction

Myocardial ischaemia causes substantial shifts in the ionic and metabolic composition of the intercellular space, notably an accumulation of K^+ (Kleber, 1984), H^+ and lactate (Cobbe & Poole-Wilson, 1980) and a reduction in Po_2 and glucose. These ionic and metabolic changes can be simulated with an appropriate salt solution and gassing conditions. Such simulated ischaemia causes relaxation followed by contraction of rings of sheep coronary artery (Kwan *et al.*, 1989c). The contraction phase of the response to simulated ischaemia is abolished by removal of the endothelium (Kwan *et al.*, 1989c) which is known to be capable of releasing a variety of vasoconstrictor and vasodilator substances (Vanhoutte *et al.*, 1986). The aim of the present experiments was to investigate the hypothesis that the mechanical response of coronary artery rings to simulated ischaemia is caused by increases or decreases in the release of mediators from the artery wall. Several mediators have been shown to be released during myocardial ischaemia including thromboxane A_2, prostacyclin, adenosine, and acetylcholine (ACh); (Yasue *et al.*, 1974; Folts *et al.*, 1976; Berne, 1980; Coker *et al.*, 1981; Hirsch *et al.*, 1981). It is also likely that there will be a release of 5-hydroxytryptamine (5-HT) (from aggregating platelets) and possibly also noradrenaline (from the sympathetic nerves). Since all of these mediators are vasoactive, a secondary aim of the present experiments is to investigate whether they can modify the mechanical response of arterial rings to simulated ischaemia.

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An abstract based on some of these results has been published (Kwan *et al.*, 1989d).

Methods

The experiments were performed on rings of sheep left circumflex coronary arteries of outer diameter, 2–2.5 mm (main circumflex trunk after its first branch). The hearts were brought to the laboratory from the abbatoir within 30 min of slaughter. Care was taken to avoid stretching and damage to the luminal surface during the clearing of the fat and adhering connective tissue. In some experiments, the endothelium was deliberately removed by gentle rubbing of the lumen of the ring with a wooden stick and the absence of endothelium was confirmed by histological examination and by the absence of relaxation with substance P. The arteries were cut into rings 4–5 mm long. Segments were suspended in a constant temperature water-jacketed 10 ml organ bath filled with Krebs-Henseleit solution (37°C) of the following composition (mM): NaCl 119, $NaHCO_3$ 25, glucose 11, KCl 4.6, $MgCl_2$ 1.2, KH_2PO_4 1.2 and $CaCl_2$ 2.5. The solution was aerated with gas mixtures containing 95% O_2:5% CO_2 (oxygenated, $Po_2 = 620 \pm 30$ mmHg, pH = 7.51 ± 0.04). The rings were suspended on a pair of stainless-steel hooks, one of which was fixed to an L-shaped rod inside the organ bath and the other to a Grass isometric transducer. Arterial rings were equilibrated in Krebs-Henseleit solution gassed with 95% O_2 : 5% CO_2 for 1 h at their optimum resting force of 1.5 g. During the equilibration period, the artery ring was washed three to four times with oxygenated Krebs solution, the tension was readjusted and the artery was allowed to relax. The isometric

contraction was calculated as force developed per cross sectional area. The cross sectional area (A) of the artery was calculated by using the equation: A = blotted weight of the artery/(h × β) where h = the distance (cm) between the two stainless steel hooks with the artery ring under optimum resting tension, and β = the density of the artery ring which was taken as 1.05 g cm^{-3}. The tissues were sensitized by repeated contraction cycles with 40 mM KCl until two consecutive identical responses were observed before the start of the experimental protocol.

The major ionic and metabolic changes that occur in myocardial ischaemia (see Introduction) have been studied by use of a physiological salt solution (PSS) with the following composition (mM): NaCl 134.5, $NaHCO_3$ 8.5, glucose 4, KCl 7.6, $MgCl_2$ 1.2, KH_2PO_4 2.4, $CaCl_2$ 1.5 and calcium lactate 1. This was pre-equilibrated with 95% N_2 : 5% CO_2 for 30 min, giving Po_2 = 8 ± 2 mmHg and pH = 6.85 ± 0.05. This solution was rapidly exchanged for the oxygenated Krebs-Henseleit solution; this procedure is termed 'simulated ischaemia'. Oxygen tension was measured with an oxygen electrode (Strathkelvin Instruments) immersed in the organ bath. Aliquots of the bathing medium were removed by syringe and the Pco_2 and pH were measured with a blood gas analyser (Radiometer, Copenhagen).

Experimental protocol

Preliminary studies on the specificity of receptor antagonists and enzyme inhibitors The concentration of the antagonists methiothepin, ketanserin, BM 13177, atropine, Sar^1-Thr^8-angiotensin II and mepyramine, used in this study was based upon the observations that this concentration of the agent could antagonize the contraction mediated by the appropriate agonist at its EC_{90} while having no apparent effect on at least three other contractile agonists on 4 to 5 preparations under the oxygenated conditions. The agonists used were 5-HT, U46619 (a stable thromboxane-mimetic) acetylcholine, angiotensin II and histamine. The concentration of propranolol used was shown to prevent completely the vasorelaxant effect produced by noradrenaline at its IC_{90}. The specificity of phentolamine and ICI 198615 could not be tested in this study as the sheep coronary artery has no α receptors (Brine *et al.*, 1979; Kwan *et al.*, 1989a) and leukotriene C_4 and D_4 had no apparent effect. The effects of trypsin, 1 u mol^{-1}, on the contractile responses to KCl, 5-HT, ACh and U46619 at their respective EC_{25}, EC_{50} and EC_{80} were tested (n = 4). Previous work carried out on this preparation in this laboratory had ascertained that indomethacin, 5 μM, did not modify the contractile response to the EC_{35} and EC_{80} of KCl (Amatya *et al.*, 1990).

Effect of simulated ischaemia on the tone of the coronary artery The solution simulating myocardial ischaemia (pre-equilibrated with 95% N_2 : 5% CO_2) was introduced and the organ bath was then bubbled continuously with 95% N_2 : 5% CO_2 for 25 min, giving Po_2 values (mmHg) of 21 ± 1, 16 ± 2, 10 ± 2, 9 ± 1, 8 ± 1 at 30 s, 2, 5, 15 and 25 min respectively after the addition of ischaemic Krebs solution. Oxygenated conditions were re-established by washing with Krebs solution aerated with 95% O_2 : 5% CO_2. The ischaemic-induced contraction was reproducible at least four times in each preparation (n = 14).

Effects of changing oxygen tension alone on the tone of coronary artery rings in ischaemic solution After the first ischaemic challenge, instead of washing the artery ring in the organ bath with oxygenated Krebs solution, the gas mixtures were changed from 95% N_2:5% CO_2 to 95% O_2:5% CO_2. The artery ring was allowed to equilibrate for 3 min and the gas mixtures were changed back to 95% N_2:5% CO_2, resulting in a Po_2 of 32 ± 8 mmHg after 30 min. This procedure was repeated three times in each preparation and the tone of the artery ring and the Po_2 in the organ bath were recorded continuously.

Effects of receptor antagonists and enzyme inhibitors on the ischaemic-induced contraction After the first ischaemic challenge, the artery ring was washed with oxygenated Krebs solution and allowed to return to baseline tone. The antagonist (or the solvent) was then added and allowed to equilibrate for 30 min under the oxygenated conditions. Resting force was then adjusted to optimal, by reducing the stretch applied, and the ischaemic challenge repeated in the continuing presence of the antagonist. The contraction developed under ischaemia before and in the presence of each agent was compared. In order to check that the change in length of the muscle during the above protocol had not influenced the results, the effect of BW 755C and indomethacin which had caused a marked change in the baseline tone were re-examined without resetting the optimal resting tension. In addition, the contractions caused by BW 755C and indomethacin were matched by an appropriate concentration of the contractile agent U46619 and the size of the ischaemic-induced contraction compared before and in the presence of U46619.

Effects of vasoconstrictor and vasodilator agonists on the ischaemic-induced contraction Following a control ischaemic challenge, the artery ring was washed with oxygenated Krebs solution, allowed to relax for 45 min and resting force readjusted if necessary. The agonist, or combination of agonists, was then added simultaneously with a second ischaemic challenge. The ischaemic-induced contraction before and in the presence of the agonist were recorded and compared.

Drugs

Adenosine, 5-hydroxytryptamine creatinine sulphate complex, acetylcholine chloride, superoxide dismutase, substance P, (±)-propranolol hydrochloride, quinacrine hydrochloride, sodium nitroprusside, atropine sulphate, Sar^1-Thr^8-angiotensin II, dimethyl sulphoxide, trypsin (type XIII), verapamil (all dissolved in distilled water), noradrenaline bitartrate (dissolved in acidic saline), indomethacin (dissolved in absolute ethanol) (all obtained from Sigma), mepyramine maleate (dissolved in distilled water, May & Baker), phentolamine maleate (dissolved in distilled water, Ciba), cromakalim (BRL 34915) (dissolved in saline, gift from Beecham), BM 13177 (4-[2-(benzene-sulphonamido)ethyl]phenoxy acetic acid, diluted in distilled water, Boehringer Mannheim GmbH), BW 755C ((3-amino-1-[*m*-(trifluoromethyl)- phenyl]-2 pyrazoline) dissolved in acidic saline, gift from Wellcome), leukotriene C_4 and D_4 (dissolved in 20% ethanol in saline, Miles Laboratories, Slough), ICI 198615 ([1-[[2-methoxy-4-[[(phenylsulphonyl)-amino]carbonyl]phenyl]methyl]-1H-indazol-6-yl]-carbonic acid cyclopentyl ester, dissolved in dimethyl sulphoxide and further diluted in distilled water, gift from Stuart Pharmaceuticals, U.S.A.), methiothepin maleate (dissolved in distilled water, Roche), ketanserin tartrate (dissolved in distilled water, Janssen), PBN (N-tert-butyl-alpha-phenylnitrone) (dissolved in saline, Sigma), U46619 (11,9 epoxymethano-PGH_2, dissolved in absolute ethanol and further diluted in distilled water, Upjohn), and iloprost (a prostacyclin mimetic) (gift from Schering). Vehicles were tested at equal volumes. Preparation of the haemolysate solution is described elsewhere (Kwan *et al.*, 1989b).

Data analysis

Results are expressed as mean ± s.e.mean. Significance tests were performed by Student's paired t test. P values of less than 0.05 were considered to be statistically significant.

Results

Endothelium

In endothelium containing rings of sheep coronary artery, set at their optimal resting force, simulated myocardial ischaemia

caused a relaxation (lasting about 5 min) followed by a sustained contraction, as previously described (Kwan *et al.*, 1989c). This contraction was 13–16% of the tension developed by the EC_{85} of KCl. In endothelium-denuded rings of sheep coronary artery, simulated myocardial ischaemia produced a relaxation but no contraction was seen (Kwan *et al.*, 1989c). With endothelium present, haemolysate 1 μl ml^{-1} produced an increase in baseline tension under oxygenated conditions. Haemolysate significantly augmented the contraction phase (Table 1) and abolished the initial relaxation produced by simulated ischaemia.

Pharmacological antagonists

Arachidonic acid pathway The combined cyclo-oxygenase and lipoxygenase inhibitor BW 755C, 5 μM and 20 μM, increased baseline tension, abolished the contraction produced by simulated ischaemia and in its place a relaxation occurred (Figures 1 and 2). The cyclo-oxygenase inhibitor, indomethacin 1 μM, also raised baseline tension. In contrast to BW 755C, indomethacin produced significant but modest inhibition of the ischaemic contraction (reduced by 32 ± 3%, Figure 2) and the ischaemic relaxation phase (from 3.8 ± 0.2 to 2.3 ± 0.1 g cm^{-2}). These effects of BW 755C and of indomethacin on the ischaemic contraction were not a consequence of

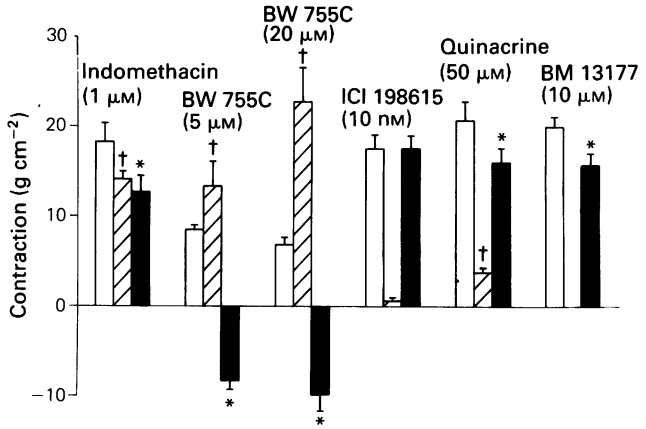

Figure 2 Effects of various pharmacological antagonists on the contraction induced by simulated ischaemia in rings of sheep coronary artery. Open columns, control ischaemic contraction; hatched columns, effects of antagonist on baseline tension; solid columns, ischaemic challenge in the presence of antagonist. * Significantly different from control ischaemic contraction: $P < 0.05$. ‡ Significant effect of antagonist alone: $P < 0.05$. ($n = 6$–10).

Table 1 Effect of various pharmacological antagonists on the ischaemic-induced contraction of circumflex coronary artery rings from sheep

	Contraction (g cm^{-2})		
	Effect of antagonist alone	*Ischaemic challenge* Control	+ Antagonist
Haemolysate (1 μl ml^{-1})	5.3 ± 0.8	19.1 ± 0.2	23.6 ± 0.9*
Trypsin (1 u ml^{-1})	0.5 ± 0.3	23.1 ± 2.1	8.7 ± 2.1*
Sar^1, Thr^8-Ag II (50 μM)	0	11.4 ± 0.5	12.2 ± 0.4
Verapamil (10 μM)	0	13.7 ± 0.7	4.7 ± 0.4*
Cromakalim (1 μM)	−7.9 ± 0.7	16.5 ± 2.0	2.9 ± 0.5*
Nitroprusside (10 μM)	0	13.6 ± 0.6	−2.5 ± 0.3*
Mepyramine (10 μM)	0	12.4 ± 0.8	12.8 ± 0.8
Atropine (1 μM)	0	11.1 ± 0.8	11.1 ± 0.7
PBN 300 (μM)	0	9.7 ± 1.0	9.8 ± 1.0
Dimethyl sulphoxide (150 u ml^{-1})	0	8.7 ± 0.4	8.4 ± 0.4
Superoxide dismutase (150 u ml^{-1})	−0.5 ± 0.7	11.2 ± 1.4	16.6 ± 2.5*

Results are expressed as mean ± s.e.mean in 6–8 preparations.
* Statistically significant difference from the control ischaemic contraction.
− indicates relaxation below baseline.

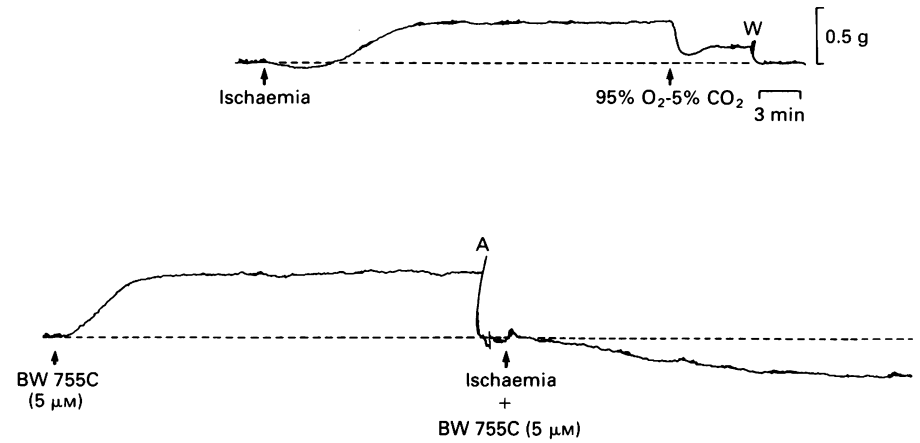

Figure 1 Effects of simulated ischaemia and of BW 755C in sheep coronary artery rings. Stimulated ischaemia produced a relaxation followed by a contraction. The contraction was partly reversed on re-admission of 95% O_2:5% CO_2 and completely reversed by returning to oxygenated Krebs solution (W) (upper panel). BW 755C caused a contraction and at A applied force was adjusted to restore the optimal resting force for this artery. The ischaemic challenge now produced marked relaxation (lower panel).

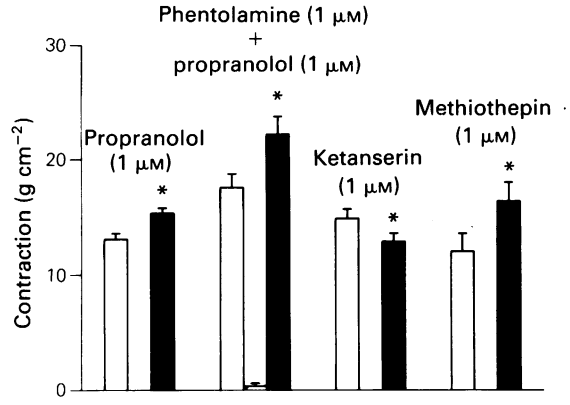

Figure 3 Effects of various pharmacological antagonists on the contraction induced by simulated ischaemia in rings of sheep coronary artery. Open columns, control ischaemic contraction; hatched columns, effects of antagonist on baseline tension; solid columns, ischaemic challenge in the presence of antagonist. * Significantly different from control ischaemic contraction: $P < 0.05$. † Significant effect of antagonist alone: $P < 0.05$. ($n = 6$–10).

the resetting of the resting tension following the change in baseline tension they had produced, since in experiments in which the tension was not reset a similar effect to that shown in Figure 2 was obtained. Thus, BW 755C, 5 μM, abolished the ischaemic contraction and caused a relaxation of $10.2 \pm 1.2\,g\,cm^{-2}$ ($n = 4$) and indomethacin, 1 μM, reduced the ischaemic contraction from 19.7 ± 2.3 to $12.6 \pm 2.4\,g\,cm^{-2}$ ($n = 4$). Moreover, when U46619 was used to match the change in baseline tension caused by BW 755C and indomethacin, ischaemia plus BW 755C caused a relaxation of $8.1 \pm 1.0\,g\,cm^{-2}$ and indomethacin reduced the ischaemic contraction from 17.3 ± 2.1 to $10.2 \pm 3.8\,g\,cm^{-2}$ ($n = 4$). The leukotriene D_4 antagonist, ICI 198615, had no effect on the ischaemic contraction (Figure 2). Moreover, leukotriene C_4 and leukotriene D_4, 0.1 nM–0.1 μM, caused neither contraction nor relaxation of sheep coronary artery rings either under their optimal resting force or when precontracted with KCl 40 mM ($n = 8$). The phospholipase A_2 inhibitor, quinacrine 50 μM, produced a small rise in baseline force and slightly inhibited the ischaemic-induced contraction (Figure 2). The ischaemic relaxation phase was significantly enhanced by quinacrine from 4.0 ± 0.3 to $5.7 \pm 0.3\,g\,cm^{-2}$. The thromboxane A_2 receptor antagonist, BM 13177 10 μM, slightly reduced the contraction produced by simulated ischaemia (Figure 2).

Catecholamines and 5-hydroxytryptamine Propranolol 1 μM, either alone or combined with phentolamine 1 μM, caused a slight but significant enhancement of the contraction caused by simulated ischaemia (Figure 3). In contrast, the initial relaxation phase of the response to simulated ischaemia was significantly inhibited by propranolol (3.9 ± 0.2 to $2.4 \pm 0.4\,g\,cm^{-2}$) or by propranolol plus phentolamine (4.0 ± 0.2 to $2.3 \pm 0.5\,g\,cm^{-2}$). Phentolamine alone had no effect. The ischaemic contraction was reduced by the 5-HT_2 receptor antagonist, ketanserin 1 μM, but was augmented by methiothepin 1 μM, an antagonist at both 5-HT_1 and 5-HT_2 receptors (Figure 3).

Peptides Trypsin, $1\,u\,ml^{-1}$, markedly inhibited the contractile response to simulated ischaemia but had no effect on baseline tension (Table 1). At this concentration, trypsin had no effect on the contractile response to KCl, 5-HT, acetylcholine or U46619. For example, in the absence and presence of trypsin the EC_{80} of KCl (35 mM) and of U46619 (2×10^{-7} M) caused contractions in $g\,cm^{-2}$ of 69.3 ± 5.1 vs 67.3 ± 6.9 and of 58.2 ± 3.3 and 59.3 ± 5.1 respectively. The angiotensin AII antagonist, Sar^1-Thr^8-angiotensin II (5 μM) was without effect on the ischaemic contraction.

Inhibitors of receptor transduction processes The inhibitors of arterial smooth muscle contraction, verapamil, cromakalim and nitroprusside all caused marked inhibition of the contraction initiated by simulated ischaemia. In the presence of sodium nitroprusside a relaxation occurred. Cromakalim, but neither of the other agents, reduced baseline tension (Table 1).

Histamine, acetylcholine, free radicals The contraction produced by simulated ischaemia was not affected by either mepyramine (10 μM) or atropine (1 μM) (Table 1). The spin trapping agent, PBN 300 μM, and the free radical scavenger dimethyl sulphoxide 20 μM, were also without effect. Superoxide dismutase, $150\,u\,ml^{-1}$, caused a slight enhancement of the contractile response (Table 1) and a very small but significant inhibition of the initial relaxation phase (from 3.4 ± 0.1 to $2.7 \pm 0.1\,g\,cm^{-2}$).

Agonists The contractile agents acetylcholine, 5-HT and U46619, each at a concentration producing approximately

Table 2 Effect of different vasoactive substances on the circumflex coronary artery rings from sheep

	Contraction ($g\,cm^{-2}$)		
	Agonist alone (a)	*Ischaemic challenge* Control	+ Agonist
Acetylcholine (0.5 μM)	49.7 ± 18.6(P) 40.1 ± 17.1(T)	26.7 ± 6.6	48.3 ± 5.2*
5-Hydroxytryptamine (4 μM)	54.1 ± 8.3(P) 37.5 ± 7.9(T)	25.4 ± 5.0	75.1 ± 4.7*
U46619 (0.3 μM)	28.2 ± 4.6	21.4 ± 6.7	96.7 ± 6.8*
Iloprost (0.14 μM)	−8.2 ± 3.5	17.2 ± 1.9	−17.7 ± 3.7*
Noradrenaline (1 μM)	−18.8 ± 3.8	17.2 ± 2.8	−10.6 ± 1.8*
Adenosine (0.5 mM)	−27.9 ± 7.0	24.4 ± 7.2	−8.1 ± 4.2*
Iloprost, 10 nM + Adenosine, 100 nM	(b)	14.7 ± 4.3	1.8 ± 1.7*
Iloprost, 10 nM + U46619, 10 nM	(b)	17.8 ± 4.2	3.9 ± 2.8*
U46619, 10 nM + 5-Hydroxytryptamine, 50 nM	(b)	17.4 ± 2.2	22.8 ± 2.7*

Results are expressed as mean ± s.e.mean in 5–8 preparations.
* Statistically significant difference from the control ischaemic contraction.
– indicates relaxation.
(a) The effects of each agonist under oxygenated conditions extrapolated from separate experiments ($n = 6$–19).
(b) Each of these combinations was shown to have no effect on the resting tension. P and T indicate phasic and tonic contractions respectively. Effects of iloprost and noradrenaline, adenosine under oxygenated conditions are expressed as decrease in force developed per cross sectional area of the 40 mM KCl precontracted artery rings.

50% maximal contraction, all markedly augmented the contraction produced by simulated ischaemia (Table 2) and abolished the ischaemic relaxation phase. Only in the case of U46619, did there appear to be potentiation of, rather than merely summation with, the ischaemic contraction. The vasodilators, iloprost, noradrenaline and adenosine, at concentrations that give approximately 25% relaxation, all abolished ischaemic contraction and relaxation below baseline was observed.

Combinations of these vasoactive substances were also investigated using concentrations that are just subthreshold. The ischaemic contraction was attenuated by the combination of two vasodilators (iloprost and adenosine) and by a vasodilator combined with a vasoconstrictor (iloprost and U46619). However a combination of two vasoconstrictors (U46619 and 5-HT) markedly enhanced the ischaemic-induced contraction (Table 2).

Discussion

Ischaemic contraction

In sheep isolated coronary artery rings, conditions simulating myocardial ischaemia produce an initial relaxation followed by a contraction. Removal of the endothelium abolishes the contractile phase of the mechanical response to simulated ischaemia. This suggests that the ischaemic contraction is caused by release of a vasoconstrictor from the endothelium, or by reduced release of a vasodilator from the endothelium. Vascular endothelium is capable of releasing endothelial-derived relaxing factor (EDRF), the actions of which are antagonized by haemoglobin (Martin *et al.*, 1985). In the sheep coronary artery, haemolysate caused contraction and augmented the contraction produced by simulated ischaemia. This suggests that there is a basal output of EDRF from the endothelium of the sheep coronary artery and that this basal EDRF attenuates the ischaemic contraction.

In the present experiments, indomethacin in a concentration that inhibits the cyclo-oxygenase enzyme (Flower *et al.*, 1972) had marked effects on basal tone and on the ischaemic contraction, suggesting that arachidonic acid metabolites may be important in mediating these responses. The contraction produced by indomethacin has been shown to be linked to inhibition of basal output of prostacyclin in these coronary arteries (Amatya *et al.*, 1988). Since indomethacin reduces the ischaemic contraction by 32%, it is likely that part of the ischaemic contraction is caused by a reduced output of prostacyclin. Hypoxia reduces prostacyclin release from the sheep coronary artery (Amatya *et al.*, 1988). BW 755C, when used in a concentration that has been reported to block both lipoxygenase and cyclo-oxygenase enzymes (Blackwell & Flower, 1978) also caused a contraction. As with indomethacin this may be attributable to inhibition of cyclo-oxygenase and a reduction in prostacyclin release. BW 755C had a much greater effect than quinacrine, the phospholipase A_2 inhibitor (Flower & Blackwell, 1976) on the ischaemic contraction, suggesting that at the concentrations used BW 755C may have additional actions, such as blockade of calcium channels. Further experiments should be carried out to ascertain if this is indeed the case. Leukotrienes do not appear to play an important role in the ischaemic contraction since neither leukotrience C_4 (LTC_4) nor LTD_4 produced contraction, and the LTD_4 antagonist ICI 198615 had no effect on the ischaemic contraction. It is possible nevertheless that other products of the lipoxygenase pathway, such as 5-hydroxyeicosatetraenoic acid (HETE) or free radicals, are responsible for the ischaemic contraction (Rao *et al.*, 1983). Endothelial cells are capable of mobilizing arachidonic acid and utilizing the lipoxygenase pathway (Thomas *et al.*, 1981; Gerritsen, 1987). Since the thromboxane antagonist BM 13177 slightly inhibited the ischaemic contraction, it is possible that there is also some activation of thromboxane production.

Noradrenaline relaxes sheep coronary artery via activation of β-adrenoceptors (Brine *et al.*, 1979). Since propranolol augmented the ischaemic contraction, it is likely that these simulated ischaemic conditions cause release of noradrenaline from intramural adrenergic nerves, as has been suggested by Borda *et al.* (1980) in the dog coronary artery. The ischaemic contraction was reduced by the 5-HT_2 antagonist ketanserin and augmented by the 5-HT_1 and 5-HT_2 antagonist methiothepin. A possible explanation for these results is that simulated ischaemia is causing release of 5-HT from the artery wall. This released 5-HT then acts on 5-HT_2 receptors in the smooth muscle to cause contraction (Brazenor & Angus, 1982) and on 5-HT_1 receptors on the endothelium to release EDRF (Cohen *et al.*, 1983). We have no direct evidence that 5-HT is present in sheep isolated coronary artery rings, but possible locations include adrenergic or other nerves (Cohen *et al.*, 1987), endothelial cells (Lee & Fanburg, 1986) and mast cells.

Trypsin reduced the ischaemic contraction by 62%, suggesting that a peptide or protein plays a role in mediating the response to ischaemia. Endothelial cells in culture can release a potent vasoconstrictor peptide, endothelin (Yanagisawa *et al.*, 1988) but as this is very slow acting it is unlikely that it could account for the rapid and reversible contraction that we observed under conditions of simulated ischaemia. This effect of trypsin was not attributable to a non specific depressant effect on vasoconstriction since the contractile effects of KCl, 5-HT, acetylcholine and U46619 were unaffected by the concentration of trypsin used. BW 755C abolished the ischaemic contraction, although as suggested above, part of it may be due to the action of a peptide or protein rather than a product of arachidonic acid metabolism. BW 755C may, therefore, be capable of preventing the action of this peptide or protein and lends support to the idea that it has actions in addition to that of enzyme inhibition in the arachidonic acid cascade. Angiotensin appears not to play a part in the ischaemic contraction since the angiotensin II antagonist Sar^1-Thr^8-angiotensin II had no effect.

The coupling processes in smooth muscle and endothelial cells can be inhibited by drugs that block membrane calcium channels (e.g. verapamil), activate K^+ channels (cromakalim) or activate guanylate cyclase (sodium nitroprusside). All these were very effective inhibitors of the ischaemic contraction. These results show that calcium is necessary for the ischaemic contraction, either because calcium is needed for the release of the vasoconstrictor or because it is needed to generate tension in the arterial smooth muscle cells.

The lack of effect of atropine, mepyramine, dimethyl sulphoxide (OH^- scavenger) and PBN (spin trapping agent) argue against an involvement of acetylcholine, histamine or free radicals in the ischaemic-induced contraction. However superoxide dismutase significantly augmented the contraction, but the mechanism of this effect was not elucidated.

Ischaemic relaxation phase

An ischaemic relaxation phase occurred both in the absence and presence of endothelium. Therefore, either it is not caused by an alteration in endothelial mediator release or different mechanisms may underlie the relaxation in intact and denuded arteries. Interestingly, haemolysate was the only one of the antagonists used that abolished this relaxation in the endothelium containing preparations. This suggests that release of nitric oxide may mediate the relaxation in intact arteries. Whether this nitric oxide is released solely from the endothelium or may also be released by other cell types merits further examination. Our results also suggest that other vasodilators such as catecholamines (relaxation reduced by propranolol or by propranolol plus phentolamine) and a cyclo-oxygenase product (relaxation reduced by indomethacin) may be released immediately following the introduction of simulated ischaemia. These vasodilators are apparently opposed by a product of the phospholipase A_2 pathway, since the relaxation was enhanced by quinacrine.

Cromakalim also enhances ischaemic relaxation, perhaps because of the marked reduction in baseline tone that is produced by cromakalim.

Interaction of agonists and the ischaemic contraction

When the heart is made ischaemic a number of mediators are believed to be released from nerves, platelets, endothelial cells and muscle. These mediators profoundly modify the response of the coronary artery to simulated ischaemia. The vasodilators adenosine, iloprost or noradrenaline abolished the contraction phase of the response to simulated ischaemia. This effect was so powerful that it occurred even at subthreshold concentrations (adenosine plus iloprost) or when a subthreshold concentration of a vasoconstrictor was added to a subthreshold concentration of a vasodilator (iloprost and U46619). In contrast, the vasoconstrictors 5-HT, acetylcholine and U46619 all increased the contraction phase of the mechanical response to ischaemia and in the case of U46619 a potentiated rather than just an additive effect was observed. This is likely to be related to the hypoxia-induced potentiation of the contractile effects of the thromboxane-mimetic previously reported by Kwan *et al.* (1989a). Thus it could be expected that a coronary artery running through an area of ischaemic myocardium would either contract or relax depending on the balance between the release of vasoconstrictor metabolites (mainly from endothelium and platelets) and vasodilator metabolites (mainly from nerve and muscle).

In conclusion, these results provide evidence that the contraction of sheep coronary artery rings initiated by simulated myocardial ischaemia is caused by the release of mediators from the endothelium and vessel wall. These mediators include a product of the lipoxygenase pathway, noradrenaline and 5-HT. The contraction is dependent on intracellular calcium and is readily prevented by vasodilators such as adenosine that are released during ischaemia.

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Specific but differential antagonism by glibenclamide of the vasodepressor effects of cromakalim and nicorandil in spinally-anaesthetized dogs

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1 Whether the vasodepressor effects of cromakalim and nicorandil, potassium channel openers, were differentially antagonized by glibenclamide, a supposed specific antagonist of potassium channel openers, was investigated in spinally-anaesthetized dogs in which arterial pressure was maintained elevated by i.v. infusion of noradrenaline.

2 Cumulative administration of cromakalim (3–100 $\mu g\,kg^{-1}$, i.v.), nicorandil (30–1000 $\mu g\,kg^{-1}$, i.v.), diltiazem (3–300 $\mu g\,kg^{-1}$, i.v.) and nitroglycerin (0.3–100 $\mu g\,kg^{-1}$, i.v.) caused dose-dependent decreases in mean arterial pressure. In dogs which received glibenclamide (3 $mg\,kg^{-1}$, i.v.), the dose-vasodepressor response curves for cromakalim and nicorandil were located on the right in parallel to the respective curves determined in control dogs, but those for diltiazem and nitroglycerin were not different. $ED_{50\%}$ values increased about 6.7 fold for cromakalim and 2.2 fold for nicorandil.

3 The depression of LV dP/dt_{max} produced by a high dose of cromakalim (100 $\mu g\,kg^{-1}$, i.v.) was abolished by glibenclamide and that produced by nicorandil was not only antagonized but converted to an increase.

4 These results suggest that the vasodepressor action of cromakalim is due predominantly to potassium channel opening, but that of nicorandil involves not only potassium channel opening but its action as a nitrate.

Introduction

Nicorandil, a new antianginal drug with coronary dilator properties (Uchida *et al.*, 1978), has a dual mechanism of action as a nitrate on the one hand and as a potassium (K)-channel opener on the other (Taira, 1987). It has been hypothesized that the action of nicorandil as a resistive vessel (arteriolar) dilator to increase coronary blood flow is due predominantly to its action as a K-channel opener (Taira, 1989). This hypothesis has been based on the close similarity between the cardiac and coronary vasodilator effects of nicorandil and cromakalim (Gotanda *et al.*, 1988; Taira, 1989). The vasodilator effects of cromakalim, a potent antihypertensive drug with vasodilator properties (Buckingham *et al.*, 1986), have recently been shown to involve K-channel opening more specifically than nicorandil (Weir & Weston, 1986). Also, cromakalim lacks the ability to increase intracellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP) as seen with nicorandil (Yanagisawa *et al.*, 1988). Recently, it has also been shown that in rats the vasodepressor effect of cromakalim on systemic arterial pressure was specifically antagonized by glibenclamide, an oral antidiabetic drug (Cavero *et al.*, 1988). Nicorandil exerts a vasodepressor effect (Uchida *et al.*, 1978; Taira *et al.*, 1979; Sakai *et al.*, 1981), and this is thought to involve two mechanisms; one is dilatation of resistive vessels caused by its action as a K-channel opener and the other is reduction of cardiac output via decreased venous return resulting from capacitive vessel dilatation, caused by its action as a nitrate. Thus, it appeared of interest to see whether the vasodepressor effects of cromakalim and nicorandil were differentially antagonized by glibenclamide. For this purpose we carried out the present experiments in spinally-anaesthetized dogs in which systemic arterial pressure was maintained elevated by noradrenaline. We used diltiazem, a calcium channel blocker, and nitroglycerin, a prototype of classical nitrates, as reference drugs. We chose diltiazem because it is highly specific as a calcium channel blocker (Fleckenstein, 1981) and nearly equipotent to nicorandil as a vasodilator (Sakai *et al.*, 1981).

Methods

Preparations

Forty-four young mongrel dogs of either sex, weighing 7 to 15 kg, were anaesthetized with pentobarbitone sodium initially at a dose of 30 $mg\,kg^{-1}$, i.v., which was supplemented when necessary. After being given nadolol (100 $\mu g\,kg^{-1}$, i.v.) and atropine (1 $mg\,kg^{-1}$, i.v.), animals were further subjected to spinal anaesthesia by an injection of 0.3% dibucaine hydrochloride solution into the cisterna magna (about 0.2 $ml\,kg^{-1}$) and both vagus nerves were cut as described previously (Cavero, 1976; Satoh *et al.*, 1985). Artificial respiration was performed with room air in a tidal volume of 20 $ml\,kg^{-1}$ at 18 breaths min^{-1} by use of a dog respirator (Sinano, SN-480-4). Body temperature was maintained at about 39°C by use of a heating pad when necessary. Systemic arterial pressure and mean systemic arterial pressure (MAP) were measured at the femoral artery by a pressure transducer (Statham, P-50) and heart rate (HR) by a cardiotachometer (San-ei, 1321) triggered by arterial pressure pulses. Left ventricular pressure (LVP) was measured by a micromanometer-tipped catheter (Mitsui, S-type) introduced into the left ventricle via the right carotid artery, and its maximum rate of rise (LV dP/dt_{max}) was obtained with an electronic differentiator (San-ei, 1323). These variables were recorded continuously on charts with rectilinear recorders (San-ei, Recti-Horiz-8S). At least a 30 min stabilization period was allowed before the experiments.

Experimental protocol

Arterial pressure of animals made spinal with dibucaine was maintained elevated by infusion of (−)-noradrenaline (0.5 $\mu g\,kg^{-1}\,min^{-1}$) into the right jugular vein with an infusion pump (Termo, STC-521). Each animal received glibenclamide at a dose of 3 $mg\,kg^{-1}$ or its solvent as control in the form of a bolus injection of a volume of 0.5 ml into the left femoral vein, after the elevated blood pressure had become stabilized (10–30 min). Then, a period of 10–30 min was allowed before

the first dose of vasodepressor drugs was administered. All vasodepressor drugs were cumulatively administered into a venous cannula placed in the right femoral vein in volumes of less than 1 ml and flushed in with 5 ml of 0.9% saline over a period of 10 s. Each dose was administered when the vasodepressor effect of the previous dose had reached a nadir; about 30 s intervals were allowed for nitroglycerin, 1–1.5 min for nicorandil, about 1.5 min for diltiazem, and about 2 min for low doses and 3–5 min for high doses of cromakalim. Cumulative dose-response curves were constructed in such a way that responses were expressed as percentages of those values obtained before the administration of the vasodepressor drugs (i.e. basal values).

Drugs

Cromakalim (Beecham), nicorandil (Chugai), diltiazem hydrochloride (Tanabe), nitroglycerin (0.05% aqueous solution in ampoule, Nippon Kayaku), glibenclamide (Hoechst Japan and Yamanouchi) and nadolol (Squibb) were gifts from each company. The following compounds were purchased: dibucaine hydrochloride (0.3% hyperbaric solution in ampoule, Teikoku Chemical), (−)-noradrenaline base (Fruka) and atropine sulphate (Wako).

Cromakalim was dissolved in 100% ethanol at a concentration of 4 mg ml^{-1} and diluted with 0.9% saline to the desired concentrations. Glibenclamide was dissolved in 100% dimethylfolmamide (DMF) at a necessary concentration in 0.5 ml. Nadolol and (−)-noradrenaline were dissolved in 0.1 N HCl-saline. Other compounds were dissolved and diluted with 0.9% saline to the desired concentrations.

Statistics

Experimental values are expressed as means ± s.e.mean. Values of LV dP/dt_{max} refer to those at the time of the peak response of MAP. The parallelism of dose-response curves was evaluated by analysis of covariance (Snedecor & Cochran, 1967). When a P value was less than 0.05, the curves were considered to be non-parallel. The doses that produced a 50% decrease in MAP ($ED_{50\%}$) were determined from the dose-response curves at three higher doses which were fitted by linear regression and the slopes of the curves refer to those of the linear regression. Statistical significance of differences between mean values was estimated by t test. A P value less than 0.05 was considered to be significant.

Results

The average values of MAP and HR of 44 dogs anaesthetized with pentobarbitone sodium and given nadolol (100 μg kg^{-1}, i.v.) and atropine (1 mg kg^{-1}, i.v.) were 144 ± 3 mmHg and 130 ± 3 beats min^{-1}. After the dogs had been subjected to spinal anaesthesia with dibucaine MAP was reduced to 73 ± 3 mmHg and HR to 121 ± 2 beats min^{-1}. With infusion of (−)-noradrenaline (0.5 μg kg^{-1} min^{-1}) MAP was maintained elevated at 163 ± 5 mmHg (referred to basal values), but HR remained virtually unchanged. A half of the 44 dogs received the solvent and the remaining half glibenclamide. The solvent had no effects on MAP and HR, or on LV dP/dt_{max} which was 2647 ± 150 mmHg s^{-1} (n = 18) before and 2642 ± 155 mmHg s^{-1} (n = 18; referred to basal values) after the solvent. Glibenclamide (3 mg kg^{-1}, i.v.) produced irregular changes in MAP and HR, but in a short time these changes disappeared. Only LV dP/dt_{max} decreased significantly but slightly; 2392 ± 133 mmHg s^{-1} (n = 18; referred to basal values) after, compared to 2694 ± 176 mmHg s^{-1} (n = 18) before glibenclamide.

In control dogs the cumulative administration of cromakalim (3–100 μg kg^{-1}; n = 6), nicorandil (30–1000 μg kg^{-1}; n = 6), diltiazem (3–300 μg kg^{-1}; n = 5) and nitroglycerin (0.3–100 μg kg^{-1}; n = 5) caused dose-dependent decreases in MAP. The results of typical experiments with cromakalim and nicorandil are shown in Figures 1 and 2. Dose-response curves for % decreases in MAP produced by these vasodepressor drugs are presented in Figure 3. MAP was decreased by about 71%, 46%, 38% and 23% with 10 fold increases in doses of cromakalim (10–100 μg kg^{-1}), nicorandil (100–

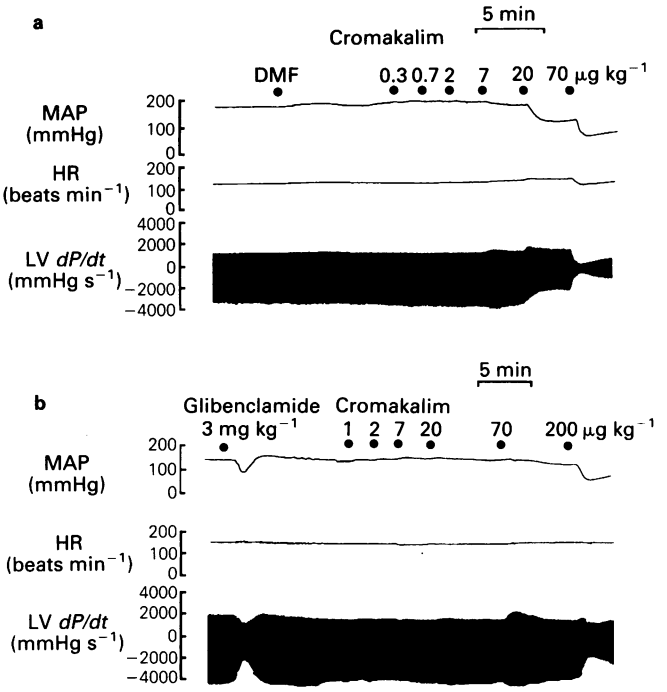

Figure 1 Effects of intravenous injections of cromakalim on mean arterial pressure (MAP), heart rate (HR) and rate of change in left ventricular pressure (LV dP/dt). (a) Control dog; (b) glibenclamide (3 mg kg^{-1}, i.v.)-treated dog. In this and subsequent figures DMF refers to dimethylfolmamide, the solvent used for glibenclamide.

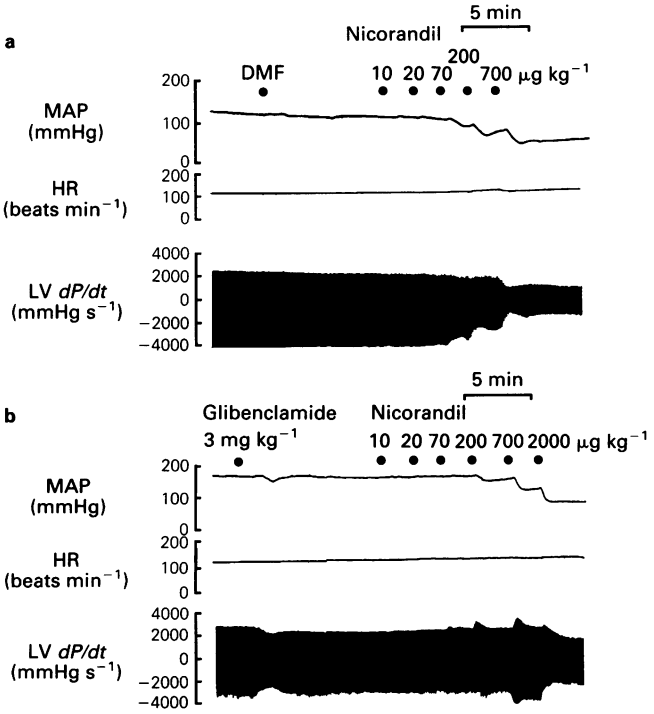

Figure 2 Effects of intravenous injections of nicorandil on mean arterial pressure (MAP), heart rate (HR) and rate of change in left ventricular pressure (LV dP/dt). (a) Control dog; (b) glibenclamide (3 mg kg^{-1}, i.v.)-treated dog.

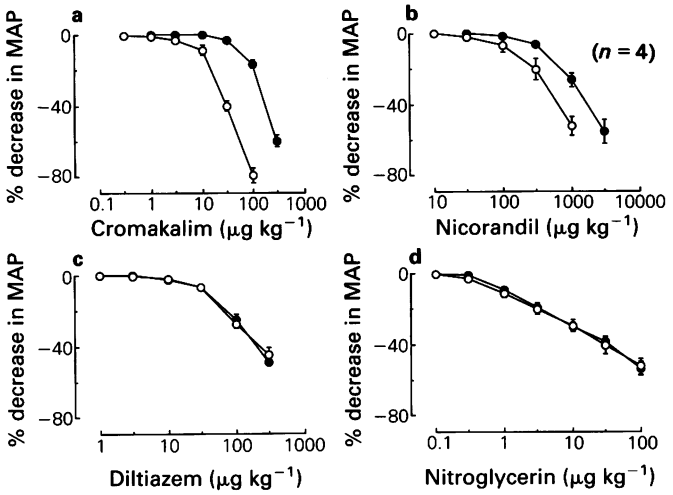

Figure 3 Effects of glibenclamide on vasodepressor responses to (a) cromakalim, (b) nicorandil, (c) diltiazem and (d) nitroglycerin. Data points represent mean from 6 dogs for cromakalim and nicorandil or 5 dogs for diltiazem and nitroglycerin. Vertical lines show s.e.mean. (○) Control dogs; (●) glibenclamide-treated dogs (3 mg kg^{-1}, i.v.).

1000 μg kg^{-1}), diltiazem (30–300 μg kg^{-1}) and nitroglycerin (10–100 μg kg^{-1}), respectively. In dogs which received glibenclamide (3 mg kg^{-1}), the vasodepressor effects of cromakalim and nicorandil were reduced (Figures 1 and 2), and the dose-response curves for cromakalim (n = 6) and nicorandil (n = 6) were shifted to the right in parallel to the respective control curves. When the ED_{50} values for the decrease in MAP were compared, cromakalim was about 33 times more potent than nicorandil. In the presence of glibenclamide, the $ED_{50\%}$ values increased about 6.7 fold for cromakalim and 2.2 fold for nicorandil (Table 1). In contrast, the dose-response curves for the % decrease in MAP produced by diltiazem (n = 5) and nitroglycerin (n = 5) were not different between control and glibenclamide-treated dogs (Table 1 and Figures 1, 2 and 3).

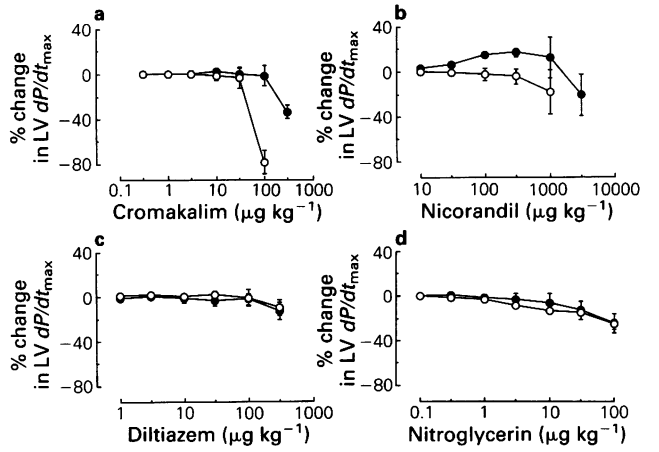

Figure 4 Effects of glibenclamide on negative inotropic actions of (a) cromakalim, (b) nicorandil, (c) diltiazem and (d) nitroglycerin. Data points represent mean from 4 dogs for cromakalim and nicorandil or 5 dogs for diltiazem and nitroglycerin. Vertical lines show s.e.mean. (○) Control dogs; (●) glibenclamide-treated dogs (3 mg kg^{-1}, i.v.).

In control dogs, cromakalim at a high dose (100 μg kg^{-1}, n = 4) markedly depressed LV dP/dt_{max}. This depression was abolished in glibenclamide-treated (n = 4) dogs. Furthermore, glibenclamide also had an inhibitory effect on the cardiodepressant effect of 300 μg kg^{-1} cromakalim. In control dogs, nicorandil (n = 4) 1000 μg kg^{-1} tended to depress LV dP/dt_{max} but not significantly, whereas in glibenclamide-treated (n = 4) dogs nicorandil (100–300 μg kg^{-1}) significantly increased LV dP/dt_{max}, but transiently. In both control (n = 5) and glibenclamide-treated (n = 5) dogs diltiazem had no effect on LV dP/dt_{max}. Nitroglycerin (3–100 μg kg^{-1}) produced a slight but significant decrease in LV dP/dt_{max} in control (n = 5) dogs, but the dose-response curves for this effect were not different between control (n = 5) and glibenclamide-treated (n = 5) dogs (Figures 1, 2 and 4).

However, none of the agents tested had a significant effect on HR except the highest dose of diltiazem (300 μg kg^{-1}); which produced a change of −13.5 ± 2.7% in control (n = 5) and −10.2 ± 4.1% in glibenclamide-treated (n = 5) dogs, respectively.

Discussion

In the present experiments on spinally-anaesthetized dogs in which arterial pressure was maintained elevated with an i.v. infusion of (−)-noradrenaline, glibenclamide (3 mg kg^{-1}, i.v.) specifically antagonized the vasodepressor effects of cromakalim (3–100 μg kg^{-1}, i.v.) and nicorandil (30–1000 μg kg^{-1}, i.v.); glibenclamide *per se* affected neither basal arterial pressure nor the vasodepressor effects of diltiazem and nitroglycerin. As glibenclamide was not potent enough to exert an antagonistic effect at low doses (0.3 or 1 mg kg^{-1}) and produced toxic effects, such as a severe fall in blood pressure and arrhythmia, at higher doses (10 mg kg^{-1}), in the present experiments we were only able to investigate the effect of glibenclamide at a single dose of 3 mg kg^{-1}. Nevertheless, the shifts of the dose-response curves for the vasodepressor effects of cromakalim and nicorandil produced by glibenclamide were parallel, suggesting that the antagonism might be competitive. Thus, the present results with cromakalim are in agreement with those obtained by Cavero *et al.* (1988) in rats, but not with those obtained by Wilson *et al.* (1988) in the same species. The lack of antagonism by glibenclamide of the effects of cromakalim in the study of Wilson *et al.* (1988) might have been due to the use of an insufficient dose of glibenclamide; they gave 100 mg kg^{-1} orally whereas Cavero *et al.* (1988) administered the same dose intravenously. The present results together with those of Cavero *et al.* (1988) strongly suggest that glibenclamide behaves as a pharmacological antagonist against K-channel openers.

The similarity of the antagonism by glibenclamide of the effects of nicorandil and cromakalim and the finding that glibenclamide was ineffective against nitroglycerin indicate that the vasodepressor effect of nicorandil is due predominantly to

Table 1 Influence of glibenclamide on the vasodepressor effects of cromakalim, nicorandil, diltiazem and nitroglycerin

	$ED_{50\%}$ (μg kg^{-1})			
	Cromakalim	*Nicorandil*	*Diltiazem*	*Nitroglycerin*
Control dogs	40 ± 4 (6)	1330 ± 360 (6)	490 ± 123 (5)	139 ± 75 (5)
Glibenclamide-treated dogs	269 ± 30** (6)	2970 ± 600* (4)	357 ± 44 (5)	108 ± 43 (5)

The doses that produced a 50% decrease in mean arterial blood pressure (MAP) ($ED_{50\%}$) were determined from the dose-response curves at three higher doses which were fitted by linear regression. Values represent mean ± s.e.mean. Numbers of dogs are given in parentheses. * $P < 0.05$, ** $P < 0.01$ against corresponding values in control dogs.

its action as a K-channel opener, as inferred previously. However, it should be noted that the shift of the dose-response curves for vasodepression induced by glibenclamide was 6.7 fold for cromakalim as against 2.2 fold for nicorandil. In other words, the antagonism by glibenclamide of the vasodepressor effect was not so effective for nicorandil as for cromakalim. Thus, it is likely that another mechanism, in all probability its action as a nitrate, is also involved in the vasopressor effect of nicorandil. The fall in systemic arterial pressure is indeed produced by dilatation of resistive vessels as well as by decreased cardiac output. The latter effect is a particular feature of nitrates. This mechanism of action of nicorandil seems to come into effect after its action as a K-channel opener had been antagonized by glibenclamide and is probably responsible for an apparent resistance to the antagonizing action of glibenclamide. The finding that the slope of the dose-response curve for the vasodepressor effect of nicorandil lies in between that of cromakalim and nitroglycerin may be also related to its dual mechanism of action. Decreases in MAP produced by 10 fold increases in the doses of cromakalim, nicorandil and nitroglycerin in the high dose range in control dogs were about 71%, 46% and 27%, respectively.

Another important finding obtained in the present experiments was that the cardiodepressant effects of cromakalim and nicorandil, as reflected by decreases in LV dP/dt_{max}, were also antagonized by glibenclamide. Decreases in LV dP/dt_{max} produced by the two drugs were in all probability a result of their negative inotropic effect, because nicorandil (Taira *et al.*, 1989; Yanagisawa *et al.*, 1988; 1989) and cromakalim (Gotanda *et al.*, 1988; 1989; Yanagisawa *et al.*, 1988; 1989) have been known to produce a negative inotropic effect both *in vivo* and *in vitro* entirely by K-channel opening. Thus, the present findings can be taken to indicate that the specific antagonism by glibenclamide of cromakalim and nicorandil occurred in left ventricular muscle cells, too. It was of further interest that the decrease in LV dP/dt_{max} produced by nicorandil was not only antagonized by glibenclamide but converted to an increase. It has been shown that nicorandil has an inhibitory action in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) phosphodiesterase (Endoh *et al.*, 1980). Inhibition of cyclic AMP phosphodiesterase produces positive inotropy. Thus, it is likely that this effect was uncovered by the inhibition of K-channel opening, reinforced by the infusion of noradrenaline and led to positive inotropy.

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Tachykinins activate guinea-pig alveolar macrophages: involvement of NK_2 and NK_1 receptors

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1 The effects of substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) were evaluated on superoxide anion (O_2^-) production by guinea-pig alveolar macrophages (AM).

2 SP dose-dependently (ED_{50} = 0.7 nM) evoked O_2^- production from guinea-pig AM; the N-terminal heptapeptide, SP(1–7), was ineffective. In the presence of thiorphan (10^{-5} M), an enkephalinase inhibitor, the stimulating effects of SP were not significantly modified. NKA and NKB were both able to induce O_2^- production from guinea-pig AM, ED_{50} values being 0.1 and 1.3 nM, respectively. Therefore, the rank order of activity of natural tachykinins was NKA > SP > NKB. Tachykinin-evoked effects were quantitatively similar to those elicited by the autacoid mediator PAF-acether and less than those induced by the synthetic peptide N-formylmethionyl-leucyl-phenylalanine (FMLP).

3 The NK_2 receptor agonist [β-Ala^8]-NKA (4–10) dose-dependently evoked O_2^- production from guinea-pig AM; the NK_1 receptor agonist [Pro^9]-SP sulphone acted only at high concentrations, while the NK_3 receptor agonist [Me,Phe^7]-NKB was ineffective.

4 These findings indicate that guinea-pig AM possess NK_2 and possibly some NK_1 tachykinin receptors and further suggest tachykinin involvement in lung pathophysiology.

Introduction

Several studies indicate that natural tachykinins may play a role in lung pathophysiology (Barnes, 1987; Lundberg & Saria, 1987; Barnes *et al.*, 1988). Among mammalian tachykinins, substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) are the most likely candidates for a neurotransmitter(s) role. SP, NKA and NKB cause, with different potencies, airway smooth muscle contraction in several animal species, increase plasma exudation in guinea-pig airways and have been implicated in lung diseases, namely asthma (Barnes, 1987; Lundberg & Saria, 1987; Sekizawa *et al.*, 1987; Rogers *et al.*, 1988; Frossard *et al.*, 1989).

The undecapeptide SP is recognized as a chemical mediator of neurogenic inflammation, since it participates in vasodilatation, increases vascular permeability and exerts modulatory effects on cells involved in inflammatory and immune processes (Lembeck & Holzer, 1979; Foreman & Jordan, 1984; Payan *et al.*, 1984).

In addition, SP degranulates rat mast cells, stimulates DNA and protein synthesis from human T lymphocytes, evokes the release of inflammatory cytokines from human blood monocytes, enhances the phagocytic activity of human neutrophils and rat peritoneal macrophages, promotes lysosomal enzyme release and oxy-radical production from human neutrophils (Bar-Shavit *et al.*, 1980; Fewtrell *et al.*, 1982; Payan *et al.*, 1983; Lotz *et al.*, 1988; Serra *et al.*, 1988).

In elicited guinea-pig peritoneal macrophages, SP induces oxy-radical production and arachidonic acid metabolite release by interacting with specific receptor sites (Hartung & Toyka, 1983; Hartung *et al.*, 1986), but no attempts were made to characterize the receptor subtype(s) mediating these effects.

We evaluated the effects of natural tachykinins on guinea-pig alveolar macrophages (AM), a cell type which plays an important role in the local inflammatory process associated with bronchial hyperreactivity in asthma (Hunninghake *et al.*, 1979).

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Previous studies have demonstrated that guinea-pig AM possess specific receptors for chemotactic formylpeptides (e.g., N-formylmethionyl-leucyl-phenylalanine; FMLP) and PAF-acether, and are capable, when challenged by these agents, to undergo a respiratory burst, which in turn leads to the production of reactive oxygen metabolites known to promote tissue damage (Daniele *et al.*, 1982; Desquand *et al.*, 1986; Henson & Johnston, 1987).

Therefore, we decided to ascertain whether natural tachykinins (SP, NKA, NKB) evoke superoxide anion (O_2^-) production from guinea-pig AM. By using synthetic peptide analogues that act as selective stimulants at tachykinin receptors (Drapeau *et al.*, 1987; Rovero *et al.*, 1989), we tried to identify which tachykinin receptor(s) mediate the activation of guinea-pig AM.

Methods

Harvest of broncho-alveolar cells and macrophage culture

Alveolar macrophages (AM) were harvested from male guinea-pigs (250–300 g), according to the method of Myrvik *et al.* (1961), with minor modifications. Briefly, the animals were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.) and the trachea was cannulated. Sixty ml of phosphate buffered saline (pH 7.4, 37°C) was infused into the lungs in 5 ml aliquots and reaspirated after gentle massage. Fluid of the two first lavages was discarded, as it was found to contain a large quantity of cells other than macrophages on microscopic examination. The lavage fluid was then centrifuged (1700 r.p.m., room temperature, 10 min) and cells were resuspended in Minimum Essential Medium with Earle's salts (MEM).

After lysis of red blood cells by hypotonic shock, cellular populations were diluted in MEM and microscopically evaluated. The preparation thus obtained consisted of more than 85% macrophages, other cells being lymphocytes and eosinophils (May-Grunwald/Giemsa staining). Cell viability was

determined by trypan blue exclusion and consistently exceeded 95%.

Aliquots of cell suspension (0.7–1 × 10^6 cells) were plated in 6-well tissue culture plates (35 mm diameter, Costar, Cambridge, U.K.) containing 2 ml of MEM supplemented with 20 mM HEPES, 2 mM glutamine and 5% foetal calf serum and were allowed to adhere for 2 h at 37°C in 5% CO_2. Thereafter, non-adherent cells were removed by washing monolayers three times with Hank's balanced salt solution. The monolayers consisted of at least 98% macrophages (May-Grunwald/Giemsa staining); cell viability (trypan blue exclusion) was approximately 95%.

Activation of guinea-pig alveolar macrophages and superoxide anion production

Adherent macrophages were incubated with SP, NKA, NKB and drugs reported to be selective NK-receptor subtype agonists: [Pro^9]-SP sulphone (NK_1 agonist), [β-Ala^8]-NKA (4–10) (NK_2 agonist) and [Me,Phe^7]-NKB (NK_3 agonist) (Drapeau *et al.*, 1987; Rovero *et al.*, 1989) up to 60 min at 37°C in a humidified atmosphere containing 5% CO_2 in the presence of cytochrome C (1 mg ml^{-1}). In some experiments, adherent AM were preincubated 30 min with thiorphan (10^{-5} M) or vehicle and then exposed, in the presence of cytochrome C, to increasing concentrations of SP for further 60 min. The stimulating effects of neuropeptides were terminated by placing the wells on ice. O_2^- production was measured by the superoxide dismutase-inhibitable cytochrome C reduction, the absorbance changes being recorded at 550 nm in a Perkin Elmer 552S spectrophotometer. O_2^- production was expressed as nmol cytochrome C reduced/10^6 cells, using an extinction coefficient of 21.1 mM (Massey, 1959).

To avoid interference with spectrophotometrical recordings of O_2^- production, monolayers were incubated in MEM without phenol red.

Data analysis

Experiments were performed in triplicate and control values (e.g., O_2^- production in the absence of any treatment) were subtracted from all determinations. All results are expressed as means ± s.e.mean. Statistical analysis was carried out by ANOVA.

Chemicals

The compounds used and their sources were: SP, SP(1–7), NKA and NKB (Peninsula); HEPES (2-4-(2-hydroxyethyl)-1-piperazino-ethansulfonic acid: Aldrich); thiorphan, superoxide dismutase, cytochrome C type VI (Sigma); FMLP (Serva); PAF-acether (Bachem); MEM with or without phenol red, glutamine and foetal calf serum (Gibco).

[β-Ala^8]-NKA(4–10) was synthesized at Menarini Laboratories, Florence (Italy); [Me,Phe^7]-NKB and [Pro^9]-SP sulphone were kindly supplied by Prof. D. Regoli, University of Sherbrooke (Canada).

Results

Substance P-induced superoxide anion generation from guinea-pig alveolar macrophages

In the concentration range 10^{-11}–10^{-6} M, SP dose-dependently evoked O_2^- production, maximal activation (2.18 ± 0.4 nmol cytochrome C reduced/10^6 cells; $n = 8$) being achieved at 10^{-7} M SP, the ED_{50} value being 0.7 nM (Figure 1a). The N-terminal heptapeptide, SP(1–7), was ineffective up to 10^{-6} M (Figure 1a).

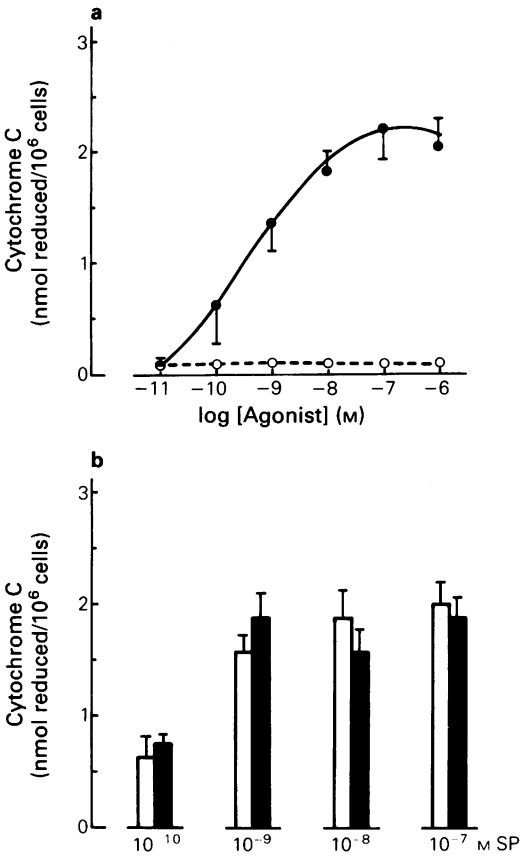

Figure 1 Superoxide anion production by guinea-pig alveolar macrophages incubated with substance P (SP). (a) Dose-response curve to SP (●; $n = 8$) and SP(1–7) (○; $n = 4$). Cells were incubated 60 min with SP or SP(1–7). Means with s.e.mean shown by vertical bars. (b) Effect of thiorphan 10^{-5} M on superoxide anion production evoked by SP; open column, substance P; solid columns, thiorphan 10^{-5} M + SP. Means with s.e.mean. (vertical bars) of three experiments. $P > 0.5$.

Activation of guinea-pig AM by two other stimuli, the autacoid mediator PAF-acether and the chemotactic peptide N-formylmethionyl-leucyl-phenylalanine (FMLP), also resulted in O_2^- production. However, while O_2^- production evoked by 10^{-6} M PAF-acether (2.05 ± 0.5 nmol cytochrome C reduced/10^6 cells; $n = 6$) was similar to that measured with SP, 10^{-7} M FMLP evoked a greater response (15.8 ± 1.2 nmol cytochrome C reduced/10^6 cells; $n = 8$) than SP or PAF-acether (data not shown).

Time-course experiments demonstrated that O_2^- generation evoked by a given concentration of SP (10^{-7} M SP) was 0.6 ± 0.4, 0.66 ± 0.29, 0.79 ± 0.5, 1.58 ± 0.2 and 1.73 ± 0.1 nmol cytochrome C reduced/10^6 cells ($n = 4$), after 5, 10, 15, 30 and 60 min, respectively. Therefore, since no significant differences were observed between the 30 and 60 min challenge period ($P > 0.05$), the 60 min period was selected for all subsequent experiments.

The enkephalinase inhibitor, thiorphan has been shown to enhance the effector responses to exogenous and endogenous tachykinins in different muscle tissues (Devillier *et al.*, 1988; Maggi *et al.*, 1989).

In the presence of thiorphan, O_2^- generation evoked by increasing concentrations of SP was not significantly modified as compared to controls (Figure 1b).

Neurokinin A and B-induced superoxide anion production from guinea-pig alveolar macrophages

The two natural tachykinins NKA and NKB stimulated guinea-pig AM (Figure 2). In the concentration range 10^{-12}–

10^{-6} M, NKA evoked O_2^- production from guinea-pig AM: maximal activation was observed at 10^{-7} M (1.89 ± 0.5 nmol cytochrome C reduced/10^6 cells; $n = 8$), the ED_{50} value being 0.1 nM (Figure 2).

NKB evoked O_2^- generation from guinea-pig AM in a dose-dependent manner: maximal activation (2.42 ± 0.7 nmol cytochrome C reduced/10^6 cells; $n = 5$) was obtained at 10^{-6} M, with an ED_{50} value of 1.3 nM (Figure 2). However, the maximum effects produced by NKA and NKB, as well as those evoked by SP, were not significantly different.

Effects of selective tachykinin-receptor agonists on guinea-pig alveolar macrophages

In the concentration range 10^{-10}–10^{-6} M, [β-Ala^8]-NKA(4–10), a selective NK_2 receptor agonist (Rovero *et al.*, 1989), evoked O_2^- production from guinea-pig AM, with maximal effects (2.52 ± 0.9 nmol cytochrome C reduced/10^6 cells; $n = 7$) at 10^{-7} M (Figure 3).

In the same concentration-range, the NK_3 receptor agonist [Me,Phe^7]-NKB (Drapeau *et al.*, 1987) was almost ineffective, while the NK_1 receptor agonist [Pro^9]-SP sulphone (Drapeau *et al.*, 1987) displayed some stimulant action at high concentrations (10^{-7}–10^{-6} M) (Figure 3).

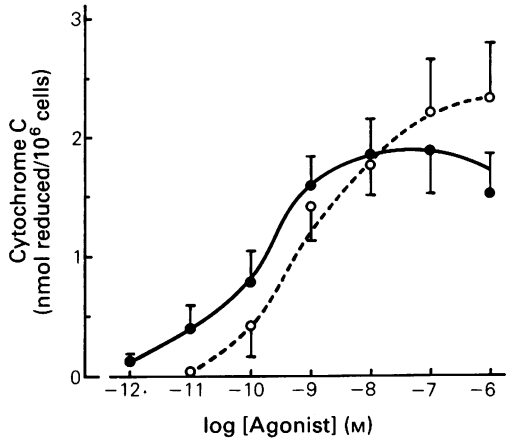

Figure 2 Superoxide anion production by guinea-pig alveolar macrophages incubated with neurokinin A (NKA) or NKB. Cells were incubated 60 min with NKA (●; $n = 8$) or NKB (○; $n = 5$). Means are shown with s.e.mean indicated by vertical bars.

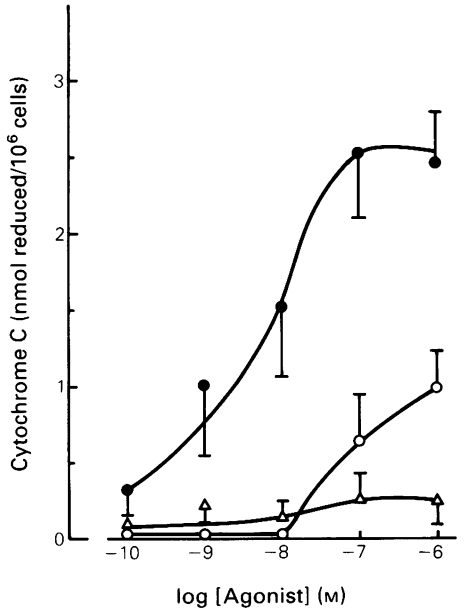

Figure 3 Superoxide anion production by guinea-pig alveolar macrophages incubated with selective tachykinin receptor agonists. Cells were incubated 60 min with [Pro^9]-SP sulphone (○; selective NK_1 agonist); [β-Ala^8]-NKA(4–10) (●; selective NK_2 agonist) or [Me,Phe^7]-NKB (△; selective NK_3 agonist). Means ± s.e.mean $n = 7$.

Discussion

These results indicate that SP, NKA and NKB are able to activate guinea-pig AM, as monitored by O_2^- production. Tachykinin-induced effects on guinea-pig AM are quantitatively similar to those evoked by the autacoid mediator PAF-acether, but less than those induced by the chemotactic peptide FMLP. The ability of FMLP to evoke a greater response in comparison with PAF-acether is similar to the situation with human neutrophils, where we have previously demonstrated that the FMLP-induced maximal response is 3–4 fold higher than that elicited by PAF-acether (Brunelleschi *et al.*, 1987). The *in vitro* concentrations at which SP activates cellular populations involved in inflammatory and immune processes greatly vary, depending on the cell type and the functional parameter evaluated. In human monocytes and guinea-pig peritoneal macrophages, the stimulant action of SP was found in the nM range (Hartung & Toyka, 1983; Lotz *et al.*, 1988), whereas mast cell degranulation occurs at μM concentrations (Shibata *et al.*, 1985). Repke & Bienert (1987) have also shown that SP degranulates rat mast cells through a receptor-independent mechanism and Bar-Shavit *et al.* (1980) have reported that enhancement of human neutrophil phagocytosis by SP resides in the N-terminal tetrapeptide, SP(1–4), which resembles tuftsin in its structure. In this study, SP stimulates O_2^- generation from guinea-pig AM at concentrations as low as 10^{-10} M ($ED_{50} = 0.7$ nM), thus suggesting the activation of a receptor-mediated mechanism. Furthermore, the N-terminal heptapeptide, SP(1–7), lacks the ability to induce O_2^- generation, thus indicating that SP-evoked effects in this cell population are mediated by the C-terminal region of the peptide.

Enkephalinase (endopeptidase 24.11), which hydrolyzes a variety of neuropeptides, exists in many peripheral tissues including lungs of different animal species (Sekizawa *et al.*, 1987); inhibitors of this metalloendopeptidase have been shown to enhance significantly tachykinin-induced effects, for example in guinea-pig trachea (Devillier *et al.*, 1988; Frossard *et al.*, 1989). However, in the guinea-pig AM, the stimulant effects of SP were not significantly modified in the presence of thiorphan, suggesting that, *in vitro*, the undecapeptide did not undergo degradation by enkephalinase.

Actually, three distinct tachykinin receptors have been recognized, each one of them having a preferential affinity for one of the three natural tachykinins (Buck & Burcher, 1986; Lee *et al.*, 1986; Regoli *et al.*, 1989).

In our experiments, NKA and NKB shared the ability of SP to induce O_2^- generation from guinea-pig AM, the former being more active ($ED_{50} = 0.1$ nM) than the latter ($ED_{50} = 1.3$ nM). Therefore, the rank order of activity of natural tachykinins (NKA > SP > NKB) might suggest that the NK_2 receptors mediate O_2^- generation in guinea-pig AM. However, the finding that SP was almost as active as NKA, does not fit with this hypothesis, because SP has been shown to have much lower affinity than NKA for NK_2 receptors in monoreceptorial assays (Buck & Burcher, 1986; Lee *et al.*, 1986; Regoli *et al.*, 1989).

Natural tachykinins at high concentrations stimulate all three receptors indiscriminately; for this reason, synthetic receptor agonists with improved or absolute selectivity for one receptor have been developed (Drapeau *et al.*, 1987; Regoli *et al.*, 1989; Rovero *et al.*, 1989).

In this study, the NK_2 receptor agonist [β-Ala^8]-NKA(4–10) dose-dependently evoked O_2^- production from guinea-pig AM, with maximal effects at 10^{-7} M; the NK_1 receptor agonist [Pro^9]-SP sulphone displayed some activity at high concentrations only, whereas the NK_3 receptor agonist [Me, Phe^7]-NKB was ineffective.

We conclude that the NK_2 receptors are the main mediators of guinea-pig AM response to natural tachykinins. The weak activity of the NK_1-selective agonist suggests the presence of a minor population of NK_1 sites on this cell population. The possibility that the effect of [Pro^9]-SP sulphone might involve activation of NK_2 or NK_3 receptors seems unlikely, because this peptide is ineffective at these sites, even at high concentrations (Drapeau *et al.*, 1987).

In conclusion, the results of this study further suggest the involvement of sensory neuropeptides in lung pathophysiology.

We wish to thank Prof. D. Regoli for kind gift of [Pro^9]-SP sulphone and [Me,Phe^7]-NKB, Dr P. Rovero for supplying [β-Ala^8]-NKA(4–10) and Mr Paolo Ceccatelli for his excellent technical assistance.

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Effects of μ-opioid receptor agonists on circulatory responses to simulated haemorrhage in conscious rabbits

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1 Cardiac output, arterial pressure, heart rate, systemic vascular conductance, respiratory rate and arterial blood P_{O_2} and P_{CO_2} were measured in unanaesthetized rabbits. Haemorrhage was simulated by inflating a cuff placed around the inferior vena cava so that cardiac output fell at a constant rate of about 8% of its resting value per min.

2 The effects of drug treatments on resting haemodynamic and respiratory variables, and on the haemodynamic response to simulated haemorrhage, were tested. The treatments were; 4th ventricular (−)-naloxone HCl (10–100 nmol), 4th ventricular H-Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH (DAMGO; 30–300 pmol), and i.v. morphine sulphate (0.5–5.0 μmol kg^{-1}). The interactions of graded 4th ventricular doses of naloxone (3–100 nmol) with the actions of DAMGO (100–300 pmol) on these responses were also assessed.

3 After sham treatments, the circulatory response to simulated haemorrhage had two phases. During the first compensatory phase, systemic vascular conductance fell, heart rate rose, and mean arterial pressure fell by only about 7 mmHg. A second decompensatory phase supervened when cardiac output had fallen by about 50%. At this point systemic vascular conductance rose abruptly and arterial pressure fell to ⩽40 mmHg.

4 Low 4th ventricular doses of naloxone (10–30 nmol) and DAMGO (30–100 pmol) had no discernible effect on the circulatory response to simulated haemorrhage. Higher doses of naloxone (30–100 nmol) and DAMGO (100–300 pmol) prevented the decompensatory phase. These high doses of naloxone and DAMGO lowered resting heart rate without affecting the other haemodynamic or respiratory variables.

5 Low doses of i.v. morphine (0.5–1.5 μmol kg^{-1}) also had no discernible effect on the circulatory response to simulated haemorrhage. Higher doses of morphine (1.5–5.0 μmol kg^{-1}) abolished the decompensatory phase. These high doses caused respiratory depression without affecting the resting haemodynamic variables.

6 The prevention of circulatory decompensation by high doses of DAMGO was reversed by 3–10 nmol of naloxone in 3 out of 4 rabbits and by 10–30 nmol of naloxone in all 4 rabbits. The decompensatory phase was, however, prevented by the combined high doses of DAMGO (100–300 pmol) and naloxone (30–100 nmol).

7 These findings provide strong evidence that activation of μ-opioid receptors in the central nervous system abolishes circulatory decompensation during acute reduction of central blood volume in conscious rabbits. This effect does not appear to be due to activation of arterial chemoreceptors or to a non-specific increase in sympathetic vasoconstrictor drive, since respiratory depression and hypertension were not observed after 4th ventricular doses of DAMGO which abolished circulatory decompensation. Our results also provide indirect confirmation of our previous finding that naloxone acts to prevent circulatory decompensation by an antagonist action at central δ-receptors.

Introduction

During acute haemorrhage or simulated haemorrhage in the conscious rabbit, arterial pressure is initially maintained by an increase in peripheral resistance but when cardiac output has fallen by about 50% the compensatory vasoconstriction fails abruptly and arterial pressure plummets (Schadt *et al.*, 1984; Ludbrook & Rutter, 1988; Ludbrook *et al.*, 1988). The initial compensatory vasoconstriction is reflex in origin and is due principally to unloading of the arterial baroreceptors (Ludbrook & Graham, 1984). There is strong evidence that its subsequent failure is triggered by a signal from the heart (Burke & Dorward, 1988; Evans *et al.*, 1989a). There is also evidence, based on the relative abilities of selective antagonists injected into the 4th ventricle to prevent its occurrence, that it is mediated by an endogenous opioid mechanism within the central nervous system which involves δ-receptors (Evans *et al.*, 1989b).

We have also found, unexpectedly, that 4th ventricular administration of the selective μ-opioid receptor agonist H-Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH (DAMGO) (Handa *et al.*, 1981) in a dose of 30 nmol prevents circulatory decompensation during simulated haemorrhage (Evans *et al.*, 1989b). We have argued that it is unlikely that this effect is mediated by blockade of δ-opioid receptors, since DAMGO is at least 500 times less potent than naloxone as a δ-antagonist (Sheehan *et al.*, 1986). The first goal of our present experiments was to test this proposition directly, by studying the interaction between DAMGO and the selective μ-receptor antagonist naloxone when they were both injected into the 4th ventricle.

When the μ-receptor agonist morphine (10–50 μg kg^{-1}) is injected into the lateral ventricle of the conscious rabbit it causes an increase in arterial pressure, associated with elevation of plasma concentrations of noradrenaline and adrenaline and with respiratory depression (May *et al.*, 1989b). This raises the possibility that 4th ventricular injection of DAMGO prevents circulatory decompensation during simulated haemorrhage because it raises sympathetic vasoconstriction drive and peripheral resistance as a result of its action on μ-receptors within the central nervous system, or because of

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the secondary effects of low arterial P_{O_2} and high P_{CO_2} on the arterial chemoreceptors. We have therefore determined the smallest dose of DAMGO administered to the 4th ventricle that would prevent circulatory decompensation during simulated haemorrhage, and measured its effects on systemic vascular conductance, respiratory rate and arterial blood gases.

We have also tested whether intravenous morphine would prevent circulatory decompensation in a dose which had no effects on the circulation or respiration, with the view of seeing whether the analgesic doses of morphine that are used in clinical practice would be likely to support arterial pressure in cases of acute blood loss.

Methods

Four New Zealand White rabbits, weighing 2.36–3.12 kg (mean 2.74), were used. The method of preparing the rabbits and the experimental protocols were similar to those described previously (Evans *et al.*, 1989a, b). The experiments were conducted in accordance with the Statement on Animal Experimentation by the National Health and Medical Research Council of Australia.

Surgical preparation

Rabbits were anaesthetized with halothane/oxygen after induction with intravenous thiopentone sodium (35–40 mg, Pentothal, Abbott) and endotracheal intubation. Buprenorphine HCl (60 μg s.c.; Reckitt & Colman) was administered post-operatively as an analgesic. Each rabbit underwent three separate operations. During the first operation an inflatable cuff was placed around the thoracic inferior vena cava (caval cuff). Two to three weeks later an electromagnetic flow probe (Biotronex BL5050) was placed around the ascending aorta. The flow probes had previously been calibrated *in vitro* by use of cellophane dialysis tubing and human whole blood with a haematocrit of 38% (Faris *et al.*, 1981). Two weeks later a polyvinyl chloride tube (Dural Plastics SV10), which had been heated and drawn out, was introduced through the atlanto-occipital ligament so that its 0.3 mm o.d. tip lay in the 4th ventricle. Its dead space of 15 μl was filled with 154 mM NaCl. The first study was done 10 days later.

Preparations for studies

These were done under local analgesia with 2% procaine HCl. The rabbit was placed in a 15 × 40 cm box fitted with a wire mesh lid, 60 min before the beginning of the study. The connecting plug for the flow probe and the ends of the tube and catheter were retrieved from their subcutaneous positions. Catheters were introduced into each central ear artery and advanced to the root of the ear, to measure arterial pressure and to collect arterial blood samples for blood-gas analysis. When necessary, a catheter was introduced into a marginal ear vein for i.v. drug administration.

Measurement of haemodynamic variables

The ear artery catheter was connected to a Statham P23Dc strain gauge zeroed 50 mm above the floor of the rabbit's box. The flow probe was connected to a Biotronex BL-613 meter to measure cardiac output. Heart rate was measured by a tachometer actuated by the flow pulse. Signals were amplified and recorded on a Grass Model 7 Polygraph.

The output from the Grass Polygraph was sent to an Olivetti M24 computer equipped with an analogue-to-digital converter which provided 10 s mean values of arterial pressure (MAP, mmHg), cardiac output (MCO, ml min^{-1}) and heart rate (MHR, beats min^{-1}). The computer also calculated 10 s means for cardiac index (MCI = MCO/body weight in kg) and systemic vascular conductance index (MSVCI = 10^2 MCI/MAP).

Respiration

A mercury-in-silastic strain gauge was placed around the thorax and connected to a bridge-circuit (Parks Electronics Laboratory, Oregon, U.S.A.), to monitor respiratory rate.

Haematocrit

Duplicate measurements (Hawksley) were made before each days' study from 0.2 ml samples of arterial blood. The haematocrit at the beginning of the first and last studies was 38.3 ± 0.6% and 35.0 ± 0.2% respectively.

Arterial blood gases

P_{aO_2} and P_{aCO_2} were measured on 0.6 ml samples of arterial blood, collected ~8 min after each drug treatment (Radiometer ABL 4 acid-base analyser, Copenhagen, Denmark).

Simulated haemorrhage

The caval cuff was inflated gradually by means of a micrometer syringe so that MCI fell at a rate of 11.97 ± 0.96 ml kg^{-1} min^{-1} per min (8.10 ± 0.11% of its resting level per min). The cuff was released when MAP had fallen to ~ 40 mmHg or when MCI had fallen to 36.8 ± 1.1% of its resting level.

Drugs

For 4th ventricular administration, (−)-naloxone HCl (Sigma) and DAMGO (Sigma) were dissolved in 154 mM NaCl at concentrations of 600 and 0.6 nmol per 15 μl respectively. For i.v. administration, morphine sulphate was used as a 10 mg ml^{-1} (15 mM) stock solution (David Bull Laboratories). All dilutions were made on the morning of the experiment, in 154 mM NaCl.

Experimental protocol

Each rabbit was studied four times at intervals of 2–7 days. In each study simulated haemorrhage (Ludbrook *et al.*, 1988) was repeated 3–5 times at intervals of not less than 90 min. When a drug was given into the 4th ventricle, the loading dose was injected in a volume of 15 μl over 1 min, 10 min before the start of simulated haemorrhage. This was followed by infusion of the same concentration at 0.75 μl min^{-1} until the caval cuff was deflated. A similar protocol was followed for i.v. administration of morphine, except that the loading dose was given in a volume of 1 ml kg^{-1} and was followed by an infusion at 50 μl kg^{-1} min^{-1}.

We first determined the lowest doses of the μ-opioid agonist DAMGO, and of the μ-selective opioid antagonist naloxone, which abolished circulatory decompensation during simulated haemorrhage. Thus in the first two studies, performed in random order, we examined the effects of 4th ventricular administration of ascending doses of naloxone (0, 10, 30, and in two cases 100 nmol) and of ascending doses of DAMGO (0, 30, 100, and 300 pmol). Given this information, we set out to determine what dose of naloxone would antagonize the effects of DAMGO on the haemodynamic response to simulated haemorrhage. We also tested whether there was an i.v. dose of the μ-receptor agonist morphine that would prevent circulatory decompensation during simulated haemorrhage, without causing respiratory depression. These second two aims were realized in the third and fourth studies, performed in random order. In the third study, the effects of the lowest effective 4th ventricular dose of DAMGO (determined from the second study) were re-tested, then the effects of combining this dose of

DAMGO with ascending 4th ventricular doses of naloxone (10, 30 and 100% of its effective dose determined in the first study). In the fourth study, the effects of ascending doses of i.v. morphine (0.5, 1.5 and 5.0 μmol kg^{-1}) were tested. In each study the effects of injecting saline vehicle, by the 4th ventricular or i.v. route, were tested first (sham treatments).

Autopsy

At the conclusion of the experiments the rabbits were killed by intravenous administration of pentobarbitone sodium (Nembutal, Ceva Chemicals). Autopsy showed that in all four rabbits the flow probe was firmly bonded to the aorta, and that the tip of the 4th ventricular catheter was properly positioned.

Statistical analysis

Analysis of data was carried out as described by Evans *et al.* (1989a, b).

Analysis of variance (ANOVA) was used to evaluate the effects of treatments on the levels of the haemodynamic and respiratory variables. The factors included rabbits, studies, drug treatments within studies, and before/after drug administration. Two separate analyses were performed. The data were first categorized by dose of drug. The interaction between dose of drug and the before/after levels of the haemodynamic variables, and the main effect of dose on the levels of the respiratory variables, were used to test for dose-dependent effects. In a second analysis, the haemodynamic and respiratory data for 4th ventricular naloxone, DAMGO and DAMGO-naloxone mixtures, and for i.v. morphine, were categorized according to sham treatments, treatments which did not abolish the decompensatory phase of the response to simulated haemorrhage, and the lowest doses that did abolish the decompensatory phase. For each variable, specific contrasts were made within the ANOVA between the levels after doses which did not prevent circulatory decompensation or those that did, and the level after sham treatment. The critical value of F was adjusted by means of the Bonferroni correction.

Regression analysis confirmed that MCI, expressed as a percentage of its pre-treatment level (%MCI), fell linearly with time during simulated haemorrhage ($r = 0.979 \pm 0.003$). As we have found previously (Evans *et al.*, 1989a), there was a near-linear relationship of MAP, MHR and MSVCI to %MCI during simulated haemorrhage, up to the point that the abrupt rise of MSVCI occurred. This enabled us to use the same graphical method for analysing the effects of opioid agonists and antagonists on the responses of MSVCI, MAP and MHR to simulated haemorrhage as we have used previously (Evans *et al.*, 1989a, b). The relationships of MAP, MHR and MSVCI to %MCI were characterized by three sets of co-ordinates: (a) Post-treatment levels, immediately before the onset of simulated haemorrhage; (b) the point at which MSVCI reached a minimum, before it rose abruptly; and (c) the final observation before the caval cuff was deflated. These co-ordinates were averaged, first within and then between rabbits, so as to distinguish the effects of sham treatments, drug treatments that did not abolish the decompensatory phase of the response to simulated haemorrhage, and those that did.

Unless otherwise indicated, the levels of the variables are tabulated as between rabbit means ± s.e.mean.

Balance of the experiment

The design of the experiment was, of necessity, only partly balanced. Although each rabbit underwent all four studies, the effects of 4th ventricular naloxone and DAMGO were always tested in the first two studies, and the 4th ventricular DAMGO-naloxone interaction and the effects of i.v. morphine in the second two studies. Also, the order of doses within each study was fixed. For these reasons we tested for possible sources of bias.

Among the four studies there was a suggestion of heterogeneity among the levels of MCI prior to sham treatment ($F = 4.1$; d.f. 3, 9; $P = 0.04$), but none was detectable in the cases of MAP, MHR, or MSVCI (P always > 0.2). Among the four studies there was no heterogeneity of respiratory rate, Pa_{O_2} or Pa_{CO_2} after sham treatment (F always < 3.5; d.f. 3, 9; P always > 0.05). The rate of fall of MCI during simulated haemorrhage was independent of studies and of treatments within studies (P always > 0.05).

Results

Effects of drug treatments on levels of haemodynamic and respiratory variables

At the beginning of each study the levels of the haemodynamic variables were within the normal range for our laboratory (Evans *et al.*, 1989a, b) (Figure 1). None of the drug treatments affected the levels of MCI, MAP or MSVCI but some affected MHR (Figure 1). Doses of naloxone (30–100 nmol) and DAMGO (100–300 pmol) that abolished the decompensatory phase of the response to simulated haemorrhage (see below), lowered MHR by 39 ± 12 and 29 ± 6 beats

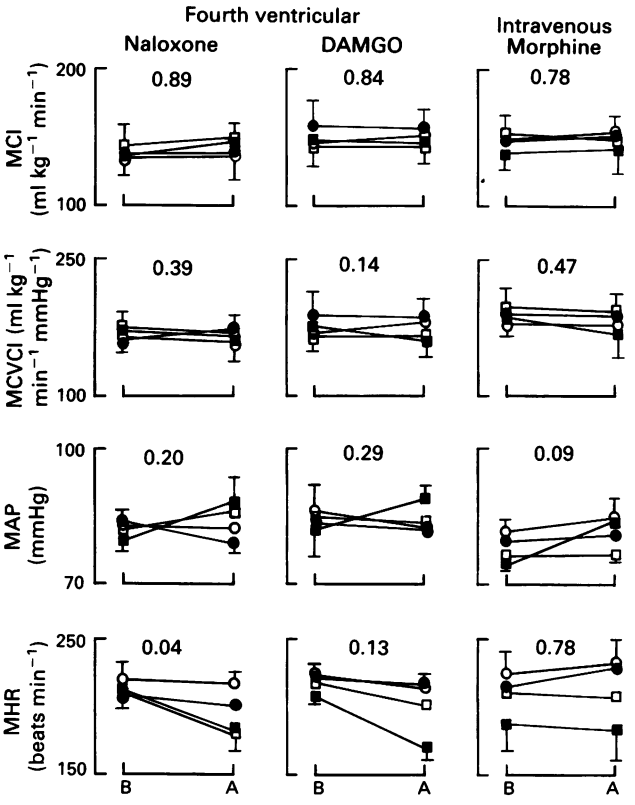

Figure 1 Effects on resting haemodynamic variables of 4th ventricular naloxone (○, saline vehicle; ●, 10 nmol; □, 30 nmol; ■, 100 nmol), 4th ventricular DAMGO (○, saline vehicle; ●, 30 pmol; □, 100 pmol; ■, 300 pmol), and i.v. morphine (○, saline vehicle; ●, 0.5 μmol kg^{-1}; □, 1.5 μmol kg^{-1}; ■, 5.0 μmol kg^{-1}). Values are 1 min averages for immediately before injection (B) and 9 min after injection (A). MCI = mean cardiac index; MSVCI = mean systemic vascular conductance index; MAP = mean arterial pressure; MHR = mean heart rate. Each point is the mean of 4 observations (except for the 100 nmol dose of naloxone, where $n = 2$). Vertical bars represent s.e.mean. Numbers on the top of each panel are P values for the interaction between dose and before/after drug administration (d.f. = 3,21 except for naloxone where d.f. = 3,17). Note the effect of naloxone on MHR.

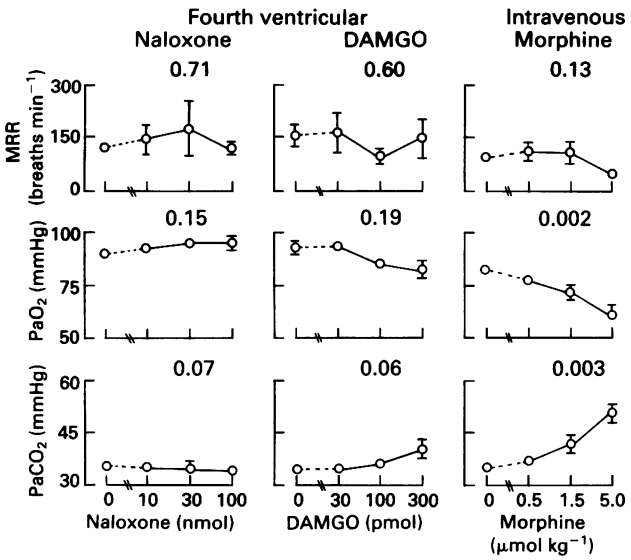

Figure 2 Effects on resting respiratory variables of 4th ventricular naloxone (0, 10, 30 and 100 nmol), 4th ventricular DAMGO (0, 30, 100 and 300 pmol), and i.v. morphine (0, 0.5, 1.5 and 5.0 μmol kg^{-1}). MRR = mean respiratory rate; Pao_2 = oxygen partial pressure of arterial blood; $Paco_2$ = carbon dioxide partial pressure of arterial blood. Each point is the mean of 4 observations (except for the 100 nmol dose of naloxone where $n = 2$) made ~8 min after injection. Vertical bars represent s.e.mean. Where no vertical bars are shown, s.e.mean is less than the symbol radius. Values on the top of each panel are P values for the main effect of dose (d.f. = 3,9 except for naloxone where d.f. = 3,7). Note the effect of morphine on Pao_2 and $Paco_2$.

min^{-1} respectively. When these doses of DAMGO and naloxone were combined, MHR was lowered by 35 ± 9 beats min^{-1} ($P < 0.01$).

The respiratory variables were also within a normal range at the beginning of each study (Figure 2). Respiratory rate was not affected by any of the treatments (Figure 2). The arterial blood gas tensions were affected only by i.v. morphine. Doses which abolished the decompensatory phase of the response to simulated haemorrhage (1.5–5.0 μmol kg^{-1}) lowered Pao_2 to 68 mmHg and raised $Paco_2$ to 46 mmHg.

Effects of 4th ventricular DAMGO or naloxone, and of i.v. morphine, on the haemodynamic responses to simulated haemorrhage

After the sham treatments, the haemodynamic response to simulated haemorrhage was always biphasic (Figure 3). During the first, compensatory, phase MSVCI fell progressively and MHR rose, while MAP fell by a grand mean of only 7 ± 2 mmHg. The second, decompensatory, phase began when MCI had fallen to 52 ± 1% of its pre-treatment level, at which stage MSVCI rose abruptly and MAP fell precipitately. The caval cuff was deflated when MAP had fallen to 37 ± 1 mmHg and MCI was 41 ± 1% of its pre-treatment level.

Low doses of naloxone (10–30 nmol) had no discernible effect on the haemodynamic responses to simulated haemorrhage. Higher doses (30–100 nmol) abolished the decompensatory phase (Figure 3). That is, throughout simulated haemorrhage there was a steady fall of MSVCI and rise of MHR. When the caval cuff was deflated, MAP had fallen by only 17 ± 2 mmHg, yet MCI was 35 ± 2% of its pre-treatment level (Figure 3).

Low doses of DAMGO (30–100 pmol) had no discernible effect on the haemodynamic response to simulated haemorrhage. Higher doses (100–300 pmol) abolished the decompensatory phase. When the caval cuff was deflated, MAP had fallen by only 7 ± 3 mmHg, yet MCI was 33 ± 3% of its pre-treatment level (Figure 3).

Low doses of i.v. morphine (0.5–1.5 μmol kg^{-1}) did not affect the haemodynamic response to simulated haemorrhage, but higher doses (1.5–5.0 μmol kg^{-1}) abolished the decompensatory phase. When the caval cuff was deflated, MAP had fallen by only 11 ± 3 mmHg, yet MCI was 35 ± 3% of its pre-treatment level (Figure 3).

Interaction between effective doses of DAMGO and ascending doses of naloxone

After sham treatment, the haemodynamic response to simulated haemorrhage followed the typical biphasic pattern (Figure 4). During the first, compensatory, phase MSVCI fell progressively and MHR rose, while MAP fell by only 9 ± 4 mmHg. The second, decompensatory, phase began when MCI had fallen to 50 ± 2% of its pre-treatment level, at which stage MSVCI rose abruptly and MAP fell precipitately. The caval cuff was deflated when MAP had fallen to 38 ± 1 mmHg and MCI was 38 ± 3% of its pre-treatment level.

As was to be expected, the previously-determined effective dose of DAMGO (100–300 pmol) abolished the decompensatory phase of simulated haemorrhage (Figure 4). When the caval cuff was deflated, MAP had fallen by only 3 ± 3 mmHg yet MCI was 36 ± 1% of its pre-treatment level.

When 10% of the effective dose of naloxone (3–10 nmol) was combined with the effective dose of DAMGO, circulatory decompensation occurred during simulated haemorrhage in three of the four rabbits. When 30% of the effective dose of naloxone (10–30 nmol) was combined with DAMGO, circulatory decompensation occurred during simulated haemorrhage in all four rabbits (Figure 4). However, when the maximum dose of naloxone (30–100 nmol) was combined with DAMGO,

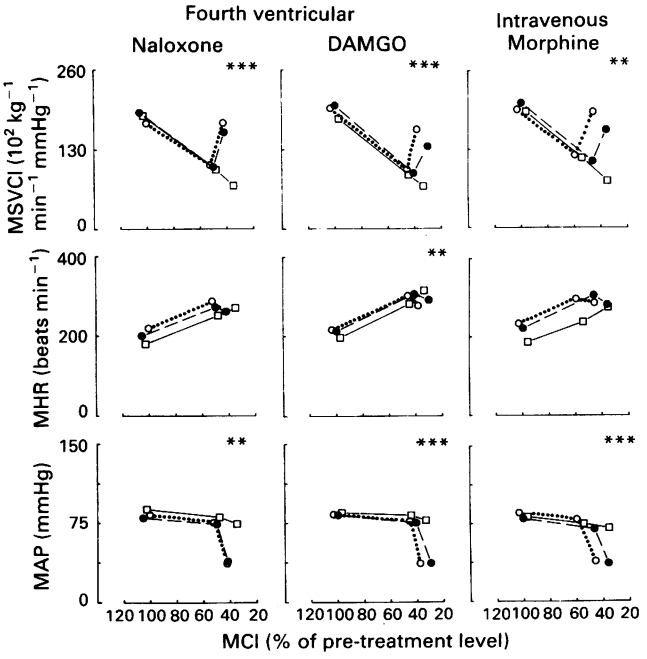

Figure 3 Haemodynamic variables during simulated haemorrhage after 4th ventricular naloxone, 4th ventricular DAMGO, and i.v. morphine. Haemodynamic variables as for Figure 1. Lines join average co-ordinates for 4 rabbits. The order of treatments was: (○·····○) saline vehicle; (●- - - -●) low doses which did not prevent circulatory decompensation; (□———□) high doses which did prevent circulatory decompensation. Low doses of naloxone, DAMGO and morphine were respectively 10–30 nmol, 30–100 pmol and 0.5–1.5 μmol kg^{-1}. High doses were respectively 30–100 nmol, 100–300 pmol and 1.5–5.0 μmol kg^{-1}. Outcome of ANOVA at d.f. 1,6 for the effects of high doses on haemodynamic variables at the end of simulated haemorrhage: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

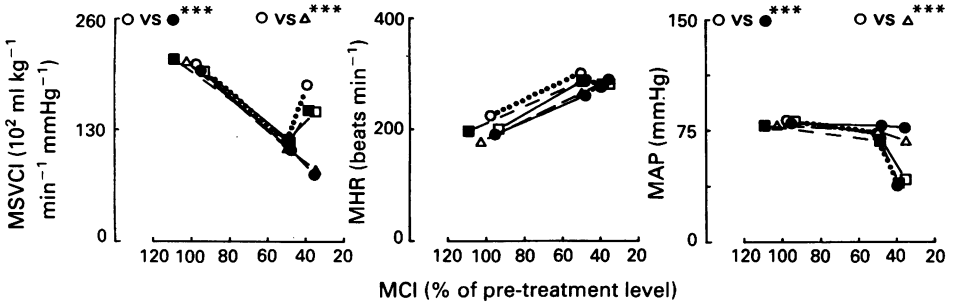

Figure 4 Haemodynamic changes during simulated haemorrhage after 4th ventricular administration of saline vehicle, the lowest dose of DAMGO which prevents circulatory decompensation, or a DAMGO-naloxone mixture. Haemodynamic variables as for Figure 1. Lines join average co-ordinates for 4 rabbits. The order of treatments (and the loading doses) were: (○·····○) saline vehicle; (●———●) the lowest effective dose of DAMGO (100–300 pmol); (□- - - -□) the lowest effective dose of DAMGO plus 10% of the lowest effective dose of naloxone (3–10 nmol); (■- - - -■) the lowest effective dose of DAMGO plus 30% of the lowest effective dose of naloxone (10–30 nmol); (△- - - -△) the lowest effective dose of DAMGO plus 100% of the lowest effective dose of naloxone (30–100 nmol). Contrasts were made within ANOVA at d.f. 1,12 between levels at the end of simulated haemorrhage after saline treatment and after each of the active treatments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

the decompensatory phase did not occur (Figure 4). When the caval cuff was deflated, MAP had fallen by only 11 ± 3 mmHg, yet MCI was $35 \pm 1\%$ of its pre-treatment level.

Discussion

We have confirmed our earlier finding that small doses (30–100 nmol) of the opioid receptor antagonist (−)-naloxone HCl, when injected into the 4th ventricle, abolish the decompensatory phase of simulated haemorrhage in conscious rabbits (Evans *et al.*, 1989a, b). We have previously provided evidence that this effect of naloxone is mediated by an antagonist action at central δ-receptors (Evans *et al.*, 1989b). We have also extended our finding that circulatory decompensation is abolished after 4th ventricular administration of 30 nmol of the μ-agonist DAMGO (Evans *et al.*, 1989b), by demonstrating that the threshold dose for this effect is as low as 100–300 pmol. We now present direct evidence that this action of DAMGO is mediated by a μ-opioid receptor mechanism within the central nervous system, and that it is independent of effects on vasoconstrictor drive, respiratory rate or arterial blood gas tensions. We have also shown that intravenous morphine will abolish the decompensatory phase of simulated haemorrhage, but only in doses that cause respiratory depression.

The action of 4th ventricular DAMGO on the response to simulated haemorrhage

Naloxone exhibits selectivity as an antagonist at μ-receptors, being some 7–30 times less potent as a δ-antagonist and 10–15 times less potent as a κ-antagonist (Magnan *et al.*, 1982; North, 1986; Smith, 1987). In view of our previous work (Evans *et al.*, 1989b), it can be assumed that when 4th ventricular naloxone prevents the decompensatory phase of simulated haemorrhage it does so by blocking δ-opioid receptors. At lower doses, it should still block μ-receptors. DAMGO is a highly specific agonist at μ-receptors, having negligible activity at κ- and δ-receptors (Handa *et al.*, 1981; Miller *et al.*, 1986). We found that 10% of the dose of naloxone which prevented circulatory decompensation during simulated haemorrhage nullified the action of DAMGO in preventing circulatory decompensation in three out of four rabbits, and 30% did so in all four rabbits (Figure 4). We interpret this as showing that the action of 4th ventricular DAMGO in preventing the decompensatory phase of simulated haemorrhage is mediated by its agonist action at μ-receptors within the central nervous system. We also found that when the entire effective dose of naloxone was combined with DAMGO, the decompensatory phase was again prevented (Figure 4). We assume that this was because the dose of naloxone was then sufficient to block δ-receptors.

In our original report that 4th ventricular DAMGO prevented the decompensatory phase of simulated haemorrhage, we used an arbitrary dose of 30 nmol (Evans *et al.*, 1989b). This caused a fall in systemic vascular conductance and a rise of arterial pressure, and it appeared to slow respiration. The effective dose-range of DAMGO that we have now established is between 100 and 300 times less, and this had no significant effect on any of the haemodynamic or respiratory variables. Indeed, in every instance, Pa_{O_2} remained well above the threshold of 50 mmHg at which arterial chemoreceptor reflex effects on the circulation can be detected in conscious rabbits (Korner, 1965). This seems to exclude an alteration in baseline vasoconstrictor drive, or excitation of arterial chemoreceptors, as the basis for the action of DAMGO in preventing circulatory decompensation.

Does activation of μ-receptors within the central nervous system occur as part of the normal response to simulated and, by extension, actual haemorrhage? We have no evidence that suggests this is so. In particular, sub-critical doses of naloxone, which would be expected to block μ- but not δ-receptors, do not appear to affect the pattern of haemodynamic response to simulated haemorrhage (Figure 3; Evans *et al.*, 1989a, b). There is some evidence, however, for an interaction between central δ- and μ-receptors in the pathogenesis of endotoxic shock. Administration of the δ-antagonist M-154,129 reversed the hypotension induced by i.v. endotoxin injection in conscious rats (Holaday *et al.*, 1982). This effect was attenuated by prior administration of the irreversible μ-antagonist β-funaltrexamine (D'Amato & Holaday, 1984). The authors proposed an allosteric interaction between μ- and δ-binding sites to explain these findings.

Intravenous administration of the μ-agonist morphine also abolished the decompensatory phase of the response to simulated haemorrhage, at threshold doses of 1.5–5.0 μmol kg^{-1} (Figure 3). These intravenous doses of morphine always caused respiratory depression (Figure 2). This suggests that, in clinical practice, systemic administration of morphine is unlikely to extend the compensatory phase of haemorrhage unless the dose is so high as to cause respiratory depression.

Is there any information about the localization within the central nervous system of these δ- and μ-receptors which modulate the haemodynamic response to simulated haemorrhage? It is reasonable to assume that when drugs are injected into the 4th ventricle their concentrations are highest in the dorsal part of the pons and medulla. Within this region lies the nucleus tractus solitarii, an important relay station for afferents from both arterial baroreceptors and cardiac receptors (Jordan & Spyer, 1986). Recent work has shown that, in

the rabbit, there is a dense population of δ-binding sites within this nucleus (May *et al.*, 1989a). It is not so clear where μ-agonists, injected into the 4th ventricle, might act to prevent circulatory decompensation during simulated haemorrhage. There are dense populations of μ-binding sites in the nucleus tractus solitarii of the rat (Mansour *et al.*, 1988) and cat (Sales *et al.*, 1988; Dashwood *et al.*, 1988). In the rabbit, μ-binding sites have been found in areas adjacent to the nucleus tractus solitarii (May *et al.*, 1989a). The cardiovascular actions of intravenous morphine were always associated with respiratory depression, whereas the corresponding actions of 4th ventricular DAMGO were not. This difference is more likely to be a function of the route of administration than of the pharmacodynamics of these compounds. There is good evidence that intravenous morphine exerts its respiratory depressant action in the hindbrain (Florez *et al.*, 1968; May *et al.*, 1989a). The development of respiratory depression is much slower when morphine is injected into the 4th ventricle, cisterna magna, or pontomedullary cistern than when it is given intravenously (Florez *et al.*, 1968; May *et al.*, 1989a). Our findings therefore suggest that the cardiovascular neurones which are sensitive to μ-agonists are located near the dorsal surface of the pontomedullary region, whereas the μ-sensitive respiratory neurones are situated more deeply. The precise identification of the sites at which δ-antagonists and μ-agonists act to prevent circulatory decompensation during simulated haemorrhage, and at which μ-agonists act to cause respiratory depression, will probably rely on micro-injection studies.

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Endothelium-dependent and BRL 34915-induced vasodilatation in rat isolated perfused mesenteric arteries: role of G-proteins, K^+ and calcium channels

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1 In the isolated perfused, noradrenaline (NA)–constricted mesenteric arteries of the rat, acetylcholine (0.003–1 nmol), histamine (0.01–10 nmol) and the calcium ionophore A23187 (0.01–1 nmol), caused endothelium-dependent vasodilatation while the vasodilatation by the K^+ channel activator BRL 34915 (0.1–1 nmol) was independent of endothelium.

2 The guanylate cyclase inhibitor, methylene blue at 10 μM did not inhibit the action of any of the vasodilators but at 50 μM reduced the vasodilator effect of acetylcholine (ACh), histamine and A23187.

3 Infusion of ouabain or perfusion with K^+-free or excess K^+ (50 mM) Krebs solution reduced the vasodilator effect of ACh, histamine and A23187, suggesting the action of these agents involves, at least in part, activation of Na^+/K^+-ATPase. The vasodilator effect of BRL 34915 was not affected by ouabain, but abolished during perfusion with Krebs solution containing excess K^+ or depleted of K^+.

4 Five structurally distinct K^+ channel blockers (apamin, crude scorpion venom, procaine, quinidine and tetraethylammonium) attenuated the vasodilator effect of ACh, histamine and A23187. The K^+ channel blockers, except apamin and crude scorpion venom, also inhibited the vasodilatation produced by BRL 34915.

5 The vasodilator effect of ACh, histamine or A23187 was not altered in mesenteric vessels of pertussis toxin-treated rats, suggesting that the K^+ channels associated with the endothelium-dependent vasodilator effect of these agents are either not coupled to G-proteins or are coupled to G-proteins that are insensitive to pertussis toxin.

6 The calcium channel blockers, diltiazem (0.1 or 1 μM), nifedipine (0.01 or 0.1 μM) or nitrendipine (1 nM) attenuated the vasodilatation produced by ACh, histamine, A23187 and also that by BRL 34915.

7 We conclude that endothelium-dependent vasodilatation induced by ACh, histamine and A23187 is mediated via activation of membrane K^+ channels and Na^+/K^+-ATPase. The K^+ channels involved in the vasodilator action of these agents are not coupled to pertussis toxin-sensitive G-proteins and appear to be regulated by Ca^{2+}.

Introduction

Vascular relaxation in response to acetylcholine (ACh) and a variety of other vasodilators has been shown to be dependent on an intact, functional endothelium (Furchgott & Zawadzki, 1980). In the rat, the endothelium-dependent nature of the vasorelaxation induced by ACh, calcium ionophore A23187 and histamine has been demonstrated in the aorta and pulmonary artery (Van De Voorde & Leusen, 1983; Chen *et al.*, 1988), perfused mesenteric (Burdet *et al.*, 1986; Furchgott *et al.*, 1987; Randall & Hiley, 1988; Bhardwaj & Moore, 1988) and tail (Spokas & Folco, 1984) arteries. These agents are believed to act through the release of endothelium-derived relaxing factor (EDRF). EDRF (and agents that cause its release) relaxes vascular smooth muscle via activation of guanylate cyclase. The evidence for this is derived from the observations that the action of agents that release EDRF is often accompanied by elevation of guanosine 3′:5′-cyclic monophospate (cyclic GMP) levels (Moncada *et al.*, 1988) and can be blocked by inhibitors of guanylate cyclase, methylene blue and oxyhaemoglobin (Murad *et al.*, 1978; Martin *et al.*, 1985). The relative insensitivity of endothelium-dependent effects to guanylate cyclase inhibitors in the rat mesenteric artery (Furchgott *et al.*, 1987) raises the possibility that this effect may be mediated via another mechanism, notably increased potassium permeability as suggested by Bolton *et al.* (1983) through activation of either the Na^+/K^+ pump (Haddy, 1978; Webb & Bohr, 1978; De Mey & Vanhoutte, 1980; Rubanyi & Vanhoutte, 1985) or directly via K^+ channels (Gordon & Martin, 1983; Petersen & Maruyama, 1984; Gebremedhin *et al.*, 1987).

ACh which acts on muscarinic receptors and certain other receptor ligands are known to elicit their effects by activation or enhancement of the activity of resting K^+ channels and recent evidence suggests that a guanine nucleotide binding-protein (G-protein) couples the specific receptors of these ligands to K^+ channels (Pfaffinger *et al.*, 1985; Andrade *et al.*, 1986; Codina *et al.*, 1987; Yamashita *et al.*, 1987; Nakajima *et al.*, 1988). Since muscarinic receptors involved in endothelium-dependent vasodilatation to ACh have also been linked to K^+ channels in bovine aortic endothelial cells (Olesen *et al.*, 1988), it is reasonable to expect modulation by K^+ channel blockers of endothelium-dependent responses. Furthermore, since G-proteins mediate the signal transduction of some K^+ channel-linked responses (Stryer & Bourne, 1986; Gilman, 1987; Sasaki & Sato, 1987), procedures that interfere with GTP-binding proteins, such as treatment with pertussis toxin, could also be expected to affect biological functions coupled to these channels. Accordingly, the objectives of the present study were two fold: first, to ascertain by use of K^+ channel blocking agents, the role of K^+ channels in endothelium-dependent vasodilatation elicited by ACh, A23187 and histamine. Second, to determine the influence of pertussis toxin treatment on the vasodilator effect of these agents in order to characterize further the contribution of G-protein coupled K^+ channels to endothelium-dependent responses. In addition, since the role of calcium in the release of EDRF is unequivocal, we

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have also examined the action of calcium channel antagonists on endothelium-dependent vasodilatation in rat mesenteric arteries.

Methods

Experiments were performed on male rats (Charles River, Indianapolis, IN) which weighed 250–300 g. Under ether anesthesia, midline laparotomy was performed on the rats and the aorta ligated both proximally and distally to the superior mesenteric artery. A small cannula was inserted into the superior mesenteric artery, and the vessels were flushed with heparinized Krebs solution (100 u ml^{-1}) and subsequently isolated, as described by McGregor (1965). Following isolation, the arterial preparation was transferred to a chamber and perfused with Krebs solution (maintained at 37°C and gassed with 95% O_2, 5% CO_2 mixture) at a constant flow rate of 5 ml min^{-1} by a peristaltic pump (Harvard Apparatus, Millis, MA). Changes in perfusion pressure were measured with a Statham pressure transducer and recorded on a Rikadenki polygraph recorder (KA-12 series; Soltec, Elcino CA). Since the flow was maintained at a constant rate, alterations in perfusion pressure were taken to reflect vascular resistance. The Krebs solution used had the following composition (mM): NaCl 118; KCl 4.7, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.5. High K^+ Krebs was prepared by substituting an equimolar amount of K^+ for Na^+, while K^+-free Krebs solution was made without any added K^+ and osmolarity was maintained with sucrose. Arterial perfusion pressure was elevated by continuous infusion of noradrenaline (NA, 2–5 μM) in the presence of ascorbic acid (20 μg ml^{-1}) as an antioxidant, or in some cases with KCl (50 mM).

Experimental protocol

Series 1 In the first series of experiments, the dependence or otherwise of the vasodilator actions of ACh, A23187 and histamine or a K^+ channel activator (BRL 34915) on an intact functional endothelium was examined. Dose-response curves were constructed to each of these substances in arteries preconstricted with NA with or without endothelium. The arteries were denuded of their endothelium by perfusion for 10 min with either distilled water (Criscione *et al.*, 1984) or 10 μM *p*-bromophenacyl bromide (*p*-BPB, Furchgott, 1983). After either treatment, the arteries were re-equilibrated in Krebs solution and subsequently tone was elevated with NA infusion. Dose-response curves to each of the vasodilators were established in this set of arteries, and the responses obtained were compared with those in control arteries. In some arteries with intact endothelium, we also examined the effect of methylene blue (MB, a guanylate cyclase inhibitor) which is widely stated to block endothelium-dependent responses of vascular tissues. In such experiments, the effects of vasodilators were tested before and after 1 h infusion of MB.

Series 2 This series of experiments was conducted in order to assess the contributions of Na^+/K^+-ATPase enzyme and K^+ channels to the vasodilator actions of the various agents used in this study. To achieve this objective, the vasodilators were tested during perfusion with either normal (with or without ouabain, 0.1 mM), 0-K^+, or excess K^+ (50 mM) Krebs solution. In experiments where normal or 0-K^+ Krebs solution was used, arterial tone was elevated with NA (2 μM). The selectivity of ouabain on Na^+/K^+-ATPase was assessed against K^+-induced vasodilatation according to Webb & Bohr (1978). Studies on the role of K^+ channels in the vasodilator action of the agents under investigation employed apamin (0.1 or 0.5 μM), crude scorpion venom (*Leiurus quinquestraitus hebraeus*, 2.5 μg ml^{-1}), procaine (0.1 or 0.5 mM), tetraethylammonium (TEA, 5 or 10 mM) or quinidine (5 or 10 μM) as inhibitors of K^+ channels. In these studies, each inhibitor was tested against the vasodilator response to a submaximal dose of each of ACh, A23187 and histamine. Each inhibitor was infused for 30 min, except TEA which (because of its quaternary ammonium chemical nature) was infused for 1 h.

Series 3 In these experiments, we examined the contribution of calcium channels to the vasodilator action of ACh, histamine, A23187 and BRL 34915 by studying the effect of calcium channel blockers (nifedipine, nitrendipine and diltiazem) on their effects; the experimental protocol was similar to that described for the K^+ channel blockers above.

Treatment of rats with pertussis toxin

Rats were injected with pertussis toxin (PTX), 25 μg 100 g^{-1} body weight intraperitoneally (i.p.) in volumes not exceeding 200 μl per rat. This dose has previously been shown (Lynch *et al.*, 1986) to be effective in causing adenosine diphosphate (ADP) ribosylation in rat hepatic plasma membranes. Control rats were administered saline. Twenty-four or seventy-two hours later, mesenteric arteries were isolated and set up as described earlier. The effect of PTX treatment was examined on the vasoconstrictor actions of NA, arginine vasopressin (AVP) and KCl for the purpose of determining the agent most suitable for elevation of arterial tone. Thereafter, vasodilator responses to ACh, histamine and A23187 during infusion of the constrictor agent were obtained in saline- (control) and PTX-treated rats, and the responses were compared. Liver plasma membranes were also prepared from both the control and PTX-treated rats for the assessment of the degree of ADP ribosylation resulting from the PTX treatment.

Drugs

The following drugs were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A.: noradrenaline bitartrate, acetylcholine bromide, arginine vasopressin, histamine dihydrochloride, apamin (from bee venom), procaine hydrochloride, crude scorpion venom (*Leiurus quinquestraiatus hebraeus*), tetraethylammonium, *p*-bromophenacyl bromide (*p*-BPB), quinidine hydrochloride, nifedipine, methylene blue, sodium nitroprusside and ouabain. Calcium ionophore A23187 was purchased from Aldrich Chem. Co, Milwaukee, WI, U.S.A., while cromakalim (BRL 34915), diltiazem and nitrendipine were obtained *gratis* from Beecham Laboratories, Bristol, TN, Marion Pharmaceutical Co, Kansas City, U.S.A., and Dr Chris Triggle respectively. NA stock solution (10^{-2} M) was prepared in 0.1 M hydrochloric acid and diluted with Krebs solution for infusion. The stock solutions of A23187, *p*-BPB, nifedipine, nitrendipine and cromakalim were prepared in ethanol, while all other compounds were dissolved in saline or Krebs solution.

Data analysis

Changes in perfusion pressure were calculated as percentage of the pressure before the administration of a vasodilator agent in the absence and during the infusion of various inhibitors and expressed as means ± s.e.mean. Differences between the mean values were determined by Student's *t* test. The differences between means were considered significant when $P < 0.05$.

Results

Effect of ACh, A23187, histamine and BRL 34915 on NA-induced arterial tone

The basal perfusion pressure in mesenteric arteries perfused with Krebs solution was 20.0 ± 2.0 mmHg ($n = 35$). In arteries with intact endothelium, infusion of NA (2–5 μM) caused a sustained elevation of arterial perfusion pressure (60.0 ± 8.0 mmHg, $n = 30$); ACh (0.003–1 nmol), histamine

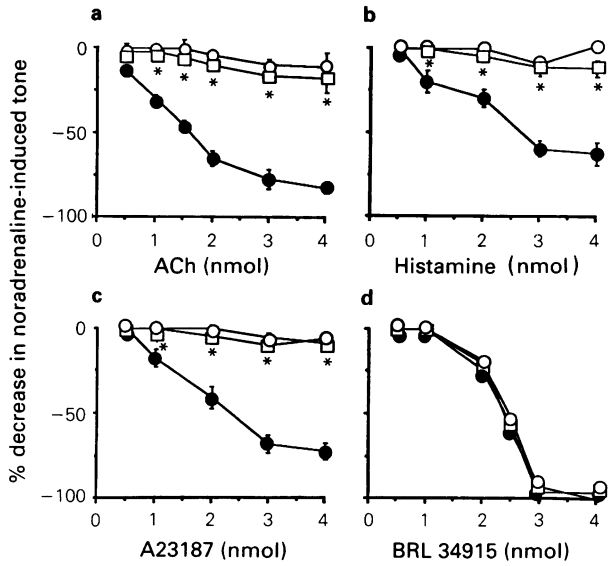

Figure 1 Dose-response curves for the vasodilatation induced by (a) acetylcholine (ACh), (b) histamine, (c) A23187 and (d) BRL 34915 in rat isolated perfused mesenteric arteries. (●) Arteries with intact endothelium; (□) and (○) respectively, represent the responses of arteries after treatment for 10 min with distilled water and *p*-bromophenacyl bromide (*p*-BPB). Each point on the graphs represents the mean and vertical lines show s.e.mean. ($n = 8$).* Denotes values obtained from arteries treated with distilled water and *p*-BPB significantly different from the corresponding values obtained in the control, untreated group ($P < 0.0005$). The doses of each agent shown are on a log scale.

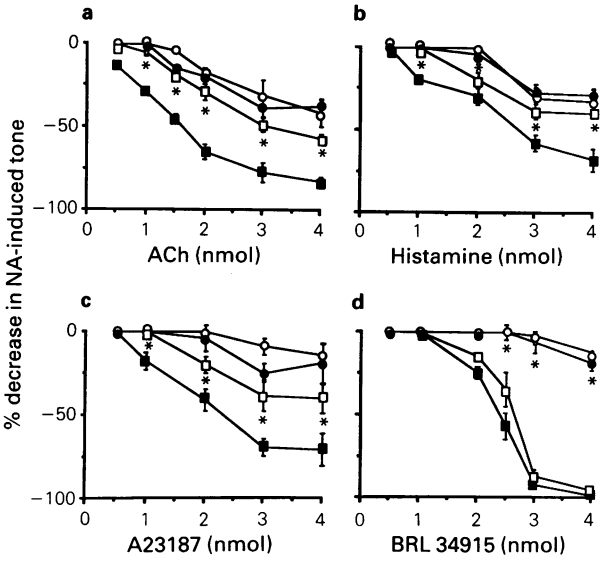

Figure 2 Effects of ouabain and of variations in Krebs solution K^+ on the vasodilatation induced by (a) acetylcholine (ACh), (b) histamine, (c) A23187 and (d) BRL 34915 in rat isolated perfused mesenteric arteries. (■) Control responses, (□) responses of arteries perfused with excess K^+ (50 mM) Krebs solution. (○) and (●) Represnt the responses obtained in K^+-free Krebs solution and ouabain infusion, respectively. Arterial perfusion pressure was elevated by an infusion of noradrenaline (NA, 2 μM), and for each point on the graph, $n = 6$. * Denotes value in the presence of ouabain, O-K^+ and 50 mM K^+ significantly different from the corresponding value obtained in the control group in the presence of 4.7 mM K^+ ($P < 0.05$).

(0.01–10 nmol), A23187 (0.01–1 nmol) and BRL 34915 (0.1–1 nmol) each caused a dose-dependent decrease in NA-induced tone. At the highest dose used, only BRL 34915 completely reversed NA-induced tone, while ACh, histamine and A23187 respectively caused 77.4 ± 5.7, 72.6 ± 6.9 and 72.4 ± 5.1% decrease in NA-induced arterial tone. In some experiments, arterial tone was raised by the infusion of AVP (1 nM). The vasodilator responses to ACh, histamine and A23187 in such arteries did not differ in magnitude from those obtained in arteries where NA infusion was used to raise arterial tone. When endothelium was removed by treatment with either distilled water or *p*-BPB (10 μM), infusion of 2–3 μM NA produced similar levels of arteriolar tone as in endothelium-intact arteries. However, both treatment procedures caused complete abolition of ACh-, histamine- or A23187-induced fall in perfusion pressure, while the fall in perfusion pressure caused by BRL 34915 was unaffected by either of the treatment procedures (Figure 1). Methylene blue (MB) did not alter the effect of any of the vasodilators at 10 μM but at 50 μM, reduced by 44.3 ± 6.4%, 36.1 ± 4.9% and 59.4 ± 5.8% respectively, the effect of ACh, histamine and A23187. MB had no effect on BRL 34915-induced vasodilatation.

Effect of vasodilators during alteration of K^+ concentration or ouabain infusion

In some experiments, arterial tone was elevated by infusion of 50 mM K^+-depolarizing Krebs solution to which prazosin (10 nM) was added to block the effect of possible indirectly-released NA from the adrenergic nerve terminals. In these conditions, the vasodilator effect of ACh, histamine, A23187 was reduced, while that of BRL 34915 was nearly abolished. A similar reduction in the effect of all the vasodilators was observed in arteries perfused with K^+-free Krebs solution (Figure 2), even though under this latter condition, the increase in perfusion pressure induced by NA was significantly greater than in normal Krebs solution. Ouabain infusion completely inhibited KCl-induced vasodilatation (Figure 3), reduced the vasodilator effects of ACh, histamine, A23187 but was ineffective against BRL 34915-induced vasodilatation.

Effect of K^+ channel blockers

The inability of ouabain or K^+ depletion (two conditions that interfere with Na^+/K^+ transport) to abolish the vasodilator effects of ACh, histamine and A23187 suggested a possible involvement of an additional mechanism in the effects of these compounds. We therefore tested the effect of five structurally distinct K^+ channel blockers (apamin, procaine, tetraethylammonium, quinidine and crude scorpion venom) on the

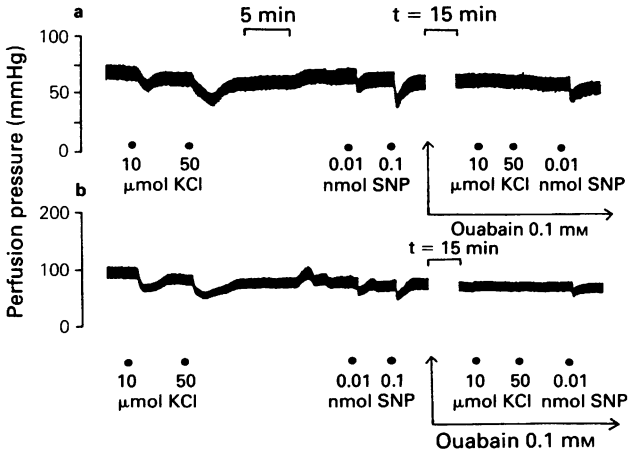

Figure 3 Representative tracings of the effect of ouabain on KCl-and sodium nitroprusside (SNP)-induced vasodilatation in mesenteric arteries perfused with K^+-free Krebs solution containing noradrenaline (2 μM), to elevate arterial tone. In (a), the endothelium was intact; while in (b) the mesenteric artery was previously infused with distilled water for 10 min to remove the endothelium.

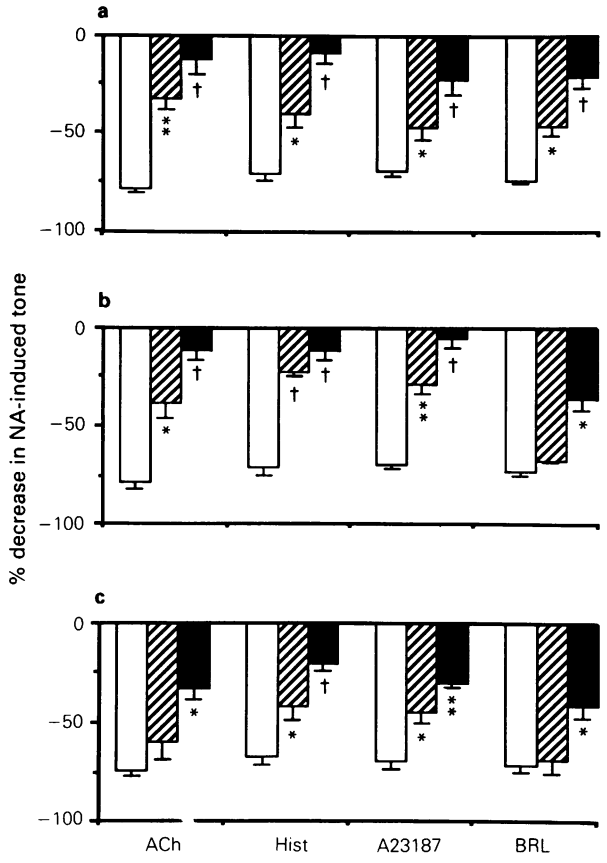

Figure 4 Effects of K^+ channel blockers, (a) procaine 0.1 μM (hatched columns) or 1 mM (solid columns), (b) quinidine 0.5 (hatched columns or 5 (solid columns) μM, and (c) tetraethylammonium 5 (hatched columns) or 10 (solid columns) mM on the vasodilator responses to acetylcholine (ACh), histamine (Hist), A23187 and BRL 34915 in perfused mesenteric arteries of the rat. In (a), (b) and (c), open columns represent vehicle-treated (control) groups. Arterial perfusion pressure was elevated by an infusion of noradrenaline (NA), and each column represents the mean response with bars showing s.e.mean. (n = 6). Statistical differences from corresponding controls are denoted by *P < 0.05, **P < 0.005 and †P < 0.0005.

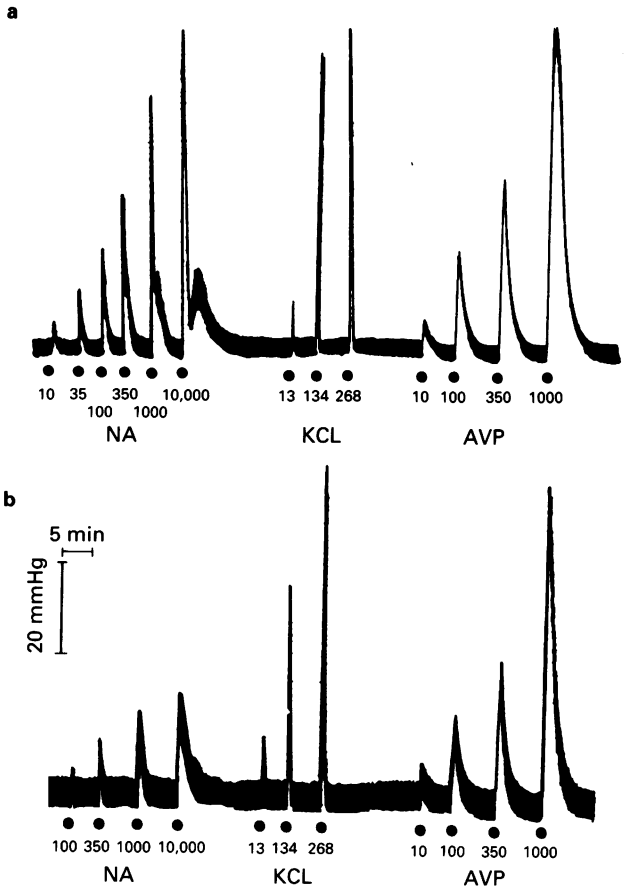

Figure 5 Representative tracings of the vasoconstrictor effects of noradrenaline (NA), arginine vasopressin (AVP) and KCl in mesenteric arteries isolated from saline- (a) and pertussis toxin- (b) treated rats. Doses for NA and AVP are indicated in pmol, while those for KCl are in μmol.

responses to the vasodilators. Procaine (0.1 or 1 mM), quinidine (0.5 or 5 μM) and tetraethylammonium (TEA, 5 or 10 mM) inhibited the vasodilator effect of ACh, histamine and A23187. The decrease in perfusion pressure induced by BRL 34915 was also attenuated by procaine, quinidine and the higher concentration of TEA (Figure 4). Two additional inhibitors, apamin and crude scorpion venom were tested on the vasodilator responses. Apamin (0.1 or 0.5 μM) and crude scorpion venom (2.0 $\mu g\,ml^{-1}$) also blocked the effects of ACh, histamine and A23187; but not that of BRL 34915 (Table 1).

Effect of ACh, histamine and A23187 in pertussis toxin-treated rats

Mesenteric arteries isolated from rats after 72 h of treatment with PTX exhibited markedly depressed responsiveness to NA (0.01–10 nmol) compared to control tissues, whereas responses to arginine vasopressin (AVP, 0.001–1 nmol) and KCl (10–300 μmol) were apparently unaffected by this treatment (Figure 5). The effect of PTX treatment was less pronounced after 24 h. For this reason, arterial tone was elevated with AVP (1 nM) infusion in situations requiring assessment of vasodilator activity. When infused, AVP (1 nM) caused a sustained increase in perfusion pressure: 48.0 ± 4.00 mmHg, n = 5 and 46.0 ± 2.0 mmHg, n = 5 respectively in saline- and PTX-treated rats. ACh (0.01 or 0.1 nmol), histamine (0.1 or

Table 1 Effect of apamin and crude scorpion (*Leiurus quinquestriatus*) venom on the vasodilator responses to acetylcholine (ACh), A23187 and BRL 34915 in isolated perfused mesenteric arteries of the rat

	% decrease in noradrenaline-induced tone			
		Apamin		*Scorpion venom*
Vasodilator	*Control*	*0.1 μM*	*0.5 μM*	*2.5 $\mu g\,ml^{-1}$*
ACh, 0.1 nmol	77.8 ± 4.3	25.6 ± 3.4*	6.2 ± 2.0†	
	80.7 ± 3.7	—	—	8.4 ± 3.9†
A23187, 0.1 nmol	75.0 ± 2.6	Not tested	Not tested	26.9 ± 3.8*
BRL 34915, 1 nmol	94.0 ± 2.2	95.8 ± 1.9	91.8 ± 1.6	
	97.1 ± 3.1	—	—	96.5 ± 2.0

Data represent the means ± s.e.mean. (n = 4). * and † denote statistically significant differences (P < 0.005 and P < 0.0005, respectively) from corresponding control values.

Table 2 Comparison of the endothelium-dependent vasodilatation induced by acetylcholine (ACh), histamine and A23187 in perfused mesenteric arteries of saline- (control) and pertussis toxin- (PTX) treated rats

Vasodilator	*Dose* (nmol)	*% decrease in AVP-induced tone* *Control*	*PTX-treated*
ACh	0.01	29.1 ± 1.7	32.6 ± 4.9
	0.1	64.9 ± 4.1	66.0 ± 6.7
Histamine	0.1	29.2 ± 4.9	30.1 ± 6.8
	1.0	58.5 ± 4.7	63.1 ± 9.5
A23187	0.03	27.7 ± 5.6	25.8 ± 6.2
	0.3	69.5 ± 4.1	67.5 ± 2.8

Data represent the mean ± s.e.mean. ($n = 5$). Statistical comparison (analysis of variance) of the control and PTX-treated revealed no significant difference. Arterial tone was elevated by an infusion of arginine vasopressin (AVP, 1 nM).

1 nmol) and A23187 (0.03 or 0.3 nmol) produced consistent, and dose-dependent vasodilator responses of similar magnitudes in saline- (control) and PTX-treated rats (Table 2).

Effect of calcium channel antagonists

Nifedipine (10 nM) did not alter NA-induced arteriolar tone, but attenuated the effects of all the vasodilator compounds (Figure 6); an effect that was mimicked by diltiazem (0.1 μM) and nitrendipine (1 nM) (see Table 3). All calcium channel antagonists tested produced a less marked effect against BRL 34915-induced vasodilatation. However nifedipine 0.1 μM or diltiazem 1 μM markedly reduced BRL 34915-induced vasodilatation, even though under these conditions, a higher NA (10 μM) concentration was required to sustain arteriolar tone (data not shown).

Table 3 Effects of diltiazem (Dilt) and nitrendipine (Nit) on the vasodilator responses of acetylcholine (ACh, 0.1 nmol), histamine (1 nmol), A23187 (0.1 nmol) and BRL 34915 (1 nmol) in isolated perfused mesenteric arteries of the rat

Inhibitor	*% decrease in noradrenaline-induced tone* *ACh*	*Histamine*	*A23187*	*BRL34915*
Dilt	20.7 ± 3.3†	24.5 ± 3.1†	9.4 ± 1.8†	39.7 ± 5.0*
0.1 μM	(72.5 ± 4.6)	(64.0 ± 2.8)	(74.4 ± 2.9)	(69.0 ± 3.0)
Nit	10.9 ± 4.0†	8.3 ± 2.2†	4.8 ± 0.9†	29.1 ± 3.6*
1 nM	(68.7 ± 2.8)	(60.2 ± 2.9)	(76.2 ± 5.0)	(70.2 ± 2.1)

Data represent the means ± s.e.mean. ($n = 6$). Statistically significant differences from corresponding control values in parentheses are denoted by $*P < 0.005$ and $\dagger P < 0.0005$. In all experiments, arterial tone was elevated by infusion of noradrenaline (2 μM).

Discussion

The contribution of EDRF to the vasodilator effects of ACh, histamine and A23187 has been documented in resistance (Byfield *et al.*, 1986; Furchgott *et al.*, 1987; Randall & Hiley, 1988; Bhardwaj & Moore, 1988) and non-resistance (Van De Voorde & Leusen, 1983; Spokas & Folco, 1984) blood vessels of the rat. This is further confirmed by the results of the present study. In addition, data from the present study also show that the endothelium-dependent responses by these agents are very sensitive to blockade by calcium channel antagonists and appear to involve activation of both Na^+/K^+-ATPase and K^+ channels which are insensitive to PTX.

Perfusion of the mesenteric arteries with either distilled water or *p*-BPB for 10 min abolished the vasodilator effect of ACh, histamine and A23187, while neither of the treatments affected the response to the K^+ channel activator, BRL 34915 suggesting that the integrity of the underlying vascular smooth muscle (VSM) cells was unaffected. This view is further supported by the observation that the responsiveness to NA infusion was not smaller (as would be expected if the VSM cells were damaged) than that in arteries with intact endothelium. Furthermore, previous work has shown short-term (10 min) perfusion with distilled water removes the endothelium from the blood vessels of the mesentery and transmission electron microscopy after such a procedure revealed no damage to the smooth muscle cells (Criscione *et al.*, 1984). Treatment with distilled water presumably caused endothelial cell (EC) loss and consequently affected both the production and release of EDRF; conversely, attenuation of endothelium-dependent vasodilatation by *p*-BPB may be related either to EC damage (Furchgott, 1983; Busse *et al.*, 1985) or to actions related or unrelated to smooth muscle cyclic GMP production (Johns & Peach, 1988). However, the failure of the guanylate cyclase inhibitor, methylene blue to attenuate the endothelium-dependent vasodilator responses suggests (Khan *et al.*, 1986) that activation of guanylate cyclase might not be an important factor in endothelium-dependent vasodilatation in rat mesenteric arteries.

The vasodilator effect of ACh before the discovery of EDRF was attributed to activation of the Na^+, K^+ pump (Haddy, 1978; Webb & Bohr, 1978; De Mey & Vanhoutte, 1980). The involvement of this pump in the vasodilator action of ACh, histamine and A23187 is evident in the present study, based

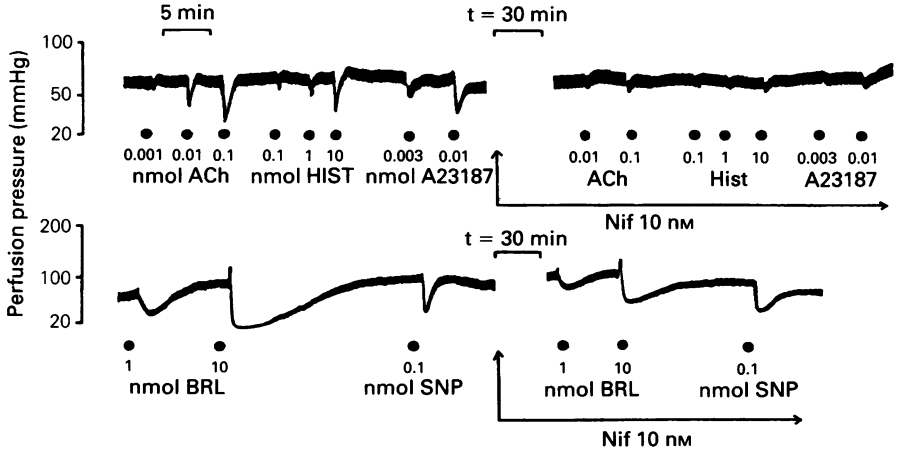

Figure 6 Representative tracings of the effects of nifedipine (Nif, 10 nM) on the vasodilator responses to acetylcholine (ACh), histamine (Hist), A23187, BRL 34915 and sodium nitroprusside (SNP, 0.1 nmol) in rat isolated perfused mesenteric arteries. Arterial tone was induced by noradrenaline (2 μM). Both the perfusion chamber and solution of nifedipine were protected from light by covering them with aluminum foil.

on the observed partial reduction in the effect of these compounds during procedures known to modify Na^+/K^+-ATPase activity *viz*: infusion of ouabain or K^+-depleted Krebs solution (Blaustein, 1977; Haddy, 1978; Webb & Bohr, 1978; Rapoport *et al.*, 1985; Feletou & Vanhoutte, 1988). The selectivity of ouabain for Na^+/K^+-ATPase activity in the present study was indicated by its ability to abolish KCl-induced vasodilatation during infusion of K^+-free Krebs solution. KCl caused vasodilatation in mesenteric arteries with or without endothelium and ouabain abolished this action under either condition, indicating that the Na^+/K^+-ATPase inhibited by ouabain is located on the VSM cells rather than the endothelial cells. That ouabain did not affect K^+ conductance directly was indicated by its failure, but not of extracellular K^+ depletion, to attenuate the vasodilator effect of the K^+ channel activator BRL 34915. Therefore, since neither ouabain nor external K^+ depletion abolished the vasodilator response to ACh, histamine or A23187, it may be concluded that an additional mechanism, possibly increase in K^+ conductance through membrane K^+ channels, contributes to the vasodilator action of these agents. This conclusion was supported by the finding that the vasodilator effect of these agents, like that of BRL 34915, was markedly reduced during elevation of arterial tone with excess K^+ (50 mM) Krebs solution.

The importance of potassium efflux in the endothelium-dependent action of vasodilator agents was suggested by Bolton *et al.* (1983). These authors concluded that EDRF increases potassium permeability in several blood vessels including guinea-pig anterior mesenteric arteries. The loss of potassium from cells occurs through membrane K^+ channels (Lattore & Miller, 1983; Peterson & Maruyama, 1984), which are known to be blocked by compounds such as apamin, procaine, quinidine, tetraethylammonium (Romey *et al.*, 1984; Cook & Haylett, 1985; Cook, 1988) and scorpion venom (Abia *et al.*, 1986; Castle & Strong, 1986). Our finding that ACh-, histamine- and A23187-induced endothelium-dependent vasodilatation was attenuated by K^+ channel blockers further supports the view that these agents increase K^+ conductance. Similar conclusions have been reached in the rabbit superior mesenteric artery (Kuriyama & Suzuki, 1978) and aortic strips (Gebremedhin *et al.*, 1987), and in bovine aortic endothelial cells (Olesen *et al.*, 1988). The vasodilator effect of BRL 34915 is endothelium-independent (present study) and has been attributed by previous studies to be due to activation of an apamin-insensitive type of K^+ channel (Allen *et al.*, 1986; Weir & Weston, 1986). Since procaine, quinidine and TEA also blocked BRL 34915-induced vasodilatation, vascular smooth muscle cells rather than the endothelium would appear to be the site of action of these K^+ channel blockers.

Many recent studies have implicated the guanine nucleotide binding proteins (G-proteins) as mediators of signal transduction of muscarinic receptor activated responses in several tissues. These include inhibition of adenylate cyclase (Jakobs *et al.*, 1979), opening of cardiac potassium channels (Pfaffinger *et al.*, 1985; Codina *et al.*, 1987), activation of phospholipase C (Sasaguri *et al.*, 1985), and opening of calcium-activated channels in lacrimal cells (Evans & Marty, 1986). In the present study, endothelium-dependent responses induced by ACh, histamine or A23187 were not altered in arteries isolated from rats treated with pertussis toxin. Since this toxin is generally used as a tool to study the role of various G proteins in membrane transduction mechanisms, our data suggest that K^+ channels associated with endothelium-dependent responses of these vasodilators are not coupled to a pertussis toxin-sensitive GTP binding protein. The finding, with respect to A23187 is in agreement with that of Flavahan *et al.* (1989) in porcine coronary arteries, but contradicts the suggestion by Sasaki & Sato (1987) that a GTP-binding protein such as G_i regulates the opening of K^+ channels coupled to receptors for dopamine, acetylcholine and histamine in the abdominal ganglion cells of the sea slug *Aplysia*. The latter authors had observed that PTX irreversibly blocked all K^+-dependent responses to these agonists. The insensitivity of the endothelium-dependent vasodilators employed in the present study to PTX is unlikely to be due to the ineffectiveness of the PTX treatment regimen since, *in vitro*, ADP-ribosylation of the liver plasma membranes isolated from the PTX-treated rats revealed no significant incorporation of radioactivity from [^{32}P]-NAD into a 41-kDa peptide as compared to hepatic plasma membranes obtained from vehicle-treated (control) rats (Bipin & Patel, personal communication).

Perhaps the most striking observation in the present study was the profound inhibitory effect of the calcium channel blockers on the vasodilator action of ACh, histamine, A23187 and BRL 34915; indicating that calcium may play a more crucial role than pertussis-toxin sensitive G-proteins in the vasodilator action of these agents in mesenteric arteries. Although the release of EDRF has been shown to be calcium-dependent (Furchgott, 1983; Singer & Peach, 1982; Long & Stone, 1985), the effectiveness of calcium channel antagonists on this process is debatable. For instance, nifedipine and verapamil have been shown to have a modest inhibitory effect in rabbit aorta (Singer & Peach, 1985), but endothelium-dependent vasodilatation in rat aorta is somewhat insensitive to calcium channel inhibition (Miller *et al.*, 1985) and activation (Spedding *et al.*, 1986). Our results, therefore, imply differences in the mechanism of endothelium-dependent vasodilatation in resistance and non-resistance vessels.

Recently, Adams *et al.* (1989) have demonstrated the existence of at least three ion transport pathways through which extracellular calcium can enter the endothelial cell: receptor-mediated calcium influx, the calcium leakage pathway and the stretch-activated calcium pathway. In the present study, calcium channel blockers attenuated the effect of both receptor- (ACh, histamine) and non-receptor (A23187, BRL 34915) activating agents, which indicates that receptor-mediated calcium influx is not of prime importance. On the other hand the electrophysiological data of Ryan *et al.* (1988) have indicated that endothelial cells lack voltage-dependent calcium channels. Therefore the VSM, rather than the endothelium would appear to be the site of action of the calcium channel blockers. This conclusion is supported by our observation that the endothelium-independent vasodilator action of BRL 34915 was also attenuated by calcium channel blockers. Moreover, Bean *et al.* (1986) have shown the presence of dihydropyridine-sensitive calcium channels in smooth muscle cells isolated from rat mesenteric arteries.

Another important finding in the present study pertains to the differential effect of PTX treatment on NA-, AVP- and KCl-induced vasoconstrictor responses. NA-, but not AVP- or KCl-induced vasoconstriction was suppressed in PTX-treated rats compared with control rats. Even though these three agents are known to require Ca^{2+} for their vasoconstrictor action, they differ remarkably in their dependence on extracellular Ca^{2+} for this effect; AVP- and KCl-induced vasoconstriction was abolished in the absence of extracellular calcium, while NA-induced vasoconstriction persisted (present study, data not shown). NA, which acts on α_1-adrenoceptors (Lynch *et al.*, 1985), and AVP on V_1-receptors (Fox *et al.*, 1987; Nabika *et al.*, 1985) and KCl, which acts directly on smooth muscle cells (Underwood *et al.*, 1988) have all been shown to be capable of inducing the breakdown of polyphosphoinositides to myoinositol trisphosphate and diacylglycerol. Therefore, the differential effects of pertussis toxin on these Ca^{2+}-mobilizing processes activated by these agents suggest that either the events associated with each of the agents are coupled to different G-proteins or only α_1-receptors, but not V_1-receptors or voltage-dependent calcium channels are coupled to PTX-sensitive G-proteins in rat mesenteric arteries.

In conclusion, this study demonstrates that endothelium-dependent vasodilator effects to ACh, histamine and A23187 are mediated via activation of K^+ efflux notably through K^+ channels and Na^+/K^+-ATPase which are regulated by calcium, and not by a PTX-sensitive G-protein. Guanylate

cyclase possibly plays a less important role in the endothelium-dependent vasodilator effects of these agents in this vascular bed.

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Caffeine-induced behavioural stimulation is dose- and concentration-dependent

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1 The relationship between plasma and brain caffeine and metabolite concentrations and behavioural stimulation was investigated over a 4 h time course.

2 CD-1 mice receiving single intraperitoneal doses of caffeine-sodium benzoate solution (caffeine doses: 0, 20, and 40 mg kg^{-1}) were evaluated in an activity monitor, and their plasma and brain caffeine and metabolite concentrations were determined by high performance liquid chromatography (h.p.l.c.).

3 Kinetic variables for caffeine at low and high caffeine doses were: volume of distribution (Vd), 1.16 and 0.88 l kg^{-1}; plasma elimination half-life ($t_{1/2}$), 1.25 and 1.62 h; brain $t_{1/2}$, 0.93 and 1.30 h; clearance, 0.64 and 0.38 l h^{-1} kg^{-1}, respectively, with Vd and brain $t_{1/2}$ differing significantly between the two caffeine doses.

4 Low-dose caffeine stimulated vertical behaviours significantly more than high-dose, during the first 150 min post-dosage; both doses stimulated vertical behaviours significantly more than vehicle treatment.

5 Low-dose and high-dose caffeine stimulated horizontal and stereotypic behaviours equivalently, during the first 150 min post-dosage; both doses stimulated these behaviours significantly more than vehicle.

6 Only later, 150 min post-dosage, did high-dose caffeine stimulate all behaviours significantly more than both low-dose and vehicle treatment; this occurred when caffeine concentrations had fallen to approximately 10 $\mu g\,g^{-1}$ in the high-dose group.

7 The maximal stimulant effects of caffeine occurred in an intermediate concentration range, between 10–20 $\mu g\,g^{-1}$, while lower and higher concentrations produced either no additional stimulation or decrements in activity.

Introduction

Caffeine appears to be a model compound to study pharmacologically-induced anxiety. The clinical syndrome of caffeinism has been described as being indistinguishable from anxiety states (Greden, 1974). A positive correlation between daily caffeine consumption and scores of anxiety was found in a group of patients with anxiety disorders (Boulenger & Uhde, 1982). Caffeine, at doses of 10 mg kg^{-1}, precipitated panic attacks in patients with pre-existing panic disorder (Charney *et al.*, 1984; 1985). Although these studies demonstrated the anxiogenic effects of caffeine, they did not define relationships between caffeine doses, plasma concentrations and anxiety.

Animal studies have begun to correlate better caffeine dosage and concentration with behavioural stimulation. Caffeine has anxiogenic effects in two animal models of anxiety, the social interaction test and the elevated plus maze test (Baldwin *et al.*, 1989). Preliminary studies in rats have examined dose-response relationships for the stimulant effect of caffeine on motor activity (Thithapandha *et al.*, 1972). More recent studies have evaluated caffeine pharmacokinetics in greater detail and have shown a relationship between brain caffeine concentrations and locomotor stimulation (Kaplan *et al.*, 1989).

Several authors have demonstrated that at higher doses (30 mg kg^{-1} caffeine or greater) there is a decline in the stimulant effects on motor and exploratory activity (Thithapandha *et al.*, 1972; Buckholtz & Middaugh, 1987; File *et al.*, 1988). In addition, at higher brain caffeine concentrations (> 20 $\mu g\,g^{-1}$) there is a decrement in motor stimulation compared to that seen at low and intermediate concentrations (Kaplan *et al.*, 1989). From these previous studies, it appears that a nonlinear relationship exists between caffeine dose and concentrations and behavioural stimulation. The purpose of this study is to delineate more carefully relationships between caffeine dose, plasma and brain concentration and behavioural stimulation.

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Materials

Anhydrous caffeine and paraxanthine were obtained from Aldrich Chemical Co. (Milwaukee, WI). Theobromine and the high-performance liquid chromatography (h.p.l.c.) internal standard beta-hydroxyethyltheophylline were obtained from Sigma Chemical Co. (St. Louis, MO). Theophylline was obtained from Chemical Dynamics Co. (So. Plainfield, NJ). Other reagents were obtained from standard commercial sources. Caffeine-sodium benzoate solution for parenteral administration (250 mg each of caffeine and sodium benzoate in 2 ml) was obtained from Quad Pharmaceuticals Inc. (Indianapolis, IN). The parenteral preparation of caffeine was initially diluted with distilled water to yield a working solution containing 6 mg ml^{-1} each of caffeine and sodium benzoate for high-dose administration. This in turn was diluted with sodium benzoate solution (6 mg ml^{-1}) for low-dose administration. The final volume of injectate was always 0.2 ml and the concentration of sodium benzoate was always 6 mg ml^{-1}.

A stock solution of caffeine (100 $\mu g\,ml^{-1}$), theophylline (50 $\mu g\,ml^{-1}$), paraxanthine (50 $\mu g\,ml^{-1}$) and theobromine (50 $\mu g\,ml^{-1}$) was prepared by weighing appropriate amounts of each standard individually and then dissolving them together in 100 ml of methanol. Another working solution of the mixed standards was prepared by a 1:10 dilution with methanol.

Methods

High-performance liquid chromatography

The h.p.l.c. instrumentation has been described previously (Kennedy *et al.*, 1987). The column (Supelco, Bellefonte, PA)

was stainless-steel, reverse-phase, 15 cm in length × 4.6 mm internal diameter packed with LC-18DB (particle size 5 μm). The mobile phase consisted of 1.75 μM aqueous orthophosphoric acid:acetonitrile:tetrahydrofuran (97:02:01). The organic and aqueous components were filtered and degassed before use. Operating conditions were: detector, 273 nm and 0.02 AUFS; mobile phase flow rate, 1.2–1.4 ml min^{-1}; injection volume varied from 5 to 30 μl.

Extractions of methylxanthines from plasma and brain

The extraction of methylxanthines (caffeine and metabolites) from plasma and brain was performed as previously described by Kaplan and coworkers (1989). Briefly, calibration curves were prepared by using varying amounts of mixed standard solution and a fixed amount of internal standard. Diluted plasma samples were extracted in 4 ml of ethyl acetate:isoamyl alcohol (98:2) by agitation in a vortex mixer. The solution was centrifuged at room temperature and the upper organic layer was transferred to a conical centrifuge tube and evaporated to dryness. The residue for h.p.l.c. analysis was reconstituted with 300 μl of mobile phase.

Mouse brain samples were weighed and homogenized with a Polytron power control unit (Brinkmann Co., Lucerne, Switzerland). The homogenate was extracted in 5 ml of chloroform, agitated in a vortex mixer and then centrifuged. The lower organic phase was transferred by pipette to conical centrifuge tubes. The same extraction procedure was performed an additional two times and the contents of the three organic phases were combined and evaporated in the conical tubes. The residue for h.p.l.c. analysis was reconstituted in the mobile phase.

Behavioural testing procedures

Mouse locomotor behaviour was measured by the Digiscan Model RXYZCM (Columbus, OH). This infrared sensor system monitors the following animal activities: horizontal activity, total distance, number of horizontal movements, horizontal movement time, vertical activity, number of vertical movements, vertical movement time, stereotypy count, number of stereotypies and stereotypy movement time. The animal monitor determines the number of times that the mouse interrupts an infrared beam in the measured direction, during a variable time interval. Stereotypic behaviour is identified if the animal repeatedly breaks the same beam or set of beams. This typically happens during grooming and head bobbing. The data for each animal are printed in separate columns representing the given monitored behaviours.

Mice were individually tested in a dimly lit sound-controlled area. They were removed from their colony and placed in the middle of the activity monitor. They were allowed to settle for at least one hour before the testing procedure began. Mice were injected intraperitoneally (i.p.) with caffeine-sodium benzoate solution to deliver either 0, 20, or 40 mg kg^{-1} of caffeine. Activity counts were cumulated and printed out at 5 min intervals. At 240 min the testing ended, and mice were decapitated for trunk blood and brain specimens. Plasma and brain specimens were prepared and extracted for methylxanthines as described above.

Kinetic study

A separate group of mice were used in the kinetic study. They were injected i.p. with caffeine, 20 mg kg^{-1} or 40 mg kg^{-1}, as described previously. At each of the following post-dosage time points, groups of three mice were decapitated, and plasma and brains were prepared for methylxanthine extraction: 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3 and 4 h.

Kinetic and behavioural statistical analyses

Mean plasma and brain caffeine concentrations at each dose were fitted to the following equation, consistent with a one-compartment open pharmacokinetic model:

$$C = C_0 e^{-kt}$$

where C is the plasma or brain concentration at time t after dosage. The fitting procedure yields parameter estimates for C_0, the extrapolated initial plasma concentration at time zero, and for k, the apparent first-order elimination or disappearance rate constant, as well as estimates of the standard error or standard deviation of each. By use of standard pharmacokinetic methods (Harmatz & Greenblatt, 1987), the parameter estimates were used to calculate the following derived kinetic variables:

$$\text{Volume of distribution (Vd)} = \frac{\text{Dose}}{C_0}$$

$$\text{Elimination half life } (t_{1/2}) = \frac{\ln 2}{k}$$

$$\text{Clearance (Cl)} = \frac{\text{Dose} \times k}{C_0}$$

By use of the standard deviations of the original parameter estimates C_0 and of k, standard deviations of the derived variables can be calculated based on error propagation methods. With their respective mean values and standard deviations, Student's *t* test was used to evaluate the effect of dose on Vd, $t_{1/2}$, and Cl for plasma caffeine, and $t_{1/2}$ for disappearance of caffeine from brain.

To distinguish early and late behavioural effects of caffeine, each behavioural score was expressed as the sum of individual scores from the time of dosage through to 150 min ('early') and from 155 to 240 min ('late'). For each summed 'early' and 'late' score, analysis of variance (ANOVA) was used to evaluate the overall significance of the effect of caffeine (0, 20, 40 mg kg^{-1}). When ANOVA was significant, the significance of individual differences was tested by Student-Newman-Keuls test.

Results

Kinetic studies

As in a previous study (Kaplan *et al.*, 1989), there were moderate variations in plasma and brain concentrations at each caffeine dose. For example, the mean plasma caffeine concentration at a dose of 40 mg kg^{-1}, at 180 min post-injection, was 10.7 μg g^{-1}. The range was 5.51 to 16.50, and the coefficient of variation was 51%. In other cases, the coefficient of variation for a given dose at a given time was as low as 3%. There was a linear relationship between plasma and brain concentrations of caffeine. The slope of the regression line passing through the origin was 1.03 ± 0.05 (mean ± s.e.). Similar linear relationships were found between plasma and brain concentrations of theophylline, paraxanthine, theobromine, with regression slopes of 0.37 ± 0.02, 0.30 ± 0.02, and 0.53 ± 0.02, respectively. These values were similar to those derived from a one-hour time course study (Kaplan *et al.*, 1989). These linear relationships show that plasma concentrations of caffeine and metabolites consistently reflected brain concentrations up to 4 h post-caffeine administration.

Plasma and brain caffeine concentrations at both doses (20 and 40 mg kg^{-1}), over the 4 h time course, are shown in Figure 1. This figure demonstrates that absolute brain concentrations consistently reflect plasma concentrations at each time point. Figure 2 shows brain concentrations of caffeine and metabolites at each dose over the 4 h study. Caffeine concentrations were consistently greater than metabolite concentrations throughout the study.

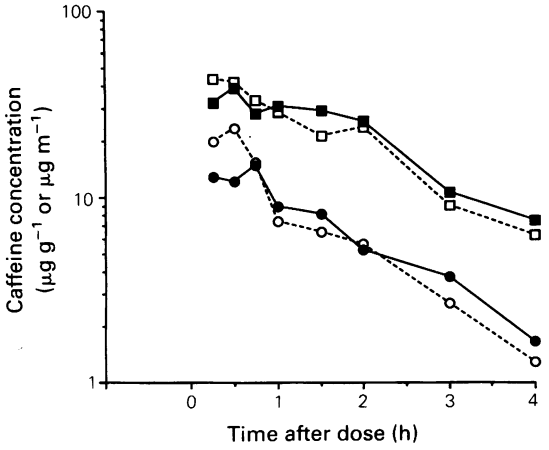

Figure 1 Plasma and brain concentrations of caffeine at both caffeine doses of 20 and 40 mg kg^{-1} over a 4 h time course. Each point represents the mean concentration for 3 animals at the corresponding time. Symbols are: after the 20 mg kg^{-1} dose, plasma (●——●); brain (○---○). After the 40 mg kg^{-1} dose, plasma (■——■); brain (□---□).

Table 1 shows values of Vd, $t_{1/2}$ and Cl determined from plasma and brain concentrations of both caffeine doses. Although differences in plasma $t_{1/2}$ and Cl between the two doses was not significant, there was a trend toward longer $t_{1/2}$ and lower Cl at the higher dose. However, there is a significant difference between the values for Vd ($P < 0.001$) and brain $t_{1/2}$ ($P < 0.01$). No differences were found between methylxanthine concentrations in animals run in the activity monitor (at 240 min) versus those used in the kinetic study, indicating that behavioural testing *per se* is unlikely to alter the kinetics of caffeine.

Table 1 Effect of dose on caffeine pharmacokinetics

	Caffeine dose	
	20 mg kg^{-1}	40 mg kg^{-1}
Volume of distribution (Vd) (l kg^{-1})	1.16* (±0.02)	0.88* (±0.01)
Plasma half-life ($t_{1/2}$) (h)	1.25 (±0.27)	1.62 (±0.54)
Clearance (l h^{-1} kg^{-1})	0.64 (±0.48)	0.38 (±0.13)
Brain half-life ($t_{1/2}$) (h)	0.93* (±0.21)	1.30* (±0.25)

* Significant differences between doses are seen with V_d ($t = 46.72$, $P < 0.001$) and brain $t_{1/2}$ ($t = 3.16$, $P < 0.01$).

Dose-behaviour relationships

Figure 3 shows horizontal activity (HA) versus time at the three caffeine doses (0, 20, 40 mg kg^{-1}). Soon after administration, low-dose and high-dose caffeine appear to stimulate activity equivalently, and both stimulate it greater than vehicle treatment. Later, this stimulant effect is sustained in the high-dose group but not in the low-dose group. As caffeine

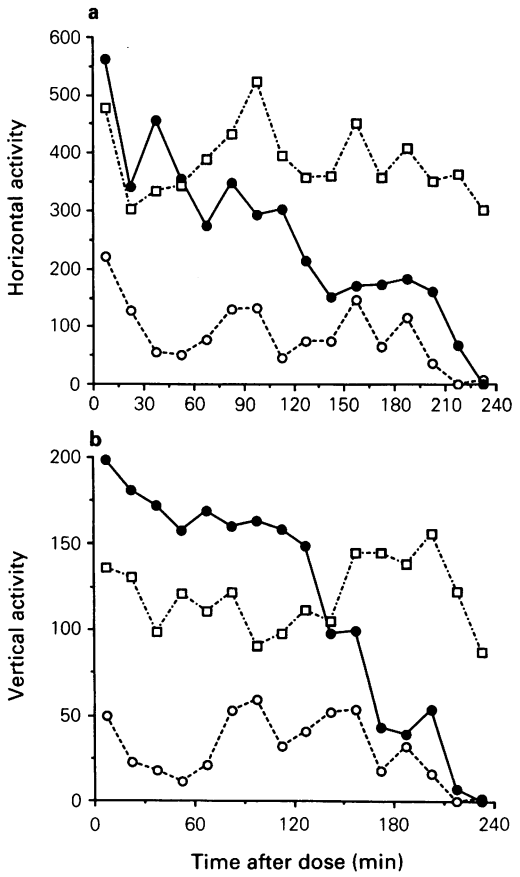

Figure 3 Horizontal activity (a) and vertical activity (b) over a 4 h time course, in animals given caffeine doses of 0, 20, and 40 mg kg^{-1}. Each point represents the mean of the behavioural parameters for 4 animals, averaged over a 15 min time period. Symbols are: caffeine dose = 0 (vehicle control) (○---○); 20 mg kg^{-1} (●——●); 40 mg kg^{-1} (□——□).

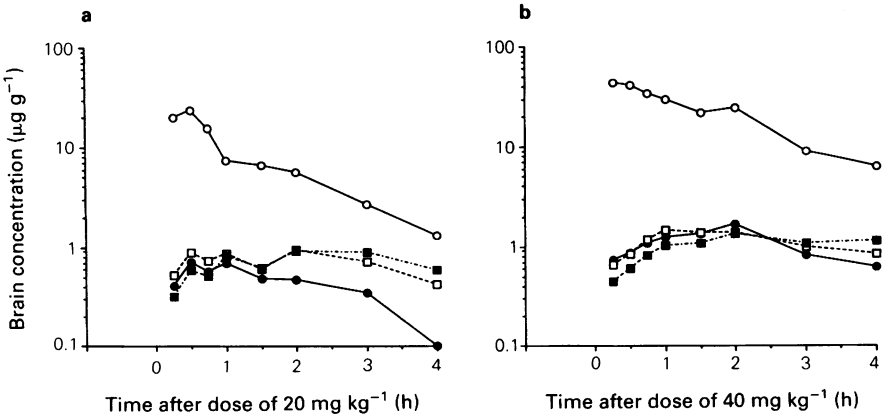

Figure 2 Brain concentrations of caffeine and metabolites of caffeine after doses of 20 mg kg^{-1} (a) and 40 mg kg^{-1} (b) caffeine over a 4 h time course. Each point is the mean concentration for 3 animals at the corresponding time. Symbols are: caffeine (○——○); theophylline (●——●); paraxanthine (□---□); theobromine (■---■).

Table 2 Statistical comparison of behaviour scores in different dosage groups

		Mean value at caffeine dose			Value of F from ANOVA	Significance of individual comparisons		
		0	20	40		20 vs 40	0 vs 20	0 vs 40
Horizontal								
Horizontal activity	Early	2947	9877	11747	5.99 ($P < 0.03$)		*	*
	Late	1112	2263	6686	15.34 ($P < 0.002$)	*		*
Horizontal movements	Early	152	596	742	6.14 ($P < 0.03$)		*	*
	Late	37	116	241	10.28 ($P < 0.005$)	*		*
Total distance	Early	341	2058	2832	5.57 ($P < 0.03$)			*
	Late	69	295	643	7.99 ($P < 0.02$)	*		*
Movement time	Early	45	289	382	5.97 ($P < 0.03$)		*	*
	Late	9	43	95	10.65 ($P < 0.005$)	*		*
Vertical								
Vertical activity	Early	1072	4817	3365	4.14 ($P = 0.053$)		*	
	Late	363	728	2379	44.67 ($P < 0.0002$)	*		*
Vertical movements	Early	261	1115	530	6.04 ($P < 0.03$)	*	*	
	Late	75	192	304	12.99 ($P < 0.003$)	*	*	*
Vertical time	Early	737	1920	2161	2.44 ($P = 0.14$)			
	Late	279	370	1080	21.65 ($P < 0.0005$)	*		*
Stereotypic								
Stereotypy count	Early	1464	5998	6897	6.51 ($P < 0.02$)		*	*
	Late	589	1320	4575	16.12 ($P < 0.002$)	*		*
Stereotypy number	Early	325	902	997	15.01 ($P < 0.002$)		*	*
	Late	120	232	554	24.06 ($P < 0.0003$)	*		*
Stereotypy time	Early	297	1112	1213	9.47 ($P < 0.007$)		*	*
	Late	129	270	874	22.20 ($P < 0.0004$)	*		*

Asterisk (*) indicates significant difference ($P < 0.05$) between behavioural scores of given dosage groups.

and metabolite concentrations decline below $4\,\mu g\,g^{-1}$ in the low dose group (Figures 1 and 2), the behavioural effect of caffeine diminishes and appears similar to vehicle treatment.

The findings were borne out statistically. Scores for early HA, up to 150 min, were not significantly different for the two caffeine doses (Table 2); both are significantly greater than scores of the vehicle-treated group. In the late period (150–480 min), the high-dose HA score was significantly greater than both the low-dose and vehicle HA scores, which were not statistically different from each other. Similar results occurred with horizontal movement, total distance, and movement time. Nearly identical trends were found with all stereotypic behaviours (Tables 2 and 3). In summary, for horizontal and stereotypic behaviours, high-dose and low-dose caffeine initially stimulated behaviour equivalently. The group receiving high-dose caffeine was more stimulated than the low-dose and vehicle groups, only after 150 min, when caffeine concentrations had fallen to a concentration of $10\,\mu g\,g^{-1}$ for this group (Figures 1 and 2).

The effects of caffeine were somewhat different when the various vertical behavioural measures were studied. For vertical movement and activity (Figure 3), early effects of low-dose treatment were greater than either vehicle or high-dose treatment. This difference was statistically significant in the case of vertical movements (Table 2). However, this trend was not seen when the parameter of vertical movement time was looked at. For all vertical measures, effects of the high-dose were significantly greater than either the low-dose or the vehicle, during the late period of treatment. These late-stage results are similar to those seen with all stereotypic and horizontal behaviours. A summary of these findings are presented in Table 3.

Table 3 Summary of caffeine dose-behavioural response relationships

	Comparison of behavioural scores in different dosage groups† Time period:	
Type of behaviour	*Early*	*Late*
Horizontal	20 = 40 Both > 0*	0 = 20 Both < 40*
Stereotypic	20 = 40 Both > 0*	0 = 20 Both < 40*
Vertical	20 > 40 > 0*	0 = 20 Both < 40*

Asterisk (*) indicates significant ($P < 0.05$) difference between behavioural scores in different dosage groups.

Kinetic-behaviour relationships

Figure 4 demonstrates the relationship between mean brain concentrations and activity scores at corresponding times. It indicates that the stimulant effects of caffeine were maximal at concentrations of approximately $10–20\,\mu g\,g^{-1}$ and that concentrations exceeding this range did not cause additional stimulation. In fact, stereotypic counts declined at the highest brain concentrations.

Discussion

Our study provides information about caffeine pharmacokinetics, and relationships between caffeine dose, concentration and behavioural stimulation. The pharmacokinetic results indicate that plasma concentrations consistently reflect brain concentrations of caffeine and metabolites over a 4 h course. This is consistent with previous results (Kaplan *et al.*, 1989) and suggests that plasma caffeine and metabolite concentrations may be accurate indicators of brain concentrations in man as well. The diminished clearance and prolonged plasma and brain half-life associated with higher doses, suggest dose-dependent caffeine kinetics, at least in mice.

Dose-response and concentration-response relationships are delineated in our study. High-dose caffeine does not stimulate activity to a greater degree than low-dose, early after treatment. In fact, low-dose caffeine stimulates certain behaviours to a greater degree than high dose. High-dose caffeine only stimulates activity more than low-dose, 150 min or more after treatment, when concentrations in the high-dose group have fallen to more intermediate levels. The graph of mean brain concentrations versus motor activity (Figure 4) plateaus at intermediate brain concentrations of caffeine, between $10–20\,\mu g\,g^{-1}$; in the case of stereotypic counts, this graph resem-

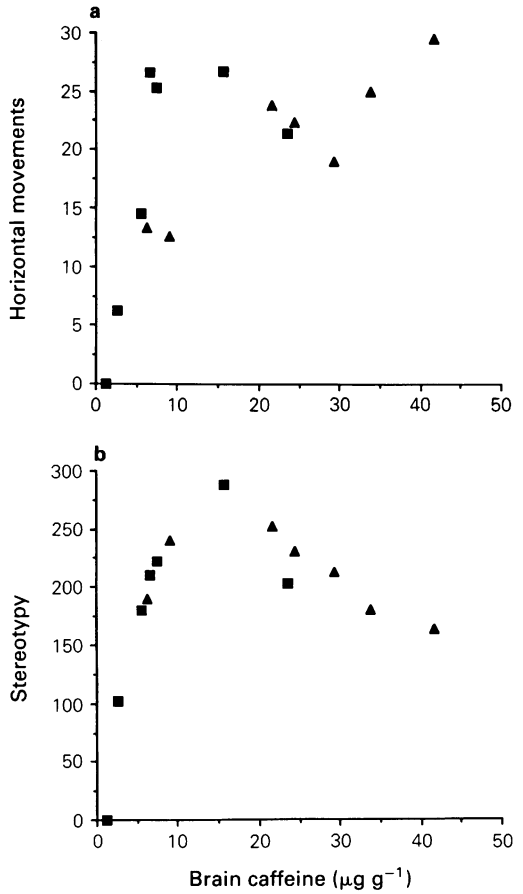

Figure 4 Mean brain caffeine concentrations (x-axis) versus horizontal movements (a) and stereotypy (b) among animals that received 20 or 40 mg kg^{-1} of caffeine. Each point is the mean value for all animals at corresponding times (30 min to 4 h). Symbols are: caffeine dose = 20 mg kg^{-1} (■); 40 mg kg^{-1} (▲).

bles an inverted U. This suggest that the maximal stimulant effects of caffeine occur in a concentration range of 10–20 μg g^{-1}. Concentrations below or above this range are associated with submaximal behavioural stimulation.

Caffeine-induced behavioural stimulation may relate to the complex effects of caffeine on central benzodiazepine, adenosine and monoamine systems (Daly *et al.*, 1981). Snyder and coworkers (1981) were the first to correlate the stimulant effects of caffeine with its antagonism of central adenosine receptors. They also demonstrated that caffeine had a weak inhibitory effect on benzodiazepine receptor binding. Several authors have confirmed that caffeine significantly alters central adenosine receptor binding (Boulenger *et al.*, 1983; Hawkins *et al.*, 1988; Daval *et al.*, 1989) and benzodiazepine receptor binding (Marangos *et al.*, 1979; Weir & Hruska, 1983; Boulenger *et al.*, 1983; Wu & Coffine, 1984; Kaplan *et al.*, 1989). Future studies can examine how various caffeine doses and concentrations affect adenosine and benzodiazepine receptor binding differently, ultimately resulting in different behavioural responses to the drug.

Our results may be useful in their application to man. As in our animal study, Bruce and coworkers (1986) showed that in man, the effects of caffeine on the central nervous system, as measured by electrophysiological changes, were maximal at intermediate plasma concentrations (between 4–6 μg ml^{-1}). High doses and plasma concentrations (10–12 μg ml^{-1}) did not produce these central effects. Therefore, a specific caffeine concentration range for maximal stimulatory effects may be found in man as well as animals. Future studies in man can be done to evaluate more carefully the relationship between caffeine plasma concentration and pharmacodynamics (e.g., mood, motor performance and autonomic effects). In this way, we can better understand pharmacologically-induced anxiety.

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Phenthonium, a quaternary derivative of (−)-hyoscyamine, enhances the spontaneous release of acetylcholine at rat motor nerve terminals

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1 A quaternary derivative of (−)-hyoscyamine, phenthonium (Phen) induced a concentration-dependent increase in the rate of spontaneous quantal release of acetylcholine (ACh) at the mammalian neuromuscular junction, as shown by intracellular recordings of the miniature endplate potentials (m.e.p.ps) in rat diaphragm muscles.

2 The prejunctional effect of Phen (10–50 μM) was reversible, unrelated to temperature (22°–35°C), unaltered by either changes in $[Ca^{2+}]_o$ or by high $[Mg^{2+}]_o$, and was not induced by membrane depolarization.

3 Simultaneously, Phen reduced the amplitude of m.e.p.ps by a postjunctional action.

4 The muscarinic agonist oxotremorine did not prevent the increase in m.e.p.p. frequency induced by Phen. Cholinesterase inhibition with neostigmine potentiated the prejunctional effect induced by a low (20 μM) but not a high (50 μM) concentration of Phen.

5 The increase in m.e.p.p. frequency induced by Phen was not influenced by previous incubation with either atropine (0.01–10 μM) or (+)-tubocurarine (0.05–0.1 μM). Each antagonist however, intensified the postjunctional effect of Phen.

6 Phen (20 μM) did not influence the quantal contents of e.p.ps in cut-muscle preparations or in the presence of high $[Mg^{2+}]_o$. A high concentration of Phen (50 μM) increased the rundown of e.p.p. trains evoked at 10–50 Hz.

7 The results indicate that the facilitatory prejunctional action of Phen cannot be explained by an antimuscarinic activity. A possible interaction of the antagonist with putative prejunctional nicotinic cholinoceptors however, was not excluded.

Introduction

The existence of prejunctional acetylcholine (ACh) receptors which modulate the transmitter release at motor nerve terminals has been suggested frequently (Miyamoto, 1978; Kilbinger, 1984; Bowman *et al.*, 1984; 1988). The type of cholinoceptor sites involved in the feedback regulation however, is controversial. Muscarinic inhibitory (Duncan & Publicover, 1979; Michaelson *et al.*, 1979; Abbs & Joseph, 1981; Somogyi *et al.*, 1987) and excitatory (Das *et al.*, 1978; Wali *et al.*, 1987; Wessler *et al.*, 1987) prejunctional cholinoceptors have been reported at vertebrate neuromuscular junctions and in the *Torpedo* electric organ. In the presence of tubocurarine, a frequency-dependent decrease of evoked ACh release was obtained which suggested the presence of nicotinic prejunctional cholinoceptors mediating a positive feedback control of evoked transmitter release (Glavinovic, 1979a; Magleby *et al.*, 1981; Gibb & Marshall, 1984; Wessler *et al.*, 1986; Matzner *et al.*, 1988). Evidence has also been presented against both muscarinic (Gundersen & Jenden, 1980; Haggblad & Heilborn, 1983) and nicotinic (Beranek & Vyskocil, 1967; Auerbach & Betz, 1971; Jones & Salpeter, 1983) prejunctional cholinergic regulation at the neuromuscular junction.

Most of the data supporting a prejunctional cholinergic modulation have been provided by experiments on the nerve evoked ACh release largely performed in the presence of either high K^+ or anticholinesterase agents. These agents could have accounted for the conflicting results (Kilbinger, 1984; Somogyi *et al.*, 1987). Few studies have presented evidence for a prejunctional cholinergic regulation of spontaneous transmitter release. In the rat diaphragm muscle, carbachol increased the frequency of miniature endplate potentials (m.e.p.ps), an effect which was blocked by tubocurarine, indicating the existence of prejunctional nicotinic receptors (Miyamoto & Volle, 1974). At frog neuromuscular junctions however, an increase in the m.e.p.p. frequency was detected in the presence of atropine, suggesting the involvement of prejunctional muscarinic modulation (Duncan & Publicover, 1979).

The present study investigated the prejunctional effects of a quaternary derivative of (−)-hyoscyamine, phenthonium (Figure 1), on transmitter release at the mammalian neuromuscular junction. This drug has antimuscarinic but no ganglionic blocking activity (Della Bella *et al.*, 1968; Teotino *et al.*, 1968), and is a noncompetitive antagonist of neuromuscular transmission (Souccar *et al.*, 1986). The results showed that phenthonium increased the spontaneous but not

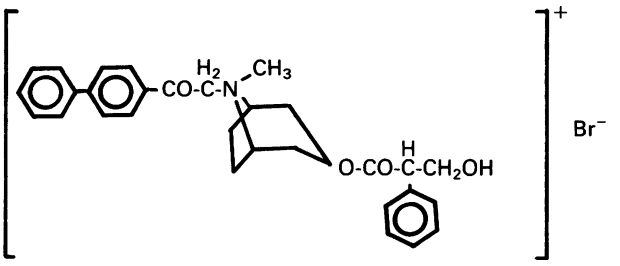

Figure 1 Chemical structure of phenthonium.

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the evoked ACh release, suggesting a prejunctional cholinergic modulation of the resting quantal transmitter release.

Methods

Phrenic nerve-diaphragm muscle preparations from female Wistar rats (200–250 g) were used. The left hemidiaphragm was pinned down, slightly stretched on Sylgard (Dow Corning), placed in a 10 ml muscle chamber and continuously perfused with physiological solution. The resting membrane potentials (RMP), miniature endplate potentials (m.e.p.ps) and endplate potentials (e.p.ps) with rise time of 1 ms or less, were recorded intracellularly at endplate regions, at room temperature (22–25°C). Glass microelectrodes containing 3 M KCl (resistance 6–10 MΩ) and a high impedance unity gain electrometer (WP Instruments 750) were employed. A silver/silver chloride electrode was used as reference. When necessary, the temperature of the bathing solution was raised by circulating heated water through a chamber beneath the bath floor.

The normal physiological solution consisted of (mM): NaCl 135, KCl 2.5, $MgCl_2$ 1, $CaCl_2$ 2, $NaHCO_3$ 15, NaH_2PO_4 1 and glucose 11, gassed with 95% CO_2 and 5% O_2 (pH 7.3–7.4). For e.p.p. recording, muscle contractions were prevented by either raising the Mg^{2+} concentration to 15 mM in normal physiological solution (Ca^{2+} = 2 mM) or by cutting the muscle fibres across their length approximately 2 mm at each extremity (Barstad & Lilleheil, 1968). Osmolarity of the saline solution was maintained by correction of the NaCl concentration.

E.p.ps were evoked by stimulating the phrenic nerve via a bipolar platinum electrode at 0.5 Hz, 0.05 ms and supramaximal voltage. Trains of 25 e.p.ps were elicited by repetitive stimulation (25, 50 and 100 Hz) at intervals of 60–90 s. For quantal content determination, at least 100 e.p.ps were recorded at each endplate, photographed from the oscilloscope and measured on film under magnification. M.e.p.ps were simultaneously recorded on a Mingograph chart recorder (Elema-Schonander, Sweden) and measured. The amplitudes of e.p.ps were corrected for non-linear summation (MacLachlan & Martin, 1981) assuming the reversal potential for ACh in the diaphragm muscle fibres as 0 mV (Alemá et al., 1981). The amplitudes of m.e.p.ps from the cut-muscle fibres were corrected to a mean RMP of −45 mV determined experimentally, using the relationship: $m.e.p.p._c$ = m.e.p.p. × 45/RMP, where $m.e.p.p._c$ and m.e.p.p. are the corrected and recorded m.e.p.p. amplitude, respectively (Thomsen & Wilson, 1983). The quantal content of the e.p.ps (m) was calculated by the direct method as the ratio of mean e.p.p. amplitude to the mean m.e.p.p. amplitude (Hubbard et al., 1969).

Materials

Drugs used were: atropine sulphate, oxotremorine sesquifumarate, neostigmine bromide, (+)-tubocurarine hydrochloride and tetrodotoxin (Sigma). Phenthonium [N-(4-phenyl)-phenacyl (−)-hyoscyamine] bromide was kindly provided by Zambon Laboratories (Italy). Stock solutions of all drugs were prepared in de-ionized water, and phenthonium was dissolved in 0.01 N hydrochloric acid (12%). All muscle preparations were allowed to equilibrate for 30 min in the normal or modified physiological solution before a control period of recording. Incubation with drugs was done by exchanging the bathing medium with a physiological solution containing appropriate concentrations of the drugs being tested.

Statistics

Normally distributed data were expressed as means ± s.e.mean, and differences among groups were determined by Student's t test. Data of non-normal distributions (m.e.p.p. frequency) were presented as geometric means ± approximate s.e.; the Mann-Whittney test was used for comparison of data (Colquhoun, 1971). Differences among data were considered significant at $P < 0.05$.

Results

Spontaneous transmitter release

In normal physiological solution, the mean RMP at room temperature (22–25°C) was −81 ± 1 mV; the frequency and amplitude of m.e.p.ps were respectively, 1.20 ± 0.15 s^{-1} and 0.76 ± 0.02 mV (139 fibres of 12 muscles). Phenthonium (Phen, 10–50 μM) did not alter the RMP, but induced a concentration-dependent increase in m.e.p.p. frequency, after 10 min. The effect was maximal within 25–30 min of incubation, being 1.99 ± 0.34 s^{-1}, 2.68 ± 0.37 s^{-1}, and 6.46 ± 0.70 s^{-1} in the presence of 10, 20 and 50 μM Phen, respectively (Figure 2). Simultaneously, the amplitude of m.e.p.ps was decreased by 30–35% of control in the presence of low concentrations of Phen (10 and 20 μM). Higher concentrations of Phen (50–100 μM) caused intense postjunctional blockade, leading to an apparent decrease of the m.e.p.p. frequency in few cells. After 5 min washout of the drug however, the postjunctional blockade was reversed and m.e.p.p. frequencies higher than 6 s^{-1} were recorded. To assess the prejunctional actions of Phen underlying the increase of m.e.p.p. frequency, a concentration of the antagonist that induced a small postjunctional blockade (20 μM) was employed in most of the following experiments.

Effect of temperature Raising the temperature of the bathing medium from 22°C to 30°C did not affect the frequency of m.e.p.ps (1.11 ± 0.24 s^{-1}, 43 fibres/4 muscles), but at 35°C frequency was increased 2.8 fold (3.40 ± 0.34 s^{-1}, 45 fibres/4 muscles). Incubation with Phen (20 μM) at both temperatures increased the frequency of m.e.p.ps to 246% and 188% of the respective control at 30°C and 35°C.

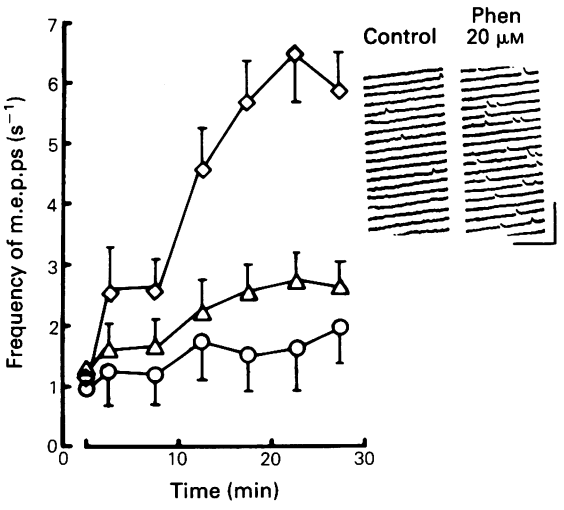

Figure 2 Time course of the effects of phenthonium (Phen; 10 μM ○; 20 μM △ and 50 μM ◇) on the frequency of miniature endplate potentials (m.e.p.ps) recorded from rat diaphragm muscle fibres at 22–25°C. Phen was incubated at time zero, and m.e.p.p. frequency was determined at 5 min intervals. Symbols and vertical bars are geometric means ± approximate s.e. of 20–60 fibres in 4 to 6 muscles. Inset: typical m.e.p.p. recordings alone and after 30 min exposure to 20 μM Phen. Calibration bars are 10 mV (control) or 5 mV (Phen) and 50 ms.

The amplitude of m.e.p.ps was not altered by raising the temperature either to 30°C (0.72 ± 0.03 mV) or to 35°C (0.70 ± 0.01 mV), and was reduced in presence of Phen (20 μM) by 33% of control at both temperatures. The RMP values recorded in the absence or presence of Phen (20 μM) were not altered by changes of temperature.

Effect of extracellular Ca^{2+} Reducing $[Ca^{2+}]_o$ in the physiological solution to 0.5 and 1.0 mM decreased m.e.p.p. frequency to respectively, $0.61 \pm 0.27\,s^{-1}$ (42 fibres/4 muscles) and $0.77 \pm 0.29\,s^{-1}$ (46 fibres/4 muscles), whereas raising the $[Ca^{2+}]_o$ to 5 mM increased the m.e.p.p. frequency to $2.41 \pm 0.43\,s^{-1}$ (39 fibres/3 muscles). Incubation with Phen (20 μM) for 30 min did not alter the frequency of m.e.p.ps in a medium containing 0.5 mM $[Ca^{2+}]_o$, but increased that recorded in presence of 1 mM $[Ca^{2+}]_o$ to 197% of control. In the presence of 5 mM $[Ca^{2+}]_o$, the effect of Phen (20 μM) did not differ from that obtained in normal $[Ca^{2+}]_o$ solution, raising the frequency of m.e.p.ps to 210% of control after 10 min. The relationship of the log m.e.p.p. frequency against the log $[Ca^{2+}]_o$ determined in the absence and presence of Phen was linear, with slope values of 0.71 and 0.74, respectively. The curve in the presence of Phen showed a parallel shift to the left of that obtained in its absence (Figure 3).

Raising the Mg^{2+} concentration (15 mM) in normal $[Ca^{2+}]_o$ (2 mM) solution did not alter the control frequency of m.e.p.ps ($1.11 \pm 0.39\,s^{-1}$, 32 fibres/3 muscles), nor did it affect the increase in m.e.p.p. frequency induced by 20 μM Phen (230% of control).

The amplitude of m.e.p.ps recorded in the presence of various $[Ca^{2+}]_o$ (0.5 mM: 0.78 ± 0.03 mV; 1 mM: 0.87 ± 0.06 mV; 5 mM: 0.82 ± 0.04 mV, $n = 114$ to 252 m.e.p.ps) did not differ from control, but it was reduced by 35% to 50% after 30 min exposure to 20 μM Phen.

No significant changes in RMP values were detected over the range of $[Ca^{2+}]_o$ tested in either controls or after incubation with Phen.

Effect of neostigmine Neostigmine (0.5 μM) caused spontaneous twitches of the muscle fibres which were prevented by tetrodotoxin (TTX, 0.5 μM). TTX (0.5–1.0 μM) alone did not affect the ability of 20 and 50 μM Phen to increase m.e.p.p. frequency to respectively, 240% and 528% of control ($1.24 \pm 0.27\,s^{-1}$; 90 fibres/4 muscles). Neostigmine (0.5 μM) did not alter the mean frequency of m.e.p.ps after 30 min ($1.22 \pm 0.17\,s^{-1}$; 129 fibres/8 muscles), although few cells presented m.e.p.p. frequencies up to $10\,s^{-1}$. Phen (20 μM) added to muscles preincubated with neostigmine (0.5 μM) increased the m.e.p.p. frequency to $4.06 \pm 0.52\,s^{-1}$ (19 fibres/7 muscles) after 15 min (Figure 4). The results were 1.5 fold higher than those obtained in presence of Phen (20 μM) alone. A higher concentration of Phen (50 μM) added after preincubation with neostigmine (0.5 μM) increased the m.e.p.p. frequency to the same levels as those found in the presence of Phen (50 μM) alone (523% of control).

The mean amplitude of m.e.p.ps determined after 30 min incubation with neostigmine (0.5 μM) was increased by 47% of control (0.70 ± 0.04 mV, $n = 210$ m.e.p.ps). Those values were not altered after 30 min exposure to neostigmine (0.5 μM) plus Phen (20 μM). On the other hand, neostigmine (0.5 μM) together with Phen (50 μM) reduced m.e.p.p. amplitude to those values obtained with Phen alone (20% of control).

Effect of oxotremorine The mean values of RMP (-81 ± 2 mV) and m.e.p.p. frequency ($1.21 \pm 0.18\,s^{-1}$; 90 fibres/6 muscles) were not altered by 1 μM oxotremorine, although m.e.p.p. amplitude was decreased by 20% of control (0.65 ± 0.08 mV). Higher concentrations of oxotremorine (5 and 10 μM) each depolarized the muscle fibres by some 20 mV causing spontaneous twitching. The frequency of m.e.p.ps however, did not differ from control over 1 h.

Previous incubation with oxotremorine (1 μM) for 30 min did not prevent the increase in m.e.p.p. frequency induced by Phen (20 μM), which reached 213% of the values determined in presence of oxotremorine alone ($1.0 \pm 0.31\,s^{-1}$, 52 fibres/4 muscles). These values did not differ from those obtained with equal concentration of Phen alone (Figure 4). The postjunctional effect of Phen (20 μM) however, was potentiated by oxotremorine (1 μM) reducing the m.e.p.p. amplitude by some 80% of control.

Effect of atropine and (+)-tubocurarine Exposure to atropine (0.01–10 μM) and (+)-tubocurarine (Tc, 0.05–0.1 μM) each for 30 min did not alter RMP or m.e.p.p. frequency; the amplitude of m.e.p.ps was reduced by 20–30% and 50–70%, respectively.

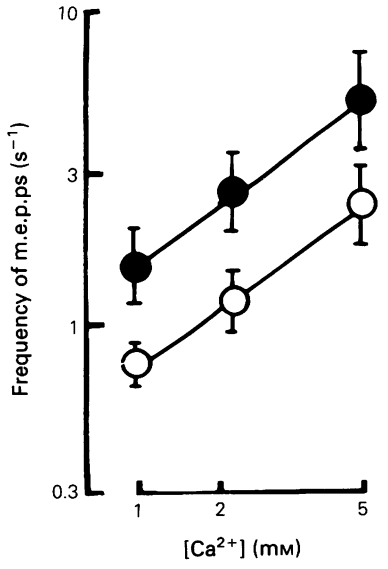

Figure 3 Effect of increasing the external concentration of Ca^{2+} (abscissa scale), on the frequency of miniature endplate potentials (m.e.p.ps, ordinate scale) of rat diaphragm muscle fibres recorded at 22°–25°C, in control preparations (○) and following 25–30 min incubation with phenthonium 20 μM (●). Symbols and vertical bars are geometric means ± approximate s.e. of 15–40 fibres in 4 muscles.

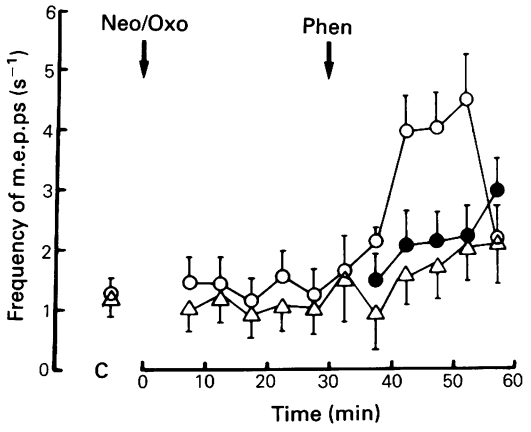

Figure 4 Time course of effects of phenthonium (Phen, 20 μM) on the frequency of miniature endplate potentials (m.e.p.ps in s^{-1}) recorded from rat diaphragm muscle fibres, in presence of Phen alone (●), and after additional preincubation with neostigmine (Neo, 0.5 μM, ○) or oxotremorine (Oxo, 1 μM, △). Incubation began at the time indicated by arrows. Control values (C) were obtained from 106 fibres/9 muscles for neostigmine, and 89 fibres/7 muscles for oxotremorine. Symbols and vertical bars are geometric means ± approximate s.e. of 15–38 fibres in 5–11 muscles.

Table 1 Effect of phenthonium on the quantal content of endplate potentials (m) determined in 'cut' diaphragm muscle fibres and in intact muscles bathed in high $[Mg^{2+}]_o$ solution

Condition	*e.p.p. amplitude* (mV)	*m.e.p.p. amplitude* (mV)	*m*
'Cut-muscle'			
Control	17.89 ± 1.97* (17/6)	0.64 ± 0.08	31.0 ± 3.9
Phenthonium (20 μM)	11.55 ± 1.71* (17/6)	0.52 ± 0.08	25.3 ± 3.8
15 mM Mg^{2+} solution			
Control	1.74 ± 0.36 (16/5)	0.37 ± 0.03	4.69 ± 0.80
Phenthonium (20 μM)	1.61 ± 0.23 (17/4)	0.38 ± 0.02	4.31 ± 0.61

* Values are means ± s.e.means with the number of fibres/number of muscle preparations in parentheses.
* Significantly different from control ($P < 0.05$).

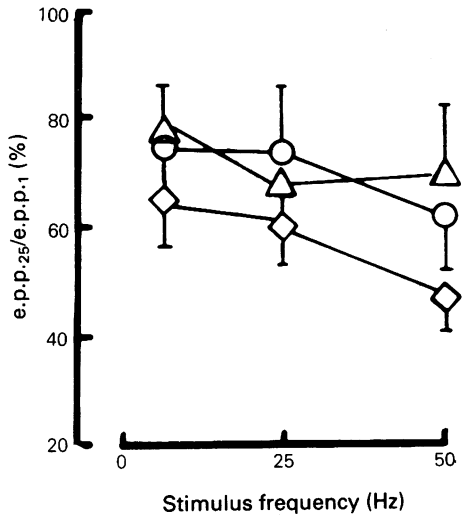

Figure 5 Decay of endplate potentials (e.p.ps) elicited at different frequencies of stimulation in cut diaphragm muscles of the rat, in the absence (○) and presence of phenthonium (20 μM, △; 50 μM, ◇). The nerve was stimulated by trains of 25 pulses each at the indicated frequency of stimulation (abscissa scale), and the e.p.p. decay was expressed as a % of the first e.p.p. amplitude (ordinate scale). Symbols are means ± s.d. of 6–12 fibres in 4 muscles.

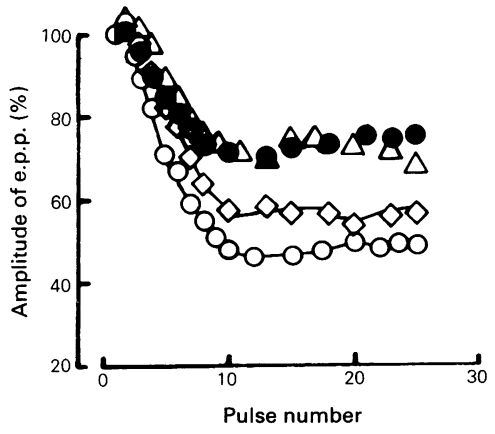

Figure 6 Time course of the decay of endplate potentials (e.p.ps) evoked by nerve stimulation, at 25 Hz, of cut diaphragm muscle fibres of the rat, obtained in the absence (●) and presence of phenthonium (20 μM – △; 50 μM – ◇) or tubocurarine (0.5 μM – ○). The decay of e.p.ps was expressed as a % of the first e.p.p. amplitude (ordinate). Symbols are means of 7–12 fibres in 4 muscles; s.d. at each point ranged from 3 to 10% and was omitted for clarity.

Addition of Phen (20 μM) to muscles preincubated with atropine (10 μM) or Tc (0.1 μM) increased the frequency of m.e.p.ps to respectively, 2.17 ± 0.39 s^{-1} (17 fibres/3 muscles) and 2.31 ± 0.57 s^{-1} (16 fibres/3 muscles) after 30 min. Those results did not differ from those obtained in the presence of Phen (20 μM) alone. The amplitude of m.e.p.ps was further reduced by adding Phen and atropine (40%) or Tc (80%). Phen (20 μM) together with higher concentrations of atropine (1 μM) or Tc (1 μM) reduced the amplitudes of m.e.p.ps to values equal to or below 0.1 mV, making the recordings unreliable.

Evoked transmitter release

To investigate the effect of Phen on the evoked transmitter release, the quantal content of e.p.ps (m) were determined in cut-muscle preparations and in intact muscles bathed in high $[Mg^{2+}]_o$ containing physiological solution. The RMP recorded from 'cut' diaphragm muscle fibres was −43 ± 1 mV (39 fibres/16 muscles), and was not altered after 30 min incubation with Phen (20 μM). The mean values of m and m.e.p.p. amplitude did not differ from controls, although the e.p.p. amplitude was reduced by some 35% (Table 1). In intact muscle fibres bathed in a high $[Mg^{2+}]_o$ medium, Phen (20 μM) did not change e.p.p. or m.e.p.p. amplitude, or m values from the respective controls (Table 1).

The effect of Phen was also examined on the decay of e.p.p. amplitudes evoked by repetitive nerve stimulation. Under control conditions, the amplitude of trains of e.p.ps induced at 10, 25 and 50 Hz in the cut rat diaphragm was initially increased transiently then decreased to a plateau level. The amplitude of the last (25th) e.p.p. expressed as % of the first e.p.p. reached 75.0 ± 7.0%; 73.1 ± 10.2% and 62.0 ± 10.1% (means ± s.d.) at respectively, 10, 25 and 50 Hz. After 30 min incubation with Phen (20 and 50 μM) the amplitude of the first e.p.p. was reduced by 40 and 46% of control values (10.03 ± 1.49 mV). Incubation with Phen (20 μM) for 30 min did not alter the decay of e.p.p. trains recorded at 10–50 Hz. However, a high concentration of Phen (50 μM) caused a faster decline of the e.p.p. trains, reaching the amplitude of the 25th e.p.p. 64.8 ± 5.8%; 60.8 ± 4.6% and 47.0 ± 4.7% of the first e.p.p. at 10, 25 and 50 Hz, respectively (Figure 5). In similar experiments, Tc (0.5 μM) induced a more intense e.p.p. rundown, reducing the amplitude of the 25th e.p.p. to 47.8 ± 5.2% and 49.5 ± 3.1% at 10 and 25 Hz respectively (Figure 6).

Discussion

A quaternary derivative of (−)-hyoscyamine, phenthonium (Phen), induced a concentration-dependent increase in the rate of spontaneous quantal transmitter release but did not affect the RMP in the rat neuromuscular junction. This prejunctional effect was reversible thus eliminating the physical disruption of the nerve terminal membrane or intraterminal organelles and persisted in the presence of tetrodotoxin, excluding an activation of the voltage sensitive Na^+ channels at the nerve terminal membrane as a mechanism of action of the drug.

Raising the temperature in control muscles increased m.e.p.p. frequency (Hubbard *et al.*, 1969), without altering the prejunctional effect of Phen. In fact, at 35°C Phen induced a smaller increase of m.e.p.p. frequency than that obtained at 22°C or 30°C, suggesting a saturation level for the facilitatory effect of Phen. This limitation was not observed after raising the $[Ca^{2+}]_o$.

Increase in the free intraterminal Ca^{2+} concentration is known to raise the m.e.p.p. frequency (Katz & Miledi, 1969; Cohen *et al.*, 1974). The intraterminal Ca^{2+} concentration in its turn depends on the ion influx and Ca^{2+} regulating systems of intracellular organelles (Blaustein *et al.*, 1978; 1980). Changes of extracellular Ca^{2+} concentration carried

out in the presence of Phen caused a proportional increase of the m.e.p.p. frequency. As shown in Figure 3, the relationship of the log of m.e.p.p. frequency against the log of Ca^{2+} concentration obtained in the presence of Phen was shifted to the left of that obtained in control muscles, without alteration of the slope. These results indicate that the agent enhances the physiological mechanism of calcium-dependent quantal transmitter release. Although the effect was abolished in presence of low extracellular Ca^{2+} concentration (0.5 mM), it was still obtained in high-Mg^{2+} solution, indicating that Phen does not increase the spontaneous acetylcholine (ACh) release by promoting the influx of Ca^{2+} in the nerve terminal. A direct stimulating action on Ca^{2+} release from its internal stores also seems unlikely, since the compound is a quaternary derivative and therefore, hardly penetrates the nerve terminal. Nevertheless, one cannot eliminate a possible disruption of the intraterminal Ca^{2+} buffering systems, indirectly triggered through interaction of Phen with prejunctional receptors on the external surface of the nerve terminal membrane, resulting in hastened exocytosis.

In view of the cholinolytic actions of Phen, the increase in m.e.p.p. frequency induced by the antagonist may arise from an interaction with prejunctional cholinoceptors, either muscarinic or nicotinic. In our experimental conditions, the muscarinic agonist oxotremorine did not influence the spontaneous ACh release, confirming other studies (Gundersen & Jenden, 1980). At concentrations that did not exert a postjunctional blockade, oxotremorine was unable to antagonize the prejunctional effects of Phen. In addition, previous incubation with atropine did not facilitate or prevent the increase of m.e.p.p. frequency induced by Phen, indicating that antimuscarinic activity does not account for the enhanced spontaneous ACh release.

Alternatively, if the antinicotinic activity of Phen is responsible for the increase of m.e.p.p. frequency, this effect might be antagonized by increasing the concentration of ACh in the synaptic cleft with anticholinesterase agents. However, paradoxical results were obtained with neostigmine which potentiated the prejunctional effect of a low (20 μM), but not of a high (50 μM) concentration of Phen. One possibility is that at the higher concentration, the antagonist may have reached its maximal enhancing capability on the spontaneous ACh release, the reason why neostigmine did not potentiate the effect. On the other hand, an eventual potentiation of the prejunctional effect may have been missed with intracellular recording techniques, because of the intense depression of m.e.p.p. amplitudes induced by high concentrations of Phen.

The increase in m.e.p.p. frequency induced by Phen was not altered in the presence of tubocurarine, whereas the postjunctional blockade was exacerbated. Due to the latter effect, higher concentrations of tubocurarine could not be tested and therefore, the involvement of prejunctional nicotinic receptors cannot be ruled out. Studies using other techniques such as chemical determinations of the ACh release may provide better evaluation of the actions of both antagonists (Wessler *et al.*, 1986).

At concentrations that increased m.e.p.p. frequency, Phen did not influence the evoked transmitter release as shown by determinations of the quantal content of e.p.ps. Equal concentrations of Phen did not alter the rundown of successively evoked e.p.ps as did tubocurarine (Figure 6). The faster rundown of the endplate current amplitudes recorded in the presence of tubocurarine have been attributed to its prejunctional actions (Glavinovic, 1979b; Magleby *et al.*, 1981; Gibb & Marshall, 1984). A faster decay of the e.p.p. amplitude was also induced by a high concentration of Phen (50 μM). Considering the intense postjunctional blockade obtained in this case, those results may not be explained solely by a prejunctional action.

In conclusion, the results showed that in the rat phrenic nerve diaphragm, Phen facilitated the spontaneous quantal release of ACh without altering the evoked transmitter release. The effect was not related to either the temperature or membrane depolarization nor did it depend on the $[Ca^{2+}]_o$. The prejunctional effect may be explained by an interaction of Phen with membrane-bound receptors since the agent does not cross the nerve terminal membrane. Our findings did not support an interaction with putative prejunctional muscarinic receptors; a possible interaction with prejunctional nicotinic cholinoceptors could not be excluded due to the technical limitations of intracellular recordings.

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Cationic factors affecting phospholipase activities from human lung

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1 The effects of varying H^+ and other cation concentrations on phospholipase activity were investigated on two particulate fractions from human lung, corresponding to the mitochondrial and microsomal fractions.

2 Three ^{14}C-labelled substrates, arachidonyl-phosphatidylcholine (PC), -phosphatidylethanolamine (PE) and -phosphatidylinositol (PI) were used.

3 For two substrates, PE and PI, hydrolysis was maximal at pH 6, with either subcellular fraction.

4 Hydrolysis of all three substrates was strongly inhibited by EDTA and EGTA (10–25 mM). Addition of 2,2-dipyridyl, *o*-phenanthroline, 8-hydroxyquinoline or desferrioxamine (10 μM–1 mM) did not inhibit but often increased hydrolysis of all substrates.

5 Addition of Zn^{2+}, as $ZnCl_2$, (10 μM–1 mM) inhibited PE and PI, but not PC, hydrolysis.

6 The phospholipase activities from human lung appear to be dependent on Ca^{2+} for maximal activity and to be inhibited by other metal ions including Zn^{2+}.

Introduction

Phospholipases liberating arachidonate from endogenous lipids play a crucial role in the overall control of eicosanoid biosynthesis (Flower & Blackwell, 1976). The eicosanoids exhibit a wide range of potent biological activities on a variety of cells in the lung and these activities are expressed in both physiological and pathological conditions (Bakhle & Ferreira, 1985). Phospholipase action has also been linked to receptor activation by many different agonists effective in lung (Fain *et al.*, 1988). Thus phospholipases are involved crucially in both inter- and intracellular signalling in lung and a fuller knowledge of lung phospholipases is essential to our understanding of pulmonary physiology and pathophysiology in man.

We have shown the presence of a phospholipase A_2 (PLA_2)-like activity and of a PLC-like activity in cell-free extracts of human lung (Bakhle & Yeats, 1986; Yeats & Bakhle, 1989b). To characterize further these activities exhibited towards phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI), we have investigated the effects of pH and of Ca^{2+} and Zn^{2+} ions on the hydrolysis of these substrates, labelled with [^{14}C]-arachidonic acid in the sn-2 position. A preliminary account of some of the results have been communicated to the British Pharmacological Society (Bakhle & Yeats, 1988; Yeats & Bakhle, 1989a).

Methods

Preparation of subcellular fractions of human lung homogenate

Samples of human lung obtained at surgery and macroscopically normal were chopped with scissors, rinsed with cold (4°C), sterile isotonic saline and homogenized in 4 volumes of cold standard buffer with 6 × 10 s bursts of a Polytron blade homogenizer. The standard buffer used throughout this work contained (mM): Tris HCl (pH 7.4) 10, sucrose 250, $CaCl_2$ 5. All processing of the lung extracts was carried out at 4°C.

Subcellular fractions were derived from the homogenate by differential centrifugation as described earlier (Yeats & Bakhle, 1989b). In this set of experiments, only the particulate fractions P_1 and P_2 were used. Mitochondrial and lysosomal enzymes were concentrated in the P_1 fraction and microsomal enzymes in the P_2 fraction (Yeats & Bakhle, 1989b). These fractions exhibited higher specific activity than the supernatant fraction (S_2 obtained after 100 000 *g* for 60 min): P_2:S_2 activity ratios were 15 for PC, 13 for PE and 360 for PI. Further details of the distribution of phospholipase activity in the various subcellular fractions have already been given (Yeats & Bakhle, 1989b).

Phospholipase assay

This assay has been fully described elsewhere (Yeats & Bakhle, 1989b). The standard incubation mixture (100 μl) contained about 2×10^5 d.p.m. of [^{14}C]-arachidonyl-PC, PE or PI (15 μM); 5 mM $CaCl_2$; 10 mM Tris HCl, pH 7.4 and various amounts of lung protein. The amount of protein depended on the substrate; with PC, 20–30 μg, and with PE and PI, 10–20 μg were used. Hydrolysis of each substrate was linearly proportional to the amount of protein used from 3–30 μg per incubation sample (data not shown). After 40 min incubation at 37°C, the reaction was stopped with 25 μl of 2 M HCl and 10 μl of 0.6 mM arachidonic acid (AA) added. Then 1 ml of the first eluting solvent (1a or 1b, see below) was added and the two phases mixed vigorously before application to the silicic acid columns for analysis.

Non-enzymic hydrolysis of phospholipid substrates was measured by incubation with buffer alone and these values subtracted from those derived from incubation with lung fractions. Where necessary the buffer contained the additional cations, chelating agents etc., under study. These additions were made up in the standard buffer to provide the required final concentrations in the incubation mixture.

Analysis of hydrolysates

Silicic acid column chromatography Glass Pasteur pipettes (145 × 5 mm) were plugged with glass wool and a slurry of silicic acid in eluting solvent 1a (hexane:dioxane:glacial acetic acid, 80:20:1, v/v) poured in to form a column 3 cm high. To these columns, the emulsified reaction mixture was applied and hydrolysis products eluted with a further 5 ml of solvent 1a. Another solvent, solvent 2, ($CHCl_3$:MeOH:H_2O, 65:35:1, v/v) was used to elute unchanged substrate.

This two solvent system was not adequate for the full analysis of PI-derived hydrolysates which contained labelled

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diglyceride (DG) as well as AA and both these products were eluted together by solvent 1a. Thus for incubations with PI, another less polar solvent, solvent 1b (hexane:dioxane:glacial acetic acid, 97:2:1, v/v) was devised and used both to prepare the columns and as the first eluting solvent, before solvent 1a. All three labelled species, AA, DG, and PI, were very effectively separated by these three solvents. From incubation samples, the radioactivity in eluate 1 (solvent 1b) was chiefly associated with marker AA (98%), that in eluate 2 (solvent 1a) with DG (93%) and in eluate 3 (solvent 2) with PI (96%), on analysis by thin layer chromatography (t.l.c., see below). Further metabolism of the product AA to prostaglandins was negligible; marker prostaglandin E_2 (PGE_2) or 6-oxo-$PGF_{1\alpha}$ was eluted with solvent 2 and less than 2% of ^{14}C eluted with solvent 2 from incubation mixtures co-chromatographed with these prostaglandins on subsequent t.l.c. Routinely, 6 ml of each solvent gave good recovery of the radioactivity in the incubation mixture applied to the column (>85% for product and >80% for substrate). The total eluate of each solvent was combined to provide a single fraction for radioactivity measurements.

Thin layer chromatography Each eluate from the column was analysed by t.l.c. in which silica gel layers on aluminium foil were used. The eluates were evaporated under reduced pressure and the residues redissolved in $CHCl_3$:MeOH (2:1, v/v, 200 μl) and applied to the t.l.c. plates with a mixture of marker compounds. Development was with two solvent systems depending on the possible contents of the eluate; to separate prostaglandin and substrate, we used $CHCl_3$:MeOH; glacial acetic acid, 90:5:5, v/v (Flower & Blackwell, 1976) and to separate AA and glycerides and prostaglandins, we used hexane:diethyl ether:glacial acetic acid, 50:50:1, v/v (Jose & Seale, 1979). After development the plates were dried, markers visualized with iodine vapour and each sample track cut into 1 cm strips. These were placed in scintillation vials with 2 ml MeOH and, after 2 h at room temperature, 8 ml of scintillant fluid added and the radioactivity measured.

Measurement of radioactivity

All radioactive samples were assayed by liquid scintillation methods with external standards used to assess quenching. Radioactivity was expressed as d.p.m.

Other assays

Protein in the lung fractions was estimated by the method of Bradford (1976).

Variation of activity with pH

Incubations at pH 4 and 5 used sodium acetate buffers; at pH 6, Tris maleate and at other pH values, Tris HCl buffers were used, all 10 mM. All pH values were measured at 37°C.

Materials

We are most grateful to Mr J. Dussek, F.R.C.S., Guy's Hospital, London for samples of human lung. The following radioactive compounds were obtained from Amersham International, Bucks.: PC, 1-stearoyl-2-[1-^{14}C]arachidonyl-3-phosphatidylcholine; PE, 1-acyl-2-[1-^{14}C]arachidonyl-3-phosphatidylethanolamine; PI, 1-stearoyl-2-[^{14}C]arachidonyl-3-phosphatidylinositol, all 58–60 mCi mmol^{-1}. Aqueous liquid scintillation cocktail, 'Optiphase Safe', was obtained from LKB (Loughborough). Other materials were obtained as follows; Tris base and marker compounds for t.l.c. from Sigma (Poole); silicic acid (Bio-Sil A), 100–200 mesh (Bio-Rad Laboratories, California, USA); t.l.c. plates, silica gel 60 on aluminium foil (Merck, Darmstadt, F.R.G.). All other chemicals were of Analar grade and supplied by BDH Chemicals (Poole).

Statistical methods

Results given are the mean values from at least six assays from at least three lungs. The paired t test was used to assess the significance of the difference between means and a value of $P < 0.05$ was taken to denote a significant difference.

Results

Analysis of phospholipid hydrolysis

Under the original conditions (pH 7.4 and 5 mM Ca^{2+}), PC and PE were hydrolysed to a single radioactive product, free AA, but PI yielded two labelled metabolites, diglyceride (DG) and, as the lesser component, free AA (Yeats & Bakhle, 1989b). Since variations in pH and other cations might affect these products differently, each product had to be measured separately. For this reason we modified the column chromatography, for PI only, to yield separate eluates for each species; AA, DG and unchanged PI. Because each lung sample was assayed separately, the effects of variations of pH, Ca^{2+} or other ionic factors on phospholipase activity were related to hydrolysis under the original conditions and expressed as a percentage of that value, referred to henceforward as 'control hydrolysis'. The mean (±s.e.mean) phospholipase activity towards PC was 15 ± 2 ($n = 5$ lungs), towards PE, 203 ± 23 ($n = 6$) and for PI, 907 ± 179 ($n = 6$) pmol min^{-1} mg^{-1} protein.

Variation of phospholipid hydrolysis with pH

Because hydrolysis of PC proceeded relatively slowly under our control conditions (about 7% of the rate of PE hydrolysis (see above)), we used only PE as the substrate for PLA_2-like activity. Over the range of pH studied (pH 4–9), PE hydrolysis was fairly constant, the maximum activity ratio being about 3 between the lowest activity at pH 4, about 50% of the control value, and at pH 6, about 150% of control (Figure 1). At the highest pH studied (pH 9), hydrolysis was only slightly reduced from the control value. This pH profile was virtually the same for the two particulate fractions (P_2, microsomal and P_1, mitochondrial and lysosomal).

Hydrolysis of PI was first measured as total hydrolysis, i.e. DG and AA together. Although fractions P_1 and P_2 again showed similar profiles (Figure 2), the magnitudes of the pH-induced changes were greater than with PE. A further difference was that activity towards PI was retained at pH values below 6. The maximum hydrolysis at pH 6 was almost three times control with the P_2 fraction and the minimum at pH 9 was about half the control value for both P_1 and P_2 fractions.

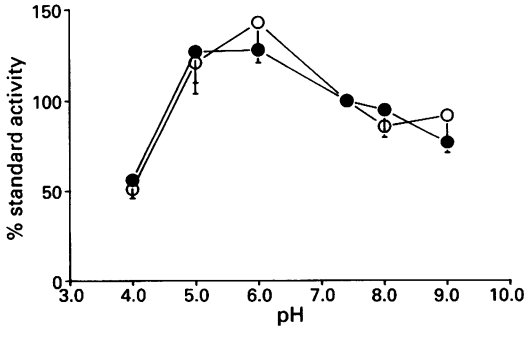

Figure 1 Variation of phosphatidylethanolamine (PE) hydrolysis with pH. The values shown are the mean hydrolysis (with s.e.mean shown by vertical bars), expressed as a percentage of the hydrolysis under control conditions at pH 7.4. Activity of both P_1 (●) and P_2 (○) fractions was almost identically affected by pH with a peak at pH 6 and a minimum value at pH 4.

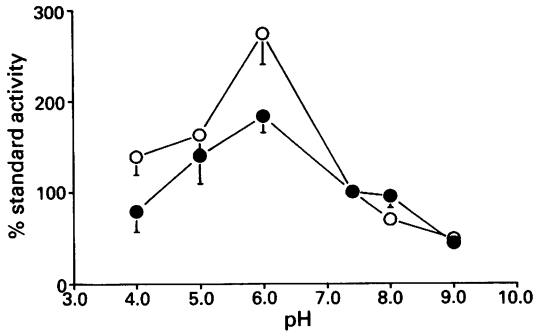

Figure 2 Variation of phosphatidylinositol (PI) hydrolysis by particulate fractions of human lung with pH. The values shown are the mean total hydrolysis, (with s.e.mean shown by vertical bars), measured as arachidonic acid plus diglyceride produced, expressed as a percentage of the total hydrolysis under control conditions at pH 7.4. Activity of both P_1 (●) and P_2 (○) fractions was highest at pH 6 and lowest at pH 9.

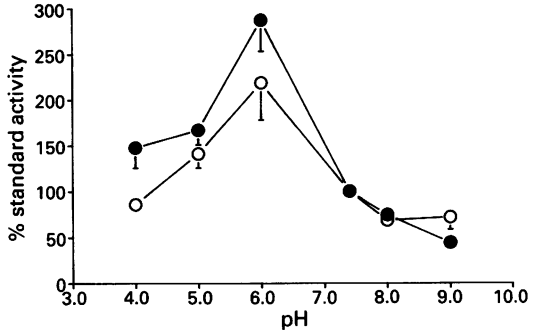

Figure 3 Variation of phosphatidylinositol (PI) hydrolysis by the P_2 (microsomal) fraction of human lung with pH. The values shown are the mean values (with s.e.mean shown by vertical bars) of arachidonic acid (AA, ○) and diglyceride (DG, ●) produced, measured separately. They are expressed as a percentage of the production under control conditions at pH 7.4. Production of DG was well above control levels at low pH but fell more sharply than that of AA at high pH.

Further analysis of PI hydrolysis into AA and DG production separately showed that DG production remained higher than that of AA at pH 6 and that it fell more steeply at high pH, as shown for fraction P_2 in Figure 3. As a result of this disparate response to pH, the AA:DG ratio increased at pH 9, from 0.3 to 0.6 with the P_1 fraction and from 0.2 to 0.3 with the P_2 fraction.

Effects of other cations on phospholipase activity

The control incubation mixture included Ca^{2+} (5 mM; final concentration) as this ion is known to be required by several phospholipases including those derived from lung (van den Bosch, 1982). Increasing the Ca^{2+} concentration to 9.5 mM increased hydrolysis of PC by the P_2 fraction to 140 ± 15% (mean ± s.e.mean) of the control value but did not change PE hydrolysis (89 ± 10%). For PI, while DG production was maintained (103 ± 3%), that of AA fell to 75 ± 9%. However because AA comprised only about 15% of the total hydrolysis products, overall hydrolysis was unchanged (99 ± 5%).

Lowering Ca^{2+} concentrations by the addition of EDTA and EGTA inhibited phospholipase activity markedly (Table 1) but, even with a considerable excess of EDTA (25 mM), the activity was never abolished. This general pattern of response to EDTA and EGTA was shown by the activities in both subcellular fractions. However, with fraction P_1, hydrolysis of PC and PE were equally inhibited by these chelating agents but, with P_2, the microsomal fraction, PE hydrolysis was more sensitive to inhibition. The PLC activity was also inhibited by EDTA, total PI hydrolysis falling to about 10% of normal values. Production of the DG component was more affected, resulting in an increase in the AA/DG ratio to 0.5 in the presence of EDTA, over the control value of 0.2.

Further analysis of the cation dependence of the phospholipase activities, in the P_2 fraction only, was attempted with the calcium salt of EDTA (CaEDTA) and other chelating agents (Table 2). In the presence of CaEDTA (5 mM), PC and PE hydrolysis by the microsomal fraction was strongly inhibited, but that of PI was increased by about 30%, with no change in AA:DG ratio.

Table 1 Phospholipid hydrolysis by particulate fractions of human lung in the presence of EDTA and EGTA

	Hydrolysis of substrate (% control value)		
	PC	*PE*	*PI*
Fraction P_1 (mitochondrial and lysosomal			
Na EDTA; 25 mM	20 ± 4	20 ± 5	—
Na EGTA; 10 mM	38 ± 8	33 ± 8	—
Fraction P_2 (microsomal)			
Na EDTA; 25 mM	38 ± 11	8 ± 2	11 ± 3
Na EGTA; 10 mM	42 ± 11	18 ± 4	—

The values in the Table are the mean (±s.e.mean) results expressed as a percentage of the corresponding control hydrolysis, from at least 6 assays from 3 lungs at each point. All the conditions yielded significant inhibition of hydrolysis. PC phosphatidylcholine; PE phosphatidylethanolamine; PI phosphatidylinositol.

Table 2 Effects of chelating agents on phospholipase activity in the P_2 (microsomal) fraction of human lung

Chelating agent (concentration)	*Hydrolysis of substrate* (% control value) *PC*	*PE*	*PI*
Ca EDTA (5 mM)	30 ± 5	15 ± 3	135 ± 10
o-Phenanthroline			
0.1 mM	159 ± 26	276 ± 23	177 ± 31
1 mM	214 ± 35	214 ± 25	219 ± 19
8-Hydroxyquinoline			
0.1 mM	—	139 ± 25	126 ± 12
1 mM	281 ± 27	182 ± 40	196 ± 37
2,2-Dipyridyl			
0.1 mM	114 ± 13 (NS)	200 ± 12	132 ± 18
1 mM	220 ± 39	177 ± 19	202 ± 28
Desferrioxamine			
1 mM	—	106 ± 10 (NS)	108 ± 2

The values in the Table are the mean(± s.e.mean) results expressed as a percentage of the corresponding control hydrolysis, from at least 6 assays from 3 lungs at each point. All the effects were significant except those marked (NS). Inhibition was only seen with CaEDTA and phosphatidylcholine (PC) and phosphatidylethanolamine (PE) hydrolysis; all of the other effects were stimulatory. PI phosphatidylinositol.

With the four other chelating agents used, a completely different profile emerged (Table 2). For any substrate with any concentration of chelator, hydrolysis catalysed by the microsomal fraction was never inhibited. Two agents, *o*-phenanthroline and 2,2-dipyridyl, were tested at a lower concentration (10 μM) with PC as substrate and were without effect (117 ± 14% and 90 ± 12% of control values respectively). Furthermore, at the higher concentration of chelator (1 mM), hydrolysis was always increased over normal values. There were minor variations within this group of chelating agents; *o*-phenanthroline was effective at 100 μM with all substrates, whereas 8-hydroxyquinoline was effective only at 1 mM. Desferrioxamine was the least effective, increasing PI hydrolysis only slightly at 1 mM. The AA:DG ratio of products of PI hydrolysis was not changed by any of this group of agents.

The effects of these four chelating agents were in marked contrast to those of EDTA and EGTA. However, the former group was used in concentrations only up to 1 mM and the latter two not below 10 mM. It was possible that the discrepant results were due to the different concentration ranges used.

To eliminate this possibility, hydrolysis of PE and PI was measured at low EDTA (1 mM) and high 2,2-dipyridyl (25 mM) concentrations. PE hydrolysis was not changed by either treatment, 99 ± 8% of the control value with 1 mM EDTA and 88 ± 10% with 2,2-dipyridyl. However, PI hydrolysis was increased by 1 mM EDTA (133 ± 5%) and by 2,2-dipyridyl (122 ± 10%) with no change in the AA:DG ratio.

In the light of these results and the report of inhibition of purified PLA_2 by Zn^{2+} ions (Finnen *et al.*, 1986), we investigated the effects of $ZnCl_2$ added to the incubation mixture. Using the P_2 fraction, hydrolysis of PC was measured at 9.5 mM Ca^{2+}, the optimal concentration for this substrate but PE and PI hydrolysis were measured at 5 mM Ca^{2+} as their hydrolysis was not affected by increased Ca^{2+}. As shown in Table 3, addition of $ZnCl_2$ to a final concentration of 100 μM slightly decreased PC hydrolysis but, at the higher concentrations, PC hydrolysis was equal to normal values. However, for PE and PI hydrolysis, the inhibition was more extensive and concentration-related. The AA:DG ratio in PI hydrolysates was clearly affected by Zn^{2+} from a control value of 0.45 (no added Zn^{2+}) to 0.77 at 100 μM and to 1.13 at 1 mM Zn^{2+}, demonstrating a relatively greater inhibition of DG than of AA production.

Table 3 Effect of added Zn^{2+} on phospholipase activity of P_2 (microsomal) fraction from human lung

Concentration of $ZnCl_2$	*Hydrolysis of substrate* (% control value) *PC*	*PE*	*PI*
10 μM	—	43 ± 3*	73 ± 9*
100 μM	85 ± 5*	28 ± 3*	38 ± 7*
1 mM	98 ± 9	28 ± 3*	26 ± 3*

* Different from control value i.e., no added $ZnCl_2$ (= 100%).
The values in the Table are the mean (± s.e.mean) results expressed as a percentage of the corresponding control hydrolysis, from at least 6 assays from 3 lungs at each point. Hydrolysis of phosphatidylcholine (PC) was much less affected by Zn^{2+} than that of either phosphatidylethanolamine (PE) or phosphatidylinositol (PI).

Discussion

In this series of experiments, we have examined the effects of some cations (including H^+) on the phospholipase activities in human lung extracts using, for the most part, the microsomal fraction (P_2). Because our lung samples derived from a genetically wild population, we did not combine samples to give a single 'average' preparation but kept each sample separate. All the assays utilized at least three different lung samples but the three lungs were not the same throughout these experiments. However, within a group of assays, e.g. pH variations, Ca^{2+} or Zn^{2+} variations, the same lung samples were used.

pH dependence

The dependence of PLA_2 activity on pH was assayed only with PE as substrate as the activity towards PC was much lower (see Figure 1 and Table 1). The results demonstrated a pH optimum at about pH 5–6, with relatively higher activity at high pH than at low pH. Both the particulate fractions tested showed a similar profile.

Our results contrast with most of the previous work on membrane-bound PLA_2. Non-lysosomal PLA_2 activities from human neutrophils (Balsinde *et al.*, 1988), rat platelets (Colard *et al.*, 1987; Blache & Ciavatti, 1987) and liver (Victoria *et al.*, 1971; Nachbaur *et al.*, 1972; Newkirk & Waite, 1973) or from polymorphonuclear leukocytes (PMNs) of rat, rabbit and man (Lanni & Becker, 1983; Chang *et al.*, 1985; Alonso *et al.*, 1986), all have pH optima between pH 7.5 and pH 9. Lysosomal PLA_2 from human liver (Loffler & Kunze, 1987) or from rabbit alveolar macrophages (Franson *et al.*, 1973) shows an acid optimum at about pH 4. Although we were able to show a relative concentration of lysosomal marker enzymes in the P_1 fraction, our results on PLA_2 activity in P_1 showed no sign of a pH optimum markedly different from that for the PLA_2 activity in the microsomal fraction (P_2). A closer analogy might be sought in non-human lung PLA_2 but, here also, the membrane-bound enzymes are clearly of neutral-basic pH optima, values ranging between pH 7.5 and pH 9.5 for rat, rabbit and dog lung (Garcia *et al.*, 1975; Longmore *et al.*, 1979; Franson & Weir, 1982; Scott *et al.*, 1987).

Our subcellular fractions may contain a number of PLA_2 enzymes with either acid or alkaline optima which might in combination yield the profiles observed. However, such a mixture might reasonably be different in mitochondrial and microsomal fractions and thus give rise to different pH-activity profiles, in contrast to our results. Another possible explanation is that, because our lung samples derived from patients, i.e. with some pathological condition, our results represent the phospholipase activities in lung responding to a pathological insult, whereas most previous studies have used normal animals or man. However, because other properties of these human lung PLA_2 and PLC activities are similar to those of normal rat lung (Yeats & Bakhle, 1989b), we consider that our samples of human lung probably represent normal tissue and, by extension, the properties of the phospholipases should also reflect that of the normal enzymes. The pH dependence of PI hydrolysis in our lung extracts was more marked with the P_2 fraction, although both fractions exhibited maximum activity at pH 6. A lysosomal PI-specific PLC from rat liver had a pH optimum of 4.8 with very little activity above pH 7.0 (Irvine *et al.*, 1978). Our findings are closer to the soluble PLCs from heart (Low & Weglicki, 1983) and lymphocytes (Carter & Smith, 1987) with maxima at pH 5.5–6.0. In the lymphocyte preparation before purification, there was a minor component with activity at pH 7.0 which may represent a membrane associated form of this PLC (Carter & Smith, 1987). However, our results could as easily reflect a mixture of PLCs with acid and acid-neutral pH optima.

Effects of Ca^{2+} and other cations

In common with many other phospholipase activities, the presence of Ca^{2+} appeared to be essential for maximal activity in human lung extracts. The increased PC but not PE hydrolysis at higher Ca^{2+} concentrations could indicate either different enzymes for each substrate or different binding requirements for the same enzyme. A full examination of Ca^{2+}-dependence is needed for both PLA_2 and PLC activities in these fractions. Drastic reductions in Ca^{2+} with excess EDTA or EGTA lead

to equally drastic reductions in activity towards all three substrates. However the relative resistance to EDTA of P_1 catalysed hydrolysis of PE and of PC hydrolysis by both P_1 and P_2 fractions would be compatible with the presence of a lysosomal, Ca^{2+}-independent PLA_2 activity (Franson *et al.*, 1973).

We attempted to explore the requirement for other cations by using other chelating agents, with greater affinity for transition metal ions. The potentiation of hydrolysis suggests the removal of an inhibitory factor which, on the basis of previous findings (Wolf & Gross, 1985; Finnen *et al.*, 1986) and our own results, could be Zn^{2+} ions. However, 8-hydroxyquinoline, 2,2-dipyridyl and *o*-phenanthroline all potentiated PC hydrolysis, but this hydrolysis was not inhibited by added Zn^{2+} ions. This could be because there were already enough Zn^{2+} ions present in the P_2 fraction to inhibit PC-hydrolysis maximally and addition of further Zn^{2+} ions was thus ineffective. Nevertheless, removal of all Zn^{2+} ions by chelation would still increase PC-hydrolysis. As a corollary, PE and PI hydrolysis would require more Zn^{2+} ions than present in P_2 for maximal inhibition and would thus appear to be more sensitive to added Zn^{2+}. Such an explanation would also be compatible with the lower PC hydrolysis in all lung fractions and with the higher concentrations of 2,2-dipyridyl and *o*-phenanthroline needed to potentiate PC hydrolysis. From our experiments, it is not possible to decide between an interaction with enzyme or with substrate as the mechanism for inhibition by Zn^{2+}, but Finnen *et al.* (1986) favour the former.

In summary, our experiments with human lung phospholipases have shown that phospholipid hydrolysis was maximal at about pH 6. This profile is unlike that of many other PLA_2 activities and may be an intrinsic property of human lung PLA_2s or, possibly, a reflection of pathology. These human lung phospholipases were also dependent on Ca^{2+} and their activity was modified by other cations, including Zn^{2+}. This last finding suggests an additional cationic control mechanism for the generation of arachidonate and diglyceride in lung and perhaps in other tissues. Our results provide more information on the properties of the phospholipases in human lung involved in liberating arachidonate and thus controlling eicosanoid biosynthesis in human lung.

These results have at least two important pharmacological corollaries. First, we can now measure the effect of activators and inhibitors of phospholipases (peptides, cytokines, lipocortins) directly on human lung enzymes without having to infer their effects from experiments with animal tissues. Second, where animal models still must be used, we can now select those species whose phospholipase activities most clearly resemble those in human lung. There are important species differences in the biosynthesis of cyclo-oxygenase products in lung (Bakhle & Ferreira, 1985) and these differences could extend to the phospholipases involved. Both considerations will allow a more accurate analysis of how human lung responds to pathological conditions such as endotoxin shock or immunological challenge and to drug treatments aimed at preventing such responses.

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Inhibition of Na^+-K^+ pump activity by divalent cations in intact peritoneal mast cells of the rat

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1 The inhibition by the divalent cations magnesium, barium and strontium and the trivalent ion lanthanum of the Na^+-K^+ pump in the plasma membrane of rat peritoneal mast cells was studied in pure mast cell populations by measurement of the ouabain-sensitive uptake of the radioactive potassium analogue, ^{86}rubidium ($^{86}Rb^+$).

2 Exposure of the cells to magnesium induced a time- and concentration-dependent decrease in the ouabain-sensitive K^+($^{86}Rb^+$)-uptake of the cells without influencing the ouabain-resistant uptake. The time-dependent decrease was apparent after incubation of the cells for 10 min or more, but no decrease was observed after 2 min incubation when the cells are supposed to be loaded with sodium due to the cell isolation procedure.

3 Barium and strontium caused concentration-dependent decreases in the ouabain-sensitive K^+-($^{86}Rb^+$)-uptake of the cells but the ouabain-resistant uptake was not changed. Half maximum decrease in the ouabain-sensitive K^+($^{86}Rb^+$)-uptake was observed with 1.8 mM magnesium, 1.2 mM barium and 0.7 mM strontium.

4 The trivalent ion lanthanum blocked almost completely the ouabain-sensitive K^+($^{86}Rb^+$)-uptake at a concentration of 1 μM as does 1 mM calcium. Combining either of these ions with magnesium had no further inhibitory effect on the ouabain-sensitive uptake.

5 In conclusion, in addition to the previously suggested modulation by calcium of the activity of the Na^+-K^+ pump, evidence is provided in this investigation that the modulation may be a more general effect of divalent and polyvalent cations present in the extracellular space through their influence on the sodium permeability of the plasma membrane.

Introduction

The secretory responses of mast cells and other tissues are enhanced by digitalis glycosides (Vizi, 1978; Frossard *et al.*, 1983; Powis, 1983; Amellal *et al.*, 1984; 1985 and references therein; Binck *et al.*, 1985). Recently, we characterized the Na^+-K^+ pump mechanism in intact peritoneal mast cells of the rat and provided evidence for modulation of the pump activity by changes in the sodium permeability of the plasma membrane by extracellular calcium (Knudsen & Johansen, 1989a,b).

This investigation was performed in order to examine whether modulation of the pump activity could also be produced by divalent or polyvalent cations other than calcium.

Methods

Isolation of mast cells

Male Sprague-Dawley rats, 320–435 g, were used for the experiments. The rats were killed by decapitation under ether anaesthesia. The mast cells were isolated by differential centrifugation of mixed peritoneal cells in a self-generating gradient of Percoll as previously described (Knudsen & Johansen, 1989a). Following the isolation procedure, the mast cells were washed and suspended in a Krebs-Ringer solution containing Bovine serum albumin, 1 mg ml^{-1}, and glucose, 1 mg ml^{-1}, final pH 7.4. The cells were kept at 4°C during the isolation procedure. A Coulter counter (Model 134, Analys Instrument AB, Sweden) was used to count the number of cells, and inspection of stained smears (Toluidine Blue) was used to determine the fraction of mast cells which was 98.4% (range: 96.5–100%).

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Incubation procedure

Mast cell suspensions pooled from 3 to 6 rats were divided into samples of the same cell density in a final volume of 0.5 ml. Each sample contained between 9.4×10^4 and 2.5×10^5 cells. The samples were equilibrated at 37°C under various experimental conditions before incubation with K^+($^{86}Rb^+$) was carried out in order to determine the Na^+-K^+ pump activity. Specific details for individual experiments are contained in the legends to figures.

Measurement of Na^+-K pump activity

The cells were incubated at 37°C for 5–10 min with and without 1 mM ouabain in a medium containing potassium (4.75 mM) and minute amounts of the potassium analogue $^{86}Rb^+$ used as a tracer. The specific activity of $^{86}Rb^+$ in the samples was mean 1.6 μCi ml^{-1} (range 0.8–1.9 μCi ml^{-1}). The incubation was stopped by addition of 9.5 ml of ice-chilled Krebs-Ringer solution to the cell suspension. In order to remove the extracellular $^{86}Rb^+$ the cell suspensions were washed twice by centrifugation at 600 *g* for 15 min at 4°C and resuspension. Following the washing procedure, cell lysis was performed by adding 1 ml of an aqueous solution of NaOH to the cell suspension (final concentration 47.6 mM). The whole sample was then transferred to counting vials and mixed with Ecoscint. Cellular K^+($^{86}Rb^+$)-uptake was measured in a Mark III Liquid Scintillation Spectrometer (Nuclear Chicago) using the preset window for ^{32}P. Counting efficiency was always greater than 95% and no corrections were made. The specific activity of $^{86}Rb^+$ in the suspending medium was used to calculate the cellular K^+($^{86}Rb^+$)-uptake. The Na^+-K^+ pump activity was equivalent to the cellular uptake of K^+($^{86}Rb^+$) corrected for the ouabain-resistant uptake of K^+($^{86}Rb^+$) taking place in the presence of 1 mM ouabain.

Measurement of cellular histamine release

Mast cells, in a final volume of 0.5 ml, were incubated at 37°C for 45 min with potassium (4.75 mM), in the absence of calcium

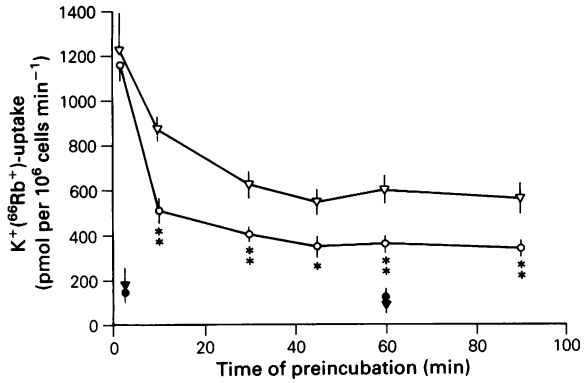

Figure 1 Time courses of changes in cellular $K^+(^{86}Rb^+)$-uptake after preincubation of the cells in presence (○) or absence (▽) of magnesium, 1.2 mM. The preincubation was performed in a calcium-free medium containing potassium, 4.75 mM, and the time of preincubation is indicated on the abscissa scale. Open symbols represent $K^+(^{86}Rb^+)$-uptake into cells preincubated in the absence of ouabain, while filled symbols show the uptake into cells preincubated in the presence of ouabain, 1 mM. The ordinate scale shows cellular $K^+(^{86}Rb^+)$-uptake. Mean values and s.e.means from five experiments are shown. * $P < 0.05$; ** $P < 0.01$).

and magnesium and in the presence of either sodium or lithium. No secretagogue was added. The incubation was terminated by the addition of 1.5 ml of ice-cold Krebs-Ringer solution to the samples. After centrifugation at 220 *g* for 10 min at 4°C, the supernatant was collected for determination of the amount of histamine released. The cell pellet was suspended in 250 µl of a physiological saline solution and boiled for 3 min. After addition of 1.75 ml of Krebs-Ringer solution and centrifugation the supernatant was collected for determination of the residual histamine. This was performed by the fluorimetric method (Shore *et al.*, 1959) omitting the extraction procedure. The histamine release was expressed as a percentage of the total histamine content of the cells.

Statistics

The Mann-Whitney U-test was used for the statistical analysis of the data. $P < 0.05$ was used as the threshold level of significance.

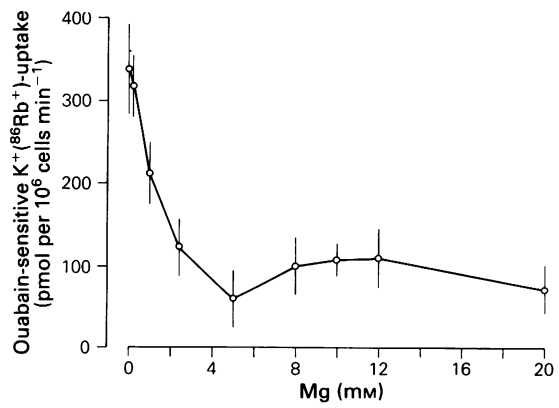

Figure 2 Dose-dependent inhibition of the Na^+-K^+ pump activity by magnesium. The cells were preincubated for 45 min in a calcium- and magnesium-free medium containing potassium 4.75 mM and then exposed to magnesium as indicated on the abscissa scale for 15 min before the activity of the pump was measured. The ordinate scale shows the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake, which is the difference in uptake into cells incubated in presence or absence of ouabain 1 mM. Mean values and s.e.means (vertical lines) from five experiments are shown.

Solutions

The Krebs-Ringer solution was calcium- and magnesium-free and had the following composition (mM): NaCl 136.8, KCl 4.75, Tris-HCl 12.5.

Changes in the concentrations of calcium and magnesium were balanced by changes in the concentration of NaCl in order to maintain isotonicity. In some of the samples in the experiments shown in Figure 6, NaCl was replaced by an equimolar concentration of LiCl.

All solutions contained bovine serum albumin, 1 mg ml^{-1}, and glucose, 1 mg ml^{-1}. The pH was adjusted to 7.4 (room temperature).

Materials

Bovine serum albumin was supplied by Sigma Chemical Company (St. Louis, U.S.A.), glucose by E. Merck (Darmstadt, W. Germany), Percoll by Pharmacia Fine Chemicals (Sweden), Ecoscint by BN Plastics (Helsinge, Denmark) and $^{86}Rb^+$ by Amersham (Buckinghamshire, U.K.). Ouabain (Mecobenzon, Denmark) and all other chemicals were of analytical grade.

Results

Exposure of mast cells to magnesium in a calcium-free medium caused a time-dependent decrease in the cellular uptake of $K^+(^{86}Rb^+)$. Except for the initial value, the decrease was significantly larger than that observed in control samples without magnesium (Figure 1). The initial values of cellular $K^+(^{86}Rb^+)$-uptake observed after a preincubation period of 2 min were approximately 1200 pmol per 10^6 cells min^{-1} both in the absence and presence of magnesium. Following the time-dependent decrease, steady state levels of cellular $K^+(^{86}Rb^+)$-uptake were found after preincubation for 45 min both in the absence and presence of magnesium. The steady state levels were (in pmol per 10^6 cells min^{-1}) 550 and 350, respectively. Magnesium had no effect on the uptake of $K^+(^{86}Rb^+)$ by the cells in the presence of ouabain.

Increasing concentrations of magnesium up to 5 mM caused a gradual decrease in the activity of the Na^+-K^+ pump (Figure 2). Beyond that concentration no further decrease was observed. Additional experiments showed that there was no effect on the ouabain-resistant uptake.

The reduced ouabain-sensitive uptake of $K^+(^{86}Rb^+)$ by mast cells when suspended in a medium containing calcium

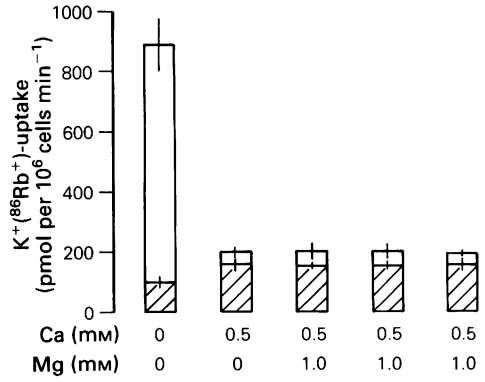

Figure 3 Effect of increasing concentrations of magnesium on the $K^+(^{86}Rb^+)$-uptake by mast cells suspended in a calcium containing medium. Before the measurements of the pump activity were performed, mast cells were preincubated for 60 min in a calcium- and magnesium-free medium containing potassium 4.75 mM and then for an additional period of 15 min with magnesium and calcium present as indicated on the abscissa scale. The ordinate scale shows the cellular $K^+(^{86}Rb^+)$-uptake. Open columns indicate the ouabain-sensitive $K^+(^{86}Rb)$-uptake. Hatched columns indicate the ouabain-resistant uptake, which takes place in the presence of ouabain 1 mM. Mean values and s.e.means (vertical lines) from five experiments are shown.

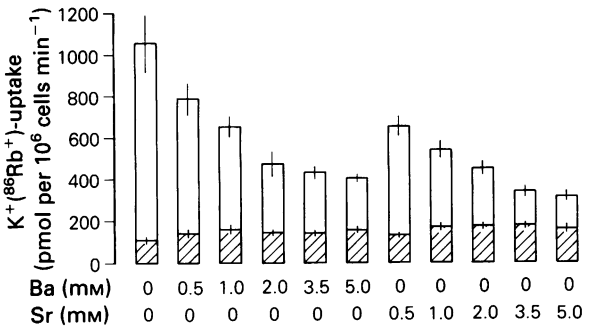

Figure 4 Effect of barium and strontium on the Na^+-K^+ pump activity (open columns) and the ouabain-resistant uptake of $K^+(^{86}Rb^+)$ (hatched columns, 1 mM ouabain). Before the measurements of the pump activity were performed, mast cells were preincubated for 45 min in a calcium- and magnesium-free medium containing potassium 4.75 mM and then for an additional period of 15 min with barium or strontium present as indicated on the abscissa. The ordinate scale shows the cellular $K^+(^{86}Rb)$-uptake. Mean values and s.e.means from five experiments are shown.

(0.5 mM) was not influenced by the presence of magnesium (0.1–10 mM) in addition to calcium (Figure 3). Neither was the ouabain-resistant uptake changed by calcium (0.5 mM) or the combined addition of calcium and magnesium (0.1–10 mM).

A concentration-dependent decrease in the activity of the Na^+-K^+ pump of mast cells was observed when the cells were suspended in a calcium- and magnesium-free solution and exposed to either barium (0.5–5 mM) or strontium (0.5–5 mM) (Figure 4). The pump activity in the absence of barium and strontium was 950 pmol per 10^6 cells min^{-1}. In presence of 5 mM of the ions the pump activity was decreased to (in pmol per 10^6 cells min^{-1}) 250 (barium) and 150 (strontium). These values seem to represent almost the maximal decrease in pump activity in presence of these ions. The ouabain-resistant uptake of $K^+(^{86}Rb^+)$ was not changed by barium and strontium.

The Na^+-K^+ pump activity of the mast cells was decreased to the same extent by lanthanum and calcium (Figure 5). The control value of the pump activity in the absence of calcium and magnesium was 660 pmol per 10^6 cells min^{-1}. In presence of lanthanum or calcium, the activity of the pump was (in pmol per 10^6 cells min^{-1}) 75 and 65, respectively. No further

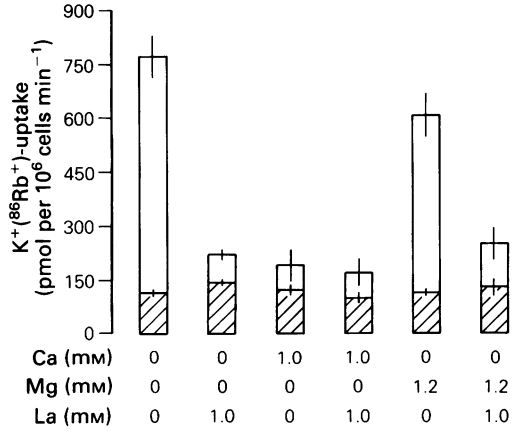

Figure 5 Effect of La^{3+} on the Na^+-K^+ pump activity (open columns) and the ouabain-resistant uptake of $K^+(^{86}Rb^+)$ (hatched columns, 1 mM ouabain). The cells were divided into three pools, which were preincubated for 60 min as indicated on the abscissa scale: (1) in the absence of calcium and magnesium, (2) in the presence of calcium 1.0 mM, and (3) in presence of magnesium 1.2 mM. All three solutions contained potassium 4.75 mM. Then $LaCl_3$ 1 μM was added, and 15 min later measurements of the cellular uptake of $K^+(^{86}Rb^+)$ were performed. Controls samples without $LaCl_3$ were run in parallel. The ordinate scale shows the cellular $K(^{86}Rb)$-uptake. Mean values and s.e.means (vertical lines) from 5–6 experiments are shown.

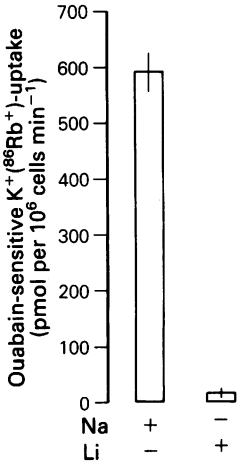

Figure 6 Effect of replacement of sodium with lithium on the Na^+-K^+ pump activity. The cells were preincubated with potassium (4.75 mM) for 45 min in the absence of calcium and magnesium and either in the presence of sodium or lithium. The ordinate scale shows the ouabain-sensitive $K^+(^{86}Rb^+)$-uptake, which is the difference in uptake by cells incubated in absence and presence of ouabain, 1 mM. Mean values and s.e.means (vertical lines) from 5 experiments are shown.

decrease was observed by simultaneous addition of both ions. While magnesium alone decreased the pump activity to 490 pmol per 10^6 cells min^{-1}, the simultaneous addition of magnesium and lanthanum caused a further decrease to 120 pmol 10^6 cells min^{-1}. In contrast, neither of the ions nor any of the ion combinations had any effect on the ouabain-resistant $K(^{86}Rb)$-uptake.

The ouabain-sensitive $K^+(^{86}Rb^+)$-uptake into mast cells incubated in the absence of divalent and trivalent cations was 590 pmol per 10^6 cells min^{-1} (Figure 6). Replacement of sodium with an equimolar concentration of lithium almost abolished the uptake and there was a tendency to an increased release of histamine as previously reported (Cochrane & Douglas, 1976; Atkinson *et al.*, 1979). In presence of sodium the non-stimulated secretion was 5.1% ± 1.9%, and in presence of lithium it was 8.2% ± 3.8% (mean values and s.e.mean from five experiments, $P < 0.1$).

Discussion

The previously observed inhibition by extracellular calcium of the Na^+-K^+ pump activity is confirmed in the present study (Knudsen & Johansen, 1989a,b). The blockade of the pump activity after 45 min exposure of the cells to lithium show that it is dependent on continuous influx of sodium. Thus, it is likely that the inhibition of calcium is due to a decreased influx of sodium and suggestive of a decreased stimulation of the pump from inside the plasma membrane. A decreased sodium permeability, measured as decreased $^{22}NaCl$ efflux due to extracellular calcium in the micromolar to millimolar range, has been observed in outside-out orientated plasma membrane vesicles from cat pancreas (Schulz & Heil, 1979). Recently, inhibition of the Na^+-K^+ pump activity by extracellular calcium in isolated myocytes from guinea-pigs has also been reported (Stemmer & Akera, 1988). These authors observed an increased turnover rate of the Na^+-K^+ pump in the absence of calcium, and this is likely to be caused by an increased stimulation of the pump due to an increased sodium permeability of the plasma membrane. Similarly, the omission of extracellular calcium increased the ouabain-sensitive uptake of $^{86}Rb^+$ into cultured rat vascular smooth muscle cells. This same treatment also caused an increase in the intracellular concentration of sodium (Kino *et al.*, 1985). Increased sodium permeability of rat heart myocytes was also the conclusion reached from the observation that, in the absence of extracellular calcium an elevated ratio of intracellular Na^+-K^+

developed in nonenergized myocytes and the finding that increased ouabain-sensitive lactate production occurred in anaerobic myocytes incubated with glucose (Hohl *et al.*, 1983). The effects of calcium on the membrane permeability may have a structural basis. It has been shown that calcium depletion of the sarcolemma of human adult cardiac myocytes induces separation between the layers of the glycocalyx and changes in the distribution of the intramembrane particles (Frank, 1983).

The inhibition of the pump activity by 1.2 mM magnesium appeared as an enhanced rate of decrease of the cellular uptake of $K^+(^{86}Rb^+)$ and a lower steady state level of $K^+(^{86}Rb^+)$ uptake when compared with the changes in the $K^+(^{86}Rb^+)$ uptake into cells incubated in the absence of both magnesium and calcium. However, the drastic decrease in the activity of the pump observed in presence of calcium 0.6–1.2 mM (Knudsen & Johansen, 1989a,b) was not observed even with higher concentrations of magnesium (Figure 2). This is compatible with the apparent lack of effect of magnesium on the pump activity in presence of calcium 0.5 mM. In contrast to our finding, a significantly lower ^{86}Rb uptake into a human atrial cell line was found in the absence of magnesium compared to the uptake in presence of magensium 0.6–1.2 mM (Borchgrevink & Ryan, 1988). Depletion of cellular magnesium is likely to inhibit the Na^+-K^+ pump activity, since the activity of an isolated enzyme preparation is known to be dependent on magnesium (Skou, 1960). The long term exposure of the human atrial cell line to magnesium-free medium (3.5 h) may cause a cellular depletion of magnesium and thus an inhibition of the uptake of ^{86}Rb as proposed by the authors (Borchgrevink & Ryan, 1988).

The dose-dependent inhibition of the pump activity by magnesium was similar to the effect of strontium and barium. The maximum inhibition was obtained with about 5 mM of each of these ions. This effect was in all three cases a less complete inhibition than that observed with calcium (Figure 3 and Knudsen & Johansen, 1989a,b). By interpolation it may be observed that half maximal decrease in the pump activity occurred in the presence of 1.8 mM magnesium, 1.2 mM barium and 0.7 mM strontium. These values may be compared with the concentration of calcium, 0.2 mM, causing half maximal inhibition under identical experimental conditions (Knudsen & Johansen, 1989a). In contrast, lanthanum ion 1 μM caused the same degree of inhibition of the pump as 1 mM calcium, but the inhibition was not further increased by incubation of the cells with both calcium and lanthanum (Figure 5). The pattern of effects on the pump activity observed with calcium and magnesium was also observed with lanthanum and magnesium, i.e. the effect of the ion combination was the same as that caused by lanthanum alone.

The mechanism previously proposed for the modulation of the pump activity by calcium is a probable explanation of the inhibitory effects of magnesium, barium, strontium and lanthanum. An increased permeability of the plasma membrane to sodium in the absence of these ions and in the absence of calcium increases the activity of the pump by stimulation of the pump from inside the plasma membrane by sodium. The addition of magnesium, barium, strontium or lanthanum to the cell suspension then decreases the permeability of the plasma membrane and thus the pump activity. This view is supported by the observation that in presence of 0.1 mM of either of these ions the permeability to sodium was decreased in plasma membrane vesicles from cat pancreas (Schulz & Heil, 1979). It is also supported by the observed time-dependent inhibition by magnesium of the $K^+(^{86}Rb^+)$-uptake by mast cells (Figure 1). The initial uptake value was not influenced by the presence of magnesium. The high level of $K^+(^{86}Rb^+)$-uptake is due to cellular accumulation of sodium during cell preparation (Knudsen & Johansen, 1989a). The rapid decrease after 10 min exposure of the cells to magnesium at 37°C may be explained by an outward transport of sodium by the pump mechanism having a higher rate than the concomitantly inward transport of sodium.

In conclusion, in addition to the previously suggested modulation by calcium of the activity of the Na^+-K^+ pump, evidence is provided by this investigation that the modulation may be a more general effect of divalent and polyvalent cations present in the extracellular space by their influence on the sodium permeability of the plasma membrane.

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Neuropeptide Y neuromodulation of sympathetic co-transmission in the guinea-pig vas deferens

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1 We examined the neuromodulatory effects of neuropeptide Y (NPY) on purinergic and adrenergic co-transmission in the guinea-pig vas deferens.

2 In superfused vas deferens preparations, NPY (0.3 μM) inhibited the stimulus-evoked overflow of both ATP and [^{3}H]-noradrenaline ([^{3}H]-NA) at 2 Hz, but only the stimulus-evoked release of [^{3}H]-NA at 20 Hz.

3 Postjunctionally, NPY greatly enhanced responses to α,β-methylene ATP and to a lesser extent to exogenous NA.

4 Preparations stimulated in organ baths showed frequency-dependent contractions to field stimulation. NPY abolished responses to field stimulation at low frequency and a small number of pulses. At high frequency (20 Hz), NPY abolished responses elicited by 10 pulses, inhibited responses by 50% at 20 pulses and had little effect on preparations stimulated for 240 pulses.

5 Our study suggests that NPY neuromodulates co-transmission in the vas deferens by inhibiting the release of ATP and NA and that these effects predominate over the postjunctional enhancement by NPY. These results also show that the physiological effect of NPY will be determined both by the frequency at which the nerves are discharging and the duration of their firing.

Introduction

Neuropeptide Y (NPY), a 36 amino acid peptide originally isolated from porcine brain (Tatemoto *et al.*, 1982), has subsequently been shown to co-exist with noradrenaline (NA) and adenosine 5'-triphosphate (ATP) in sympathetic nerves in several tissues including the vas deferens (Lagercrantz, 1976; Fried *et al.*, 1978; 1985; Stjärne *et al.*, 1986). Recently, NPY has been shown to be concomitantly released along with the co-transmitters ATP and NA in the guinea-pig vas deferens (Kasakov *et al.*, 1988).

ATP and NA have been shown to be co-transmitters in the vas deferens. Upon release they produce a biphasic contraction, with ATP mediating the fast twitch phase and NA the sustained second contraction (Sneddon & Burnstock, 1984; Sneddon & Westfall, 1984; Stjärne & Åstrand, 1985). NPY upon release does not produce a contraction, but rather it acts prejunctionally to inhibit NA release, whereas postjunctionally, NPY modulates the action of NA and ATP by enhancing their action on their receptors (Lundberg & Stjärne, 1984; Lundberg *et al.*, 1984; Stjärne & Lundberg, 1986; Stjärne *et al.*, 1986). In this respect, NPY acts as a neuromodulator rather than a transmitter in the vas deferens. This appears to be the case too in many blood vessels, although NPY has been claimed to be a transmitter in some (Lundberg & Tatemoto, 1982; Fried *et al.*, 1986; Edvinsson *et al.*, 1987).

The aim of this study was to examine pre- and postjunctional effects of NPY on both purinergic and adrenergic transmission. As the pre- and postjunctional effects of NPY are antagonistic, it was also hoped to examine which action of NPY predominated at various stimulation parameters in order to ascertain possible physiological roles for NPY.

Methods

Superfusion studies

Male guinea-pigs (250–400 g) were stunned and exsanguinated. The prostatic portions of the vasa deferentia were excised and stripped of connective tissue. Following incubation (60 min) with [^{3}H]-NA, the isolated preparations (10–20 mg wet weight) were placed between pairs of platinum ring electrodes (15 mm apart) and were allowed to equilibrate, with one end tied to the holder and the other attached to a Grass force-displacement transducer (FT03 C). Contractions were measured as changes in isometric tension by a Washington data recording system. The preparations, at an initial tension of 0.5–1 g, were superfused by a Watson-Marlow peristaltic pump at a flow rate of 2.5 ml min^{-1} with a modified Krebs solution (MKS) (mM: NaCl 133, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 2.5, NaH_2PO_4 1.4, $NaHCO_3$ 16.3, glucose 7.7 and ascorbic acid 0.1 and gassed continuously with 5% CO_2 in O_2 at 36°C). After a 90 min equilibration period the superfusate was collected before, during and after stimulation in polypropylene vials for subsequent determination of [^{3}H]-NA and ATP levels. The preparations were stimulated electrically twice, at 9 and 45 min with trains of 240 rectangular pulses of 0.1 ms duration, 50 V, and either at 2 or 20 Hz, delivered by Grass SD9 stimulators. Perfusate was collected for periods corresponding to the length of the stimulus train which was 120 s at 2 Hz and 12 s at 20 Hz. Results are expressed as the ratio of responses to the second stimulation (S_2) versus responses to the first stimulation (S_1), that is, the S_2/S_1 ratio. Control values for S_2/S_1 ratios were obtained when the second stimulation was carried out in the absence of any drug. Experimental data were obtained by superfusion of the preparation with MKS containing NPY, 6 min before the second stimulation. This superfusion continued until the end of the experiment. Tissues from different animals, but from the same batch, were used to assess the effects of NPY.

ATP overflow

ATP overflow from the sympathetic nerves supplying the vas deferens was assayed by the luciferin-luciferase technique described by Stanley & Williams (1969). Briefly, 50 mg of luciferin-luciferase was dissolved in 12.5 ml of sterile water and left in the dark for 2 h to equilibrate. Under these conditions the assay was capable of measuring ATP levels as low as 30 fmol. Before the experiment, a calibration curve for ATP was prepared to be used as a standard. Automatic injection of 200 μl of luciferin-luciferase solution to 100 μl of superfusate or

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standards produced bioluminescence which was measured by a Packard Picolite Luminometer. The MKS was assayed for ATP content. This was used as a blank and subtracted from each sample.

[^3H]-NA overflow

After preparation (described above), the tissues were incubated for 60 min in continuously gassed MKS containing 0.1 nM tritium-labelled noradrenaline ([^{3}H]-NA) (two preparations in 5 ml medium) at 36°C. After being rinsed five times with MKS, the preparations were placed between the stimulating electrodes and superfused to wash out the excess [^{3}H]-NA during the 90 min equilibration period. Samples (2.5 ml for 2 Hz and 0.25 ml for 20 Hz) were added in a ratio of 1:3 to Optiphase mp (LKB) scintillation cocktail and ^{3}H-radioactivity in the samples was counted in a Beckman 7500 liquid scintillation system. The superfusion was stopped immediately after the end of the experiment and the preparations weighed and dissolved in 0.5 ml Optisolv (LKB) tissue solubilizer. The total tissue [^{3}H]-NA radioactivity was counted as described above. The results were calculated as the percentage fractional rate (FR %; mean ± s.e.mean) of the basal release and stimulation-evoked release of [^{3}H]-NA, which were calculated according to Alberts *et al.* (1981). Previous investigations in rodent vas deferens have shown that [^{3}H]-NA is the major constituent of the total radioactivity released upon stimulation of the tissue (Beattie *et al.*, 1986; Kapocsi *et al.*, 1987). Hence, the evoked efflux of [^{3}H]-NA reflected a quasi-physiological release of NA (Reichenbacher *et al.*, 1982).

Pharmacological study in the organ bath

Prostatic portions of the vas deferens were tied to tissue holders and placed in 5 ml organ baths containing MKS and equilibrated for 90 min.

Responses to α,β-methylene ATP and NA After the equilibration period either NA or α,β-methylene ATP (α,β-mATP) at bath concentrations of 30 μM and 1 μM respectively were added for short periods every 15 min. The tissues were washed as soon as a maximum response was reached to prevent desensitization. α,β-mATP was used as it is a stable analogue of ATP. After the control responses to agonists had been established, NPY was added 6 min before a further addition of NA or α,β-mATP. Results were calculated as the percentage response to each agonist relative to the previous response when no drug was present.

Responses to field stimulation After the equilibration period the preparations were electrically stimulated at 50 V, 0.1 ms at varying frequencies and duration of stimulation. The effects of NPY were tested in four different sets of experiments: (1) the frequency of stimulation was varied from 4 to 20 Hz and the number of pulses kept constant at 20. Concentrations of NPY used varied from 0.3 nM to 0.3 μM; (2) the frequency was kept constant at 20 Hz and the number of pulses altered from 10 to 240. Concentration of NPY used was 0.3 μM; (3) the frequency was kept constant at 4 Hz and the number of pulses altered from 10 to 240. Concentration of NPY used was 0.3 μM; (4) the tissues were stimulated every 30 s, 20 pulses at either 4 or 20 Hz. The NPY (0.3 μM) was added between stimulation trains immediately before the next stimulation train.

Analysis of results

The effects of NPY were assessed by the comparison of S_2/S_1 ratios for the experimental data versus S_2/S_1 ratios for the control data. The results for ATP overflow, [^{3}H]-NA overflow and mechanical responses were evaluated by Student's *t* test for unpaired observations. Results from the pharmacological study were evaluated by Student's *t* test for paired observations. In both cases, a probability less than 0.05 was considered significant.

Drugs

Neuropeptide Y (NPY), (−)-noradrenaline HCl (NA), α,β-methylene ATP Li (α,β-mATP) and luciferin-luciferase were obtained from Sigma. Tritiated (−)-noradrenaline [^{3}H]-NA (specific activity 1624.3 Gbq mmol^{-1}) was obtained from New England Nuclear. NPY and α,β-mATP were first prepared as stock solutions in distilled water. NA was prepared in a 0.4% ascorbic acid solution to prevent oxidation. Luciferin-luciferase was prepared in sterile water.

Results

Field stimulation on superfused preparations

Control responses The contractile response of the vas deferens to electrical field stimulation (EFS) at 2 Hz (stimulation period lasting 2 min) exhibited two distinct phases: an initial twitch response, which reached its maximum in 2–3 s and then faded rapidly, and a second slow phase which reached its maximum in 10–15 s and whose amplitude slowly decreased, but which lasted for the duration of the stimulation period. The second stimulation period, 30 min later, exhibited similar contractions but both phases were slightly reduced in amplitude. However, this reduction was not significant (S_2/S_1 ratio for the first phase was 0.81 ± 0.09, and for the second phase was 0.84 ± 0.06, $n = 6$) (Figure 1). During both stimulation periods there was a marked rise in [^{3}H]-NA and ATP overflow over basal levels. [^{3}H]-NA overflow differed little between the two stimulation periods (S_2/S_1 ratio was 1.04 ± 0.04, $n = 8$) (Figure 2). However, ATP overflow during the second phase was reduced (S_2/S_1 ratio was 0.51 ± 0.04, $n = 11$) (Figure 3), indicating that the amount of ATP released during the first period of stimulation is far greater than that actually required to elicit a contraction.

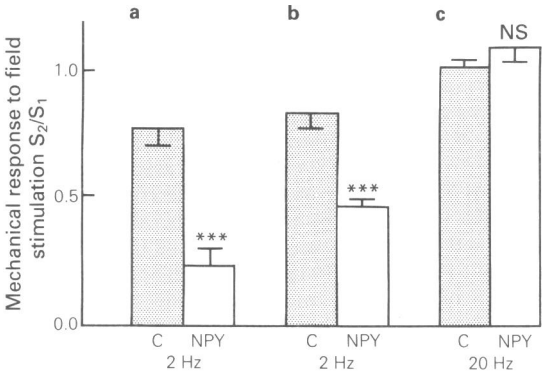

Figure 1 Effects of neuropeptide Y (NPY) on the mechanical response of the guinea-pig superfused vas deferens to electrical field stimulation. Alterations in the mechanical responses are shown by comparing the control S_2/S_1 ratio when no drug was present throughout the experiment with the S_2/S_1 ratio obtained when the drug was added to the superfusate 6 min before the second stimulation. (a) The purinergic response at 2 Hz in the presence of NPY (0.3 μM) compared with control (C, stippled columns). (b) The adrenergic response at 2 Hz in the presence of NPY (0.3 μM) compared with control. (c) The total response at 20 Hz in the presence of NPY (0.3 μM) compared with control. Each column is the mean of at least 6 observations and vertical lines indicate s.e.mean. Significance of drug effects was determined by Student's *t* test for unpaired observations (NS = not significant, *** $P < 0.001$).

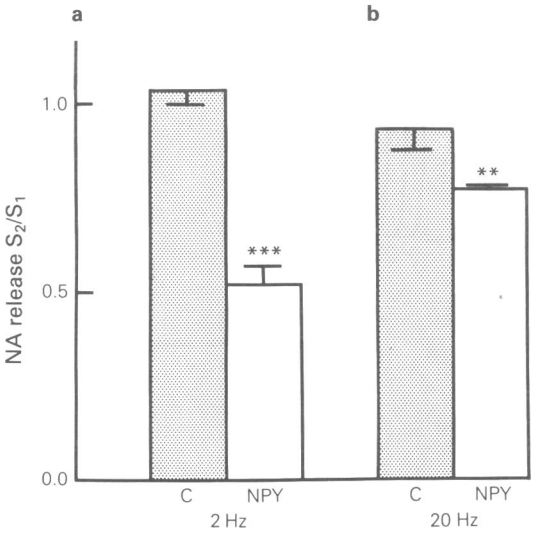

Figure 2 Effects of neuropeptide Y (NPY) on the overflow of [^{3}H]-noradrenaline ([^{3}H]-NA) from the superfused guinea-pig vas deferens induced by electrical field stimulation. Alterations in the overflow of [^{3}H]-NA are shown by comparing the control S_2/S_1 ratio when no drug was present throughout the experiment with the S_2/S_1 ratio obtained when the drug was added to the superfusate 6 min before the second stimulation. (a) Overflow at 2 Hz in the presence of NPY (0.3 μM) compared with control (C, stippled columns). (b) Overflow at 20 Hz in the presence of NPY (0.3 μM) compared with control. Each column is the mean of at least 6 observations and vertical lines indicate s.e.mean. Significance of drug effects was determined by Student's t test for unpaired observations (** $P < 0.01$, *** $P < 0.001$).

At 20 Hz (stimulation period lasting 12 s) the distinction between the two phases was not clear, therefore mechanical responses are given as the overall magnitude of the response and not divided into two distinct phases. The magnitude of the response elicited by the second stimulation was unchanged (S_2/S_1 ratio was 1.02 ± 0.03, $n = 6$) (Figure 1). Again, during both stimulation periods, there was a marked rise in [^{3}H]-NA and ATP overflow over basal levels. [^{3}H]-NA overflow elicited by stimulation was unchanged during the second stimulation (S_2/S_1 ratio was 0.93 ± 0.05, $n = 9$) (Figure 2). ATP overflow was only slightly diminished during the second stimulation (S_2/S_1 ratio was 0.78 ± 0.09, $n = 11$) (Figure 3).

Neuropeptide Y The addition of NPY (0.3 μM) to the superfusate 6 min before the second stimulation had no effect on the baseline tension of the preparations. At 2 Hz NPY inhibited both the purinergic and adrenergic phases of the response. The S_2/S_1 ratio for the purinergic phase of the response was 0.24 ± 0.07 ($n = 6$) (Figure 1) and for the adrenergic phase it was 0.51 ± 0.05 ($n = 10$) (Figure 1). At this frequency NPY inhibited the stimulus-evoked overflow of both [^{3}H]-NA (S_2/S_1 ratio was 0.40 ± 0.09, $n = 10$) (Figure 2), and ATP (S_2/S_1 ratio was 0.28 ± 0.07, $n = 6$) (Figure 3). At 20 Hz NPY had no effect on the overall magnitude of the response (S_2/S_1 was 1.10 ± 0.06, $n = 8$) (Figure 1). At this frequency NPY significantly inhibited the overflow of [^{3}H]-NA (S_2/S_1 ratio was 0.77 ± 0.01, $n = 8$) (Figure 2). However, it was without significant effect on ATP overflow (S_2/S_1 ratio was 0.72 ± 0.08, $n = 6$) (Figure 3).

Responses in the organ bath

Responses to exogenously added NA (30 μM) and α,β-mATP (1 μM) did not show tachyphylaxis, provided that once the tissues had reached their maximum response they were washed immediately. NPY (0.3 μM) added 6 min before the addition of NA produced a significant increase in the response to a value $109 \pm 1\%$ ($n = 6$) (Figure 4) of the control level. NPY produced a larger increase in the response to α,β-mATP to $153 \pm 11\%$ of the control value ($n = 6$) (Figure 4).

The ability of NPY to inhibit responses to electrical field

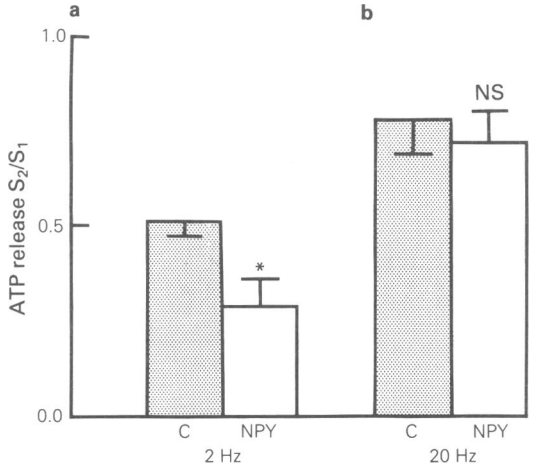

Figure 3 Effects of neuropeptide Y (NPY) on the overflow of ATP from the guinea-pig superfused vas deferens induced by electrical field stimulation. Alterations in the overflow of ATP are shown by comparing the control S_2/S_1 ratio when no drug was present throughout the experiment with the S_2/S_1 ratio obtained when the drug was added to the superfusate 6 min prior to the second stimulation. (a) Overflow at 2 Hz in the presence of NPY (0.3 μM) compared with control (C, stippled columns). (b) Overflow at 20 Hz in the presence of NPY (0.3 μM) compared with control. Each column is the mean of at least 6 observations and vertical lines indicate s.e.mean. Significance of drug effects was determined by Student's t test for unpaired observations (NS = not significant, * $P < 0.05$).

stimulation (EFS), 4, 10 and 20 Hz for 20 pulses, was tested over a range of concentrations (0.3 nM–0.3 μM). In six different tissues NPY, from a threshold concentration of 3 nM, inhibited responses to EFS in a concentration-dependent manner (Table 1). Thus, NPY (0.3 μM) added 6 min before the

Table 1 % inhibition produced by neuropeptide Y (NPY) of responses to electrical field stimulation at various frequencies (0.1 ms, 50 V, 20 pulses)

	NPY concentration			
Frequency	0.3 nM	3 nM	30 nM	0.3 μM
4	17 ± 5	22 ± 2	78 ± 11	100 ± 0
10	5 ± 3	12 ± 4	38 ± 7	51 ± 11
20	5 ± 3	8 ± 3	31 ± 5	35 ± 5

Values are mean $\pm$ s.e.mean, $n = 4$.

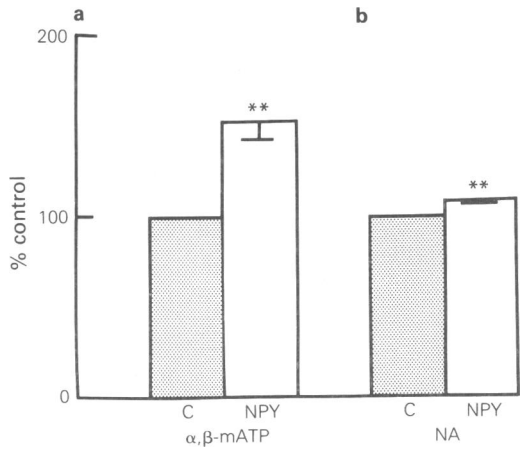

Figure 4 Effects of neuropeptide Y (NPY) on the response of guinea-pig isolated vas deferens to noradrenaline (NA) and exogenous α,β-methylene ATP (α,β-mATP). Responses to drugs are compared with the control value (C, stippled columns). (a) Responses to α,β-mATP in the presence of NPY (0.3 μM). (b) Responses to NA in the presence of NPY (0.3 μM). Each column is the mean of 6 observations and vertical lines indicate s.e.mean. Significance of drug effects was determined by Student's t test for paired observations (** $P < 0.01$).

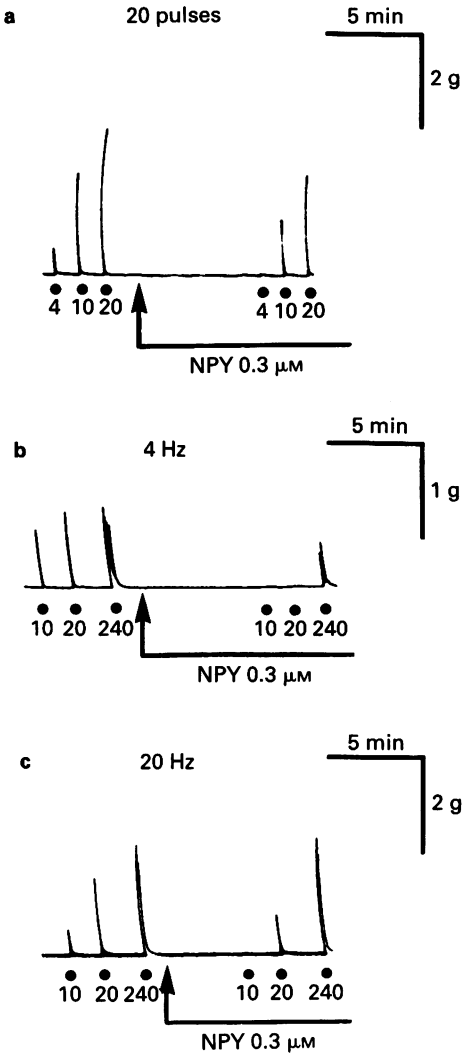

Figure 5 Effects of neuropeptide Y (NPY, 0.3 μM) on responses to electrical field stimulation (EFS) on isolated vas deferens in the organ bath. (a) Effect of NPY on responses with constant pulse number and with the frequency varying from 4 to 20 Hz. (b) Effect of NPY on responses at constant frequency (4 Hz) and the number of pulses varying from 10 to 240. (c) Effect of NPY on responses at constant frequency (20 Hz) and the number of pulses varying from 10 to 240.

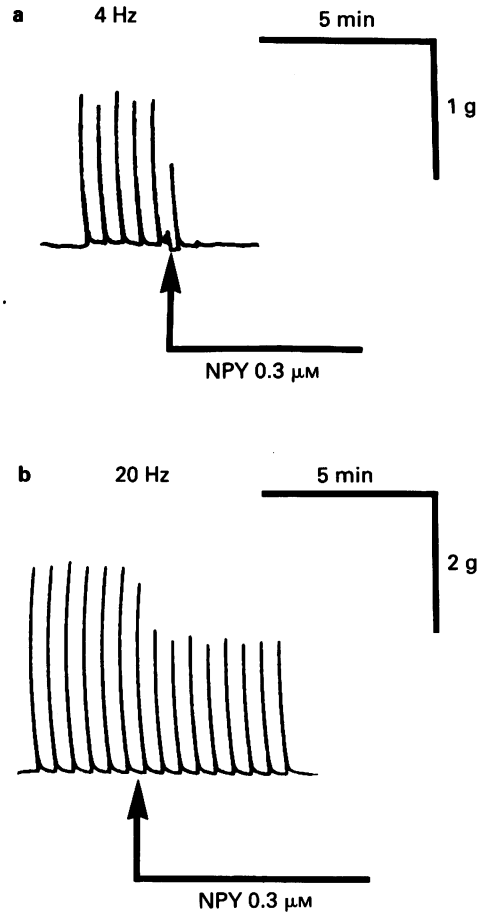

Figure 6 Effects of neuropeptide Y (NPY, 0.3 μM) on responses to continuously stimulated isolated vas deferens in the organ bath. (a) Effects of NPY added between stimulation trains at 4 Hz, 20 pulses. (b) Effects of NPY added between stimulation trains at 20 Hz, 20 pulses.

frequency-response curve, obtained by use of 20 pulses, abolished responses to 4 Hz, but inhibited responses at 10 and 20 Hz (Figure 5). NPY (0.3 μM) abolished responses to 4 Hz, using 10 and 20 pulses, and inhibited responses to 50% of control at this frequency for 240 pulses (Figure 5, Table 2). Against stimulation at 20 Hz, NPY abolished responses to 10 pulses, inhibited responses to 20 pulses to about 50% of the control level and slightly enhanced responses to stimulation for 240 pulses (Figure 5, Table 2).

Table 2 % inhibition produced by neuropeptide Y (NPY, 0.3 μM) of responses to electrical field stimulation (4, 20 Hz, 0.1 ms, 50 V, 10–240 pulses)

	Number of pulses		
Frequency	10	20	240
4	100 ± 0	100 ± 0	48 ± 6
20	100 ± 0	51 ± 5	−5 ± 2

Values are mean ± s.e.mean, $n = 4$.

The effects of NPY (0.3 μM) on six tissues stimulated every 30 s, 20 pulses at either 4 or 20 Hz, was tested. The NPY was added immediately before the next stimulation train. By employment of these parameters, NPY was shown to inhibit responses to EFS immediately. After 60 s, responses to 4 Hz were abolished, whereas at 20 Hz responses were inhibited to about 65% of the control level (Figure 6).

Discussion

This study presents direct evidence that NPY is capable of modulating purinergic and adrenergic transmission both pre- and postjunctionally in the guinea-pig vas deferens. This is in agreement with an earlier study by Lundberg *et al.* (1984) in the guinea-pig, which also demonstrated that NPY inhibited adrenergic and purinergic transmission in the vas deferens by a prejunctional action.

There has been much discussion on the possible physiological roles of NPY in sympathetic transmission. One popular hypothesis, especially for blood vessels, is that NPY promotes economy of the motor transmitters ATP and NA. This is achieved in that the initial effect of NPY has been suggested to be an enhancement of the responses of the transmitters via a postjunctional effect and a secondary delayed prejunctional effect to inhibit the release of the co-transmitters, ATP and NA (Stjärne *et al.*, 1986).

However, the results in this study on the guinea-pig vas deferens do not fit in with this hypothesis. NPY added during a stimulation train showed no enhancement of response, but rather a successive inhibition of responses at both low and high frequency. Our results show that at nearly all the stimu-

lation parameters employed, NPY caused an inhibition of sympathetic transmission, indicative that the prejunctional inhibition predominated over postjunctional enhancement. Only at 20 Hz and 240 pulses was NPY shown not to inhibit, but to enhance slightly responses to EFS.

Another possible mechanism of action for NPY is that the postjunctional enhancement of transmitter action predominates at low concentrations of NPY and that prejunctional inhibition is not seen until higher concentrations of NPY are used (Wong-Dusting & Rand, 1988). However, this was shown not to be the case in this study, as over the range of concentrations tested (0.3 nM–0.3 μM), NPY was not shown to enhance responses. What was shown was that from a threshold concentration of about 3 nM, NPY concentration-dependently inhibited responses to EFS. Similar results were found in the mouse vas deferens (Stjärne *et al.*, 1986), where it was demonstrated that even at low concentrations NPY did not enhance responses to EFS, although in this tissue postjunctional enhancement was more marked than in the present study. This is in contrast to the situation in many blood vessels where NPY has been shown to enhance responses to EFS, but to inhibit the release of NA (Pernow *et al.*, 1986; Wong-Dusting & Rand, 1988).

The inhibition of transmission by NPY has been shown in this study to be dependent on both the frequency and duration of stimulation. Thus, NPY inhibition is maximal at low frequency and a low number of pulses. This is likely to be due to the decreased modulation of transmitter release seen at higher frequencies and duration of stimulation for both purinergic (Ellis & Burnstock, 1988) and adrenergic transmission (Vizi, 1979).

The question still remains to be answered on the physiological function of NPY neuromodulation. Lundberg *et al.* (1986) found that NPY release from pig spleen was favoured by high frequency and short bursts of stimulation, although Lundberg and his colleagues still demonstrated that NPY release was seen at low frequency (2 Hz) stimulation. NPY release has also been shown to occur from the guinea-pig vas deferens at 9 Hz, 540 pulses (Kasakov *et al.*, 1988). It therefore seems likely that NPY is co-released with ATP and NA over a range of stimulation frequencies and that the physiological effect of NPY in the guinea-pig vas deferens will predominantly be one of inhibition of sympathetic transmission.

The observed action of NPY is likely to be the resultant enhancement of transmission via its postjunctional action and reduction of transmission via its prejunctional action. The resultant response may depend on the geometry of the neuroeffector junction. Where the minimum neuromuscular separation of the junction is relatively large (60–600 nm), as is the case in blood vessels, and the co-transmitters need to diffuse a long way to reach receptors on the effector cells, one could argue that it makes physiological sense to have a mechanism that enhances the postjunctional actions of the transmitters with NPY, a more slowly degradable molecule. On the other hand, where the junction is narrow (10–20 nm), for example in the vas deferens, one could argue that inhibition of transmitter release is a more physiologically viable action for NPY release from the nerve. Thus the physiological role for NPY at sympathetic junctions could be considered as a device for conservation of co-transmitters, enhancing transmitter action in the blood vessels and inhibiting their release in densely innervated tissues such as the vas deferens.

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Endogenous nitric oxide as a modulator of rabbit skeletal muscle microcirculation *in vivo*

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1 Intravital microscopy of rabbit tenuissimus muscle microvasculature was used for *in vivo* studies of the role of endogenous nitric oxide (NO) in local vascular control. Derivatives of arginine were applied topically in order to modulate the formation of NO from L-arginine.

2 L-N^G-monomethylarginine (L-NMMA) (10–100 μM), but not D-N^G-monomethylarginine (D-NMMA), dose-dependently reduced microvascular diameters. The vasoconstriction induced by L-NMMA (100 μM) was prevented by pretreatment with L-arginine (1 mM) but not with D-arginine (1 mM). Intravenous infusions of L-arginine (300 mg kg^{-1}) reversed the effect of L-NMMA (100 μM). L-Arginine or D-arginine applied topically at 1 mM *per se* had no effect on microvascular diameters.

3 Vasodilatation by acetylcholine (0.03–3 μM) was significantly inhibited by L-NMMA (100 μM), whereas vasodilatation by adenosine (0.1–100 μM) or sodium nitroprusside (100 nM) was not affected.

4 The hyperaemic response after tenuissimus muscle contractions induced by motor nerve stimulation was unaffected by the presence of L-NMMA (100 μM).

5 Aggregates of platelets and white blood cells were seen in venules during superfusion with L-NMMA (100 μM), but not with D-NMMA (100 μM).

6 Our results suggest that endogenous NO formed from L-arginine is a modulator of microvascular tone and platelet and white cell-vessel wall interaction *in vivo*. Nitric oxide does not, however, appear to play a role in the mediation of functional hyperaemia in this tissue.

Introduction

Since Furchgott & Zawadzki (1980) reported that acetylcholine-induced vasodilatation is mediated by an endothelium-derived relaxing factor (EDRF) it is becoming increasingly clear that the endothelium plays a crucial role in the regulation of vascular tone. Vasodilatation by a number of endogenous vasodilators (e.g. histamine, bradykinin, thrombin, adenosine 5'-triphosphate (ATP), acetylcholine and substance P) (Furchgott, 1983) and flow-dependent dilatation (Holtz *et al.*, 1984) are now known to be mediated via EDRF, which induces vascular smooth muscle relaxation by increasing intracellular levels of guanosine 3':5'-cyclic monophosphate (Rapoport & Murad, 1983).

Recently, EDRF has been identified as nitric oxide (NO) (Ignarro *et al.*, 1987; Kahn & Furchgott, 1987; Palmer *et al.*, 1987), which is produced by vascular endothelium (Amezcua *et al.*, 1988; Sakuma *et al.*, 1988). Macrophages also produce nitrite and nitrate (Iyengar *et al.*, 1987), which are now known to be formed via the generation of NO (Marletta *et al.*, 1988). More recently the central nervous system (Garthwaite *et al.*, 1988; Knowles *et al.*, 1989) and neutrophils (Rimele *et al.*, 1988; McCall *et al.*, 1989) have also been shown to produce NO.

The NO formed from these different sources is synthesized from the amino acid L-arginine, and in all studied tissues L-N^G-monomethylarginine (L-NMMA) is a stereospecific inhibitor of the enzyme(s) responsible for this synthesis (Furchgott & Vanhoutte, 1989; Moncada *et al.*, 1989). Using L-NMMA, Rees *et al.* (1989) demonstrated that endothelium-derived NO plays a role in the regulation of blood pressure and in the hypotensive action of acetylcholine in the anaesthetized rabbit.

We have used L-NMMA to study the involvement of endogenous NO in the regulation of skeletal muscle microcirculation under resting conditions and in the mediation of functional hyperaemia *in vivo*.

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Methods

Male New Zealand White rabbits (0.8–1 kg, $n = 24$) were anaesthetized with 20% Urethane (1.5 g kg^{-1} b.w., i.v.). Breathing was facilitated by the insertion of a tracheal cannula. Catheters were inserted in the carotid artery and the jugular vein for blood pressure recordings and administration of additional anaesthetic, respectively.

The tenuissimus muscle of the left hindleg was prepared for intravital microscopy according to Lindbom *et al.* (1982). Briefly, an incision was made along the saphenous vein, the fascia was cut and the overlying tissue lifted to expose the tenuissimus muscle. An incision caudally to the saphenous vein was made for the insertion of a transillumination system. Intravital microscopy was performed by means of a Leitz Biomed microscope with a movable stage and a salt water immersion lens (SW X25, NA 0.60).

The muscle was continuously superfused with a modified Krebs-Henseleit solution (composition, mM: KH_2PO_4 1.2, $NaHCO_3$ 25.0, NaCl 118.4, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.19) at 37°C, equilibrated with 5% CO_2 in nitrogen. The hind part of the animal was kept in a sealed plastic box continuously flushed with nitrogen so as to keep tissue oxygen tensions at normal, relatively low levels. Drugs were dissolved in water and administered diluted in the superfusate proximal to the muscle by a microinjection pump (CMA 100, Carnegie Medical, Stockholm). For intravenous administration, drugs were dissolved in 1 ml of superfusion medium and administered via the jugular cannula.

To obtain access to the tenuissimus motor nerve, an incision was made slightly caudal to the left hip joint. The sciatic nerve bundle was exposed under the anterior limit of the biceps femoris muscle. The nerve to the tenuissimus muscle

was isolated, and crushed proximally between a pair of forceps. Platinum electrodes were inserted under the nerve and insulated by a plastic cover. The electrodes were connected to a Grass Instruments S4 stimulator via a stimulus isolation unit.

The vascular structures were filmed on videotape by a Panasonic VW-1550 videocamera and a Panasonic AG-6010 videorecorder, yielding a final magnification on the TV screen of 1250×. Vessel inner diameter was measured off-line from the videotape recordings.

Measurements of red cell velocity in the arterioles was performed on-line by means of cross correlation technique (Wayland & Johnson, 1967). Two small fibreoptic filaments were interposed in the light path of the phototube immediately before the camera, thus guiding the two samples of light to two photomultiplier diodes. The time delay between identical photometric signals was computed by a cross-correlator, IPM Velocity Tracker Mod-102B. The volume flow was calculated from the velocity and diameter values.

The preparation was allowed to rest for 30 min to obtain stable microcirculatory conditions. After a 1 min control period, drugs were added to the superfusion solution and allowed to exert their effects until a steady state had been obtained (6–20 min). The mean of 6 diameter measurements during 1 min was used for further calculations. Dose-response curves were cumulative.

For studies of functional hyperaemia the animal was positioned in the experimental setup and allowed to rest for 30 min to obtain stable microcirculatory conditions. Control values were obtained by measuring vascular parameters for 2 min immediately before motor nerve stimulation. Stimulation (5 Hz, 0.05 ms, 0.5–5 V) was then applied for 60 s. Voltage was set at 1.0 V above the threshold for muscular contractions as observed macroscopically. During the 1 min stimulation no measurements could be made due to the movements of the muscle. Measurements were obtained every 10 s during the first 2 min following stimulation, thereafter at every 30 s up to 5 min and at 10 and 15 min after the end of stimulation. The paired t test was used for statistical calculations of data. Data are expressed as mean ± s.e.mean.

Drugs

Urethane, acetylcholine, adenosine, L-arginine hydrochloride and D-arginine hydrochloride were purchased from Sigma, St. Louis, Missouri, U.S.A.; heparin from Kabi Vitrum, Stockholm, Sweden; L-N^G-monomethylarginine (L-NMMA) and D-N^G-monomethylarginine (D-NMMA) were synthesized as described (Patthy *et al.*, 1977), and used as acetate salts. Sodium nitroprusside was from F. Hoffman-La Roche & Co., Basel, Switzerland.

Results

L-NMMA (10–100 μM), applied topically, caused dose-dependent reductions of vascular diameters in the skeletal muscle microcirculation. At 100 μM the diameter of transverse arterioles (control diameter 8–25 μm) and their first order side branches, the terminal arterioles (control diameter 2–7 μm), decreased to 64 ± 4% ($P < 0.01$, $n = 9$) and 80 ± 6% of control ($P < 0.05$, $n = 6$), respectively (Figure 1). Blood flow in transverse arterioles was reduced to 48 ± 8% of control ($P < 0.005$, $n = 8$) by the presence of L-NMMA (100 μM). Venules (control diameter 10–40 μm) within the microvascular network of the tenuissimus muscle constricted (to a diameter of 82 ± 6% of control, $P < 0.05$, $n = 10$) in response to L-NMMA (100 μM). The maximal effect of each concentration was obtained within 15 min after starting the superfusion. D-NMMA (100 μM) did not affect the tone of arterioles (Figure 2) or venules (data not shown).

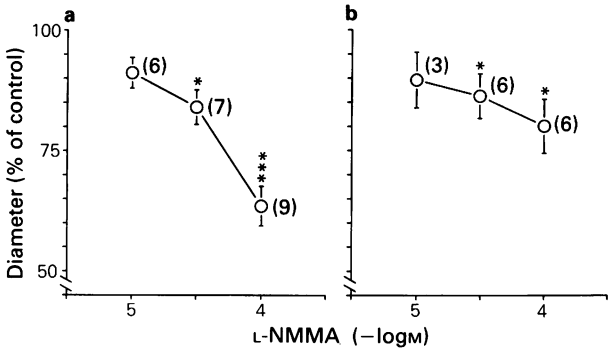

Figure 1 Log dose-response curves for the vasoconstriction induced by topically applied L-N^G-monomethylarginine (L-NMMA) (10–100 μM) in transverse (a) and terminal arterioles (b) of the rabbit tenuissimus muscle. Data (means with s.e.mean shown by vertical bars) are values obtained at the end of a 15 min superfusion and expressed as % of control. The asterisks denote a significant difference (*$P < 0.05$ and ***$P < 0.001$) from control values. Numbers in parentheses indicate number of observations.

The natural amino acid L-arginine (1 mM), administered topically 15 min prior to and during superfusion with L-NMMA (100 μM), prevented the constrictor effect of L-NMMA. In contrast, topical D-arginine (1 mM), the unnatural isostere, did not alter the vasoconstrictor effect of L-NMMA (Figure 2). Topically applied L-arginine or D-arginine (1 mM) *per se* were without effect on transverse arteriolar diameters (98 ± 2%, $n = 4$ and 97 ± 1% of control, $n = 3$, respectively).

In four experiments, L-arginine (300 mg kg^{-1}) was administered systemically via a cannula in the jugular vein after the vasoconstrictor effect of topical L-NMMA (100 μM) had been established. The vasoconstriction by L-NMMA (77.5 ± 3% of control diameter in transverse arterioles, $P < 0.01$) was promptly reversed upon i.v. infusion of L-arginine (to 94 ± 4% of control diameter). The infusion of L-arginine had no discernible effect on systemic blood pressure.

Topical application of acetylcholine (0.03–3 μM) evoked dose-dependent arteriolar dilatation which was progressively

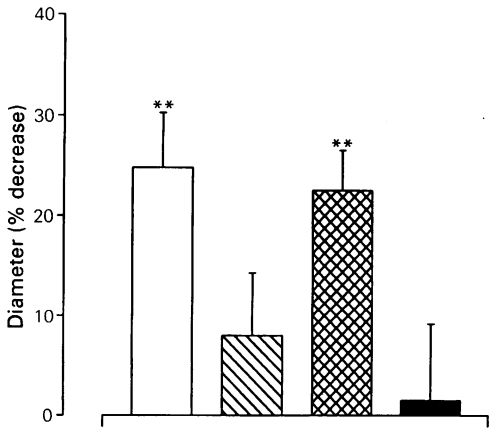

Figure 2 Effects of arginine derivatives on diameters of transverse arterioles in rabbit tenuissimus muscle. Data expressed as % decrease from control diameters induced by 15 min of topical superfusion of either L-N^G-monomethylarginine (L-NMMA) alone (100 μM; open column), or combined with L-arginine (1 mM; hatched column) or D-arginine (1 mM; crosshatched column). Also shown is the lack of effect of D-NMMA (100 μM; solid column). Mean values with s.e.mean shown by vertical bars, $n = 4$. The asterisks denote significant differences from control values (**$P < 0.01$).

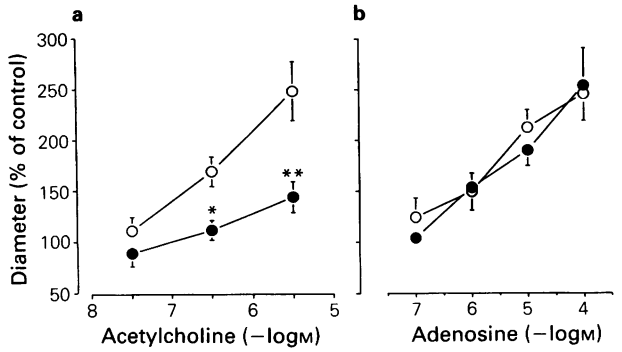

Figure 3 Log dose-response curves for the vasodilatation induced by acetylcholine (a) and adenosine (b), expressed as diameter relative to control, in transverse arterioles in the absence (○) and presence (●) of L-N^G-monomethylarginine (100 μM). Mean values with s.e.mean indicated by vertical bars, $n = 5$–6. The asterisks denote significant differences between the two curves (**$P < 0.01$; *$P < 0.05$).

antagonized by L-NMMA (100 μM) (Figure 3a). In contrast, the dose-response curve for vasodilatation induced by topical adenosine (0.1–100 μM) was not significantly altered by the presence of L-NMMA (100 μM) (Figure 3b). Likewise, L-NMMA (100 μM) failed to affect sodium nitroprusside-induced dilatation of transverse and terminal arterioles.

The effect of L-NMMA treatment on functional hyperaemia was investigated by use of motor nerve stimulation (1 min at 5 Hz, 0.05 ms and 0.5–5 V) which induces selective skeletal muscle contractions in the tenuissimus and a submaximal hyperaemic response (Persson *et al.*, 1988). During resting conditions the blood flow was 1.3 ± 0.7 nl s^{-1} (nanoliters per second) in transverse arterioles. After stimulation the blood flow increased to a peak flow of 7.6 ± 4.6 nl s^{-1} (507 ± 63% of control, $P < 0.001$, $n = 7$) in transverse arterioles and returned to resting values within 15 min. After motor nerve stimulation in the presence of topical L-NMMA (100 μM), peak blood flow increased to 6.1 ± 3.7 nl s^{-1}, a value not significantly different from control stimulation. Furthermore, L-NMMA did not alter the subsequent post-contraction hyperaemia (Figure 4).

In resting conditions some leukocytes moving off the axial stream can be seen to roll along the venular endothelium or even stick to it for a short while. Topical application of L-NMMA (100 μM) did not change the number of cells rolling or sticking. However, during superfusion with L-NMMA, aggregates of white blood cells and platelets were occasionally formed, adhering to the vessel wall and sometimes occluding the vessel before they were spontaneously washed away by the blood stream. Aggregates, which were observed only in venules, were formed at a rate of 3–6 per 10 min.

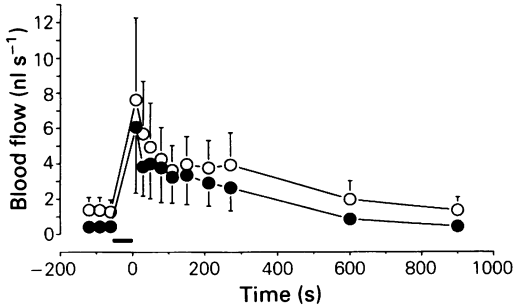

Figure 4 Blood flow in transverse arterioles of tenuissimus muscle, including a brief period (60 s as indicated by horizontal bar) of motor nerve stimulation (0.05 ms, 5 Hz, 0.5–5 V) in the absence (○) or presence (●) of L-N^G-monomethylarginine (100 μM). Mean values with s.e.mean indicated by vertical bars, $n = 7$.

Discussion

Recent studies have shown that L-arginine is the precursor of endothelium-derived NO and that its derivative L-NMMA is a stereospecific inhibitor of NO formation (Palmer *et al.*, 1988; Sakuma *et al.*, 1988). Thus, the findings that L-NMMA, but not D-NMMA, dose-dependently reduced microvascular diameters and that L-arginine, but not D-arginine, either by topical pretreatment or intravenous administration, abolished the effect of L-NMMA in skeletal muscle microcirculation strongly indicate the presence of endogenous NO derived from L-arginine in the rabbit skeletal muscle microcirculation. The stereospecific interaction by L-arginine and its methylated derivative L-NMMA on the putative NO formation is in good agreement with earlier studies (Palmer *et al.*, 1988; Sakuma *et al.*, 1988). The finding that L-arginine itself had no effect on vascular parameters implies either that the synthesis of NO from L-arginine is a substrate-inhibited process or that there are sufficient amounts of L-arginine to saturate the NO-forming enzyme in the anaesthetized rabbit (Rees *et al.*, 1989). Another possibility is that endothelium-dependent vasodilators might act via an allosteric activation of the enzyme. The arteriolar constriction *in vivo* by topical L-NMMA, observed in our experiments emphasizes that the blood pressure-increasing effect of systemic L-NMMA (Rees *et al.*, 1989) must, to a large extent, be dependent on an actual change in peripheral vascular tone rather than on alterations in central cardiovascular regulation.

The inhibition of acetylcholine-induced vasodilatation by L-NMMA in the present study agrees well with *in vitro* studies in which NO was detected in the effluent from the acetylcholine-treated rabbit heart (Amezcua *et al.*, 1988) and from *ex vivo* measurements from vascular tissue obtained from animals treated with L-NMMA (Rees *et al.*, 1989). In the present study, the major part of the acetylcholine-induced response was inhibited, although some of the response remained (Figure 3a). This might be attributable either to an insufficient local concentration of L-NMMA or to the presence of an endothelium-derived hyperpolarizing factor, different from NO (Feletou & Vanhoutte, 1988; Chen *et al.*, 1988).

Vasodilatation by nitroprusside or adenosine was unaffected by the presence of L-NMMA, suggesting that vasoconstriction by L-NMMA was not due to a general, unspecific smooth muscle contractile action, and confirming an intact responsiveness of the vascular smooth muscle to nitrous vasodilators such as NO in the presence of L-NMMA (Rees *et al.*, 1989).

Endogenous adenosine influences basal vascular tone and partly mediates functional hyperaemia in the tenuissimus muscle (Gustafsson *et al.*, 1987; Persson *et al.*, 1988). Since L-NMMA failed to modify the vasodilatation by exogenous adenosine, the L-NMMA effect on vascular tone must be independent of basal adenosine action, which is in agreement with the suggestion that adenosine is an endothelium-independent vasodilator (Furchgott, 1983). Although adenosine partly mediates functional hyperaemia in the tenuissimus, the greater part of the hyperaemic response is intact in the presence of specific and effective adenosine antagonists (Persson *et al.*, 1988). Since L-NMMA lacked effect on functional hyperaemia, NO appears a less likely candidate as a mediator in the non-adenosine part of the hyperaemic response, either directly or as a consequence of adenosine release. However, additional studies are required to exclude such a role for NO.

In vitro data have shown that endothelium-dependent relaxation is more prominent in arteries than in veins. In agreement with this, our data indicate that the resting tone of venules in the microcirculation *in vivo* is dependent on endothelium-derived NO, but to a lesser extent than that of arterioles. The aggregates induced by L-NMMA in venules were similar to those observed during application of substance P. Such aggregates have been shown to consist of leukocytes and platelets (Öhlén *et al.*, 1989). This suggests either that NO

has an antiaggregatory role or that a charged compound like L-NMMA can induce release of tachykinins. Since D-NMMA lacked the aggregatory effect, an unspecific release of tachykinins seems unlikely. Furlong *et al.* (1987) and Hogan *et al.* (1988) have shown that EDRF has an antiaggregatory effect on platelets *ex vivo*.

In summary, our observations indicate that endogenous NO might be a significant modulator of the microvascular tone in skeletal muscle. Furthermore, our data extend the observations which indicate that L-arginine is the precursor for endothelium-derived NO, and that vasodilatation induced by acetylcholine, but not adenosine or nitroprusside, is dependent on the generation of NO from L-arginine.

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Effects of butanedione monoxime on neuromuscular transmission

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1 The amplitude of endplate potentials was increased by concentrations of butanedione monoxime (BDM, 5–20 mM) that typically caused muscle paralysis.

2 Although BDM slowed the decay of spontaneous miniature endplate currents, the effect was insuffient to explain most of the large increase in amplitude of endplate potentials.

3 The quantal content of endplate potentials was increased by BDM in a reversible, concentration-dependent manner.

4 The frequency of miniature endplate potentials was not changed by 10 mM BDM in the presence of normal or raised potassium concentrations, indicating that BDM does not change quantal content by a direct effect on calcium channels or on steady-state intracellular calcium concentration.

5 A change in the time course of the extracellularly recorded nerve terminal action potential caused by BDM was similar to the change produced by 4-aminopyridine (4-AP).

6 The increase in quantal content produced by BDM was only slightly reduced in the presence of 1 mM tetraethylammonium (TEA) but was significantly reduced in the presence of 0.5 to 1 mM 4-AP.

7 It was concluded that BDM blocks a 4-AP-sensitive potassium conductance in motor nerve terminals and, by increasing the duration of the action potential in this way, increases evoked transmitter release.

Introduction

The chemical phosphatase, butanedione monoxime (BDM), has been shown to depress contraction in both skeletal (Mulieri & Alpert, 1984) and cardiac (Li *et al.*, 1985) muscle. It has been suggested that this effect is related to an action on calcium channels, e.g. block of calcium fluxes across the muscle membrane in response to depolarization (Wiggens *et al.*, 1980) or inhibition of calcium release from the sarcoplasmic reticulum (Fryer *et al.*, 1988). The calcium current in the surface membrane of mammalian skeletal muscle is indeed blocked by BDM (Fryer *et al.*, 1988). Because of the possibility that BDM might also block calcium channels in nerve terminals responsible for transmitter secretion, we have explored its effect on neuromuscular transmission. Far from depressing neuromuscular transmission, BDM caused a large increase in transmitter secreted in response to an action potential; this effect is probably related to an action on potassium channels.

Methods

The effects of BDM on transmitter secretion were examined in several different nerve-muscle preparations from mice; extensor digitorum longus (EDL), triangularis sterni and phrenic nerve-diaphram. The latter two preparations were used because they are relatively thin and it was hoped that a more homogeneous distribution of drugs amongst the muscle fibres would result. Nerve-muscle preparations were pinned to a block of Sylgard (Dow Corning) in a tissue bath and superfused for 30 min with a control solution containing either (mM): NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, TES buffer 2 (pH 7.4), or NaCl 125, $NaHCO_3$ 25, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1; pH 7.4 when bubbled with a gas mixture containing 5%CO_2 and 95%O_2. No obvious differences were observed between results obtained with either of these two control solutions.

Endplate potentials (e.p.ps) were evoked by stimulating the nerve through a suction electrode with pulses of 0.2 to 1 ms duration. Contraction was suppressed when necessary either by raising the magnesium concentration in the bath solution or by crushing the muscle fibres on either side of the endplate region. Spontaneous miniature endplate potentials (m.e.p.ps) and e.p.ps were recorded with conventional intracellular microelectrodes filled with 3 M KCl. Miniature endplate currents were recorded either by a two electrode point voltage clamp or extracellularly by blunt microelectrodes filled with control solution and pressed against the muscle in the region of a nerve terminal. Endplate currents and nerve terminal action potentials were recorded extracellularly. Experiments were done at room temperature (20–25°C).

Results

Addition of BDM to the bath solution at concentrations from 5 to 20 mM produced a rapid decrease in the twitch tension produced in response to nerve stimulation. However, the drug rarely caused complete depression of twitches within 30 min in whole muscles, presumably because the concentration attainable in deeper fibres was limited by slow diffusion through the tissue. It was therefore usually necessary to use solutions containing a raised magnesium concentration or to crush muscles on either side of the endplate region in order to obtain completely paralysed preparations. In the presence of BDM, it was possible to record action potentials in muscle fibres in response to nerve stimulation (Figure 1). Such observations indicated that excitation-secretion coupling in the nerve terminal, as well as muscle action potentials, were preserved in preparations paralysed with BDM.

In the presence of BDM, e.p.ps were not only still present but their amplitude was considerably increased, as illustrated in Figure 2. In this preparation, muscle fibres were crushed to prevent contraction before exposure to BDM. E.p.ps recorded in the presence of 10 mM BDM were approximately 5 times larger than control e.p.ps. This effect was readily reversible when the solution containing BDM was washed out of the bath.

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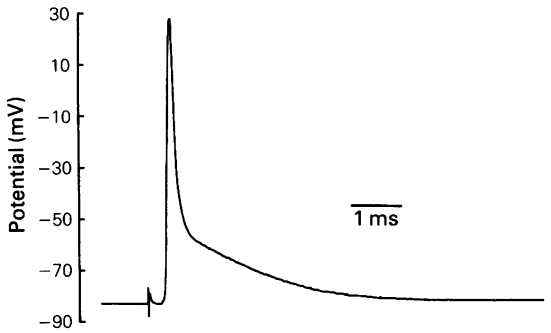

Figure 1 An action potential, evoked by motor nerve stimulation, recorded intracellularly from a surface muscle fibre in a rat extensor digitorum longus (EDL) muscle immobilised with 20 mM butanedione monoxime (BDM). There was a small amount of movement from deeper fibres but this was insufficient to dislodge the electrode during repeated stimuli.

This increase in e.p.p. amplitude could be due to presynaptic or postsynaptic effects of the drug, e.g. an increase in transmitter release or an increase in the postsynaptic response to acetylcholine. The latter possibility was tested by recording currents generated by single quanta of acetylcholine.

Postsynaptic effect of BDM

The effect of BDM on miniature endplate currents (m.e.p.cs) is illustrated in Figure 3.

In the presence of BDM (10 mM), the decay of m.e.p.cs remained exponential but the decay time constant increased on average from 1.2 ms to 2.2 ms. This effect was readily reversible and could be reproduced several times at the same endplate during successive exposures to the drug (see, for example, Figure 5). This increase in the decay time constant could be responsible for a small part of the increase in e.p.p. amplitude (Gage & McBurney, 1973), but was not sufficient to account for the 4 to 5 fold increase in e.p.p. amplitude illustrated in Figure 2. The postsynaptic effect of BDM, which may have been due to an interaction with acetylcholinesterase (Wilson & Ginsberg, 1955), was not investigated further, but was used as a convenient indicator of the presence of BDM at a synapse being investigated.

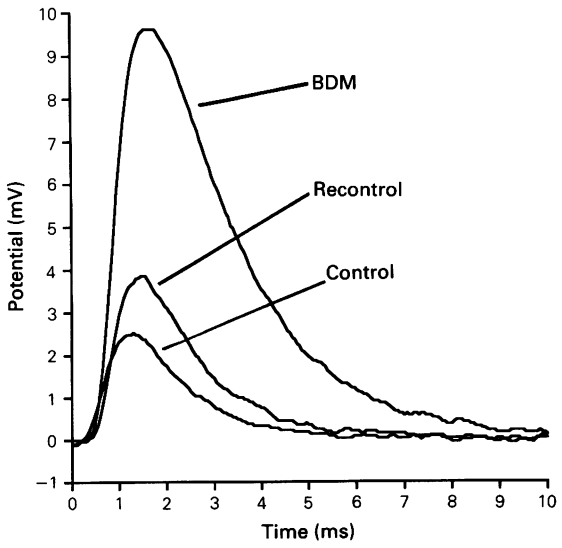

Figure 2 Butanedione monoxime (BDM) increases endplate potential (e.p.p.) amplitude. E.p.ps were recorded in an extensor digitorum longus (EDL) preparation in which the fibres had been crushed to prevent twitching. The bathing solution contained 14 mM magnesium and 2 mM calcium. Traces show a representative e.p.p. before (control), during (BDM) and after (recontrol) exposure to 10 mM BDM.

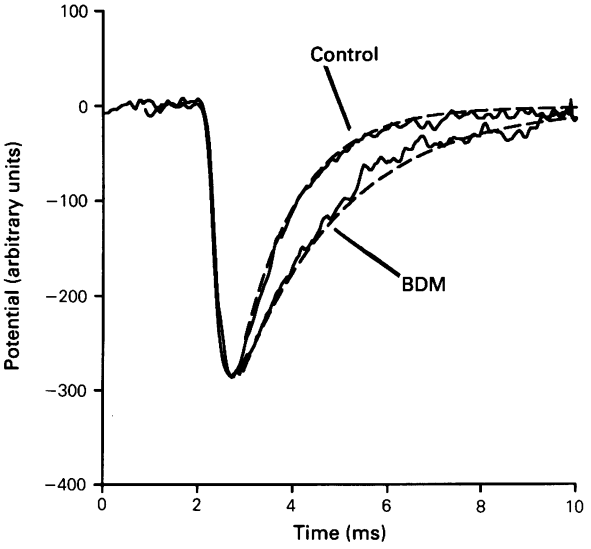

Figure 3 Postsynaptic effect of butanedione monoxime (BDM). Spontaneous miniature endplate currents (m.e.p.cs) were recorded extracellularly at an endplate in a mouse triangularis sterni preparation. The currents shown are averaged m.e.p.cs recorded in control solution ($n = 14$), and in the presence of 10 mM BDM ($n = 14$). The decays were fitted with single exponentials with time constants of 1.2 ms and 2.2 ms in the control and BDM solutions, respectively.

Presynaptic effects of BDM

Quantal content of e.p.ps The increase in the amplitude of e.p.ps (Figure 2) is likely to be due largely to an increase in the quantal content. This is difficult to measure accurately when the quantal content is high because of non-linear summation, which is particularly troublesome in crushed fibre preparations in which the resting membrane potential is usually greatly reduced. The effect of BDM on the quantal content of e.p.ps was measured, therefore, in solutions containing raised magnesium and reduced calcium concentrations, so that quantal content could be estimated from the proportion of 'failures' of e.p.ps in response to presynaptic action potentials (Del Castillo & Katz, 1954). This method provides an accurate measure of presynaptic changes, uncomplicated by postsynaptic effects, provided that quantal responses are distinguishable from noise.

Concentrations of BDM from 5 to 20 mM produced increases in the quantal content of e.p.ps. The results shown in Figure 4 were obtained at one junction in a diaphragm preparation, in a solution containing 10 mM magnesium and 0.5 mM calcium. The increase in quantal content was proportional to drug concentration over the range examined.

Frequency of m.e.p.ps An increase in resting intracellular calcium concentration could be responsible for this increase in quantal content. If so, BDM would be expected to cause an increase in the frequency of m.e.p.ps. However, when BDM was added at concentrations up to 20 mM to the normal extracellular solution there was no appreciable effect on the frequency of m.e.p.ps. Even when m.e.p.p. frequency was elevated in a solution containing 15 mM potassium and 8 mM calcium, when modulation of calcium channels would be expected to have a large effect on m.e.p.p. frequency (Cooke & Quastel, 1973), addition of BDM to the extracellular solutions had little effect, as illustrated in Figure 5.

It can be seen that the frequency of m.e.p.ps, measured in 20 s time bins during addition and removal of 10 mM BDM, showed no abrupt change in the presence of BDM, although the postsynaptic effect, monitored by simultaneous recording of m.e.p.ps, confirmed the presence of BDM at the junction very soon after addition of the drug.

Another possible explanation for the effect of BDM on the quantal content of e.p.ps might be a change in the action

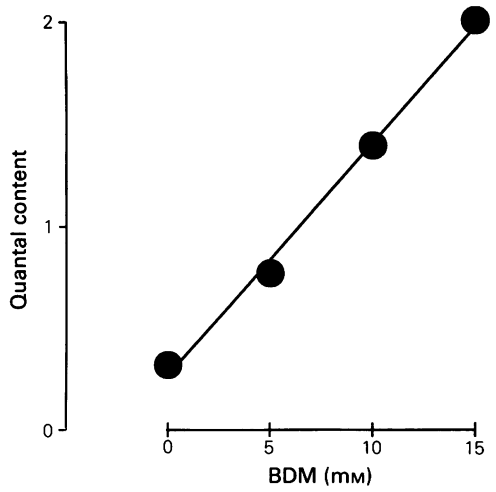

Figure 4 Butanedione monoxime (BDM) increases the quantal content of endplate potentials (e.p.ps) and the effect is concentration-dependent. Quantal content of e.p.ps was measured by the method of failures in a phrenic nerve-diaphragm preparation bathed in a solution containing 10 mM magnesium and 0.3 mM calcium (bicarbonate buffer). The standard errors of the means were smaller than the size of the symbols used.

potential in the presynaptic nerve terminals. Indeed, BDM did produce some change in the time course of the nerve terminal action potential, as can be seen in Figure 6.

The increase in the size of the e.p.c. in 10 mM BDM was accompanied by a delay and broadening of the second part of the presynaptic current. This prolongation is very similar to that observed with drugs that block potassium channels (Burley & Jacobs, 1981; Brigant & Mallart, 1982).

In order to explore the possibility that BDM increases quantal content by blocking potassium channels, its effect following exposure to two well characterised potassium channel blockers was examined. In the presence of 1 mM tetraethylammonium (TEA), a concentration that has been shown to be near maximal in increasing quantal content (Saint *et al.*, 1987), BDM still caused an increase in quantal content similar to that seen in the absence of TEA. At 7 endplates in which quantal content was depressed in a solution containing 10 mM magnesium and 0.5 mM calcium, exposure to 10 mM BDM increased quantal content on average by a factor of 6.7 ± 1.0 (mean ± 1 s.e.mean). In the presence of 1 mM TEA, 10 mM BDM increased quantal content on average by 5.8 ± 2.0.

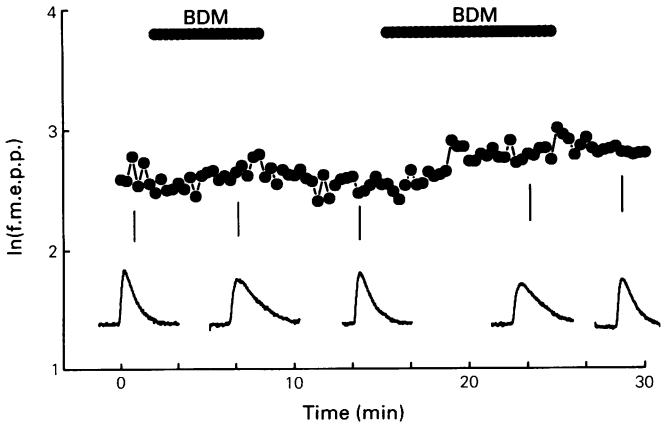

Figure 5 The lack of effect of butanedione monoxime (BDM) on miniature endplate potential (m.e.p.p.) frequency. The frequencies, measured in 20 s time bins at a single junction in a phrenic nerve-diaphragm preparation, are plotted semilogarithmically. The bathing solution contained 15 mM potassium and 8 mM calcium and the preparation was exposed to a solution containing 10 mM BDM during the times indicated by the horizontal bars. Averaged m.e.p.ps recorded at the times indicated are shown below.

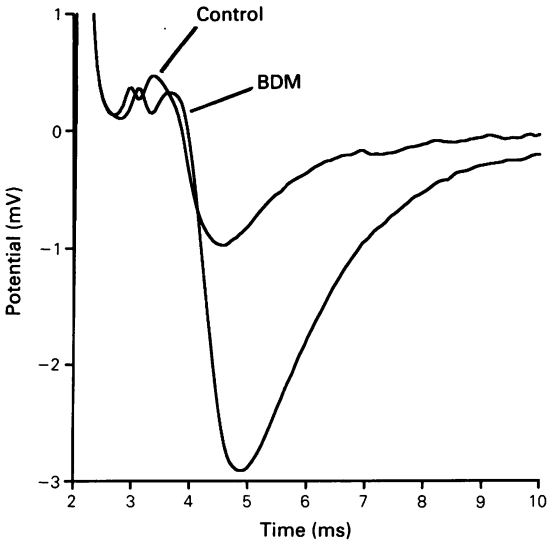

Figure 6 Effect of butanedione monoxime (BDM) on extracellularly recorded nerve terminal action potentials. Endplate currents (e.p.cs) were recorded with an extracellular electrode placed over an endplate in a mouse triangularis sterni preparation. The bathing solution contained 15 mM magnesium and 2 mM calcium (TES buffer). The addition of BDM produced a change in the time course of the nerve terminal action potential accompanied by a large increase in the amplitude of the e.p.c.

These increases in quantal content in the presence and absence of TEA were not statistically significant at the 5% level (Student's *t* test).

In contrast, the effect of BDM on quantal content was clearly depressed in the presence of 4-aminopyridine (4-AP). At 7 junctions in another 3 diaphragm preparations, 10 mM BDM increased quantal content by a factor of 5.6 ± 1.5. At another 7 junctions in the same 3 preparations, 10 mM BDM increased quantal content by a factor of only 1.5 ± 0.2 in the presence of 0.5 mM 4-AP. This depression of the effect of BDM by 4-AP was significant at the 5% level (Student's *t* test). The occlusion of the effects of 4-AP and BDM on quantal content was demonstrated clearly at 2 other junctions which survived multiple solution changes (Table 1). Before exposure to 4-AP, 10 mM BDM increased quantal content by factors of 4.9 and 4 whereas, in the presence of 0.5 mM 4-AP, quantal content increased by factors of only 1.6 and 1.3.

Discussion

The results indicate that BDM, at concentrations that effectively depress muscle contraction, does not depress transmitter secretion from motor nerve terminals. It can be concluded therefore that BDM does not depress the calcium current

Table 1 Reduction in the effect of butanedione monoxime (BDM) on quantal content in the presence of 0.5 mM 4-aminopyridine (4-AP) in two phrenic nerve-diaphragm preparations

	Control	*BDM*	*Control + 4-AP*	*BDM + 4-AP*
Prep 1	0.052	0.257	2.72	4.48
Ratio		4.9		1.6
Prep 2	0.098	0.391	3.97	5.31
Ratio		4.0		1.3

Solutions contained 10 mM magnesium and 0.3 mM calcium. In control and the BDM (10 mM) solution, quantal content was calculated from failures whereas, in solutions containing 4-AP, quantal content was calculated from the ratio of average e.p.p. and m.e.p.p. amplitudes.

responsible for transmitter secretion. In fact, BDM produces a large increase in quantal content of e.p.ps without affecting the frequency of m.e.p.ps. The latter observation makes it unlikely that BDM alters intracellular calcium concentration by changing the characteristics of calcium channels or the storage of intracellular calcium. On the other hand, prolongation of the presynaptic action potential would be expected to increase evoked but not spontaneous transmitter release. Indirect evidence that BDM produces the increase in quantal content in this way is presented: BDM alters the time course of the nerve terminal action potential and is less effective in increasing quantal content in the presence of 4-AP. In view of these observations, it seems very likely that most of the increase in quantal content caused by BDM is related to a depression of a potassium conductance in the motor nerve terminal that is sensitive to 4-AP. It may be that this potassium channel is functionally depressed when dephosphorylated.

Whatever the mode of action of BDM on transmitter release, the persistence of quantal secretion at neuromuscular junctions in which muscle fibres are immobilised provides a new preparation for quantitative studies of synaptic transmission and electrical activity, such as asymmetric charge movement in skeletal muscle fibres.

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Cardiovascular effects of central injection of acetylcholine in anaesthetized dogs: a role for vasopressin release

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1 The effect of an intracisternal injection of 20 $\mu g\,kg^{-1}$ of acetylcholine was studied on systolic and diastolic blood pressures, heart rate, and plasma levels of noradrenaline, adrenaline, vasopressin, plasma renin activity and atrial natriuretic factor in chloralose-anaesthetized dogs, 8 of which were normal and 7 with diabetes insipidus (deprived of vasopressin secretion by surgical lesion of the hypothalamoneurohypophysial system).

2 Acetylcholine significantly increased systolic and diastolic blood pressures in both groups of animals. However, the rise in blood pressure was significantly shorter lived in the dogs with diabetes insipidus.

3 Acetylcholine significantly increased plasma levels of noradrenaline but not adrenaline in control animals and in dogs with diabetes insipidus. Noradrenaline and adrenaline responses after acetylcholine were not different in the two groups of animals.

4 Acetylcholine induced a significant increase in vasopressin plasma levels only in control animals while in dogs with diabetes insipidus vasopressin remained at nearly undetectable levels.

5 Acetylcholine significantly increased atrial natiuretic factor plasma levels only in control dogs.

6 Although plasma renin activity increased in both groups of animals after the i.c. injection of acetylcholine, this change was not significant in any group.

7 These results suggest that, in the anaesthetized dog, the central injection of acetylcholine induces a rise in blood pressure through both an increase in sympathetic outflow and a release of vasopressin.

Introduction

Since the early observation by Shu *et al.* (1935) of the rise in blood pressure elicited by an intracisternal injection of acetylcholine in the anaesthetized dog, many studies have described the cholinergically mediated pressor response induced by the central administration of acetylcholine or related drugs (cholinoceptor agonists or acetylcholinesterase inhibitors) in several species including rat, cat and dog (for review see Brezenoff & Giuliano, 1982). In contrast, the peripheral mechanisms mediating such cholinergic cardiovascular effects remain poorly known. The main one generally put forward is a centrally-evoked cholinergic activation of the sympathetic outflow; however, beside this enhancement of the peripheral noradrenergic activity, the putative effects of other systems remain unclear: a role for vasopressin release is debated and, to our knowledge, the possible role of other peripheral vasoactive systems like plasma renin or atrial natriuretic factor has not been investigated.

The purpose of the present study was to investigate the respective roles of peripheral noradrenaline, adrenaline, vasopressin, plasma renin activity and atrial natriuretic factor in mediating the central cholinergic pressor response. Therefore, we studied the cardiovascular responses and the plasma level variations of noradrenaline, adrenaline, vasopressin, plasma renin activity and atrial natriuretic factor elicited by an intracisternal administration of acetylcholine (20 $\mu g\,kg^{-1}$) in anaesthetized control dogs and in dogs with diabetes insipidus induced by surgical section of the supraoptico-hypophysial tract.

Methods

General procedure

The experiments were performed between 08 h 00 min and 12 h 00 min in mongrel dogs of either sex weighing 15 to 24 kg. The dogs were deprived of food the morning of the experiment but had free access to water *ad libitum* in order to be normally hydrated. Animals were anaesthetized with chloralose (100 mg kg^{-1}, i.v.), intubated and ventilated with an Ideal Palmer respirator. Animal temperature was maintained constant with an electric blanket. A catheter introduced into the left femoral artery was connected to a Statham P23 Db transducer to monitor systolic and diastolic blood pressures. Heart rate was counted on the electrocardiogram (lead II). These parameters were continuously recorded on a Philips recorder.

A single dose of 20 $\mu g\,kg^{-1}$ of acetylcholine chloride (Lemotte and Boinot Labs) dissolved in a 0.9% w/v NaCl solution (saline) was injected into each dog by the intracisternal (i.c.) route. I.c. injections were performed with a 18 gauge needle introduced percutaneously. This dose was chosen after preliminary experiments because it induces, according to the present protocol, a reproducible rise in blood pressure of about 30 mmHg. This effect was blocked by intracisternal atropine (1 $\mu g\,kg^{-1}$) (unpublished data). The volume of the injection was no larger than 0.5 ml; before injection of acetylcholine, each animal received a preliminary i.c. injection of the same volume of saline to confirm the absence of cardiovascular effects of a sham injection.

A second catheter, introduced into the right femoral artery, was used for the collection of blood samples in order to measure noradrenaline, adrenaline, vasopressin, plasma renin activity and atrial natriuretic factor plasma levels. Such blood samples were collected 5 and 1 min before and 2, 10, and 30 min after the i.c. injection of acetylcholine.

Dogs with diabetes insipidus: surgical procedure

Dogs were deprived of vasopressin secretion by surgical lesion of the hypothalamo-neurohypophysial system. The general procedure for inducing diabetes insipidus in dogs has been described previously (Montastruc *et al.*, 1980). Briefly, under intravenous sodium thiopentone anaesthesia (12.5 mg kg^{-1}), the hypothalamus was reached after cutting away the soft palate and collapsing the bone along the sphenoethmoidal suture. Sectioning of the supraoptico-hypophysial tract was

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performed with a cataract lancet between the adenohypophysis and the supraoptic nucleus. The aperture was then filled with acrylic resin. Increases in urine excretion (+2 to 61 daily in different animals; $P < 0.05$ in comparison with normal dogs) were seen 15 to 20 days after surgery. Surgery was performed several months (at least three) before the present study. The selectivity of the surgical lesion which induces the diabetes insipidus is illustrated by previous work from our laboratory (Baïsset & Montastruc, 1957), showing that adrenalectomy improves the polyuric-polydipsic syndrome of these animals. This effect is ascribed to the suppression of the diuretic effects of cortisol and suggests that the surgical procedure does not interfere with the integrity of the adenohypophysial-adrenocortical axis.

Measurement of plasma hormone levels

Blood samples were obtained from the femoral artery and the volume (10 ml) was immediately replaced by 10 ml isotonic saline. All hormonal measurements were assessed blind. For catecholamine determination, blood was collected in lithium-heparin tubes containing 10 mM sodium metabisulphite; for vasopressin and plasma renin activity determinations, blood was collected in lithium heparin-tubes and for atrial natriuretic factor blood was collected in tubes containing EDTA. Blood samples were then centrifuged at 2000 *g* for 10 min at 0°C; plasma was stored at −80°C.

Catecholamines were selectively isolated from the sample at 0°C, in the dark, by adsorption to activated alumina, then eluted with 0.1 M perchloric acid. Dihydroxybenzylamine was used as an internal standard to monitor recovery from this extraction step. Noradrenaline and adrenaline were assayed by high pressure liquid chromatography using electrochemical (amperometric) detection: the working electrode potential was set at 0.65 V against a Ag-AgCl reference electrode. Catecholamines were separated on a C_{18} Waters column (3.9 × 150 mm) at a constant flow rate of 1 ml min^{-1}. The electrochemical detector response was linear for concentrations ranging from 0.17 nM to 0.60 μM; $r = 0.997$ for noradrenaline, $r = 0.922$ for adrenaline. In these conditions the detection limit was 0.3 nM. Inter-assay coefficients of variation for plasma catecholamines performed over 5 days were, for the high levels, 11% for noradrenaline and 12% for adrenaline. The intra-assay coefficients of variation over three assays were 1% for noradrenaline and 7% for adrenaline.

Other hormones were measured by radioimmunoassay. For vasopressin the radioimmunoassay used after bentonite extraction (Skowsky *et al.*, 1974) had a sensitivity of 0.3 pg ml^{-1}. The recovery of vasopressin was 75% and intra and interassay coefficients of variation were 3 and 11%, respectively. The antibodies were a gift of L. Keil (Keil & Severs, 1977). Atrial natriuretic factor radioimmunoassay was made after SEPAK extraction as previously described (Gutkowska *et al.*, 1986). The antibodies were a gift of J. Gutkowska. The recovery for added atrial natriuretic factor was 85%. Intra and interassay coefficients of variations were 10%, and sensitivity 1.2 pg ml^{-1}. Plasma renin activity was measured as previously described (Vincent *et al.*, 1972) with antibodies raised in the laboratory. Intra and interassay coefficients of variations were 1% and 2.5% respectively and sensitivity 20 ng l^{-1} min^{-1}.

Statistical analysis

Non-parametric tests were used for statistical comparisons. The Mann-Whitney U test was performed to compare the mean values of cardiovascular parameters and hormonal plasma levels between the group of control animals and the group of dogs with diabetes insipidus. This test was also used to compare the mean cardiovascular and hormonal changes induced by the i.c. injection of acetylcholine in the 2 groups of animals. Friedman's test was performed in each group to compare cardiovascular and hormonal values before and after the i.c. injection of acetylcholine.

Results

Eight control dogs (5 females, 3 males, mean weight: 19.4 ± 2.1 kg) and 7 animals with diabetes insipidus (4 females, 3 males, mean weight: 22.3 ± 1.7 kg) were studied in the present experiments.

Cardiovascular responses to intracisternal injections of acetylcholine

Systolic and diastolic blood pressures and heart rate were similar under basal conditions in control animals and dogs with diabetes insipidus (Table 1). Although mean values of systolic blood pressure appeared to be lower in dogs with dia-

Table 1 Mean systolic and diastolic blood pressure and heart rate of control animals and dogs with diabetes insipidus 5 and 1 min before and 2, 10 and 30 min after the intracisternal injection of acetylcholine

Acetylcholine (20 μg kg^{-1}, i.c.)		*Before injection* 5 min	1 min	*After injection* 2 min	10 min	30 min	*Friedman's test*
Control animals ($n = 8$)	Systolic blood pressure (mmHg)	163 ± 15	167 ± 16	191 ± 16	187 ± 17	174 ± 17	$P < 0.001$
	Diastolic blood pressure (mmHg)	68 ± 4	69 ± 4	91 ± 4	91 ± 4	76 ± 5	$P < 0.01$
	Heart rate (min^{-1})	116 ± 9	112 ± 9	119 ± 10	123 ± 8	120 ± 10	NS
Animals with diabetes insipidus ($n = 7$)	Systolic blood pressure (mmHg)	142 ± 9	142 ± 9	169 ± 12	157 ± 9	143 ± 9	$P < 0.01$
	Diastolic blood pressure (mmHg)	71 ± 5	71 ± 5	85 ± 4	79 ± 4	72 ± 5	$P < 0.01$
	Heart rate (min^{-1})	122 ± 13	123 ± 13	130 ± 12	135 ± 12	130 ± 13	NS

There was no difference between control animals and dogs with diabetes insipidus at baseline, before the injection (Mann and Whitney U test). Acetylcholine induced a significant increase in systolic and diastolic blood pressures in both groups of animals (Friedman's test). This response was shorter lived in the dogs with diabetes insipidus: 30 min after acetylcholine, blood pressure had returned to basal values in the dogs with diabetes insipidus but not in the control group (see Figure 1).

betes insipidus, this difference was not significant and this observation agrees with previous results from our laboratory (Montastruc & Dang-Tran, 1984).

In the control group, systolic and diastolic blood pressures increased after the i.c. injection of acetylcholine within 2 min ($P < 0.001$ and $P < 0.01$, respectively). Heart rate slightly and non significantly increased after the i.c. injection of acetylcholine (Table 1).

In the group of dogs with diabetes insipidus, systolic and diastolic blood pressure significantly increased ($P < 0.01$). The moderate increase which was observed in heart rate was not significant (Table 1).

When the cardiovascular responses of the dogs with diabetes insipidus were compared to those of the controls, the initial rise in systolic and diastolic blood pressures (2 min after the i.c. injection of acetylcholine) were similar in both groups (Figure 1). However the pressor effect was shorter in duration in the group of animals with diabetes insipidus: 30 min after the i.c. injection, the mean increase in systolic blood pressure induced by acetylcholine was significantly smaller in the dogs with diabetes insipidus than in the control animals ($P < 0.05$); a difference was also observed in the changes in diastolic blood pressure, the increase being significantly smaller in the animals with diabetes insipidus 10 and 30 min after the i.c. injection of acetylcholine ($P < 0.05$). No difference existed in the changes in heart rate between the two groups of animals.

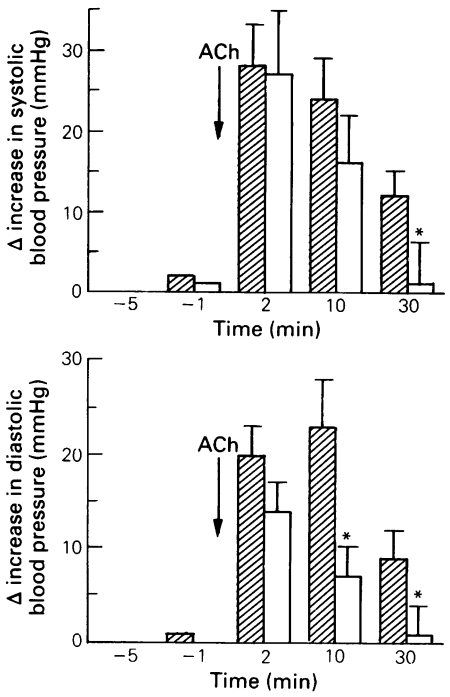

Figure 1 Mean increases in systolic and diastolic blood pressures in control animals (hatched columns) and dogs with diabetes insipidus (open columns) 2, 10 and 30 min after the intracisternal injection of 20 $\mu g\,kg^{-1}$ of acetylcholine (ACh) (basal values 5 and 1 min before the injection of acetylcholine). Notice that, in dogs with diabetes insipidus, the rise in systolic blood pressure observed 30 min after the i.c. injection of acetylcholine was significantly smaller than in control animals; the same difference was also observed for the rise in diastolic blood pressure which was significantly smaller in the dogs with diabetes insipidus 10 and 30 min after the i.c. injection of acetylcholine (*$P < 0.05$, Mann-Whitney U test).

Effects of i.c. injections of acetylcholine on hormonal plasma levels (Table 2)

Noradrenaline and adrenaline plasma levels were assessed only 5 min before and 2 and 30 min after the i.c. injection of acetylcholine. Vasopressin, plasma renin activity and atrial natriuretic plasma levels were assessed 5 and 1 min before and 2, 10 and 30 min after the i.c. injection of acetylcholine.

Before the injection of acetylcholine, the basal plasma levels of noradrenaline, adrenaline, plasma renin activity and atrial natriuretic factor were not significantly different in control dogs and animals with diabetes insipidus. On the contrary, as expected, vasopressin plasma levels were nearly undetectable in the group of animals with diabetes insipidus and the difference was highly significant from the control group ($P < 0.01$).

The i.c. injection of acetylcholine significantly increased noradrenaline plasma levels in control animals and in dogs with diabetes insipidus ($P < 0.02$) (Table 1). This increment did not differ from one group to the other.

Table 2 Mean plasma levels of noradrenaline, adrenaline, vasopressin, atrial natriuretic factor and plasma renin activity in control animals and dogs with diabetes insipidus 5 and 1 min before and 2, 10 and 30 min after the intracisternal injection of acetylcholine

Acetylcholine (20 $\mu g\,kg^{-1}$, i.c.)		*Before injection* 5 min	1 min	*After injection* 2 min	10 min	30 min	*Friedman's test*
Control animals	Noradrenaline (pg ml^{-1})	376 ± 94	—	485 ± 97	—	436 ± 133	$P < 0.02$
	Adrenaline (pg ml^{-1})	85 ± 23	—	92 ± 29	—	77 ± 20	NS
	Vasopressin (pg ml^{-1})	13.5 ± 6.4	13.7 ± 4.4	41.3 ± 9.1	53.5 ± 10.8	35.1 ± 6.4	$P < 0.01$
	Atrial natriuretic factor (pg ml^{-1})	102 ± 15	108 ± 13	134 ± 13	145 ± 23	126 ± 16	$P < 0.02$
	Plasma renin activity (ng l^{-1} min^{-1})	150 ± 36	167 ± 45	175 ± 52	201 ± 57	200 ± 46	NS
Animals with diabetes insipidus	Noradrenaline (pg ml^{-1})	191 ± 30	—	364 ± 52	—	304 ± 123	$P < 0.02$
	Adrenaline (pg ml^{-1})	86 ± 35	—	110 ± 44	—	161 ± 63	NS
	Vasopressin (pg ml^{-1})	0.5 ± 0.4	0.6 ± 0.3	0.7 ± 0.3	1.4 ± 0.8	1.4 ± 0.8	NS
	Atrial natriuretic factor (pg ml^{-1})	101 ± 9	97 ± 10	121 ± 20	128 ± 11	110 ± 11	NS
	Plasma renin activity (ng l^{-1} min^{-1})	176 ± 35	183 ± 40	191 ± 47	276 ± 77	339 ± 99	NS

At baseline, before the injection, hormonal plasma levels were not significantly different between the two groups of dogs except for vasopressin which was significantly reduced in the dogs with diabetes insipidus ($P < 0.01$, Mann-Whitney U test). Acetylcholine significantly increased noradrenaline plasma levels in both control dogs and animals with diabetes insipidus. Acetylcholine significantly increased vasopressin and atrial natriuretic factor plasma levels only in control dogs. The increases observed in plasma renin activity after acetylcholine did not reach statistical significance (Friedman's test).

No significant effect of acetylcholine was observed on adrenaline plasma levels in either group of dogs (Table 1).

In the control group, the i.c. injection of acetylcholine induced a marked and significant increase in vasopressin plasma levels ($P < 0.01$). In contrast, no change was observed in the group of animals with diabetes insipidus.

The i.c. injection of acetylcholine also significantly increased atrial natriuretic factor plasma levels in the control animals ($P < 0.02$). This effect was not observed in the group of animals with diabetes insipidus.

The i.c. injection of acetylcholine increased the plasma renin activity in both groups of animals but this rise was not significant in any group.

Discussion

The i.c. injection of acetylcholine ($20\,\mu g\,kg^{-1}$) in anaesthetized dogs induced a significant rise in blood pressure. This is in good agreement with the large number of experiments reporting the same results in different species with different cholinoceptor agents (acetylcholine, cholinesterase inhibitors, cholinoceptor agonists) administered through the central route (for review, see Brezenoff & Giuliano, 1982). Conversely, in the present experimental model, the i.c. injection of acetylcholine did not significantly modify the heart rate of the dogs. A slight tachycardia was observed in both groups of animals but this response was not constant and not significant. In fact, in the literature, no clear response can be found and variable heart rate changes, tachycardia as well as bradycardia, have been reported after central injection of cholinomimetic agents in different species (see Brezenoff & Guiliano, 1982). Anaesthetic conditions and interspecies differences may explain some of these conflicting data.

In control dogs and animals with diabetes insipidus, acetylcholine induced a significant rise in noradrenaline plasma levels. This rise illustrated the well known enhanced peripheral noradrenergic activity observed after central cholinergic stimulation which is abolished by the section of the spinal cord rostral to the spinal sympathetic outflow (Krstić & Djurković, 1978), by chemical ablation of the peripheral sympathetic nervous system by 6-hydroxydopamine (Kaul & Grewal, 1972), ganglionic transmission blockade (Della Bella *et al.*, 1964) or peripheral α-adrenoceptor blockade (Krstić & Djurković, 1978). These experiments demonstrated that an increased sympathetic outflow mediates the cardiovascular effects of central cholinergic activation. In the present experiments, a similar initial rise (2 min after the i.c. injection of acetylcholine) in blood pressure and noradrenaline plasma levels was observed in the control animals and the dogs with diabetes insipidus. This observation suggests that the mechanisms underlying these responses may be similar in the two groups of dogs. However, it must be emphasized that, in these animals, the respective contribution of the vascular conductance and of the cardiac output was not assessed. Therefore the possibility remains that the 'same' change in blood pressure in the two groups of animals was achieved with different contributions from changes in these two factors.

Unlike noradrenaline, adrenaline plasma levels were not significantly modified by the i.c. injection of acetylcholine. In fact, this lack of effect in adrenaline output by the adrenal medulla agrees with the observation that adrenalectomy, adrenal demedullation or denervation of the gland do not affect the rise in blood pressure produced by i.v. physostigmine (see Brezenoff & Giuliano, 1982).

Conflicting reports regarding the ability of phentolamine to block the pressor effect of centrally administered cholinergic drugs raises the question whether factors other than the increase in sympathetic tone could mediate this effect. The role of vasopressin has been suggested by some authors and questioned by others. In our experiments, at rest, vasopressin plasma levels were rather high in control animals. This can be explained by the fact that nearly all anaesthetics release vasopressin (Heller, 1951). In dogs with diabetes insipidus, the very low plasma levels of vasopressin confirmed the efficacy of the surgical procedure. The present data provided two results which favour a role for vasopressin in mediating part of the central pressor effect of acetylcholine.

First, we observed in control animals that acetylcholine induced significant and prolonged rises in peripheral vasopressin plasma levels. This is consistent with the well documented central cholinergic regulation of vasopressin release *in vitro* (Sladek & Joynt, 1979) and *in vivo* (Kuhn, 1974). It has long been accepted that the level of circulating vasopressin needed to produce a significant pressor response is higher than that required for maximal antidiuretic effect (Sawyer, 1961). In the present experiments, the amount of vasopressin released by the i.c. injection of acetylcholine was compatible with a cardiovascular and pressor effect (Rossi & Schrier, 1986).

A second argument implicating vasopressin in the central pressor cholinergic effect was that the hypertension observed after the i.c. injection of acetylcholine was shorter in dogs with diabetes insipidus than in controls. The role of the baroreflex in explaining this difference must be considered. Brain acetylcholine modulates baroreceptor cardiac reflexes (see Brezenoff & Guiliano, 1982) and reciprocal influences between endogenous vasopressin and baroreflex mechanisms have been pointed out (see Gardiner & Bennett, 1986). It is therefore possible that, in dogs with diabetes insipidus, the shorter duration of the cholinergic pressor response was due to an abnormality of the baroreflex. To our knowledge, the baroreflex has not been studied in dogs with surgical diabetes insipidus. However, previous works from our laboratory (Baïsset & Montastruc, 1978) showed that the acute hypertension induced by the depressor buffer nerves section was similar in normal dogs and dogs with surgical hypothalamic diabetes insipidus. Moreover, it was noticed, in the present experiment, that no difference in heart rate changes occurred between the two groups of dogs after the i.c. injection of acetylcholine. These observations indirectly suggest that dogs with surgical diabetes insipidus do not present major alterations in their baroreflex. Conversely, as expected, basal vasopressin plasma levels of dogs with diabetes insipidus were nearly undetectable in these animals and the i.c. injection of acetylcholine did not induce any significant rise of vasopressin. At the same time, the duration of the pressor cholinergic response was significantly shorter in the same animals. These data suggest that, besides a similar increase in the sympathetic outflow in the two groups of animals, the difference in the cholinergic release of vasopressin could explain the longer duration of the pressor response in the normal dogs. This putative role of vasopressin has been variously assessed in the literature: Buccaffusco & Brezenoff (1979) reported than in anaesthetized rats, the pressor response to the central injection of carbachol was unaltered by hypophysectomy. Kawashima *et al.* (1987) showed that a vasopressin antagonist did not attenuate the pressor or cardiac effects of physostigmine in normal or hypertensive rats. Such results were not in favour of a significant role of vasopressin. However, several other authors reported data that support the role of vasopressin in mediating some aspects of the central pressor cholinergic response. Varagić & Milić (1957) were the first to propose that a release of vasopressin in pressor amounts might be involved in the cardiovascular response to physostigmine in the rat. Hoffman *et al.* (1977) and Hoffman (1979) also observed pressor responses with antidiuresis after the injection of carbachol into the third ventricle of conscious rats. Bisset & Chowdrey (1984) showed that in the anaesthetized rat, intraventricular injections of carbachol produced a profound antidiuretic response associated with a pressor effect. Although these authors did not provide quantitative data on this question, they concluded that 'since the pressor response to carbachol was absent or attenuated in the Brattleboro rat, this too may be mediated, at least partly, by release of vasopressin'. Thus, it is possible to suggest that the cholinergic-induced

pressor and antidiuretic responses are not independent mechanisms.

It is not clear if the central effects of acetylcholine on vasopressin release and blood pressure share common pharmacological mechanisms or not. It is generally accepted that acetylcholine stimulates vasopressin release from the hypothalamohypophysial system through nicotinic receptors (Milton & Paterson, 1974) rather than muscarinic ones. In contrast, the nature of the central cholinergic pressor response is said to be mainly of the muscarinic type (see Brezenoff & Giuliano, 1982) and, in our model i.c. atropine abolishes the pressor effect of the central injection of acetylcholine (data not shown). According to these data, the cholinergic cardiovascular and endocrinological responses could then be separable and would correspond to different pharmacological mechanisms. However, several authors have reported that, *in vivo*, atropine was able to block the release of vasopressin (Kuhn, 1974; Bisset & Chowdrey, 1984) and to block simultaneously both hormonal and pressor responses elicited by central cholinergic agents (Hoffmann *et al.*, 1977). Therefore, it is possible that both responses are mediated, at least partly, by muscarinic receptors.

The i.c. injection of acetylcholine induces a significant rise in plasma atrial natriuretic factor in control animals. This rise was smaller and did not reach significance in dogs with diabetes insipidus. This increase in atrial natriuretic factor plasma levels can be regarded as a release in response to the pressor effects of the i.c. injection of acetylcholine. The mechanisms regulating atrial natriuretic factor release from the heart are not well understood. However, several factors are known to induce it, such as an increase in atrial pressure (Lang *et al.*, 1985), noradrenaline and other pressor agents (Garcia *et al.*, 1986; Baranowska *et al.*, 1987). A possible interaction between the endocrine systems of the heart and the pituitary gland has been debated (see Hoffman & Keiser, 1989). A direct role of the adenohypophysis has not been clearly demonstrated and there is evidence that our surgical procedure to induce diabetes insipidus spares the adenohypophysis (see methods of Baïsset & Montastruc, 1957). On the contrary, vasopressin has been shown to stimulate atrial natriuretic factor release directly or secondarily to its effect on blood volume (Manning *et al.*, 1985; Ogawa *et al.*, 1986). Therefore, in our control animals, at least three different factors could explain the rise in atrial natriuretic factor plasma levels: the rises in blood pressure, noradrenaline and vasopressin plasma levels. Furthermore, the importance of vasopressin in the *in vivo* release of atrial natriuretic factor was underlined by the fact that in dogs with diabetes insipidus, deprived of vasopressin secretion, although acetylcholine moderately increased plasma atrial natriuretic factor, this rise was not significant. This could be due to the lack in such animals of a direct effect of vasopressin or to the shorter pressor response observed in dogs with diabetes insipidus.

In the present experiments, plasma renin activity was not significantly modified by the i.c. injection of acetylcholine in control animals and dogs with diabetes insipidus. This result is somewhat surprising since many morphological, physiological and pharmacological data suggest an involvement of the sympathetic nervous system in the mechanism of renin release (Vander, 1965; Wagermark *et al.*, 1968; for review see Reid *et al.*, 1978) and since the i.c. injection of acetylcholine increased sympathetic outflow of our animals. In urethane-anaesthetized rats, Alexandre *et al.* (1970) reported that physostigmine ($75\,\mu g\,kg^{-1}$, i.v.) increased both blood pressure and plasma renin activity. This increase in plasma renin activity was attributed to the increased sympathetic activity elicited by the anticholinesterase. We did not observe the same results in dogs. However, although the phenomenon did not reach statistical significance, it can be noticed that plasma renin activity was frequently increased in dogs after the central injection of acetylcholine. Moreover, this response was more pronounced in dogs with diabetes insipidus than in control dogs. This last result can be related to the fact that several investigators have observed that vasopressin inhibits renin secretion (Bunag *et al.*, 1967; Vander, 1968). The increase in renin secretion after intravenous isoprenaline is greater in hypophysectomized dogs than in control animals (Reid *et al.*, 1972), a result ascribed to the fact that isoprenaline is a potent stimulus for vasopressin secretion (Schrier *et al.*, 1972). Therefore, it can be suggested that in our control animals, the release of vasopressin induced by acetylcholine may have acted as an inhibitory factor to renin secretion, a fact which did not occur in dogs with diabetes insipidus.

In conclusion, the present data suggest that, besides an increase in the sympathetic outflow, the rise in blood pressure observed after central stimulation of the cholinergic systems can be related to an increase in vasopressin release, while atrial natriuretic factor and plasma renin activity seem to have no or minor roles.

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Continuous fluorimetric assessment of the changes in cytoplasmic calcium concentration during exposure of rat isolated myocardium to conditions of simulated ischaemia

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1 The cytoplasmic calcium concentration ($[Ca]_c$) of rat isolated atrial myocardium was assessed with the dye indo-1. Dye-loaded atria were superfused with physiological salt solution and excited with radiation at 360 nm, while epifluorescence emissions were collected simultaneously at 400 nm and 500 nm. The ratio of these emissions was used as a measure of $[Ca]_c$.

2 Dye-loaded atria showed a phasic rise and fall in $[Ca]_c$ with each applied electrical pacing stimulus. The amplitudes of systolic increments in tension and $[Ca]_c$ were augmented by the presence of isoprenaline.

3 Atria superfused with a solution the composition of which resembled that found extracellularly in regions of ischaemia rapidly lost systolic increments in tension and $[Ca]_c$, while end-diastolic $[Ca]_c$ and tension gradually rose.

4 The presence of lactate (20 mM) or flufenamate (5 μM) in the superfusate during simulated ischaemia aggravated the rises in both end-diastolic tension and end-diastolic $[Ca]_c$. Inclusion in the superfusate of sulphinpyrazone (50 μM) or glucose (20 mM) protected against some of the deleterious effects of lactate seen during simulated ischaemia.

Introduction

A raised cytoplasmic concentration ($[Ca]_c$) has been demonstrated during periods of depletion of the myocardial stores of adenosine 5'-triphosphate (ATP), and this occurs during ischaemia, for example (Barry *et al.*, 1987; Lee *et al.*, 1987; 1988; Eisner *et al.*, 1989; Kohmoto & Barry, 1989; Morris *et al.*, 1989). These observations have been made with non-toxic fluorescent dyes, such as indo-1, that are sensitive to even the very low levels of $[Ca]_c$ that normally prevail during diastole. Indo-1 may be loaded into cardiac myocytes via an intact sarcolemma; but in order to take full advantage of the spectral properties of this dye, fluorescence needs to be measured at 2 emission wavelengths simultaneously. In that way a rise and a subsequent fall in $[Ca]_c$ may be demonstrated with each heart beat (Barry *et al.*, 1987; Lee *et al.*, 1987; 1988; Stern *et al.*, 1988; Eisner *et al.*, 1989; Kohmoto & Barry, 1989; Morris *et al.*, 1989).

The present experiments have used isolated atrial myocardium from the rat, superfused with a solution the composition of which resembles that found extracellularly in regions of ischaemia. This has permitted some previously described functional and biochemical abnormalities that occur during this type of injury (Northover, 1987; 1989; Northover & Northover, 1988) to be correlated with contemporary changes in $[Ca]_c$ measured with indo-1. It has also permitted a re-examination of the effects of drugs that were shown previously to be protective in this model of cardiac injury.

Methods

Male rats of the Sprague-Dawley strain weighing 340–480 g were killed by a blow to the head. The heart was excised quickly, the atria separated from the ventricles and the right atrium sutured via its epicardial surface to a flat rectangular plastic holder (11 mm × 50 mm) containing a pair of platinum wires that were insulated, except where the tips made contact with the epicardium. The atrium was stimulated throughout an experiment at 4 Hz with 2 ms square wave pulses, each of 10 V. Atria were bathed, unless otherwise stated, with normal superfusate (NS) of the following composition (mM): NaCl 138, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 0.5, $NaHCO_3$ 10, glucose 10 and gassed with 95% O_2 plus 5% CO_2. A thread sutured to the left atrial appendage was connected to a force-displacement transducer (type SB-IT, Nihon Khoden) at a diastolic tension of 100 mg. Tension records were made on moving paper via a d.c. amplifier (type 3552, Cardiovascular Instruments) coupled to a heated stylus recorder (type 5041, Lectromed). Atria were allowed to equilibrate in NS for 1 h before dye-loading.

Dye-loading

A 50 μl aliquot of a 1 mM stock solution of the acetoxymethyl ester of indo-1 (Sigma) in dimethyl sulphoxide was first mixed with 0.2 ml of a 0.3% solution in dimethyl sulphoxide of the non-ionic detergent Pluronic F-127 (a polyoxyethylene/polyoxypropylene co-polymer, supplied by Mazer Chemicals). This mixture was then shaken with 10 ml of NS to give a 5 μM dye solution. Atria were incubated for 1 h at 34°C in this dye solution. Ester solubility in an aqueous medium, and hence the ease of ester penetration of the sarcolemma and subsequent hydrolysis to indo-1 within the cell, have been shown to be increased in the presence of Pluronic (Poenie *et al.*, 1986; Schackman & Chock, 1986; Himpens & Somlyo, 1988; Owen, 1988).

Fluorescence

Dye-loaded atria, on the plastic holder, were positioned diagonally across a standard 1 cm square-section quartz cuvette and thermostated at 34°C in a specially designed fluorimeter equipped with one excitation monochromator and with two emission monochromators, each with a photon-counting photomultiplier tube (Alphascan System, Photon Technology International). Epifluorecence emissions were collected digitally at 60 data points s^{-1} from the front face and one side face of the cuvette. Fresh superfusate, bubbled continuously with 95% O_2 plus 5% CO_2 in an external reservoir, was pumped at 15 ml min^{-1} to the bottom of the cuvette via narrow-bore tubing. Superfusate was sucked off from the top of the cuvette. Inflow and outflow tubes and the wire electrodes were kept out of the light paths used by the fluorimeter.

Excitation was at 360 nm, and the two emission monochromators were set at 400 nm and 500 nm respectively. Grynkiewicz *et al.* (1985) showed that the intensity of fluorescence (F) emitted by indo-1 is increased by calcium in a concentration-dependent manner at 400 nm, but diminished at 500 nm, and that the ratio of emission intensities at these two wavelengths (F400/500) provides a measure of the free calcium concentration. Before dye-loading, the combined tissue and instrumental autofluorescence signals were recorded and filed in computer memory, and then routinely deducted from signals obtained later with atria in the dye-loaded state. However, tissue autofluorescence needed to be recorded during each experimental intervention, as it varied under different conditions of superfusion. Autofluorescence-corrected signals from dye-loaded atria were typically about 5 fold greater than tissue autofluorescence intensity.

Simulated ischaemia

In order to simulate ischaemic conditions the NS was replaced by a superfusate of abnormal composition (Ferrier *et al.*, 1985), containing KCl 7 mM, racemic sodium lactate 20 mM and no glucose. The pH was reduced to 6.4 by reducing the $NaHCO_3$ to 4 mM. Hypoxia was produced by replacing O_2 by N_2 (containing 0.0002% or less of O_2, British Oxygen Company).

Drugs

Sulphinpyrazone and flufenamate were gifts from Ciba-Geigy and Parke Davis & Company respectively. Both were dissolved in water with a small excess of Na_2CO_3 and the solution adjusted to neutrality with acetic acid.

Results

Effects of isoprenaline

Dye-loaded atria in NS displayed a rise in the F400 signal and a simultaneous fall in the F500 signal during each systole, causing marked positive changes to occur in the calculated F400/500 ratio, as shown in Figure 1.

Isoprenaline (1 μM) caused positive inotropism when added to atria bathed with NS. Systolic developed tension became maximal within the first 2 min, then faded somewhat before reaching a plateau (Figure 2). Isoprenaline increased the rate of both systolic tension development and subsequent relaxation. The peak F400/500 ratio reached during systole and the rate of diastolic decline in this ratio were also augmented by isoprenaline, and both became maximal soon after starting exposure to this agent. End-diastolic values of both tension and the F400/500 ratio were unaltered by isoprenaline (Figure 2).

Exposure to simulated ischaemia

Atria that were exposed to conditions of simulated ischaemia (SI) showed a decline in systolic developed tension within 2 min, and after 6 min systolic developed tension was no longer detectable (Figure 3). Despite this, the systolic increments in the F400/500 ratio after 6 min of exposure to SI averaged 76% of the value in NS (Figure 3). Over the next few min, however, atrial contracture developed, accompanied by a slow rise in the end-diastolic F400/500 ratio, together with further diminution in the systolic increments in the F400/500 ratio (Figure 3). By the end of 14 min of exposure to SI the peak systolic and end-diastolic values of the F400/500 ratio were no longer different from each other. By the end of 20 min both had become similar to peak systolic values previously observed in NS (Figure 3), and by the end of 30 min both had become significantly higher than peak systolic values previously observed in NS.

Atria that were exposed to conditions of lactate-free SI, like those exposed to full lactate-containing SI, lost all systolic developed tension within the first 6 min (Figure 4). However,

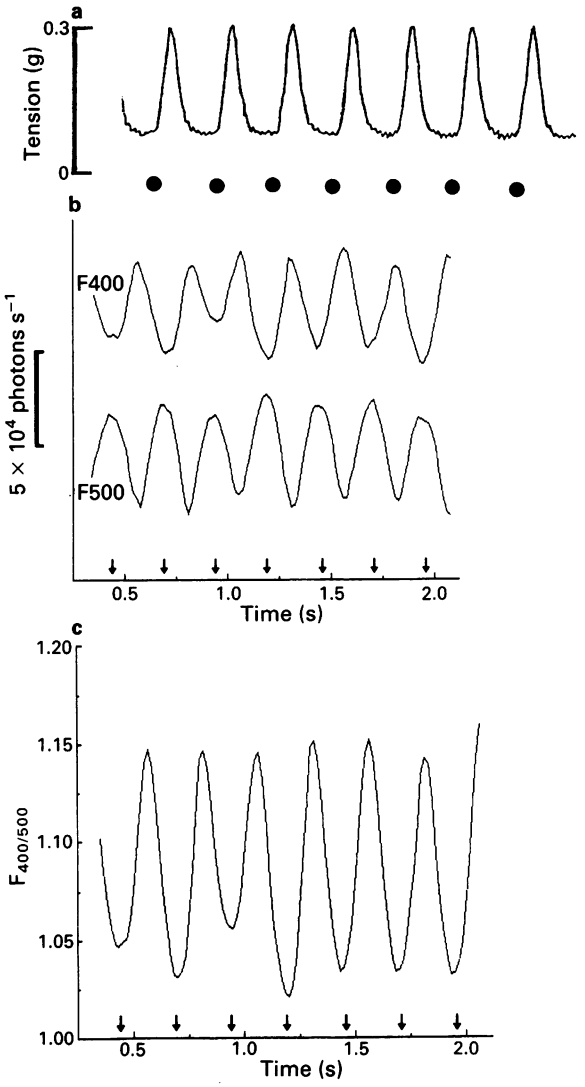

Figure 1 Changes in tension (a), in intensity of fluorescence emitted by indo-1 at 400 nm (F400) and 500 nm (F500) (b) and in the F400/500 ratio (c) during the same 7 consecutive systolic-diastolic cycles from a representative dye-loaded atrium while in normal superfusate and stimulated at the points marked in panel (a) and at the arrows in panels (b) and (c).

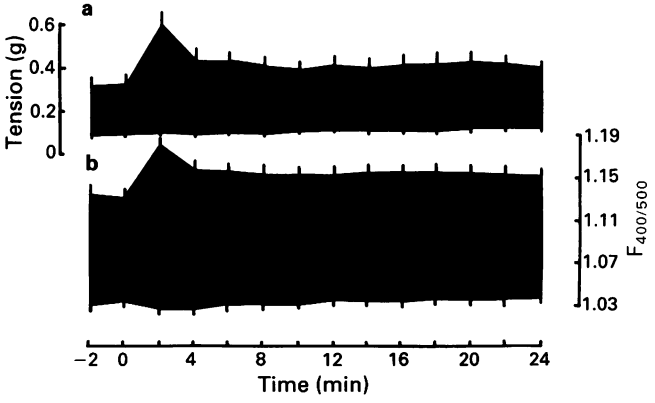

Figure 2 Mean tension (a) and contemporary mean ratios of fluorescence emissions at 400 nm and 500 nm (F400/500) (b) of 20 systolic-diastolic cycles from each of 10 dye-loaded atria exposed to normal superfusate which also contained isoprenaline (1 μM) from time zero onwards. The upper edges of each panel represent peak systolic values and the lower edges end-diastolic values. Vertical bars represent s.e.mean.

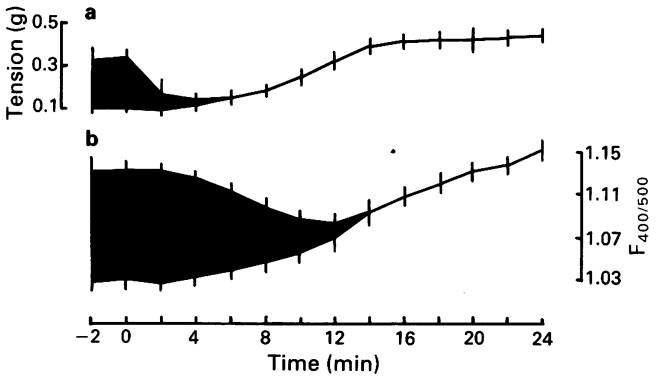

Figure 3 Mean tension (a) and contemporary mean ratios of fluorescence emissions at 400 nm and 500 nm (F400/500) (b) of 20 systolic-diastolic cycles from each of 10 dye-loaded atria exposed to normal superfusate until time zero and to conditions of simulated ischaemia (with 20 mM lactate) thereafter. The upper edges of each panel represent peak systolic values and the lower edges end-diastolic values. Vertical bars represent s.e.mean.

subsequent contracture development during lactate-free SI was milder than during lactate-containing SI. During exposure to the former condition, in contrast to the latter, the systolic increments in the F400/500 ratio were preserved, even after contracture had become established (Figure 4). End-diastolic values of the F400/500 ratio rose significantly during exposure to lactate-free SI (Figure 4), but the rise was significantly less than that seen during lactate-containing SI (Table 1).

Glucose was absent during conditions of full SI. Addition of glucose (20 mM) to the superfusate during SI protected atria against contracture development, almost abolished the accompanying rise in end-diastolic F400/500 ratio, but did not protect against the diminution in systolic developed tension that was seen during SI (Figure 5 and Table 1). Moreover, the presence of this concentration of glucose only slightly delayed the disappearance of the systolic increments in the F400/500 ratio usually produced during the first 14 min of SI. This can be seen by comparing the F400/500 values 10–18 min after starting SI in Figures 3 and 5.

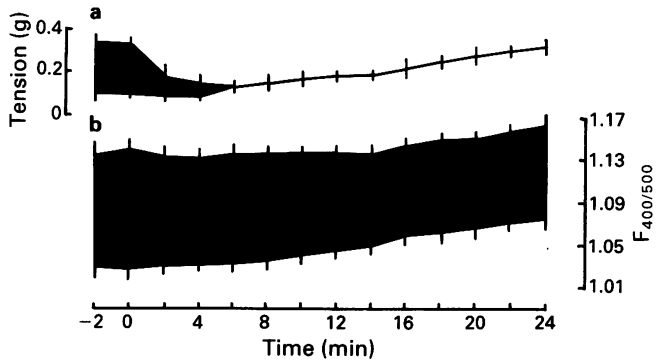

Figure 4 Mean tension (a) and contemporary mean ratios of fluorescence emissions at 400 nm and 500 nm (F400/500) (b) of 20 systolic-diastolic cycles from each of 10 dye-loaded atria exposed to normal superfusate until time zero and thereafter to lactate-free simulated ischaemia. The upper edges of each panel represent peak systolic values and the lower edges end-diastolic values. Vertical bars represent s.e.mean.

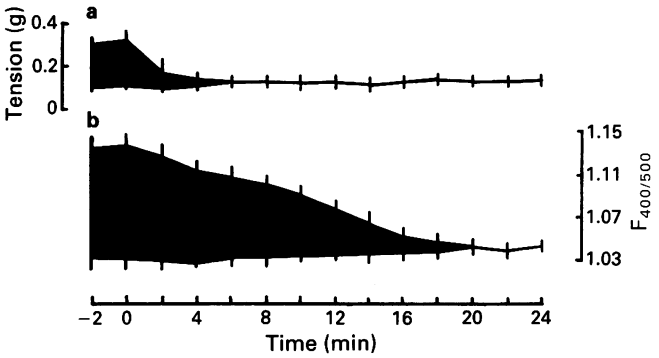

Figure 5 Mean tension (a) and contemporary mean ratios of fluorescence emissions at 400 nm and 500 nm (F400/500) (b) of 20 systolic-diastolic cycles from each of 10 dye-loaded atria exposed to normal superfusate until time zero and thereafter to simulated ischaemia with glucose (20 mM) added. The upper edges of each panel represent peak systolic values and the lower edges end-diastolic values. Vertical bars represent s.e.mean.

Effects of sulphinpyrazone and flufenamate

Addition of sulphinpyrazone (50 μM) to the superfusate protected atria against contracture development during SI (Table 1). There was protection also against the SI-induced rise in the end-diastolic F400/500 ratio (Table 1), although not against the early loss of systolic increments in atrial tension, and to only a slight extent against the loss of systolic increments in the F400/500 ratio (Table 1). Sulphinpyrazone failed to protect against any of the mechanical or optical changes induced by exposure to lactate-free SI (Table 1). Pretreatment of atria with flufenamate (5 μM) aggravated the development of contracture and the associated rise in the end-diastolic F400/500 ratio during exposure of SI. These effects of flufenamate were displayed equally prominently during lactate-free and lactate-containing SI (Table 1). Addition of sulphinpyrazone or flufenamate to NS failed to modify systolic developed tension, end-diastolic tension, or the F400/500 ratio measured at the peak of systole or at the end of diastole.

Discussion

The present work has revealed that atrial muscle loaded with indo-1 and superfused with NS displays a phasic rise and fall in the F400/500 ratio in response to each applied electrical stimulus. Previous workers have obtained similar findings

Table 1 Effects of different superfusates on the mechanical and optical responses of dye-loaded atria

	Superfusate			*Tension* (mg)		*F400/500 ratio*	
	Glucose (mM)	*Lactate* (mM)	*Drug* (μM)	*End-diastolic*	*Systolic increment*	*End-diastolic*	*Systolic increment*
NS	10	0	—	102 ± 11**	218 ± 29*	1.030 ± 0.012**	0.104 ± 0.015**
SI	0	20	—	386 ± 40*	12 ± 17**	1.097 ± 0.016*	0.013 ± 0.018*
SI	0	0	—	204 ± 35**†	15 ± 18**	1.057 ± 0.013**†	0.085 ± 0.020**†
SI	20	20	—	115 ± 18**	−1 ± 12**	1.036 ± 0.014**	0.025 ± 0.013
SI	0	20	Sulphinpyrazone (50)	238 ± 30**	2 ± 8**	1.048 ± 0.017**	0.054 ± 0.012**
SI	0	20	Flufenamate (5)	465 ± 46**	−3 ± 5**	1.131 ± 0.015**	−0.003 ± 0.019
SI	0	0	Sulphinpyrazone (50)	227 ± 25**	16 ± 14**	1.042 ± 0.012	0.051 ± 0.014
SI	0	0	Flufenamate (5)	449 ± 51†	11 ± 20**	1.132 ± 0.018†	0.008 ± 0.011†

Tabulated values represent means ± s.e.mean of 20 systolic-diastolic cycles from each of 10 atria after exposure to the stated conditions for between 14 and 15 min. A significant difference exists ($P < 0.05$) between a value marked * and corresponding values marked **, and also between members of a corresponding pair of values marked †. NS: normal superfusate; SI: conditions of simulated ischaemia.

with both intact ventricular myocardium and isolated ventricular myocytes, and have concluded that this optical phenomenon represents the systolic rise and diastolic fall in $[Ca]_c$ upon which contraction and relaxation depend (Dubell & Houser, 1987a; Lee *et al.*, 1987; 1988). Both the systolic rise in the F400 signal and simultaneous fall in the F500 signal also reflect the rise in $[Ca]_c$. However, calculated changes in the ratio of emission intensities at 400 nm and 500 nm respectively were larger than the changes observed in either the F400 or the F500 signal separately, and the ratio has been shown to be less subject to interference and motion artefact (Lee *et al.*, 1987; 1988; Eisner *et al.*, 1989). For these reasons the F400/500 ratio, rather than its components was used routinely in the present work. Nevertheless, when changes in this ratio resulted from an experimental intervention, concomitant, albeit smaller, changes were detected in both the F400 and F500 signals. Grynkiewcz *et al.* (1985) showed that aqueous solutions of indo-1 gave F400/500 ratios that accurately reflected the ambient free calcium concentration. In the myoplasm, however, the likely presence of residual unhydrolysed indo-1 ester (Dubell & Houser, 1987b; Lee *et al.*, 1987; 1988; Peeters *et al.*, 1987; Tamura *et al.*, 1989), plus the probable binding of some of the hydrolysed indo-1 to structural and other cytoplasmic components (Owen & Shuler, 1989), make the calculation of absolute $[Ca]_c$ values from the F400/500 ratios rather questionable (Dubell & Houser, 1987b; Cork *et al.*, 1989; Tamura *et al.*, 1989). Nevertheless, changes that appear in the F400/500 ratio of indo-1 loaded myocardium are probably directly proportional to the accompanying changes in $[Ca]_c$ (Barry *et al.*, 1987; Dubell & Houser, 1987a; Lee *et al.*, 1987; 1988). Actual F400/500 ratios rather than derived $[Ca]_c$ values are presented in this study. However, it seems safe to assume that changes in the former faithfully monitored any changes that occurred in the latter.

Isoprenaline produced a positive inotropic response when applied to atria superfused with NS in the present experiment. Fading of the early inotropic response, despite the continued presence of isoprenaline, was paralleled by a similar change in systolic increments in the F400/500 ratio (Figure 2). This suggests that fading of the response was likely to have been due, in part at least, to diminishing systolic rises in $[Ca]_c$. Previous workers have demonstrated that fading of the inotropic response to isoprenaline was associated with diminishing sensitivity of the contractile proteins to $[Ca]_c$ (Ray & England, 1976; Endoh & Blinks, 1988), but the extent to which this latter process operated in the present experiments is unknown.

Exposure of the rat atrium to conditions of SI previously was shown to elicit a wide range of mechanical, electrical, biochemical and structural abnormalities (Northover, 1987; 1989; Northover & Northover, 1988). The tension changes shown in Figure 3 confirm these earlier findings and permit comparisons to be made with contemporary changes in $[Ca]_c$. Clearly, the major loss of systolic developed tension preceded comparable diminution in the systolic increments in $[Ca]_c$. This suggests that early loss of systolic developed tension during SI is attributable, in the main, to reduced sensitivity of the contractile proteins to $[Ca]_c$. Others have reached similar conclusions using other myocardial preparations (Allen & Orchard, 1983; Allen *et al.*, 1989), but doubts remain about which of the changes in the chemical composition of the myoplasm during SI is responsible for this loss of sensitivity to $[Ca]_c$ (Godt & Nosek, 1989). Stern *et al.* (1988), on the other hand, concluded that failure of systolic shortening during SI is accounted for mainly by a loss of the systolic increments in $[Ca]_c$. The latter workers, however, used single isolated cardiomyocytes at zero tension, which may explain these atypical findings.

Exposure to SI in the absence of lactate was associated with a loss of systolic developed tension similar to that seen in the presence of lactate (Figures 3 and 4). During the former, as opposed to the latter condition, little diminution in the systolic increments in the F400/500 ratio occurred. This is further evidence that the loss of systolic developed tension during SI primarily reflects an insensitivity of the contractile proteins to $[Ca]_c$. Why the presence of lactate during SI was necessary for the loss of systolic increments in $[Ca]_c$ is not known. Depletion of the myocardial store of ATP is known to be less severe when rat atria are subjected to SI in the absence rather than the presence of lactate, and this difference has been attributed to the ability of lactate to inhibit glycolysis (Northover, 1989). The ATP derivable from glycolysis in the absence of added lactate, therefore, may have been sufficient to preserve systolic rises in $[Ca]_c$ under anaerobic conditions. Allen & Orchard (1983) also noted that small amounts of ATP derived from glycolysis were able to preserve systolic rises in $[Ca]_c$ under conditions of hypoxia, or under the influence of respiratory poisons such as cyanide, even when systolic increments in myocardial tension had already failed.

The importance of a rising end-diastolic $[Ca]_c$ value in the production of an ischaemic contracture is still debated. Clearly, however, the technique used to study $[Ca]_c$ needs to be sensitive to Ca at normal end-diastolic levels. This may not have been true with some aequorin luminescence measurements, and is in contrast to measurements based upon indo-1, as discussed by Eisner *et al.* (1989).

Both end-diastolic tension and end-diastolic $[Ca]_c$ gradually rose during exposure to SI in the present experiments, confirming previous findings based upon other models of ATP-depletion (Barry *et al.*, 1987; Eisner *et al.*, 1989; Kohmoto & Barry, 1989; Morris *et al.*, 1989). Both rises had a similar time course, suggesting a causative connection between them. This was reinforced by the fact that during lactate-free SI, during glucose-containing SI, as well as during SI conducted in the presence of sulphinpyrazone, the rises in end-diastolic tension and the F400/500 ratio were less than those seen during the usual conditions of glucose-free SI in the presence of lactate. The presence of glucose, however, only slightly delayed the SI-induced decline in the systolic increments in the F400/500 ratio, and this may be seen by comparing Figures 3 and 5. This finding was unexpected since previous work showed that the presence of glucose (20 mM) almost fully protects atria against the depletion of ATP normally found at the end of 15 min of SI (Northover, 1989). Systolic increments of $[Ca]_c$ may decline during some types of SI, therefore, even without depletion of the ATP stores. During hypoxia the free energy change resulting from the hydrolysis of ATP has been calculated to decline due to the accumulation of catabolites of ATP (Kammermeier *et al.*, 1982; Allen & Orchard, 1983). Kentish & Allen (1986) were unable to account for the extent of the decline in systolic developed tension during ischaemia solely in terms of the decrement in the calculated free energy change of ATP hydrolysis. Under certain circumstances, however, it is possible that even normal stores of ATP may be insufficient to preserve normal systolic rises and falls in $[Ca]_c$. Sulphinpyrazone is another agent that is known to protect atria against ATP-depletion during the first 15 min of SI (Northover, 1989). Sulphinpyrazone, like glucose, only slightly delayed the SI-induced reduction in systolic increments in the F400/500 ratio in the present experiments (Table 1). Stern *et al.* (1988) suggested that the failure of systolic increments in $[Ca]_c$ during SI was due to shortening of action potential duration. Atrial action potentials certainly shorten during the first 15 min of SI (Northover, 1987). Since sulphinpyrazone has been shown to protect against this shortening (Northover, 1987) but failed to protect significantly against the loss of the systolic increments in $[Ca]_c$ seen after 15 min of SI in the present experiments, the loss of the systolic increments in $[Ca]_c$ would seem to depend mainly upon factors other than action potential shortening, at least in rat atria.

Previous work with this model of myocardial injury has shown that the presence of lactate during SI enhances the observed shortening of atrial refractory periods and slowing of conduction velocity (Northover, 1989). The fact that the presence of lactate during SI enhanced the rise in end-diastolic $[Ca]_c$ in the present experiments is consistent with a raised

$[Ca]_c$ being responsible for these electrical disturbances. This suggestion gains additional support from the fact that the presence of glucose during SI protected the myocardium not only against the rise in end-diastolic $[Ca]_c$ in the present experiments (Table 1), but also against the shortening of atrial refractory periods and the slowing of conduction velocity in previous work (Northover, 1989). Possible mechanisms whereby the changes in $[Ca]_c$ that are seen during SI may provoke these electrical disturbances have been discussed previously (Northover, 1987; 1989; Northover & Northover, 1988).

The ability of sulphinpyrazone in the present experiments to protect atria against the SI-induced rises in both end-diastolic tension and end-diastolic $[Ca]_c$ constitutes further evidence that the rising end-diastolic $[Ca]_c$ contributes to contracture development. This is reinforced by the fact that under conditions of lactate-free SI sulphinpyrazone no longer was able to prevent either of these changes, whereas flufenamate was still able to potentiate both changes. The protective effects of sulphinpyrazone and the deleterious effects of flufenamate previously were shown to include the SI-induced shortening of atrial refractory periods and slowing of conduction velocity (Northover, 1987; 1989; Northover & Northover, 1988). This earlier work indicated that sulphinpyrazone protects the ischaemic myocardium by preventing the inhibition of anaerobic glycolysis by lactate. Anaerobic ATP production from endogenous stores of glycogen effectively slows the decline in ATP at the start of ischaemia. Glycolytic ATP production fails, however, if lactate is plentiful. Sulphinpyrazone under these circumstances can preserve this route of ATP production. Flufenamate, on the other hand, aggravates the decline in ATP stores during ischaemia by disrupting the net synthesis of ATP. The fact that sulphinpyrazone in the present experiments protected against the SI-induced rise in end-diastolic $[Ca]_c$, and that flufenamate had an opposite effect, is consistent with the rising end-diastolic $[Ca]_c$ during SI being responsible for the electrical disturbances noted in previous studies.

In conclusion therefore, the present experiments provide evidence that during SI the early losses of atrial systolic tension development do not reflect the contemporary changes in $[Ca]_c$, whereas the more slowly developing contracture reflects the progressive rise in end-diastolic $[Ca]_c$. The alterations in end-diastolic $[Ca]_c$ show a time-course that also suggests that they are responsible, in part at least, for the accompanying slowing of action potential conduction velocity and shortening of refractory periods. Sulphinpyrazone has been shown to protect against all of the changes produced by SI with the exception of the early losses of systolic developed tension. Evidence both from this and previous studies suggests the ability of sulphinpyrazone to protect against the disturbances of calcium metabolism during SI is partly explained by an ability to protect against early ATP depletion.

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Characterization of the adenosine receptor in porcine coronary arteries

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1 Relaxant responses of ring preparations from porcine ventricular coronary arteries to adenosine and various stable adenosine analogues were investigated *in vitro*.

2 The adenosine analogues did not produce contraction but elicited almost complete relaxation of coronary arteries preconstricted with 3 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), even after removal of the endothelium.

3 The order of potency, was 5'-N-ethylcarboxamide-adenosine (NECA) > 2-(2-phenylethylamino)5'-N-ethylcarboxamide-adenosine (2-PEA-NECA) > 2-phenylamino-adenosine (CV-1808) > N^6-[R(−)-1-phenyl-2-propyl]adenosine (R-PIA) > N^6-[S(+)-1-phenyl-2-propyl]adenosine (S-PIA) > N_6-cyclopentyl-adenosine (CPA) > adenosine > ATP = ADP which suggested the presence of adenosine A_2-receptor subtypes.

4 There was an excellent correlation between the calculated pD_2 values on coronary arteries and the pK_D values at adenosine A_2 binding sites, whereas no correlation was obtained when the pD_2 values were compared to the pK_D values at adenosine A_1-binding sites on membranes from porcine striata.

5 The relaxant effects of adenosine and its analogues were competitively antagonized by 8-(*p*-sulphophenyl)-theophylline (8-SPT), producing pA_2 values similar to the respective pK_D value of the antagonist at adenosine A_2 binding sites.

6 It is suggested that the porcine coronary artery possesses adenosine A_2 receptors which seem to be similar to the adenosine A_2 binding site in pig striatum, whereas no evidence was obtained for the presence of adenosine A_1 receptors.

Introduction

Adenosine receptors mediate several physiological effects, the most important of which may be the restoration of a balance between metabolic demand and supply (Newby, 1984). According to the classification proposed by Burnstock (1978) the pharmacological responses to adenosine are mediated through extracellular adenosine (P_1) receptors which have been further subdivided into two types of membrane bound adenosine receptors, namely adenosine A_1 and A_2 receptors both of which are coupled to adenylate cyclase via a G regulatory protein. While the A_1 subtype of adenosine receptor mediates inhibition of adenylate cyclase, the A_2 subtype induces activation of the enzyme (Van Calker *et al.*, 1978; Londos *et al.*, 1980). Although antagonists with adenosine A_1 receptor selectivity, and only little effect on phosphodiesterase, have become available, classification of adenosine receptor subtypes is still based predominantly on the relative potencies of various adenosine analogues (Stone, 1984; Bowmer & Yates, 1989). At the A_1 receptor subtype, N^6 substituted analogues, e.g., N^6-[1-phenyl-2-propyl]adenosine (PIA), are more potent than 5' substituted analogues, such as 5'-N-ethylcarboxamide-adenosine (NECA), with the reverse being true at the A_2 receptor subtype. By use of this classification, the presence of A_2 subtype receptors has been demonstrated in canine (Kusachi *et al.*, 1983; Nakazawa & Mustafa, 1988), bovine (Mustafa & Askar, 1985) and human coronary arteries (Ramagopal *et al.*, 1988). The results of the present study add support to the contention that responses to adenosine of porcine coronary arteries are also mediated mainly, if not exclusively through A_2 adenosine receptors while there is no evidence for the presence of A_1 adenosine receptors in this vascular smooth muscle.

Methods

Binding assay

A crude membrane preparation was prepared from porcine striata as described by Bruns *et al.* (1986). Briefly, striata were disrupted in 10 volumes of ice-cold 50 mM Tris HCl buffer (pH 7.4) for 30 s with a Polytron PT-10/35 homogenizer (Brinkmann) at setting 5–6 and centrifuged at 50,000 *g* for 10 min. The supernatant was discarded and the pellet was resuspended with a Polytron in 10 volumes of ice-cold Tris HCl buffer as above and recentrifuged. The final pellet was resuspended at 1 g 5 ml^{-1}, and stored in plastic vials at −70°C until used. All incubations were carried out in triplicate for 45 min at 37°C in TRF 95 vials. The final volume of 1 ml contained: Tris HCl buffer (50 mM Tris HCl + 10 mM $MgCl_2$, pH 7.4), 4 nM [^{3}H]-NECA, 1 μg ml^{-1} adenosine deaminase, 50 nM N^6-cyclopentyladenosine (CPA, to eliminate A_1 receptor binding), substance under investigation and pig striatum membranes (5–6 mg of original tissue weight). CPA was omitted from those studies where both A_1 and A_2 components of binding were determined. The incubation was terminated by addition of about 5 ml ice-cold buffer (50 mM Tris HCl + 154 mM NaCl, pH 7.4) and filtration through Whatman GF/B filter sheets, which were then further rinsed with about 5 ml ice-cold buffer. The damp filters were put into scintillation vials, scintillation fluid (5 ml Controgel) was added and the vials incubated at 37°C for 30 min and shaken. After the contents of the vials had been cooled down to room temperature the radioactivity was measured in a beta counter.

Organ bath studies

Porcine hearts were obtained from a local slaughter house within 30 min after death and transported to the laboratory where the ventricular coronary arteries were carefully removed and placed in Krebs-Henseleit solution (composition in mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, EDTA 0.03) gassed with 5% CO_2 in oxygen. The arteries were cut into rings (2–3 mm) and stainless steel hooks were inserted through the lumen, allowing them to be suspended for isometric tension recording in 10 ml organ baths containing Krebs-Henseleit solution at 37°C, bubbled continuously with 5% CO_2 in O_2. Changes in the tone of the preparations were recorded isometrically with electromechanical transducers (Statham model UC3) and a potentiometric recorder. At the beginning of the experiments,

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the rings were stretched to an initial tension of about 1.5 g and allowed to equilibrate and establish baseline tension for 2 to 3 h in the bathing medium, which was changed every 15 min. During this time the preparations were stimulated twice with KCl (30 mM) and the baseline tension was adjusted to about 1 g. After repeated changes of the bathing solution the tissues were exposed to 3 μM prostaglandin $F_{2\alpha}$, which induced a stable submaximal contraction of about 80% of maximum response. Concentration-response curves to adenosine analogues were then determined in the absence and presence of antagonists. Inhibitors were added 30 min before the agonists to the organ baths and remained in contact with the tissue when the responses to agonists were tested. At the end of each concentration-response curve papaverine (300 μM) was added to induce complete relaxation of the arterial rings. This effect was taken as 100% relaxation. In each experiment 6 rings of the same artery were investigated at the same time, one of which was used as a control preparation, to correct for any sensitivity changes during the experiment.

Data analysis

Non-specific binding of [^{3}H]-NECA was defined as binding in the presence of 100 μM NECA. Specific binding was about 85–90% of the total binding. The experimental data were fitted to a two-site model yielding Hill coefficients of 1.0. The regression algorithm applied was derived from the Downhill Simplex Method of Multidimensions (Press *et al.*, 1987). The 95% confidence limits for the estimated parameters B_{max_1}, B_{max_2}, $IC_{50}(1)$, $IC_{50}(2)$ and non-specific binding were obtained by maximizing and minimizing each of the 5 parameters, with the restriction that the Chi square value of the residuals was kept within the 95% confidence limits, by use of the same algorithm mentioned above.

The confidence limits for the calculated curves depicted in the figures were obtained from the envelopes of all calculated curves obtained in the process of estimating the confidence limits for the 5 parameter estimates. They depict the '95% confidence channel', that implies the course of the 'true' curve, assuming the two-site model to be applicable.

Concentration-response curves on coronary arteries were analysed with a non-linear computer programme, and E_{max} (maximal effect) and pD_2 values (negative logarithm of the molar concentration of the agonist producing 50% of E_{max}) were derived from this analysis. Apparent pA_2 values were calculated according to the equation $pA_2 = pA_x + \log(x - 1)$, where pA_x is the negative logarithm of the molar concentration of the antagonist used and x is the ratio of equieffective concentrations of the agonist in the presence and absence of the antagonist (Arunlakshana & Schild, 1959; Furchgott, 1972). Data are given as means ± s.e.mean, statistical analysis was performed by Student's *t* test.

Drugs

The following pharmacological agents were used: CHA (N^6-cyclohexyladenosine, Boehringer, Mannheim, F.R.G.), NECA (5'-N-ethylcarboxamide-adenosine, Byk-Gulden, Konstanz, F.R.G.), adenosine (Fluka, Buchs, Switzerland), CPA (N^6-cyclopentyladenosine), R-PIA (N^6-[R(−)-1-phenyl-2-propyl] adenosine), S-PIA (N^6-[S(+)-1-phenyl-2-propyl]adenosine, Research Biochemicals, Incorp., Wangen, Switzerland), ADP (adenosine diphosphate), ATP (adenosine triphosphate), $PGF_{2\alpha}$ (prostaglandin $F_{2\alpha}$), forskolin, isoprenaline sulphate, papaverine hydrochloride, (Sigma, Munich, F.R.G.), 8-SPT (8-(*p*-sulphophenyl)-theophylline-monohydrate), CV-1808 (2-phenylamino-adenosine) and 2-PEA-NECA (2-(2-phenylethylamino)5'-N-ethylcarboxamide-adenosine, European Patent No. 277 917, 1988) were synthesized at Sandoz Ltd., Basel, Switzerland.

Both prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and forskolin were dissolved in ethanol and diluted in distilled water to give a 1 mM solution containing 10% ethanol. Samples of this solution were stored at −20°C until use. CV-1808 was dissolved in 50% ethanol containing 25% N-methylpyrrolidone (v/v), to give a 1 mM solution containing 1.5% ethanol and 1% N-methylpyrrolidone, CPA and 2-PEA-NECA were dissolved in polyethylene glycol (PEG) 400 and diluted with distilled water to give a 1 mM solution containing 60% PEG 400. CHA, R-PIA and S-PIA were dissolved in dimethyl sulphoxide (DMSO) and diluted in distilled water to give a 1 mM solution containing 10% DMSO. Further dilutions were performed in either 5% glucose or physiological salt solution. All compounds, except papaverine, forskolin and $PGF_{2\alpha}$, were dissolved just before use. Drug concentrations are given as molar concentrations throughout.

Results

Agonism

Under control conditions neither adenosine nor CPA elicited a contractile response of porcine coronary arteries. Even after activation of adenylate cyclase by isoprenaline (1 μM) or forskolin (0.1 and 1 μM), CPA like adenosine elicted no contractile response but induced an additional relaxation (Figure 1). Consequently, only for relaxant responses were concentrations-response curves to the various adenosine analogues determined, using arterial preparations precontracted with 3 μM $PGF_{2\alpha}$ (Figure 2). In preliminary experiments it was observed that removal of the endothelium from the arterial preparations, by rubbing the interior of the ring with a wooden matchstick, did not modify the relaxant response to adenosine. Therefore no special attention was paid to preserve the endothelium when the relaxant activities of the various adenosine analogues were investigated. All the compounds tested induced nearly complete relaxation of rings from porcine ventricular coronary arteries. Comparison of the calculated pD_2 values on coronary vascular smooth muscle with the pK_D values determined on membranes from pig striatum

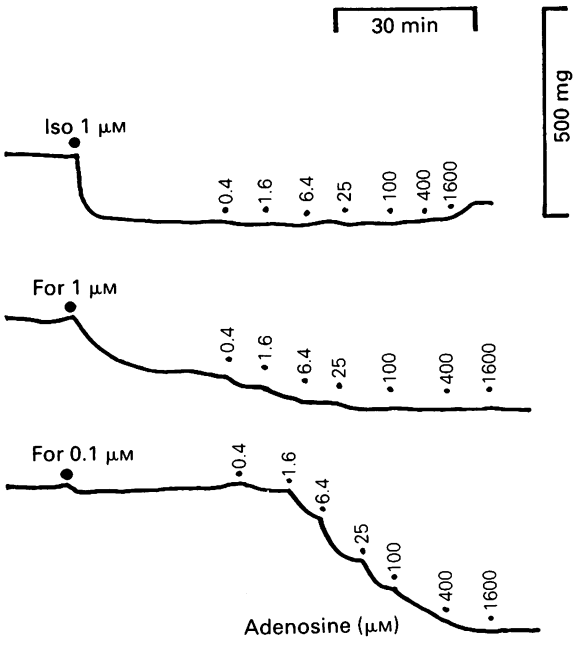

Figure 1 Representative tracing of responses to adenosine of rings from porcine ventricular coronary arteries without and after activation of adenylate cyclase with isoprenaline (Iso, 1 μM) or forskolin (For, 1 and 0.1 μM).

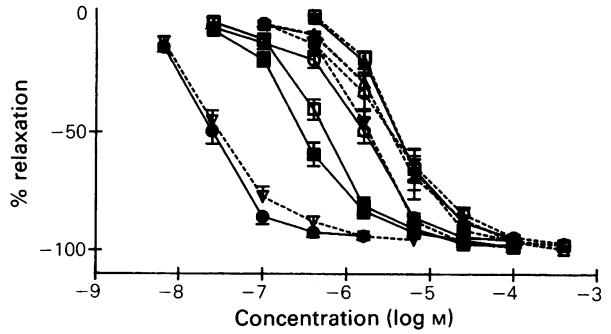

Figure 2 Cumulative concentration-response curves for 5'-N-ethylcarboxamide-adenosine (NECA) (●———●, $n = 7$), 2-(2-phenylethylamino) NECA (2-PEA-NECA) (▽- - - -▽, $n = 10$), 2-phenylamino-adenosine (CV-1808) (■———■, $n = 8$), N^6-[R(−)-1-phenyl-2-propyl]adenosine (R-PIA) (□———□, $n = 5$), N^6-[S(+)-1-phenyl-2-propyl]adenosine (S-PIA) (○———○, $n = 6$), N^6-cyclopentyladenosine (CPA) (▼- - - -▼, $n = 13$), N^6-cyclohexyladenosine (CHA) (△- - - -△, $n = 4$), adenosine (○- - - -○, $n = 13$), ATP (△———△, $n = 8$) and ADP (□- - - -□, $n = 10$) on rings from procine ventricular coronary arteries stimulated with 3 μM $PGF_{2\alpha}$. The effects are expressed as percentages of the relaxant response to 300 μM papaverine. The points represent means and vertical lines s.e.mean.

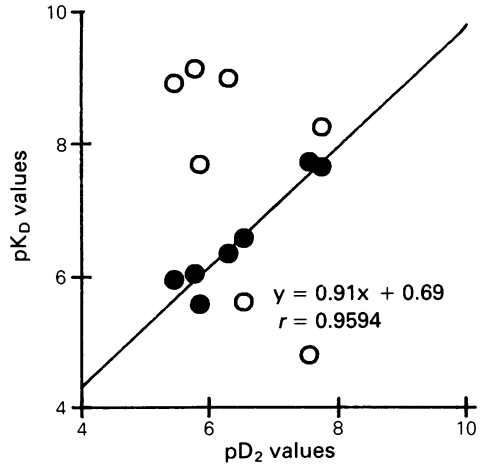

Figure 3 Correlation between drug potencies for relaxation of porcine coronary arteries (pD_2 values, abscissa scale) and (pK_D values, ordinate scale) at adenosine A_1 (○) and A_2 (●) binding sites in pig striatum. Data were compared by linear regression analysis. The correlation coefficient and regression line for the correlation of pD_2 values and A_2 binding are indicated; $P < 0.001$.

Table 1 Affinity parameters (pD_2 values) and intrinsic activity for various adenosine analogues of rings from pig ventricular coronary arteries and pK_D values for binding at A_1 and A_2 binding sites in membranes from pig striatum

	pD_2 values	Intrinsic activity	pK_D values A_1	A_2
Adenosine	5.51 ± 0.13	94.2 ± 1.3 (13)	*	*
ADP	5.38 ± 0.05	95.8 ± 1.0 (10)	*	*
ATP	5.39 ± 0.10	94.6 ± 1.3 (8)	*	*
CHA	5.46 ± 0.11	99.3 ± 0.5 (4)	8.91	5.95
CPA	5.77 ± 0.07	98.5 ± 0.5 (13)	9.13	6.03
CV-1808	6.53 ± 0.07	98.5 ± 0.2 (8)	5.61	6.57
2-PEA-NECA	7.54 ± 0.08	95.8 ± 1.0 (10)	4.81	7.72
NECA	7.74 ± 0.08	96.5 ± 1.3 (4)	8.25	7.64
R-PIA	6.29 ± 0.07	97.0 ± 1.0 (5)	8.99	6.34
S-PIA	5.85 ± 0.07	98.0 ± 1.1 (6)	7.69	5.57

Data are given as mean ± s.e.mean, number of determinations in parentheses.
* Since binding studies were performed in the presence of adenosine deaminase, pK_D values for adenosine, ADP and ATP were not determined.

Table 2 Apparent pA_2 values for antagonism of various adenosine analogues by 8-(*p*-sulphophenyl)-theophylline (8-SPT) on rings from porcine ventricular coronary arteries

	pA_2 values
Adenosine	4.91 ± 0.21 (6)
ADP	4.60 ± 0.14 (7)
ATP	5.13 ± 0.11 (8)
CHA	4.94 ± 0.13 (8)
CPA	5.02 ± 0.11 (8)
CV-1808	5.35 ± 0.15 (8)
2-PEA-NECA	5.08 ± 0.09 (5)
NECA	5.31 ± 0.07 (8)
R-PIA	5.03 ± 0.08 (8)
S-PIA	4.93 ± 0.16 (8)

Data shown are means ± s.e.mean, number of determinations in parentheses.

revealed a significant correlation between pD_2 values and the pK_D values at the A_2 binding sites ($P < 0.001$), whereas there was no correlation when the pD_2 values were compared to the pK_D values for A_1 binding sites (Figure 3, Table 1).

Antagonism

The non-selective adenosine receptor blocker 8-SPT consistently produced a parallel displacement of the concentration-responses curves of all adenosine analogues tested. The calculated pA_2 values for 8-SPT (Table 2) were statistically not different from the pK_D value of 5.55 ($n = 3$) determined for binding of 8-SPT at A_2 binding sites, suggesting that all agonists acted through stimulation of A_2-receptors.

Discussion

The aim of these experiments was to characterise the subtype of adenosine receptor(s) present in porcine coronary arteries by use of adenosine and different stable adenosine analogues. The order of potencies for the relaxant activities of the agonists used in our study, NECA > 2-PEA-NECA > CV-1808 > R-PIA > S-PIA > CPA > adenosine > ATP = ADP, suggested that the responses in this tissue are mediated by adenosine A_2 receptors. Further evidence for this was obtained when the calculated pD_2 values were compared to the pK_D values determined at A_1 and A_2 binding sites on membranes from porcine striata. There was an excellent correlation between the pD_2 values on coronary arteries and the pK_D values at A_2 binding sites. Whereas no correlation could be obtained when the pD_2 values were compared to the pK_D values at A_1 binding sites. In agreement with results obtained from other studies on coronary arteries of various species, such as dog, bovine and man (Kusachi *et al.*, 1983; Mustafa & Askar, 1985; Nakazawa & Mustafa, 1988; Ramagopal *et al.*, 1988), the present results suggest that the adenosine-induced dilatation of pig coronary arteries is also mediated through adenosine A_2 receptors. However, in contrast to that found for canine coronary arteries (Houston *et al.*, 1985), the adenosine-mediated relaxation of pig coronary arteries was not endothelium-dependent. Similar findings have been obtained for human (Ramagopal *et al.*, 1988), bovine (Mustafa & Askar, 1985) and monkey coronary arteries (Toda, 1983). The adenosine receptor blocker 8-SPT antagonized the relaxations induced by adenosine and the various analogues in a competitive manner and with similar potency, suggesting that the relaxations are mediated by a homogeneous population of adenosine receptors. Moreover, the pA_2 values calculated for antagonism of adenosine and the various analogues by 8-SPT

were statistically not different and similar to the pK_D value of 8-SPT at the A_2 binding site, suggesting that the responses are mediated by adenosine A_2 receptors.

In conclusion, the data presented in this study suggest that the porcine coronary artery possesses only adenosine A_2 receptors which mediate dilatation and seem to be similar to the adenosine A_2 binding site in pig striatum, whereas no evidence was obtained for the presence of adenosine A_1 receptors.

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Tetrahydroaminoacridine (tacrine) stimulates neurosecretion at mammalian motor endplates

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1 Tacrine (20 μM) induced, like 4-aminoquinoline (4-AQ, 200 μM), the appearance of a population of miniature endplate potentials (m.e.p.ps) with more than twice the normal amplitude or time-to-peak. The times-to-peak of nerve impulse-evoked endplate potentials were not similarly affected.

2 Cholinesterase inhibition by edrophonium (25 μM) did not prevent tacrine or 4-AQ from inducing this population of m.e.p.ps.

3 Nerve-muscle preparations in which the normal calcium-sensitive quantal release of acetylcholine had been blocked by botulinum neurotoxin type A also responded to tacrine by an increase in the frequency of giant or slow m.e.p.ps.

4 Reduction of the temperature from 30° to 14°C reduced the frequency of giant or slow m.e.p.ps induced either by tacrine or by 4-AQ. A similar effect was obtained by colchicine (5 mM). This supports the idea that proximo-distal axonal transport is required for the secretory activity.

5 The neurosecretion evoked by tacrine could explain the therapeutic effects of the drug claimed in the treatment of Alzheimer's type of dementia.

Introduction

Tacrine (1,2,3,4-tetrahydro-9-aminoacridine) is a drug which recently has achieved international interest because of claims that it is capable of relieving some of the symptoms that characterize Alzheimer's type of dementia (Summers *et al.*, 1981; 1986). Chemically, a similarity exists between tacrine and 4-aminoquinoline (4-AQ). In previous studies (Molgó & Thesleff, 1982; Molgó *et al.*, 1982; Thesleff *et al.*, 1983) it has been shown that 4-AQ selectively stimulates the spontaneous release from mammalian motor nerve terminals of large quanta of acetylcholine (ACh) which are recorded postjunctionally as giant or slow-rising miniature endplate potentials (m.e.p.ps) at the motor endplate. This release is, in contrast to that giving rise to normal-sized m.e.p.ps and to stimulus-evoked endplate potentials (e.p.ps), insensitive to extra- and intraterminal Ca^{2+} concentrations (Molgó & Thesleff, 1982; Thesleff *et al.*, 1983). A similar type of spontaneous, calcium-insensitive quantal secretion of ACh has previously been described at growth cones of embryonic cholinergic neurones in culture (Hume *et al.*, 1983; Young & Poo, 1983). Slow-rising, large-amplitude and calcium-insensitive m.e.p.ps are also present at endplates with regenerating motor nerve terminals (Bennett *et al.*, 1973; Colméus *et al.*, 1982). They are particularly prominent in muscles chronically paralysed by botulinum toxin (Colméus *et al.*, 1982; Kim *et al.*, 1984), tetrodotoxin (Gundersen, 1987) or curare (Ding *et al.*, 1983). It has been suggested (Thesleff *et al.*, 1989; 1990) that the source for such potentials are the large, dense-cored synaptic vesicles which, in addition to ACh, contain various neuropeptides believed to have trophic actions on the muscle cell (see, Ochs, 1988).

The present study was designed to see if tacrine, because of its chemical similarity to 4-AQ, also had a stimulatory action on the frequency of large or slow-rising m.e.p.ps and thereby possibly on the exocytotic release of the content of large, dense-cored synaptic vesicles with postsynaptic trophic functions. If that were the case it might explain the favourable effects of tacrine in Alzheimer's disease.

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Methods

The experiments were performed on the isolated phrenic nerve-hemidiaphragm preparation of adult NMRI male mice of about 30 g body weight. The nerve-muscle preparation was removed from animals killed by cervical dislocation. In order to record e.p.ps without muscle contraction the 'cut muscle' preparation was used in some experiments (Barstad, 1962, and as modified by Glavinović, 1979).

A few experiments were carried out with *Clostridium botulinum* toxin type A (haemagglutinin + neurotoxin) paralysed isolated extensor digitorum longus muscles of Sprague-Dawley rats with a body weight of about 200 g. The toxin was dissolved in phosphate buffer solution (Ambache, 1949) and injected as a single dose of 0.25 ml which represents approximately 60 pg of the purified neurotoxin, into the anterolateral region of the right hindleg of the rat, superficial to the extensor digitorum longus muscle. As shown by Kim *et al.* (1984), this type of toxin administration causes complete paralysis of the muscle and induces, within 7–10 days the appearance of giant or slow m.e.p.ps at a frequency of 0.1–1 Hz. The rats were killed by diethylether before removal of the muscle.

The excised muscles were pinned at their resting length through their tendons to a Sylgard plate, transferred to a temperature-controlled (±0.5°C) 25 ml bath, in which they were superfused at a rate of 4–5 ml min^{-1} with aerated (95% O_2 : 5% CO_2) physiological solution of the following composition (mM): NaCl 135, KCl 5 (2.5 for the 'cut muscle' preparation, see Glavinović, 1979), $CaCl_2$ 2, $MgCl_2$ 1, Na_2HPO_4 1, $NaHCO_3$ 15 and glucose 11, pH 7.2–7.3; temperature 30°C, unless otherwise stated. In experiments not involving nerve stimulation, tetrodotoxin (TTX) at a concentration of 5×10^{-7} M was present in the medium to prevent action potentials from appearing in response to giant m.e.p.ps.

Electrophysiological recordings

Potentials were recorded intracellularly with conventional glass capillary micro-electrodes filled with 2 M K^+-citrate (resistances 5–8 $\times 10^6 \Omega$). Synaptic potentials were displayed on analogue and digital storage Tektronix D13 and 5223 oscilloscopes. The noise level of the recording circuit was less than 50 μV. A bipolar platinum wire electrode was used for

nerve stimulation. Synaptic potentials were analysed for frequency, amplitude and time-to-peak with an ABC 80 microcomputer with Data Disk 82 double floppy disk unit as described in detail by Colméus et al. (1982) and Kim et al. (1984). The amplitude of the potentials was calculated for a resting membrane potential of −75 mV and an ACh reversal potential of 0 mV (Katz & Thesleff, 1957). At each endplate synaptic potentials were recorded during a period of 1–4 min.

Drugs and chemicals

Tacrine was obtained from Janssen, Belgium; 4-AQ was kindly supplied by Professor P. Lechat (Institut de Pharmacologie, Paris); TTX was from Sigma, U.S.A.

Results

Tacrine (20 μM) or 4-AQ (200 μM) induced, within minutes of their application to the isolated mouse nerve-hemidiaphragm preparation, the appearance of a population of m.e.p.ps with increased amplitude or slower time-to-peak (Figure 1). M.e.p.ps with amplitudes larger or times-to-peak longer than two times the modal values for m.e.p.ps in control muscles were designated as slow m.e.p.ps (Kim et al., 1984). Such slow m.e.p.ps constituted on the average 1.8 ± 0.43% (mean ± s.e.mean; number of observations is given in the legend of Figure 2) of all m.e.p.ps (n = 2847) in untreated muscles and occurred at a mean frequency of 0.03 ± 0.01 Hz. In the presence of tacrine they numbered 14.3 ± 1.89% of the total population of m.e.p.ps and occurred at a frequency of 0.4 ± 0.12 Hz. In the presence of 4-AQ (200 μM) slow m.e.p.ps constituted 25.6 ± 2.71% of the total population and occurred at a frequency of 0.6 ± 0.07 Hz. The total m.e.p.p. frequency increased from a control value of 1.1 ± 0.09 Hz to 2.3 ± 0.13 Hz with 4-AQ and to 2.3 ± 0.41 Hz with tacrine. The histograms in Figure 2 illustrate m.e.p.p. amplitude and time-to-peak distributions before and in the presence of tacrine or 4-AQ, respectively.

At lower concentrations of tacrine (10 μM) the aforementioned effects were less marked while concentrations higher than 100 μM reduced the amplitude of all m.e.p.ps apparently by a postjunctional action. This effect of tacrine was use-dependent, i.e. more marked following nerve stimulation or the addition of an acetylcholinesterase inhibitor to the preparation. Hence, we tentatively attribute it to blockade of the open ion channel of the nicotinic ACh receptor (see, Colquhoun, 1986), but that remains to be established by direct recordings of channel life-times. 4-AQ behaved similarly at concentrations above 200 μM. For those reasons in all subsequent experiments we used 20 μM tacrine or 200 μM 4-AQ.

Tacrine and 4-AQ are both weak acetylcholinesterase inhibitors (see Ashford et al., 1989; Molgó & Thesleff, 1982) and it was therefore of interest to determine if this property of the drug might have contributed to the appearance of large or slow m.e.p.ps. Experiments were done on preparations pretreated with the acetylcholinesterase inhibitor, edrophonium (25 μM). In the presence of edrophonium alone 4.9 ± 1.97% of the 842 m.e.p.ps recorded in 12 fibres were slow at a mean frequency of 0.1 ± 0.04 Hz while the addition of tacrine increased those numbers to 30.1 ± 4.3% of 712 m.e.p.ps and to 0.5 ± 0.1 Hz in 7 fibres. With 4-AQ the values were 31.0 ± 5.8% of 579 m.e.p.ps and 0.5 ± 0.12 Hz in 7 fibres

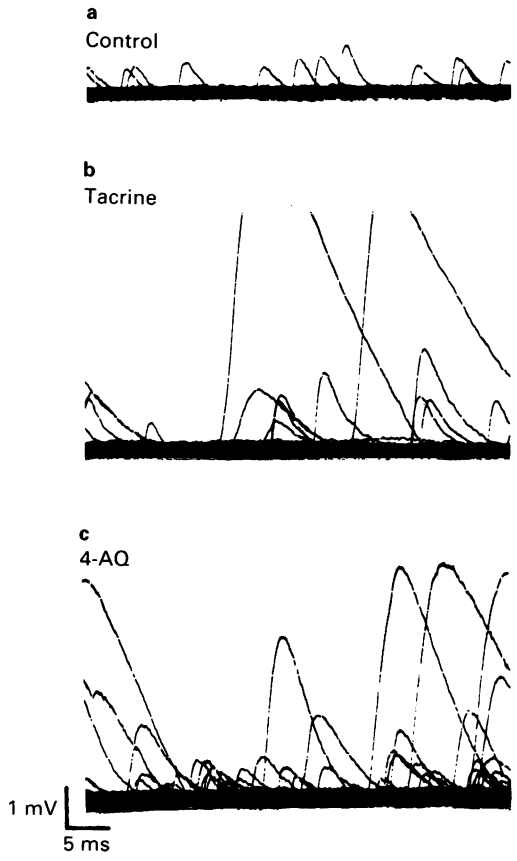

Figure 1 Superimposed oscilloscope tracings showing spontaneous m.e.p.ps in the mouse nerve-hemidiaphragm preparation: (a) in an untreated control muscle shows normal sized, fast-rising m.e.p.ps; (b) in the presence of tacrine (20 μM) and (c) in the presence of 4-aminoquinoline (4-AQ, 200 μM), illustrating the appearance of a population of giant or slow m.e.p.ps.

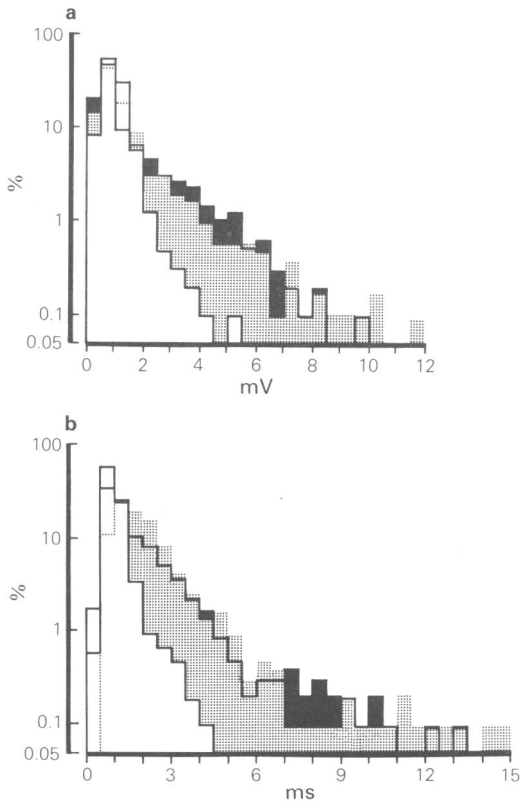

Figure 2 Histograms showing m.e.p.p. amplitude (a) and time-to-peak (b) distributions in untreated control muscle (open areas), in the presence of 20 μM tacrine (stippled areas) and in the presence of 200 μM 4-aminoquinoline (4-AQ, solid areas). To emphasize the presence of a population of giant or slow-rising m.e.p.ps the percentage values are plotted on a logarithmic scale. Each histogram consists of the following: numbers of m.e.p.ps/no of fibres/no of muscles. For untreated controls 2847/40/7; for tacrine 1641/18/3 and for 4-AQ 2088/18/3.

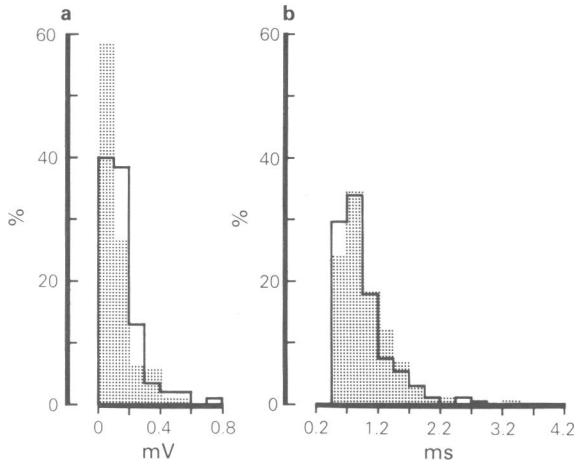

Figure 3 Amplitude (a) and time-to-peak (b) histograms of m.e.p.ps in the presence of 5 mM colchicine (open areas) and after the addition of 20 μM tacrine (stippled areas). Note, that colchicine has prevented tacrine from inducing a population of giant or slow m.e.p.ps.

and therefore the presence of edrophonium did not prevent tacrine or 4-AQ from inducing slow m.e.p.ps.

Large dense-cored synaptic vesicles are formed in the Golgi apparatus of the neuronal cell body and transported to the terminals by proximo-distal axonal flow (Ochs, 1974). Therefore, the hypothesis that such vesicles are responsible for the release of ACh giving rise to the slow m.e.p.ps requires that procedures which block axoplasmic transport also block the appearance of slow m.e.p.ps. Such procedures include low temperature and agents (e.g. colchicine) which aggregate microtubules (Ochs, 1974). Lowering the temperature from 30°C to 14°C reduced slow m.e.p.p. frequency from a mean of 0.03 Hz to a mean of 0.001 Hz in untreated muscles and from 0.4 to 0.02 and from 0.6 to 0.03 Hz in the presence of tacrine and 4-AQ, respectively. Colchicine (5 mM) prevented tacrine from inducing slow m.e.p.ps as shown by the amplitude and time-to-peak histograms of Figure 3. A similar effect was observed on slow m.e.p.ps induced by 4-AQ (not shown). Hence, temperature-sensitive processes and microtubuli are necessary for ACh release which gives rise to giant or slow m.e.p.ps.

As previously mentioned, the ACh release mechanism which accounts for slow m.e.p.ps is insensitive to calcium and

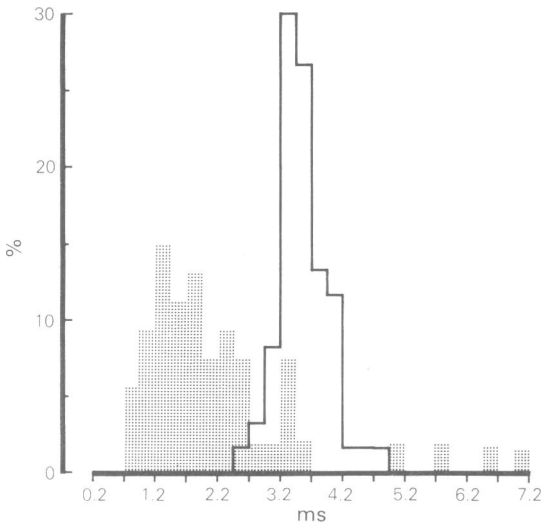

Figure 4 Time-to-peak histogram of e.p.ps (open areas) and m.e.p.ps (stippled areas) from the same fibre of a mouse 'cut nerve-hemidiaphragm muscle' in the presence of tacrine (20 μM).

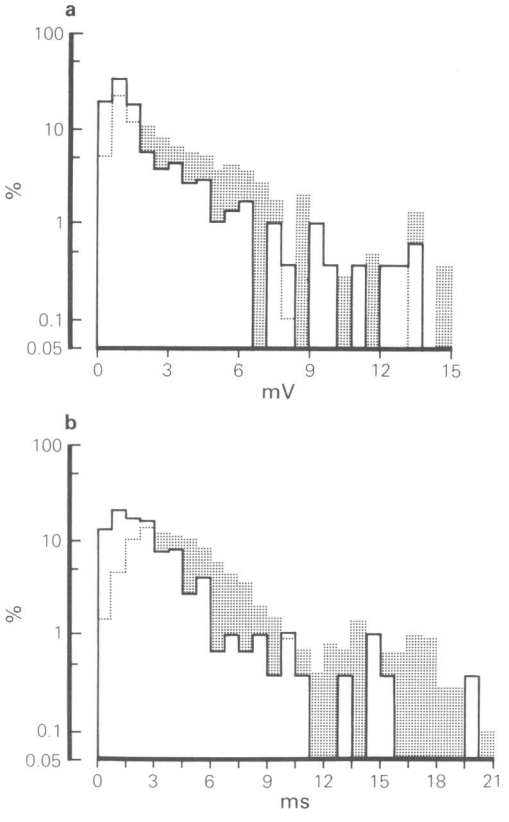

Figure 5 Amplitude (a) and time-to-peak (b) distributions of m.e.p.ps in rat extensor digitorum longus muscles paralyzed 12 or 13 days previously by botulinum neurotoxin type A. The histograms are before (open areas) and after (stippled areas) the addition of tacrine (20 μM) to the preparation. Note, log scale for percentage values.

therefore nerve impulses fail to release such quanta of ACh (Thesleff, 1986). To test if that also applied to slow m.e.p.ps induced by tacrine we compared, in 'cut muscle' preparations, times-to-peak of nerve stimulus-evoked endplate potentials (e.p.ps) with those of m.e.p.ps in the presence of tacrine. As seen from the histograms in Figure 4, times-to-peak of e.p.ps had a uniform, narrow distribution while those of spontaneous m.e.p.ps differed widely as can be expected from the appearance of a population of slow m.e.p.ps.

Botulinum neurotoxin type A selectively blocks the quantal release of ACh which is calcium-dependent and which causes normal m.e.p.ps and e.p.ps. Giant or slow m.e.p.ps on the other hand are unaffected by the toxin (Dolly *et al.*, 1987). In fact their frequency gradually increases with the time of paralysis (Kim *et al.*, 1984). Figure 5 illustrates amplitude and time-to-peak distributions of m.e.p.ps in rat extensor digitorum longus muscles paralysed for 12–13 days by botulinum neurotoxin A. Many of the 280 potentials in the 11 fibres investigated fulfilled the criteria for giant or slow m.e.p.ps and the addition of tacrine to the preparation enhanced their frequency from 0.2 ± 0.06 to 0.7 ± 0.11 Hz (13 fibres). Similar results have previously been reported for 4-AQ (Thesleff *et al.*, 1983).

Discussion

The results obtained show that tacrine stimulates the spontaneous secretion of large quanta of ACh which give rise to giant or slow-rising potentials. In that respect it acts similarly to 4-AQ but is about ten times more potent. If this type of ACh release, as proposed by Thesleff *et al.* (1989; 1990), reflects the secretion of substances with trophic actions on the post-junctional target cell, the observation is of considerable

interest. It might explain the claimed effectiveness of tacrine in alleviating symptoms of Alzheimer's disease.

Studies at other kinds of synapses (especially non-cholinergic) are required to determine whether tacrine and 4-AQ evoke the spontaneous release of transmitter quanta which might be related to the exocytosis of contents of large, dense-cored synaptic vesicles. In support of such a possibility it has recently been reported that tacrine stimulates the release of endogenous 5-hydroxytryptamine and dopamine from rat brain tissue in a calcium-independent manner (Robinson *et al.*, 1989).

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Influence of capsaicin-sensitive afferent neurones on the acid secretory responses of the rat stomach *in vivo*

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1 The influence of capsaicin-sensitive afferent neurones in modulating acid-secretory responses has been investigated in the continuously perfused stomach of the anaesthetized rat.

2 Ablation of primary afferent neurones, after systemic neonatal pretreatment with high doses of capsaicin, did not modify acid responses to direct stimuli of the oxyntic cell with histamine ($5\,mg\,kg^{-1}$), pentagastrin ($20\,\mu g\,kg^{-1}$) or carbachol ($4\,\mu g\,kg^{-1}$).

3 Acid responses to hypoglycaemia induced by insulin ($0.3\,iu\,kg^{-1}$) were not influenced by systemic capsaicin pretreatment or by acute coeliac ganglionectomy. Vagotomy abolished this secretory response.

4 The increase in acid output induced by gastric distension ($20\,cmH_2O$) was abolished by systemic neonatal capsaicin pretreatment. Likewise, vagotomy and acute coeliac ganglionectomy eliminated this secretory response.

5 Acute intragastric infusion with high doses of capsaicin inhibited the acid responses to distension but failed to modify the increase in acid output induced by insulin.

6 Local application (7–14 days before) of capsaicin to the coeliac ganglion abolished the acid response to gastric distension. This lack of secretory response was not the result of a nonspecific destruction of the ganglion, since changes in intragastric pressure after electrical stimulation of the coeliac ganglion were unaffected by such treatment.

7 These observations indicate that peripheral capsaicin-sensitive sensory neurones, located both in the gastric mucosa and in the coeliac ganglion, play a physiological role in the acid secretory responses to gastric distension.

Introduction

Capsaicin, the pungent component of red peppers of the genus *Capsicum*, has become widely used as a pharmacological tool to assess the involvement of sensory neurones in biological functions (Buck & Burks, 1986; Holzer, 1988). When administered acutely, capsaicin induces a potent stimulation of small diameter unmyelinated sensory nerve fibres (C-fibres), followed by desensitization to the drug and by a prolonged blockade of transmission of stimuli. If administered to newborn rats, capsaicin induces a permanent and selective degeneration on unmyelinated primary afferent neurones. The presence of capsaicin-sensitive nerve fibres has been demonstrated in the gastric mucosa (Sharkey *et al.*, 1984; Green & Dockray, 1988) and there is growing evidence that these neurones participate in various aspects of the pathophysiology of the stomach such as changes in motility (Holzer *et al.*, 1986), blood flow regulation (Lippe *et al.*, 1989; Pique *et al.*, 1989) or the maintenance of the integrity of the gastric mucosa (Holzer & Sametz, 1986; Esplugues & Whittle, 1989a; 1990).

In the present study we have used capsaicin, administered either systemically or locally, as a tool to analyse indirectly the participation of capsaicin-sensitive afferent nerve fibres in the regulation of acid secretion in the anaesthetized rat. We have studied the influence of capsaicin desensitization on the effects of three direct stimuli of the oxyntic cell (pentagastrin, histamine and carbachol) and, furthermore, of CNS-mediated stimuli of gastric secretion, following hypoglycaemia induced by either insulin or gastric distension. In addition, we have investigated the effects of acute coeliac ganglionectomy and the local application of capsaicin to the caeliac ganglion on these responses.

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Methods

Animals

Wistar rats of either sex, on their second day of life, received $50\,mg\,kg^{-1}$ of capsaicin by subcutaneous injection in a volume of $10\,\mu l\,g^{-1}$. This treatment has been shown to lead to functional ablation of primary afferent neurones (Buck & Burks, 1986; Holzer, 1988). Control animals received equal volumes of vehicle (10% ethanol, 10% Tween 80 and 80% saline, vol/vol/vol). All injections were performed under ether anaesthesia. The rats were then grown to adulthood and used for the experiments when weighing 230–260 g. Animals were fasted for 24 h before the experiments, but received drinking water *ad libitum*.

Measurement of gastric acid secretion

The procedure followed was that described by Ghosh & Schild (1958). In brief, animals were anaesthetized with urethane ($1\,g\,kg^{-1}$, i.p.) and the trachea intubated. A soft catheter was inserted into the stomach through an incision in the cervical oesophagus and held in place by a ligature. After a laparotomy, a second polyethylene cannula was inserted into the stomach via an incision in the duodenum, held in place by two ligatures, and led outside the abdominal wall. At the beginning of the experiment, the stomach was flushed with 50–100 ml of saline to remove any solid content. Once the surgical preparation had been completed a period of 1 h was allowed for stabilization. Thereafter, the stomach cavity was perfused via the oesophageal cannulae with saline at room temperature at a rate of $0.9\,ml\,min^{-1}$. The gastric effluent was collected continuously and separated in 20 min collections.

H^+ output was determined by automatic titration (Radiometer, Copenhagen, Denmark) of the perfusate to pH 7 with 0.01 N NaOH and expressed as μEq $100\,g^{-1}$ $20\,min^{-1}$ (when referring to the total acid output of a particular period of 20 min) or μEq $100\,g^{-1}$ $120–160\,min^{-1}$ (when referring to the total H^+ production over the time span examined, 120 or 160 min, after stimulation of acid secretion).

Stimulation of acid secretion

Once the gastric acid output had remained constant for 40 min it was considered as basal or unstimulated acid secretion. Thereafter, three types of experiments were performed according to the type of stimulus of gastric acid secretion. In the first series of studies, histamine ($5\,mg\,kg^{-1}$), pentagastrin ($20\,\mu g\,kg^{-1}$) or carbachol ($4\,\mu g\,kg^{-1}$) were injected (i.p.) as a bolus. All three substances are direct stimulants of the oxyntic cell (Debas, 1987).

In the second series of experiments, gastric acid secretion was stimulated by the hypoglycaemia induced by the administration of insulin ($0.3\,iu\,kg^{-1}$, i.p.).

In the last series of studies, gastric distension was used as the stimulant of acid output. Distension of the stomach was obtained by placing the tip of the antroduodenal cannula 20 cm above the perfused stomach, where it was maintained for the remainder of the experimental period.

To analyse further the involvement of sensory nerves in acid secretory responses, in some rats the vagus was cut bilaterally on the midcervical portion of the nerve, or the coeliac ganglion was carefully removed under a stereomicroscope. In addition, in one set of experiments a solution of capsaicin (1% ethanol, 99% saline; $0.1\,mg\,ml^{-1}$) or vehicle was perfused intragastrically for 10 min ($0.9\,ml\,min^{-1}$) immediately before the stimulus of acid secretion. In all cases each preparation was used for a single response only, with treatment being allocated randomly between preparations.

Local treatment with capsaicin of the coeliac ganglion

After pretreatment with atropine ($0.5\,mg\,kg^{-1}$, i.p.), rats were anaesthetized with sodium pentobarbitone ($70\,mg\,kg^{-1}$, i.v.). The coeliac ganglion was exposed and a small piece of gauze soaked in capsaicin solution, or solvent (10% Tween 80, 90% olive oil) was left on the ganglion complex for 30 min and then removed. After carefully cleaning with sterile swabs, the abdominal laparotomy was closed and the animals received antibiotic protection (ampicilin, i.m.). Acid secretory responses to gastric distension were evaluated 7–15 days after surgery.

In order to assess the specificity of the changes on acid secretion that follow the local application of capsaicin to the coeliac ganglion, we have also analysed its consequences on the gastric contractility changes induced by the electrical stimulation of the coeliac ganglion. After ligating the oesophagus, 5 ml of saline was instilled into the gastric lumen via a 25-gauge needle inserted through the forestomach wall. As described before (Esplugues & Whittle, 1989b), changes in intraluminal gastric pressure were recorded from a catheter introduced into the stomach through the pyloric sphincter via the duodenum, and ligated in place. The pyloric catheter was connected to a pressure transducer connected to a chart-recorder. Experiments started once the basal intragastric pressure had remained constant for at least 15 min. Field electrical stimulation of the coeliac ganglion was provided by a Grass SD5 stimulator via two platinum electrodes, placed 0.5 cm apart. Three trains of 300 square-wave pulses of 0.5 ms duration at 8 Hz were applied at supramaximal voltage. A period of 15 min was allowed between stimulations. Results, expressed as the difference (in mmHg) between basal and stimulated gastric pressure, were the overall mean score value of the three stimulations for each experiment. The response to electrical stimulation after applying lidocaine (1%) to the ganglion complex was used to confirm that the effects were nerve-mediated.

Drugs

Capsaicin was purchased from Fluka Chemic AG. Histamine, carbachol and Tween 80 were obtained from Sigma Chemical Co. Pentagastrin (Peptavlon, ICI) and insulin (Humulina NPH, Lilly) were obtained from clinically available preparations. All drugs, except capsaicin, were dissolved in saline and administered in a volume of $1\,ml\,kg^{-1}$.

Statistical analysis

All data are expressed as mean ± s.e.mean. Student's t test was used to analyse the results statistically. P values of less than 0.05 were taken as significant.

Results

Basal acid secretion

There were no significant differences in the rates of basal acid secretion between control animals ($4.2 \pm 0.7\,\mu$Eq $100\,g^{-1}$ $20\,min^{-1}$, $n = 40$) and animals which had been treated with capsaicin as neonates ($3.7 \pm 0.5\,\mu$Eq $100\,g^{-1}$ $20\,min^{-1}$, $n = 22$). Furthermore, neither bilateral vagotomy ($3.4 \pm 0.6\,\mu$Eq 100^{-1} $20\,min^{-1}$, $n = 12$) nor acute coeliac ganglionectomy ($3.2 \pm 0.5\,\mu$Eq $100\,g^{-1}$ $20\,min^{-1}$, $n = 11$) exerted any significant influence on the levels of basal acid output when compared to those present in the control rats.

Acid responses to histamine, pentagastrin or carbachol

The intraperitoneal injection of histamine ($5\,mg\,kg^{-1}$), pentagastrin ($20\,\mu g\,kg^{-1}$) and carbachol ($4\,\mu g\,kg^{-1}$) induced rapid increases in acid output by the stomach which peaked 40 min (pentagastrin and carbachol) or 60 min (histamine) later (Figures 1 and 2). Thereafter, acid secretion decreased progressively, and by the end of each experimental period, the levels of acid production were not significantly different from those of basal conditions. The values of total acid output and of peak secretory response for histamine ($91.5 \pm 12.5\,\mu$Eq $100\,g^{-1}$ $120\,min^{-1}$ and $26.7 \pm 4.8\,\mu$Eq $100\,g^{-1}$ $20\,min^{-1}$ respectively, $n = 9$) were not significantly different from those obtained after administration of pentagastrin ($113 \pm 16.9\,\mu$Eq $100\,g^{-1}$ $120\,min^{-1}$ and $32.5 \pm 4.6\,\mu$Eq $100\,g^{-1}$ $20\,min^{-1}$ respectively, $n = 10$). The total acid output after the administration of carbachol was $149.4 \pm 25\,\mu$Eq $100\,g^{-1}$ $160\,min^{-1}$ ($n = 7$) whereas the peak secretory response was $35.7 \pm 4\,\mu$Eq $100\,g^{-1}$ $20\,min^{-1}$.

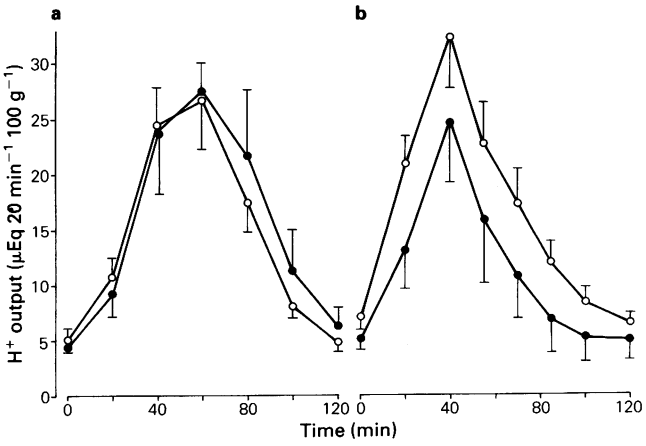

Figure 1 Stimulation of acid secretion elicited by a bolus injection (i.p.) of (a) histamine ($5\,mg\,kg^{-1}$), or (b) pentagastrin ($20\,\mu g\,kg^{-1}$) in the continuously perfused stomach of the anaesthetized rat: control (○) and neonatal capsaicin pretreatment (●). Each point represents the mean of 3 to 10 experiments and the vertical lines show s.e.mean.

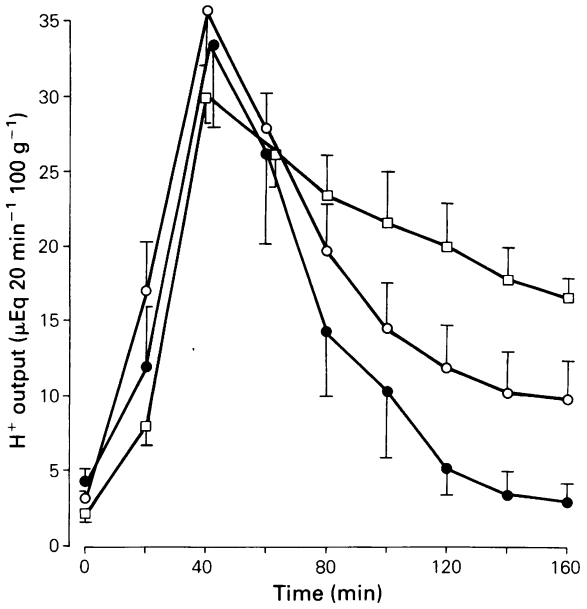

Figure 2 Stimulation of acid secretion elicited by a bolus injection (i.p.) of carbachol ($4\,\mu g\,kg^{-1}$) in the continuously perfused stomach of the anaesthetized rat: control (○), neonatal capsaicin pretreatment (●), and coeliac ganglionectomy (□). Each point represents the mean of 3 to 7 experiments and the vertical lines show s.e.mean.

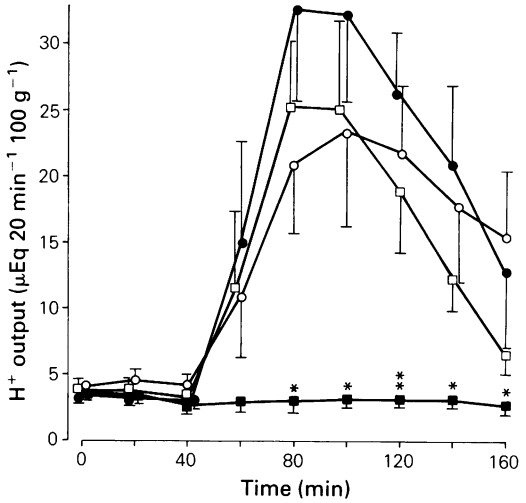

Figure 3 Stimulation of acid secretion elicited by a bolus injection of insulin (0.3 iu kg^{-1}, i.p.) in the perfused stomach of the anaesthetized rat: control (○), neonatal capsaicin pretreatment (●), coeliac ganglionectomy (□) and bilateral vagotomy (■). Each point represents the mean of 4–7 animals and the vertical lines show s.e.mean. The level of significance of the difference from control is shown $*P < 0.05$; $**P < 0.001$.

Systemic neonatal treatment with capsaicin did not modify the responses elicited by any of the three direct secretagoges employed in our experiments. The values of total acid output and of peak secretory response were, respectively, $100.1 \pm 26.4\,\mu Eq\ 100\,g^{-1}\ 120\,min^{-1}$ and $27.7 \pm 3.5\,\mu Eq\ 100\,g^{-1}\ 20\,min^{-1}$ for histamine ($n = 3$); $73.8 \pm 27.8\,\mu Eq\ 100\,g^{-1}\ 120\,min^{-1}$ and $24.5 \pm 4.4\,\mu Eq\ 100\,g^{-1}\ 20\,min^{-1}$ for pentagastrin ($n = 3$); and $107.7 \pm 17\,\mu Eq\ 100\,g^{-1}\ 160\,min^{-1}$ and $35.5 \pm 6\,\mu Eq\ 100\,g^{-1}\ 20\,min^{-1}$ for carbachol ($n = 3$). The removal of the coeliac ganglion ($n = 3$) in control, non-capsaicin treated, animals did not significantly influence the values of total acid output ($163.8 \pm 22\,\mu Eq\ 100\,g^{-1}\ 160\,min^{-1}$) or of peak secretory response $30 \pm 1.8\,\mu Eq\ 100\,g^{-1}\ 20\,min^{-1}$) induced by carbachol (Figure 2).

Acid responses to insulin

As is shown in Figure 3, the secretory effects of insulin (0.3 iu kg^{-1}) were only significantly evident 60 min after its i.p. administration, and a period of 100 min was needed to obtain the peak acid response. Although acid production decreased thereafter, at the end of the experimental period (160 min) the levels of acid output were still significantly ($P < 0.05$) higher than those present in basal, non-stimulated, conditions.

The values of total acid output and of peak secretory response induced by insulin ($123.1 \pm 31\,\mu Eq\ 100\,g^{-1}\ 160\,min^{-1}$ and $23.4 \pm 7.1\,\mu Eq\ 100\,g^{-1}\ 20\,min^{-1}$ respectively, $n = 7$) were neither significantly affected by neonatal treatment with capsaicin ($155 \pm 57\,\mu Eq\ 100\,g^{-1}\ 160\,min^{-1}$ and $32.6 \pm 6.9\,\mu Eq\ 100\,g^{-1}\ 20\,min^{-1}$, $n = 5$) nor by acute coeliac ganglionectomy ($105.9 \pm 26\,\mu Eq\ 100\,g^{-1}\ 160\,min^{-1}$ and $25.2 \pm 5\,\mu Eq\ 100\,g^{-1}\ 20\,min^{-1}$, $n = 5$). Cervical vagotomy ($n = 4$), however, abolished the secretory response induced by insulin with a total acid output of $23.7 \pm 4.2\,\mu Eq\ 100\,g^{-1}\ 160\,min^{-1}$ ($P < 0.01$) and rates of acid secretion in all $20\,min^{-1}$ periods measured not significantly different from those present in basal, pre-stimulated, conditions.

Acid secretion stimulated by gastric distension

Distension of the rat stomach *in vivo* ($n = 7$) with an intragastric pressure of $20\,cmH_2O$ produced a progressive increase in acid secretion, which peaked 140 min later with a H^+ production of $23.9 \pm 5.7\,\mu Eq\ 100\,g^{-1}\ 20\,min^{-1}$. The total acid output during these experiments was $114.9 \pm 28.2\,\mu Eq\ 100\,g^{-1}\ 160\,min^{-1}$ (Figure 4).

Neonatal pretreatment with capsaicin, acute coeliac ganglionectomy and bilateral vagotomy abolished ($P < 0.01$) the acid secretory responses to gastric distension, with values of total acid output of $19.7 \pm 7.4\,\mu Eq\ 100\,g^{-1}\ 160\,min^{-1}$ ($n = 5$), $22 \pm 5.4\,\mu Eq\ 100\,g^{-1}\ 160\,min^{-1}$ ($n = 3$) and $24.6 \pm 5.6\,\mu Eq\ 100\,g^{-1}\ 160\,min^{-1}$ ($n = 5$) respectively. In the three cases, the rates of acid output during all the 20 min periods studied were not significantly different from those present in basal, non-stimulated, conditions.

Effects of the local application of capsaicin to the coeliac ganglion

As shown in Figure 5, the local application of capsaicin to the coeliac ganglion resulted in the abolition of the secretory response to gastric distension when studied 7–14 days later. The total acid output for the 160 min period of distension was

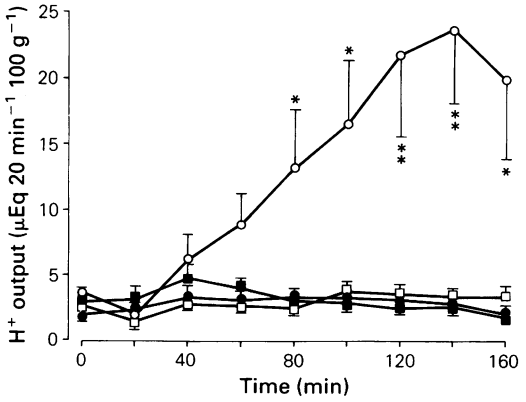

Figure 4 Stimulation of acid secretion elicited by the gastric distension ($20\,cmH_2O$) of the perfused stomach of the anaesthetized rat: control (○), neonatal capsaicin pretreatment (●), coeliac ganglionectomy (□) and bilateral vagotomy (■). Each point represents the mean of 3 to 7 animals and the vertical lines show s.e.mean. For reasons of clarity $*P < 0.05$; $**P < 0.001$ versus the other three groups.

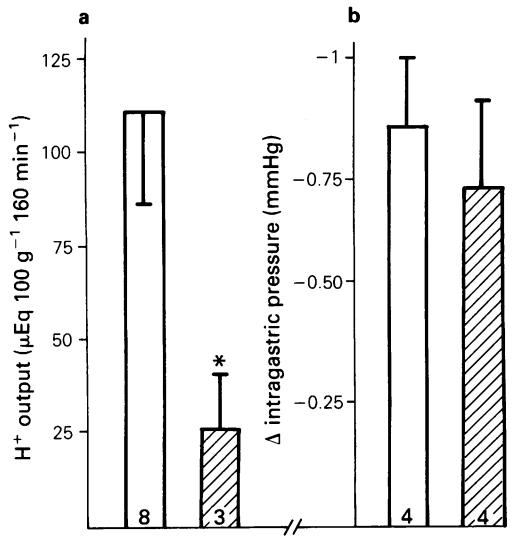

Figure 5 Effects of the local application on the coeliac ganglia of capsaicin (hatched columns), or vehicle (open columns), on (a) the acid response to intragastric distension (20 cmH_2O); and (b) the changes in intragastric pressure after the electrical stimulation of the coeliac ganglia. Each column represents mean with s.e.mean shown by vertical bars, and level of statistical difference from vehicle-treated group is shown by * $P < 0.05$. Numbers inside columns represent the number of animals used.

$110.8 \pm 25\,\mu$Eq $100\,g^{-1}$ ($n = 8$) in vehicle treated rats compared to $26.1 \pm 12.3\,\mu$Eq $100\,g^{-1}$ in animals receiving topical capsaicin ($n = 3$, $P < 0.05$).

In rats receiving a local application of capsaicin to the coeliac ganglion, the values of total acid production induced by distension were not significantly different from those in animals that had undergone vagotomy, acute coeliac ganglionectomy or systemic neonatal capsaicin administration.

To evaluate the possibility that a non-selective destruction of the coeliac ganglion by the local application of capsaicin could account for the differences in the acid response to distension, changes in intragastric pressure during electrical stimulation of the coeliac ganglion were determined. Basal intragastric pressure was similar in animals receiving the topical application of vehicle (4.3 ± 0.2 mmHg, $n = 4$) or capsaicin (4.1 ± 0.3 mmHg, $n = 4$). Electrical stimulation to the ganglion caused a rapid, frequency-dependent and reproducible diminution in intragastric pressure that lasted for 45–60 s, slowly returning to basal levels thereafter. The highest frequency used (8 Hz) induced similar reductions of intragastric pressure after local application of capsaicin (0.7 ± 0.2 mmHg, $n = 4$) or vehicle (0.8 ± 0.1 mmHg, $n = 4$) (Figure 5). In all cases, the topical application of lidocaine to the ganglion caused a sustained rise in intragastric pressure and completely blocked the relaxation induced by the electrical stimulation of the coeliac ganglion, thus confirming that such relaxation was nerve-mediated.

Effects of intraluminal capsaicin on acid secretion

The intraluminal infusion of high doses ($0.1\,mg\,ml^{-1}$, $0.9\,ml\,min^{-1}$) of capsaicin for 10 min abolished the acid responses to gastric distension (Figure 6). This was not due to a nonspecific effect of capsaicin on H^+ secretion since acid responses to insulin were not affected by such treatment.

Discussion

We have analysed the changes induced by pretreatment with capsaicin, both systemically and locally, on the acid secretory responses of the rat stomach *in vivo*. In our study basal gastric acid secretion and acid responses to direct stimulation of the oxyntic cell (Debas, 1987) with histamine, pentagastrin and carbachol were not affected by neonatal treatment with high doses of capsaicin. Such treatment has been shown to lead to the functional ablation of primary afferent neurones (Buck & Burks, 1986; Holzer, 1988). Previous studies on the effects of systemic capsaicin pretreatment on gastric acid secretion have shown a similar lack of effect on basal acid secretion, although, in contrast, the acid secretory responses to histamine (Alfondi *et al.*, 1986) or pentagastrin (Dugani & Glavin, 1986) were greatly reduced. Differences in the experimental protocol, anaesthesia and particularly, in the doses employed of the different secretagogues may account for the discrepancies.

Systemic capsaicin crosses the blood-brain barrier (Buck & Burks, 1986; Holzer, 1988). Thus it could be possible that central processes of sensory neurones involved in acid secretory responses were modified. In the present investigation two different stimuli of gastric acid secretion, through a CNS action have been employed, insulin-hypoglycaemia and gastric distension (Tache, 1987). The influence of neonatal systemic capsaicin pretreatment on the responses to both stimuli has been contradictory. Whereas acid-secretory responses to insulin administration were unaffected, responses to gastric distension were completely absent in capsaicin-pretreated rats. Differences in the nervous centres involved and its sensitivity to capsaicin treatment cannot be excluded. However, another explanation for such divergence could be based in the nature of the stimulus involved. Insulin administration induces a diminution of the level of glucose in plasma which, subsequently, is directly identified by the brain and leads to a vagally mediated increase in acid secretion. Distension of the stomach is detected by gastric mechanoreceptors which then, possibly through capsaicin-sensitive afferent sensory neurons, transmit the information to the CNS and trigger a vagal stimulus of acid secretion. The destruction by capsaicin of peripheral afferent neurones, rather than a central effect, could therefore explain the inhibition of the acid secretory response to gastric distension.

The recent finding that systemic capsaicin pretreatment decreased the acid-secretory responses induced by 2-deoxy-D-glucose (Evangelista *et al.*, 1989) could contradict our results with insulin. The actions of 2-deoxy-D-glucose on gastric acid secretion have been compared with those of insulin, since this analogue of glucose cannot be metabolized and stimulates the release of endogenous insulin by the pancreas (Debas, 1987; Tache, 1987). An involvement of capsaicin-sensitive neurones in the mechanisms leading to insulin release cannot be excluded. However, the effects of 2-deoxy-D-glucose are more complex and a direct action on hypothalamic, diencephalic and medullary sites in the CNS, as well as hepatic sites in the periphery have also been described (Debas, 1987; Tache, 1987). It is therefore feasible that any of these specific actions of 2-deoxy-D-glucose, either central or peripheral, could be significantly influenced by capsaicin pretreatment, thus eliminating the acid secretory response.

Direct application of capsaicin leads to a selective and prolonged blockade of the sensitive afferent fibres located in a peripheral tissue or nerve (Buck & Burks, 1986; Holzer, 1988). Therefore, it provides a useful tool for analysing the anatomical pathways by which the activation by gastric distension of capsaicin sensitive afferent neurones leads to the stimulus of acid secretion. The local application of capsaicin to the vagus nerve has been shown to reduce by 40% the acid secretory responses to acute (6 min) gastric distension. Bilateral vagotomy, which will sever both afferent and efferent neurones, equally reduced acid-responses to distension to a level not significantly different from those present in rats treated with topical capsaicin on the vagus. On this basis, Raybould & Tache (1989) have proposed that capsaicin-sensitive vagal afferent fibres mediate the vagal portion of the secretory response to gastric distension. Our findings with a longer period of distension do not exclude this idea, but suggest that capsaicin-sensitive afferent fibres mediating acid responses to distension are also located in the coeliac ganglion. Local application of capsaicin to the coeliac ganglion or acute gangli-

onectomy, abolished acid secretory responses to distension. Acid responses to insulin were not influenced by these two procedures, therefore implying a specific involvement of the coeliac ganglion in the acid responses to distension. The possibility of a non-specific destruction of the whole of the nervous tissue located in the ganglion has been excluded, since changes in intragastric pressure after the electrical stimulation of the ganglion were not altered by the topical application of capsaicin. It is also relevant that local treatment with capsaicin of the coeliac ganglion, as well as acute ganglionectomy, has been shown to abolish reflex cardiovascular changes after the application of warm fluids to the mucosal surfaces of the rat stomach, jejunum or ileum (Rozsa *et al.*, 1988). The degree of inhibition of the acid response after the local application of capsaicin to the coeliac ganglion was not different from that obtained after systemic treatment. This observation thus indicates that most capsaicin-sensitive neurones involved in the secretory response to distension are located in the coeliac ganglion or, at least, that the integrity of those present is necessary for a correct response to distension.

The hypothesis that a peripheral effect, rather than a central action, of capsaicin is mainly responsible for its inhibitory effect on the acid responses to gastric distension is further reinforced by the fact that intragastric infusion with high doses of capsaicin prevents the increase in acid output induced by distension. This was not due to a nonspecific inhibitory effect of capsaicin on the gastric mucosa, since acid responses to insulin and carbachol were not affected by such treatment (data not shown). Furthermore, it would suggest that gastric distension is detected by mucosal receptors, and would represent a pharmacological proof of the presence of capsaicin-sensitive afferent fibres in the gastric mucosa. In keeping with this idea, recent studies by Lippe *et al.* (1989) have shown that intragastric perfusion with lower doses of capsaicin could facilitate the elimination of the acid present in the stomach, independently of its endogenous or exogenous origin, by mechanisms still unknown. This effect requires the integrity of afferent neurones, since it was absent after neonatal treatment of the rats with high doses of capsaicin. These functional studies thus confirm previous investigations, using immunohistochemical techniques for neuropeptides, which localized such neurones in close association with blood vessels of the gastric mucosa of the rat (Sharkey *et al.*, 1984; Green & Dockray, 1988).

In conclusion, the present findings suggest that capsaicin-sensitive afferent neurones mediate acid responses to gastric distension, but not to hypoglycaemia or direct stimulation of the oxyntic cell with histamine, pentagastrin or carbachol.

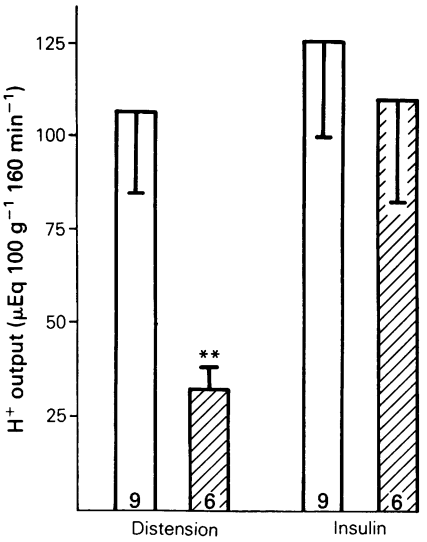

Figure 6 Effects of the intragastric infusion with capsaicin (0.1 mg min^{-1}, 10 min, hatched columns) or vehicle (open columns) on the stimulation of acid secretion in the anaesthetized rat by gastric distension (20 cmH_2O) or insulin (0.3 iu kg^{-1}, i.p.). Each column represents mean with s.e.mean shown by vertical bars, and the level of statistical difference from vehicle-treated group is shown by ** $P < 0.01$. Numbers inside the columns represent the number of animals used.

Such neurones would be located in the gastric mucosa and the coeliac ganglion and would be susceptible to the action of local capsaicin. The present observations also imply that ingestion of food rich in capsaicin, if the amount were high enough, could modify the acid secretory responses of the stomach. This may be a relevant observation when it is considered that such food has for long been included (Soll, 1989) among the aetiological factors in different gastrointestinal disorders such as dyspepsia and peptic ulcer disease.

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The effects of granisetron, ICS 205-930 and ondansetron on the visceral pain reflex induced by duodenal distension

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1 Distension of the duodenum in anaesthetized rats, by rapid application of intraluminal pressures (10–75 cmH_2O), evoked falls in diastolic blood pressure and intragastric pressure.

2 The distension-induced responses were blocked by pretreatment with morphine (20 $mg\,kg^{-1}$, s.c.), an action reversible by injection of naloxone (5 $mg\,kg^{-1}$, i.v.).

3 Bilateral cervical vagotomy reduced the distension-evoked fall in intragastric pressure but had no effect on the corresponding fall in blood pressure.

4 Granisetron or ICS 205-930 (1–1000 $\mu g\,kg^{-1}$, i.v.) had no effects on duodenal intraluminal pressure, but reduced the responses to distension with a bell-shaped dose-response relationship. Ondansetron (1–1000 $\mu g\,kg^{-1}$, i.v.) did not reduce the reflex responses.

5 These results show that the 5-HT_3 receptor antagonists used exerted different effects on the reflex responses to duodenal distension.

Introduction

Distension of the human biliary system (Gaensler, 1951), ileum (Lipkin & Sleisenger, 1958) or colon (Latimer *et al.*, 1979; Swarbrick *et al.*, 1980) evokes abdominal pain. In anaesthetized animals, the effect of distension of hollow organs can be assessed by measuring a pseudoaffective reflex. Woodworth & Sherrington (1904) described pseudoaffective reflexes as '... mimetic movements simulating expression of certain affective states' and these include movement of the head towards the stimulus, dilatation of the pupils, vocalisation and transient changes in arterial blood pressure.

In anaesthetized ferrets and cats, distension of the biliary system increased blood pressure (Cervero, 1982; 1983) and firing of high threshold afferent fibres which respond only to noxious distension. In anaesthetized rats, distension of the jejunum (Lembeck & Skofitsch, 1982), renal pelvis (Brasch & Zetler, 1982) or ileum (Clark & Smith, 1985) induced a decrease in blood pressure in 60–70% of animals. This response could be blocked by morphine, an action reversed by naloxone. Capsaicin, which selectively destroys unmyelinated afferent fibres (Buck & Burks, 1986), also abolished the response to distension of the rat jejunum (Lembeck & Skofitsch, 1982) and no response could be evoked in rats treated neonatally with capsaicin (Skofitsch & Lembeck, 1980). Thus it is possible that distension-induced changes in blood pressure can be used to assess pain in anaesthetized animals.

In the present study with anaesthetized rats, duodenal distension evoked a fall in blood pressure and intragastric pressure. It has been shown that 5-hydroxytryptamine (5-HT) can be released by increasing the intraluminal pressure of the small intestine (Bülbring & Crema, 1959; Burks & Long, 1966). A potential role of endogenous 5-HT in modulating distension-induced reflexes was therefore examined by use of the selective 5-HT_3 receptor antagonists granisetron (BRL 43694; Sanger & Nelson, 1989), ICS 205-930 (Richardson *et al.*, 1985) and ondansetron (GR 38032F; Butler *et al.*, 1988). Preliminary results have been previously reported to the British Pharmacological Society (Moss & Sanger, 1987).

Methods

Following overnight fasting, male Wistar rats (200–300 g) were anaesthetized by intraperitoneal injection of 25% urethane (6 $ml\,kg^{-1}$). The right jugular vein and a carotid artery were catheterised to facilitate drug injection and blood pressure recording respectively; the trachea was cannulated to maintain a clear airway. The whole stomach was cannulated via the oesophagus and tied distal to the pyloric sphincter. The stomach was filled with 5 ml 0.9% w/v saline to incresae resting gastric tone. A 3–4 cm segment of duodenum was isolated by ligature immediately below the stomach, with major blood vessels and nerves remaining intact. This segment was flushed with saline and connected orally via a 37°C heating coil to a series of reservoirs containing 0.9% w/v saline and set at various heights above the animal. Drainage from the duodenum was achieved aborally. Animals were allowed 30 min recovery after surgery, before the experiment was started.

Pressure-response curves to duodenal distension were constructed using rapid application of pressures of 10, 25, 50 or 75 cmH_2O; each increase in pressure was maintained for 20 s before the pressure was removed and the duodenum drained of fluid. In order to measure the effects of drugs, distension with 75 cmH_2O was applied every 5 min until constant responses were achieved. Drugs were then injected and the distensions repeated at 5 min intervals.

Granisetron, ICS 205-930, ondansetron (1–1000 $\mu g\,kg^{-1}$) or ((3α-tropanyl)-1H-indole-3-carboxylic acid ester) saline (0.9% w/v in an equivalent volume) were given intravenously (i.v.) via the jugular vein and their effects monitored up to 20 min following administration. Morphine (20 $mg\,kg^{-1}$; Evans Medical) was administered subcutaneously (s.c.) and then naloxone (5 $mg\,kg^{-1}$, i.v.; Endo) 25 min later. All drugs were dissolved in saline; 5-HT_3 receptor antagonists were synthesized by Beecham Pharmaceuticals.

Responses to duodenal distension following drug administration were expressed as a percentage of the response immediately prior to dosing (taken as 100% response). Means were compared by a one sample t test and considered significantly different if $P < 0.05$. The effects at 20 min (expressed as a percentage of the maximum inhibition obtained) of granisetron and ICS 205-930 on the fall in blood pressure were also subjected to regression analysis and analysis of variance using methods outlined by Draper & Smith (1981). ID_{50} values were calculated as the dose required to give 50% of the actual maximum blocking effect obtained with the compound.

Results

Duodenal distension for 20 s, by use of rapid application of pressures in the range 10–75 cmH_2O, induced a decrease in diastolic blood pressure. This decrease was rapid in onset, the maximum fall lasting for 10–15 s with a return to normal pressures occurring 1–2 min later. The initial amplitude of the

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response was related to the pressure applied to the duodenum (Figure 1). Distension of the duodenum also induced a fall in intragastric pressure which occurred at pressures of 25 cmH_2O and above (Figure 1). Compared with the fall in blood pressure, this fall was less rapid in onset, reaching a maximum after approximately 30 s, recovering over the subsequent 1–2 min. These blood and gastric pressure responses to duodenal distension were present in 80% of rats. Except where stated, those that did not respond were discarded. In all subsequent experiments, a distension pressure of 75 cmH_2O was used.

Following bilateral cervical vagotomy, a small fall was seen in resting blood pressure and intragastric pressure but not in the intraluminal pressure of the duodenum. The distension-evoked fall in intragastric pressure was unaffected in 3 rats, reduced by 60–90% in 7 rats and abolished in 4 rats even after the baseline intragastric pressure had been raised with the further addition of saline to the stomach (1.5 ml). The fall in blood pressure in response to distension was unaffected by vagotomy in all of these rats ($n = 14$).

Morphine (20 mg kg^{-1}, s.c.) had no effects on resting blood, intragastric or intraduodenal pressure, but prevented or greatly reduced the distension-evoked fall in blood and intragastric pressure; these effects were highly significant 15–25 min after dosing ($P < 0.005$; $n = 6$; Figure 2). Administration of naloxone (5 mg kg^{-1}, i.v.) at 25 min reversed the action of morphine (Figure 2).

Granisetron (1–1000 $\mu g\,kg^{-1}$, i.v.) was without effect on resting blood, intragastric or intraduodenal pressure. However, granisetron (1–100 $\mu g\,kg^{-1}$, i.v.) reduced the distension-evoked fall in blood pressure and at a dose of 10 $\mu g\,kg^{-1}$ i.v. reduced the fall in intragastric pressure (Figures 3 and 4). The most consistently effective dose of granisetron was 10 $\mu g\,kg^{-1}$; reductions were seen in all animals tested. Higher doses produced higher (50 $\mu g\,kg^{-1}$) or less consistent (100 $\mu g\,kg^{-1}$) antagonism of the fall in blood pressure, but with no reduction in the fall in intragastric pressure. Granisetron at 1000 $\mu g\,kg^{-1}$ increased or had no effect on the fall in blood and intragastric pressures in 4 of 7 and 3 of 7 animals respectively. Regression analysis of the effect of granisetron (1, 10 and 50 $\mu g\,kg^{-1}$) on the fall in blood pressure revealed a significant correlation (Figure 4; $F = 5.006$, $n = 19$, $P < 0.03$). Taking a maximum effect of granisetron at 50 $\mu g\,kg^{-1}$, then an ID_{50} value was calculated as 1.2 (95% confidence limits: 0.00026–140) $\mu g\,kg^{-1}$.

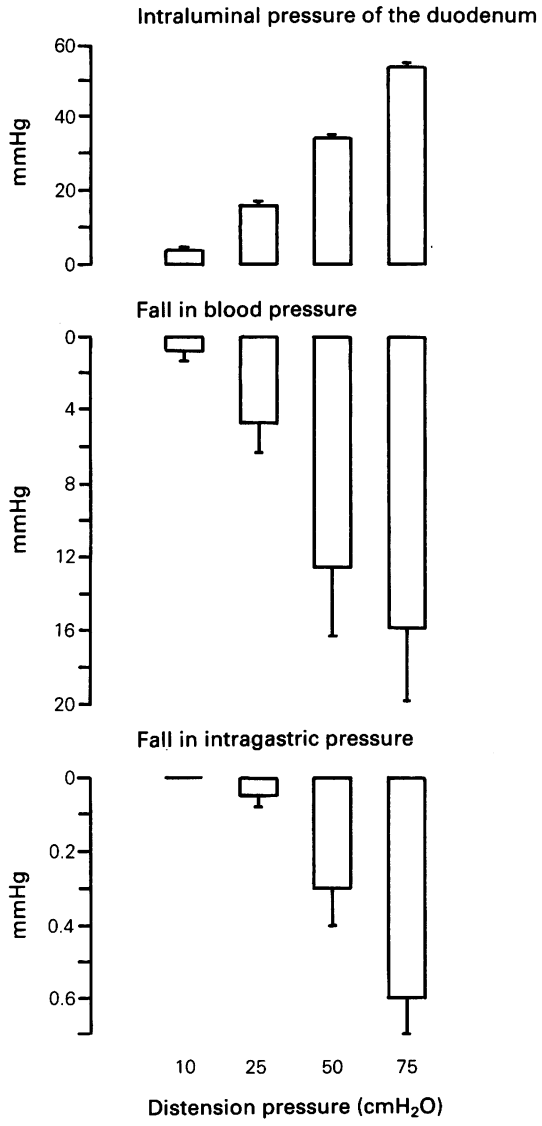

Figure 1 Effect of duodenal distension using pressures of 10, 25, 50 and 75 cmH_2O for 20 s every 5 min on the intraluminal pressure of the duodenum, diastolic blood pressure and intragastric pressure ($n = 6$). In each case, the maximum pressure change evoked by duodenal distension was recorded. Results are expressed as means (columns) with standard errors indicated by vertical bars.

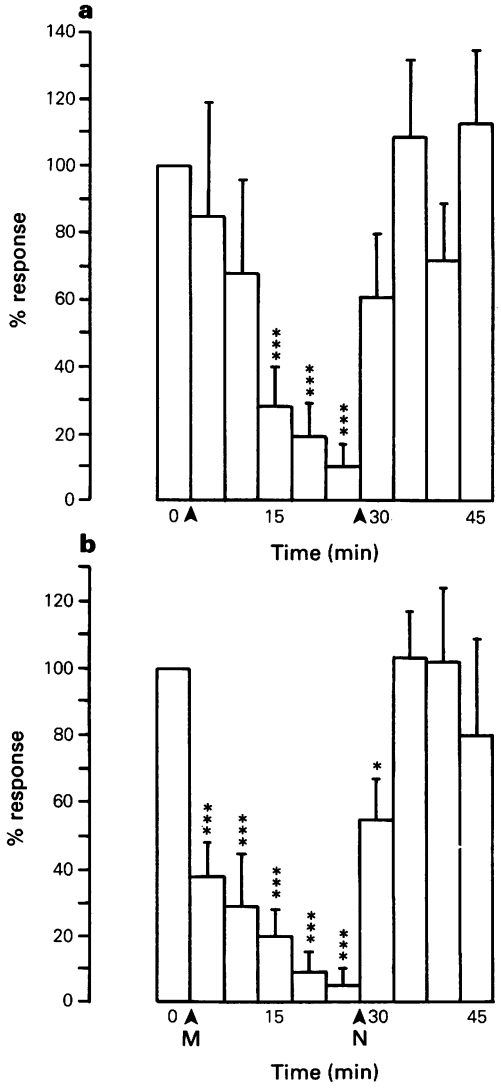

Figure 2 Antagonism by morphine (20 mg kg^{-1}, s.c.) and its reversal by naloxone (5 mg kg^{-1}, i.v.) on the fall in (a) blood pressure and (b) intragastric pressure induced by duodenal distension with 75 cmH_2O for 20 s every 5 min. Morphine (M) and naloxone (N) are given at the arrows. The first column represents the pre-morphine control response taken as 100%. Results are expressed as means (columns) with standard errors shown by vertical bars. * $P < 0.05$; *** $P < 0.005$, with one sample t test ($n = 6$).

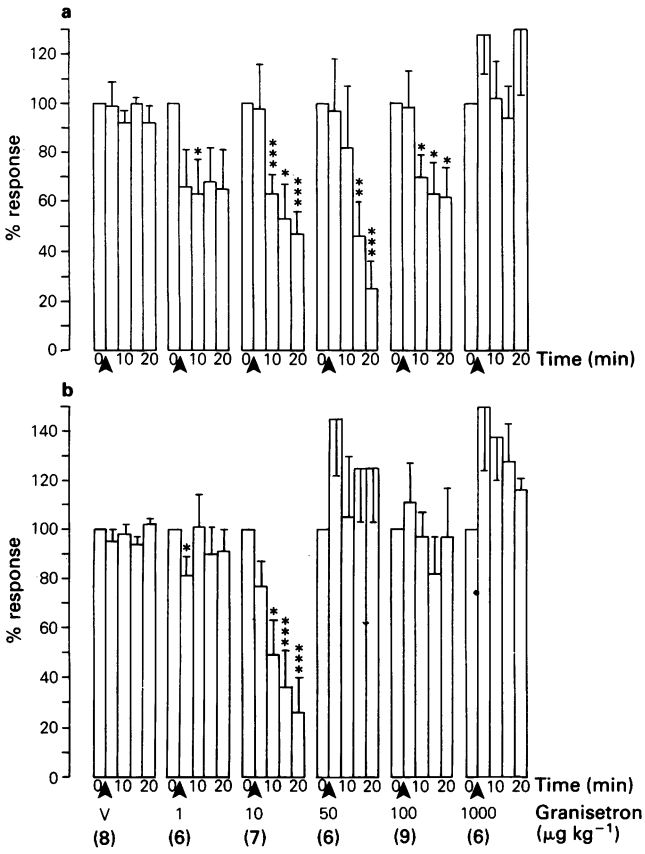

Figure 3 Effects of granisetron (1–1000 $\mu g\,kg^{-1}$, i.v.) or vehicle (V = 0.9% w/v saline) administered after a control distension (arrow) on the fall in (a) blood pressure and (b) intragastric pressure induced by duodenal distension with 75 cmH_2O for 20 s every 5 min. The first column in each group represents the pre-dose control response taken as 100%. Results are expressed as means (columns) with standard errors shown by vertical bars. * $P < 0.05$; *** $P < 0.005$, with one sample t test. Numbers in parentheses indicate the number of rats used.

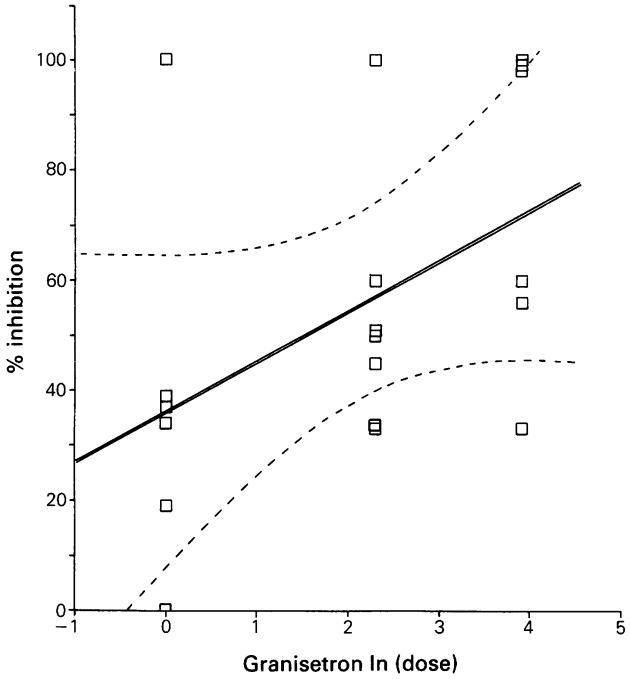

Figure 4 Regression analysis of the effect of granisetron (1, 10 and 50 $\mu g\,kg^{-1}$) on the fall in blood pressure induced by duodenal distension with 75 cmH_2O for 20 s every 5 min. The dose is plotted on a natural log scale and the effect of granisetron as a percentage of the maximum inhibition of the distension-evoked response. Squares represent each data point; $n = 6$ for 1 $\mu g\,kg^{-1}$, $n = 7$ for 10 $\mu g\,kg^{-1}$ and $n = 6$ for 50 $\mu g\,kg^{-1}$. Dotted lines represent 95% confidence limits. Regression line has equation $Y = 9.2 + 36.2$ ($F = 5.006$, $n = 19$, $P < 0.03$).

ICS 205-930 (1–1000 $\mu g\,kg^{-1}$, i.v.) had no effect on resting blood, intragastric or intraduodenal pressure. As with granisetron, ICS 205-930 (10 and 100 $\mu g\,kg^{-1}$, i.v.) reduced the responses to duodenal distension by varying degrees; ICS 205-930 1 $\mu g\,kg^{-1}$ was inactive and 1000 $\mu g\,kg^{-1}$ increased the distension-evoked blood and intragastric pressure responses in, respectively, 5 of 6 and 3 of 6 rats (Figure 5). When the effect of ICS 205-930 (1, 10 and 100 $\mu g\,kg^{-1}$) on the fall in blood pressure was subjected to regression analysis, there was no statistically significant correlation ($F = 2.315$, $n = 15$, $P = 0.15$).

Ondansetron (1–1000 $\mu g\,kg^{-1}$, i.v.) had no effects on resting intragastric or duodenal pressure. However, 100–1000 $\mu g\,kg^{-1}$ ondansetron caused the resting blood pressure to become irregular in 11 out of 22 animals receiving these doses. Furthermore, in 7 of 11 rats dosed with 1000 $\mu g\,kg^{-1}$, ondansetron consistently caused a rapid but transient fall in blood pressure of 16 ± 2 mmHg. Overall, these effects on blood pressure were so marked in these animals ($n = 11$) that it was impossible to test the effect of ondansetron on their response to duodenal distension. In those rats where distension evoked responses could be reliably measured after dosing with ondansetron, there was no statistically significant inhibitory effect of ondansetron on the responses to duodenal distension at any one of the doses tested (1–1000 $\mu g\,kg^{-1}$, i.v.; Figure 6); a lower dose (0.1 $\mu g\,kg^{-1}$; $n = 2$, data not shown) was also inactive. As with the highest dose of granisetron and ICS 205–930 (1000 $\mu g\,kg^{-1}$, i.v.), ondansetron increased the intragastric responses to distension in some animals, but increases also occurred at all other doses tested (3 of 6 rats at 1 $\mu g\,kg^{-1}$, 3 of 9 at 10 $\mu g\,kg^{-1}$, 4 of 7 at 100 $\mu g\,kg^{-1}$ and 2 of 4 at 1000 $\mu g\,kg^{-1}$). Ondansetron also caused small increases in the distension-induced fall in blood pressure at 1 and 100 $\mu g\,kg^{-1}$, i.v. Because of these effects, ondansetron (100 $\mu g\,kg^{-1}$) was tested in 4 rats which failed to respond to duodenal distension; 20 min after dosing, duodenal distension evoked a fall in blood pressure in 2 animals and a fall in intragastric pressure in the other two.

Discussion

In this study, distension of the duodenum has been used as a model for visceral pain in the urethane-anaesthetized rat. Distension evoked falls in blood and intragastric pressure, the magnitude of which was dependent on the degree of pressure used, as previously shown in the jejunum (Lembeck & Skofitsch, 1982). By contrast, the renal pelvis may have an all-or-none response to distension (Brasch & Zetler, 1982).

Following bilateral cervical vagotomy, the distension-evoked fall in blood pressure was unchanged. This was in agreement with previous observations on rat ileum and jejunum (Clark & Smith, 1985; Lembeck & Skofitsch, 1982), suggesting that the neurones involved run in the sympathetic nerves. In contrast, the fall in intragastric pressure produced by duodenal distension was usually greatly reduced by vagotomy; in some animals the response was abolished. These results contrasted with those in the anaesthetized ferret where vagotomy did not affect the gastric relaxation evoked by duodenal distension (Andrews & Lawes, 1984).

The falls in blood and intragastric pressures induced by duodenal distension were blocked by morphine, an action reversed by naloxone. Similar distension pressure within the intestine of conscious rats or rabbits also elicits a morphine-sensitive behavioural response indicative of pain or discomfort (Jensen *et al.*, 1988; Colburn *et al.*, 1989). The fall in blood pressure induced by intestinal distension in anaesthetized rats

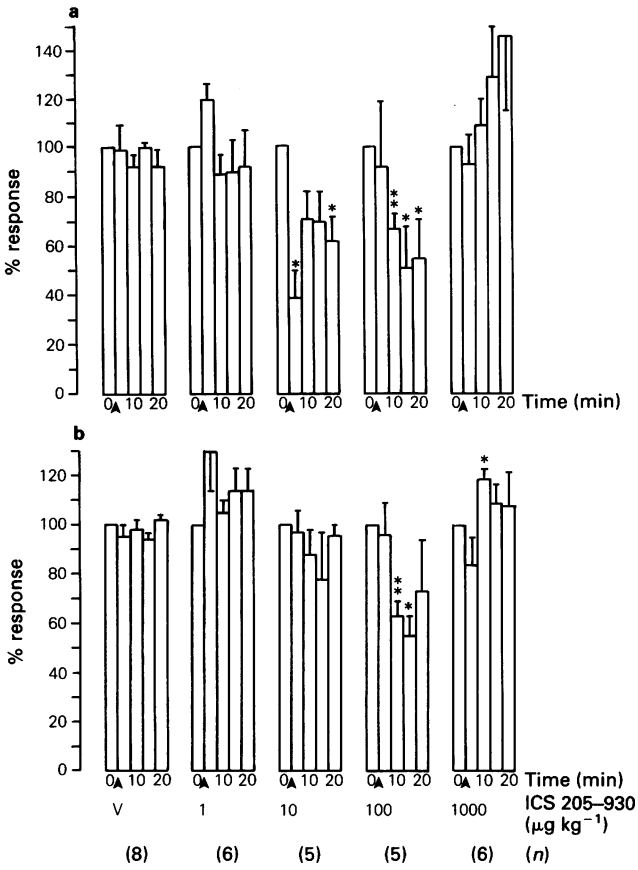

Figure 5 Effect of ICS 205-930 (1–1000 $\mu g\,kg^{-1}$, i.v.) or vehicle (V = 0.9% w/v saline) administered after a control distension (arrow) on the fall in (a) blood pressure and (b) intragastric pressure induced by duodenal distension with 75 cmH_2O for 20 s every 5 min. The first column in each group represents the pre-dose control response taken as 100%. Results are expressed as means (columns) and standard errors (vertical bars). * $P < 0.05$; ** $P < 0.01$, with one sample t test. Numbers in parentheses indicate the number of rats used.

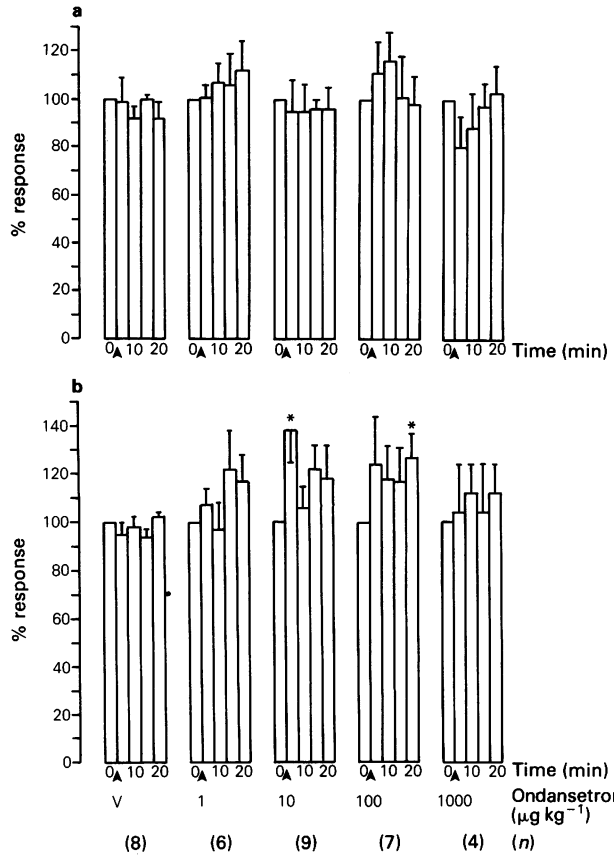

Figure 6 Effect of ondansetron (1–1000 $\mu g\,kg^{-1}$, i.v.) or vehicle (V = 0.9% w/v saline) administered after a control distension (arrow) on the fall in (a) blood pressure and (b) intragastric pressure induced by duodenal distension with 75 cmH_2O for 20 s every 5 min. The first column in each group represents the pre-dose control response taken as 100%. Results are expressed as means (columns) and standard errors (vertical bars). * $P < 0.05$ with one sample t test. Numbers in parentheses indicate the number of rats used.

may, therefore, form part of a pain response (see Introduction).

Granisetron and ICS 205-930 reduced, but did not abolish the responses to duodenal distension. For both compounds, the dose-response curve was bell-shaped. Such curves have previously been reported for ondansetron, granisetron and ICS 205-930 in rat models of anxiety (Costall *et al.*, 1988a) and psychosis (Costall *et al.*, 1988b), and for ondansetron against cytotoxic drug-induced emesis in cancer patients (Kris *et al.*, 1988). The mechanisms which underlie bell-shaped curves are often unknown, but one possibility is that two actions are involved, with the higher doses reducing the activity of the lower doses. With this in mind, interpretation of an ID_{50} value must be treated with caution. Nevertheless, the ID_{50} for granisetron in the present experiments (1.2 $\mu g\,kg^{-1}$, i.v.) is similar to the ID_{50} obtained for granisetron against the 5-HT_3 receptor-mediated Bezold-Jarisch reflex in anaesthetized rats (0.7 $\mu g\,kg^{-1}$, i.v., Sanger & Nelson, 1989). For ICS 205-930, the ability to antagonize the distension-evoked reflex was less consistent and the effective doses (10 and 100 $\mu g\,kg^{-1}$; not 1 $\mu g\,kg^{-1}$, i.v.) are greater than the range of active doses detected in the Bezold-Jarisch reflex test (ID_{50} = 0.4 or 1.4 $\mu g\,kg^{-1}$, i.v., Richardson *et al.*, 1985; Sanger, 1990; $ID_{100} > 10\,\mu g\,kg^{-1}$, i.v., Cohen *et al.*, 1989).

Ondansetron did not reduce the distension-evoked falls in either blood or intragastric pressures. This observation is in marked contrast to the effects of granisetron or ICS 205-930 and conflicts with the potent ability of this compound to antagonize the Bezold-Jarisch reflex in anaesthetized rats (ID_{50} against 2-methyl-5-HT = 0.4 $\mu g\,kg^{-1}$, i.v., Butler *et al.*, 1988; ID_{50} against 5-HT = 3.6 $\mu g\,kg^{-1}$, i.v., Sanger, 1990) and to antagonize the 5-HT_3 receptor in general. Our observations may be explained if a novel 5-HT_3 receptor subtype is involved, if certain compounds have properties which are additional to their ability to antagonize 5-HT_3 receptors and/or if the compounds have different pharmacodynamic profiles. Evidence in favour of a pharmacodynamic difference between the compounds has been suggested by Cohen *et al.* (1989), who found that ondansetron antagonism of the 5-HT-evoked Bezold-Jarisch reflex was of shorter duration than that of ICS 205-930. However, against 2-methyl 5-HT, the duration of action of ondansetron seems to be greater than 2 h (Butler *et al.*, 1988). Alternatively, our experiments show that ondansetron tended to increase the response to duodenal distension at lower doses than granisetron and ICS 205-930. If the affinity of ondansetron for a receptor causing an increase in the distension-induced reflex is higher than its affinity for a receptor which would inhibit the reflex, then no change or an increase in the distension-evoked reflex would be seen. For granisetron and to a lesser extent, ICS 205-930, these affinities could be reversed, resulting in a bell-shaped dose-response curve. For either of these activities, the compounds could, therefore, act at the 5-HT_3 receptor, at an as yet unclassified 5-HT_3 receptor subtype or at a non-5-HT_3 receptor. Further analysis is clearly required, so that these suggestions can be tested.

The site of action for granisetron and ICS 205-930 is not known. It is unlikely to be a direct effect on the smooth muscle of the blood vessels and the stomach. Neither compound affected resting blood pressure or intragastric pressure and granisetron has no effect on the fall in blood pressure

evoked by vagal stimulation (Sanger & Nelson, 1988). If granisetron and ICS 205-930 act by antagonizing a 5-HT receptor, this may be present on the peripheral afferent nerve fibres, being activated by 5-HT released from enterochromaffin cells by distension (Bülbring & Crema, 1959). Alternatively, they may also act within the central nervous system or spinal cord. Cramer & Rademaker (1989) found that in anaesthetized rats, ICS 205-930 (9 μmol kg^{-1}, i.p.) reduced the fall in blood pressure evoked by noxious distension of the proximal ileum, but did not affect the afferent nerve activity arising from the ileum. A site of action outside the gut was, therefore, suggested.

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Angiotensin converting enzyme inhibitors potentiate the bronchoconstriction induced by substance P in the guinea-pig

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1 The effects of intravenous captopril and enalaprilic acid on the increase in pulmonary inflation pressure induced by different bronchoconstrictor agents were evaluated in the anaesthetized guinea-pig.

2 Captopril and enalaprilic acid (1.6–200 $\mu g\,kg^{-1}$) enhanced dose-dependently the bronchoconstriction (BC) induced by substance P. The threshold effective dose was 1.6 $\mu g\,kg^{-1}$ and maximal potentiation over the control response was more than 400% for both agents. Enalaprilic acid was also assayed for serum and lung angiotensin converting enzyme (ACE) inhibition in anaesthetized guinea-pigs. This drug produced a dose-dependent inhibition of ACE in both tissues, with ED_{50}s of 7.6 and 9.4 $\mu g\,kg^{-1}$, respectively: this inhibitory activity was positively correlated to substance P potentiation.

3 Captopril (8–1000 $\mu g\,kg^{-1}$) enhanced dose-dependently the BC induced by capsaicin. The threshold effective dose was 40 $\mu g\,kg^{-1}$ and maximal potentiation about 90%.

4 Captopril (200–1000 $\mu g\,kg^{-1}$) did not affect BC induced by bradykinin. However, this response was markedly enhanced (about 200%) by captopril 200 $\mu g\,kg^{-1}$ in propranolol-pretreated animals.

5 Captopril and enalaprilic acid (200–1000 $\mu g\,kg^{-1}$) slightly (20–40%) but significantly enhanced the BC induced by 5-hydroxytryptamine. However, this response was potentiated to the same extent by a dose of prazosin, which produced a degree of hypotension similar to that observed after administration of the ACE inhibitors.

6 In conclusion, ACE inhibitors potentiate the BC induced by substance P and, to a minor extent, that induced by capsaicin in the anaesthetized guinea-pig. Potentiation of substance P is well correlated with ACE inhibition in guinea-pig serum and lungs. These experimental results may offer a mechanistic interpretation of cough and bronchial hyperreactivity observed in patients receiving treatment with ACE inhibitors.

Introduction

Angiotensin converting enzyme (ACE) inhibitors are being used increasingly in the treatment of hypertension and heart failure. One of the most recently recognized side-effects of these agents is the occurrence of a persistent dry cough (Coulter & Edwards, 1987), which is apparently associated with bronchial hyperreactivity (Bucknall *et al.*, 1988). Both cough and hyperreactivity are prominent features of bronchial asthma. Therefore these symptoms may result from the 'unmasking of asthma' by ACE inhibitors, as previously suggested by Salena (1986). ACE is identical with kininase II, an enzyme that degrades bradykinin, and this kinin may activate local axon reflexes by stimulating C-fibre afferent nerve endings, ultimately leading to bronchoconstriction (BC) and airway inflammation (Barnes, 1986). Moreover, ACE is capable of catalyzing the metabolism of substance P, one of the inflammatory and bronchoconstrictor tachykinins widely distributed throughout the respiratory system of various species (Cascieri *et al.*, 1984). In view of the above, we examined the effects of captopril and enalaprilic acid (MK 422, the active parent diacid of enalapril) on the response induced by different bronchoconstrictor agents: substance P, capsaicin (the pungent principle of hot peppers of the genus *Capsicum*, which selectively releases neuropeptides, including substance P, from C-fibre afferents), bradykinin and 5-hydroxytryptamine (5-HT).

Methods

Male Dunkin-Hartley guinea-pigs, body weight 300–400 g, from Rodentia, Torre Pallavicina (Bergamo), were used. The animals were anaesthetized with ethyl urethane (1.2 $g\,kg^{-1}$ i.p.). A tracheal cannula was connected to a Rodent Ventilator (U. Basile, Italy) calibrated at 3–4 ml and 60 r.p.m. and spontaneous respiration was abolished by gallamine 3 $mg\,kg^{-1}$ i.v. Pulmonary inflation pressure (PIP) and mean blood pressure were measured via Bentley Trantec 800 transducers, connected to a side-arm of the tracheal cannula and to a carotid artery respectively, and recorded on a Gemini recorder (U. Basile). After an equilibration period of at least 20 min, bronchoconstrictor agents were injected intravenously at doses aimed at producing an increase in PIP of approximately 10 cmH_2O. Capsaicin (1–3 $\mu g\,kg^{-1}$) and 5-HT (3–15 $\mu g\,kg^{-1}$) were administered repeatedly at 10 min intervals until the response was stable. Bradykinin (BK, 3 $\mu g\,kg^{-1}$) and substance P (10 $\mu g\,kg^{-1}$) were injected only twice, before and after the administration of the test drugs, at a 30 min interval. BK (0.5 $\mu g\,kg^{-1}$) was also tested in the presence of propranolol (1 $mg\,kg^{-1}$ i.p. just before the anaesthetic, plus 1 $mg\,kg^{-1}$ i.v. 20 min before the first challenge with BK). The test drugs or the vehicle were injected intravenously 2 min before the bronchoconstrictor challenge. Each animal received only a single administration of vehicle or drug. Percentage changes in the response to bronchoconstrictor agents (post-treatment versus pretreatment increase in PIP) were calculated. Changes in drug-treated groups were compared with those in vehicle-treated animals by means of Student's unpaired t test. A P value of 0.05 or less was taken to be significant.

In another experimental series the dose-response (increase in PIP) curves to substance P and 5-HT were evaluated in the presence and absence of enalaprilic acid, 200 $\mu g\,kg^{-1}$ i.v. Substance P was injected at increasing doses (1–30 $\mu g\,kg^{-1}$), at intervals of 5 min between successive administrations. After 1 h the dose-response curve was repeated, 2 min after administration of vehicle or enalaprilic acid. 5-HT (1–30 $\mu g\,kg^{-1}$) was injected at 2 min intervals between doses. About 20 min after completion of the first dose-response curve, a second curve was constructed, 2 min after the injection of vehicle or enalaprilic acid. The bronchoconstrictor responses to substance P and 5-HT after the administration of enalaprilic acid were

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compared with those obtained in vehicle-treated animals (Student's unpaired t test). In separate groups of animals, the effects of enalaprilic acid on serum and lung ACE activity were evaluated. Enalaprilic acid was used for this purpose, because plasma and tissue ACE inhibition induced by this agent is stable on storage, contrary to that of captopril (Cohen & Kurz, 1983). The animals were prepared as described above. After 20 min of equilibration, vehicle or a single dose of enalaprilic acid (1.6–1000 $\mu g\,kg^{-1}$) was injected i.v. Between 2 and 3 min after administration, thoracotomy was performed, blood was drawn from the heart and the lungs were excised. Serum and lungs (after perfusion with normal saline through the pulmonary artery) were frozen at −20°C. ACE activity was evaluated by incubating aliquots of serum or lung homogenates with the artificial substrate hippurylglycylglycine in 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonate) buffer, pH 8, containing 300 mM sodium chloride and 400 mM sodium sulphate (total volume 70 μl). After 60 min at 37°C, the reaction was stopped by adding 20 μl 0.5 N perchloric acid and 410 μl distilled water. Enzymatically released hippuric acid was extracted into 500 μl ethyl acetate, 100 μl of which were brought to dryness in a stream of air, at 37°C. Residues were dissolved in 500 μl mobile phase (see below) and analysed by h.p.l.c. on a reverse phase column (Nucleosil C_{18}, 5 μm, 15 × 0.46 cm, HPLC Technology) connected to a model 6000 A pump (Waters-Millipore) through a model U6K injector (Waters-Millipore). Peaks eluted isocratically with 20% acetonitrile in 50 mM potassium phosphate pH 3 (flow 1 ml min^{-1}, pressure 2000 psi ca.) were detected at 228 nm by means of a LC-85B spectrophotometric detector (Perkin-Elmer). Parallel analysis of standards revealed an overall 75% recovery of added hippuric acid. Activity was calculated as units (u, μmol hippuric acid formed per min) per ml (g) of serum (fresh tissue) at 37°C. Percentage inhibition of serum and lung ACE in drug-treated animals when compared to controls was calculated. ED_{50}s and 95% confidence limits were calculated by means of linear regression analysis of the dose-response curves.

Drugs used were: ethyl urethane (Fluka), gallamine triethiodide, (±)-propranolol hydrochloride, 5-hydroxytryptamine creatinine sulphate and bradykinin triacetate (Sigma), capsaicin (Serva), substance P (kindly donated by Dr P. Rovero, Menarini), prazosin hydrochloride (Pfizer), captopril (Squibb) and enalaprilic acid (Merck, Sharp & Dohme).

Results

Effects of bronchoconstrictor agents

Dose-response curves were constructed for the four bronchoconstrictor agents. Neither the maximal responses (ranging between 40 and 50 cmH_2O) nor the slopes of the curves were significantly different, as estimated by analysis of variance (data not shown). The doses of the agonists employed in the subsequent experiments and their bronchoconstrictor effects, before and after vehicle administration, are shown in Figure 1. The aim was to obtain a BC of similar magnitude with all four agonists, in order to compare the effects of ACE inhibitors on the different responses. This aim was mainly achieved with substance P, capsaicin and bradykinin, since the BC induced by these agents was similar ($P > 0.05$ by analysis of variance). On the other hand, the BC obtained with 5-HT was definitely more intense. This is due mainly to the fact that the response to a fixed dose of 5-HT tended to increase slowly: thus, when the response was stabilized, usually after 4–6 administrations, the BC was generally increased to a considerable extent with respect to the first response. As also shown in Figure 1, the second response to substance P was slightly, but significantly reduced, while no significant difference was found between the increases in PIP evoked by the two successive challenges with capsaicin, 5-HT and BK. In contrast, in the presence of propranolol, the second response to BK was only 60% of the first response, suggesting that under these experimental conditions the airways exhibit tachyphylaxis to BK.

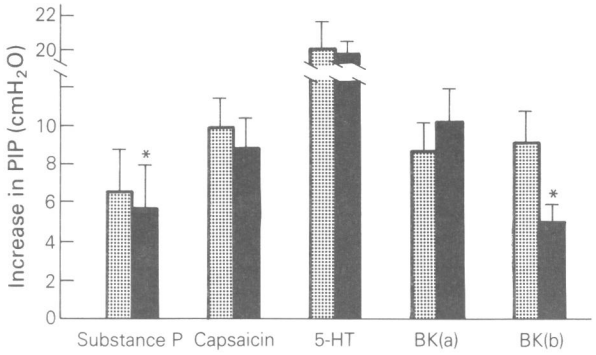

Figure 1 Effects of vehicle administration on the bronchoconstriction (increase in pulmonary inflation pressure, PIP) induced by substance P (10 $\mu g\,kg^{-1}$), capsaicin (1–3 $\mu g\,kg^{-1}$), 5-hydroxytryptamine (5-HT, 3–15 $\mu g\,kg^{-1}$) and bradykinin (BK, 3 $\mu g\,kg^{-1}$) in (a) anaesthetized guinea-pigs and by BK (0.5 μg kg) in (b) anaesthetized guinea-pigs pretreated with propranolol. The first column (stippled) represents the response observed before administration of the vehicle; the second column (solid) represents the response observed two min after vehicle administration. Mean values are presented and bars show s.e.mean (n = 5–10). * $P < 0.05$ by Student's paired t test.

Effects of captopril

Generally speaking, captopril produced an increase in the amplitude of the majority of the bronchoconstrictor responses, while their onset rate and duration were not apparently affected, as seen in Figure 2. Therefore, we always refer to changes in the amplitude of the response, if not otherwise stated.

Captopril potentiated dose-dependently the response to substance P (Figure 3a) and capsaicin (Figure 3b). In the case

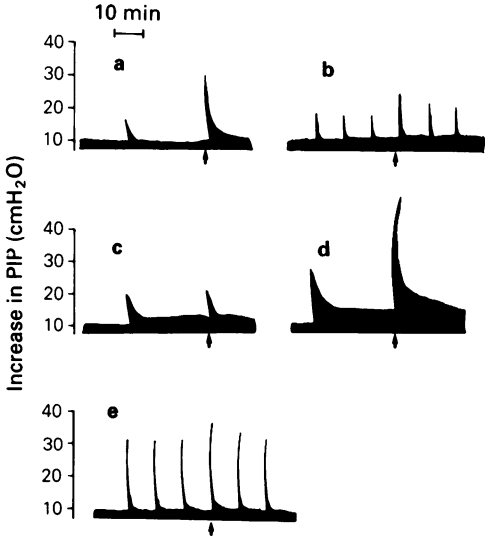

Figure 2 Representative tracings showing the effects of captopril (200 $\mu g\,kg^{-1}$ i.v.), administered at the arrow, on the bronchoconstriction evoked by substance P (10 $\mu g\,kg^{-1}$) (a), capsaicin (3 $\mu g\,kg^{-1}$) (b), bradykinin (c: 3 $\mu g\,kg^{-1}$, in the absence of propranolol; d: 0.5 $\mu g\,kg^{-1}$, in the presence of propranolol) and 5-hydroxytryptamine (10 $\mu g\,kg^{-1}$) (e) in the anaesthetized guinea-pig. Capsaicin and 5-hydroxytryptamine were administered repeatedly at 10 min intervals, until a stable response was obtained, while substance P and bradykinin were injected only twice, at an interval of 30 min. Captopril was given 2 min before the bronchoconstrictor agents.

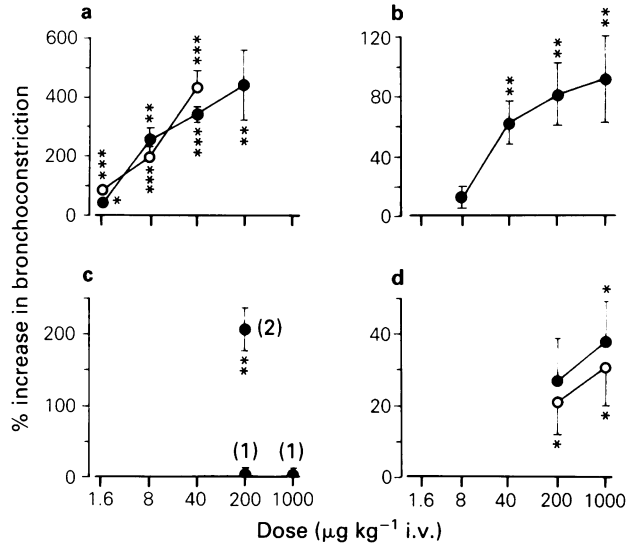

Figure 3 Effects of captopril (●) and enalaprilic acid (○) on the bronchoconstriction induced by (a) substance P ($10\,\mu g\,kg^{-1}$), (b) capsaicin ($1–3\,\mu g\,kg^{-1}$), (c) bradykinin ($3\,\mu g\,kg^{-1}$, in the absence (1) and $0.5\,\mu g\,kg^{-1}$ in the presence (2) of propranolol) and (d) 5-hydroxytryptamine ($3–15\,\mu g\,kg^{-1}$) in the anaesthetized guinea-pig. The test drugs were injected intravenously 2 min before the bronchoconstrictor challenge. Results are expressed as % increase over control responses, shown in Figure 2. Mean values are presented with vertical lines showing s.e.mean ($n = 4–10$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by Student's unpaired t test.

of substance P, the threshold effective dose was $1.6\,\mu g\,kg^{-1}$ and maximal effect was a 442% increase over control response; in the case of capsaicin, the threshold dose was $40\,\mu g\,kg^{-1}$ and maximal effect was 92%. 5-HT was potentiated significantly (38%) by captopril only at the dose of $1000\,\mu g\,kg^{-1}$ (Figure 3d). On the other hand, captopril $200–1000\,\mu g\,kg^{-1}$ did not potentiate BK in the absence of propranolol, while at $200\,\mu g\,kg^{-1}$ it did potentiate this kinin (207%) in β-blocked animals (Figure 3c).

Effects of enalaprilic acid

The effects of enalaprilic acid on substance P- and 5-HT-induced BC, shown in Figure 3a and d respectively, are essentially similar to those of captopril. Enalaprilic acid was assayed also for its ability to inhibit serum and lung ACE under the same experimental conditions used for the BC experiments (Figure 4a): a dose-dependent inhibition of ACE was found at doses of $1.6–1000\,\mu g\,kg^{-1}$ in both tissues. The ED_{50}s and 95% confidence limits were 7.6 (3.6–15.5) $\mu g\,kg^{-1}$ for serum and 9.4 (2.5–31.5) $\mu g\,kg^{-1}$ for lung ACE inhibition. As seen in Figure 4b, substance P potentiation appeared well correlated to ACE inhibition, although this correlation was significant only for serum ACE.

In a separate group of animals the effects of enalaprilic acid, $200\,\mu g\,kg^{-1}$, on the dose-response curves to substance P and 5-HT were evaluated. Substance P was markedly potentiated along the whole dose-response curve (Figures 5 and 6b).

In this case, time-integrated changes in PIP (between 0 and 5 min) were affected more intensely than the maximal amplitude of BC, since the duration of the response was increased in a more substantial manner than the BC peak (Figures 5 and 6b). On the other hand, enalaprilic acid produced a slight potentiation of 5-HT: a significant effect on either BC peak or time-integrated change in PIP was observed only at a dose of $10\,\mu g\,kg^{-1}$ of the agonist (36%, $P < 0.05$).

Hypotensive effects of ACE inhibitors and effects of prazosin on bronchoconstriction

Both captopril and enalaprilic acid produced a dose-dependent hypotensive action in the anaesthetized guinea-pig,

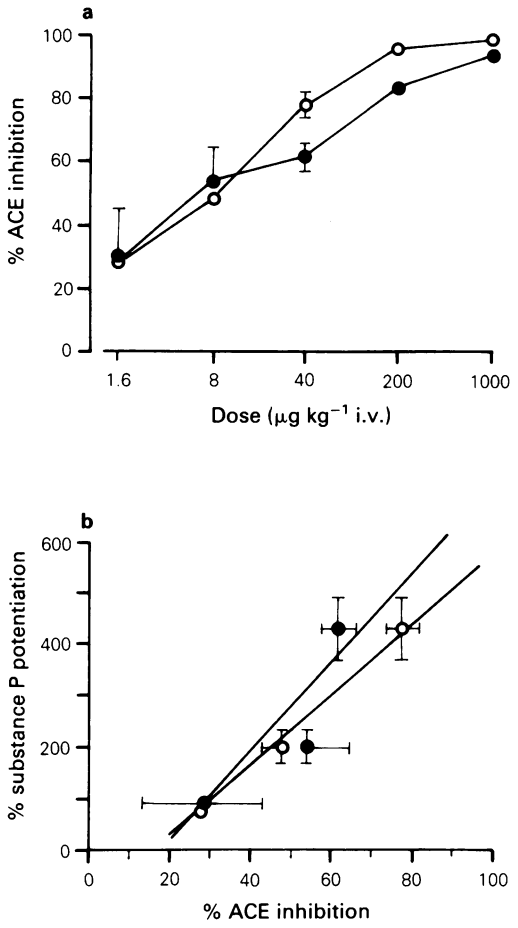

Figure 4 Effects of enalaprilic acid on serum (○) and lung (●) angiotensin converting enzyme (ACE) activity (a) and correlation between this ACE-inhibitory activity and potentiation of substance P-induced bronchoconstriction (b) in the anaesthetized guinea-pig. The test drug was administered 2–3 min before bleeding and lung excision. Mean values and s.e.mean (vertical lines) are presented ($n = 3$). ACE activity in control guinea-pig was: $6.28 \pm 0.28\,u\,ml^{-1}$ (serum) and $8.60 \pm 3.40\,u\,g^{-1}$ (lungs). Mean values of ACE inhibition induced by enalaprilic acid were correlated to mean values of substance P potentiation, induced by corresponding doses of the drug, which are shown in Figure 3a. The correlation coefficients were $r = 0.997$ for ACE inhibition in serum ($P < 0.05$) and $r = 0.885$ for ACE inhibition in lungs ($P > 0.05$).

with enalaprilic acid being slightly more potent than captopril (Figure 7). In order to ascertain the influence of hypotension on BC, the effects of prazosin on the response to bronchoconstrictor agents were evaluated. The drug was administered at a dose of $200\,\mu g\,kg^{-1}$, which caused a $44 \pm 2\%$ ($n = 18$) fall in mean blood pressure, while having no effect on basal PIP. Prazosin caused a slight but significant ($P < 0.05$) enhancement of the BC induced by capsaicin ($30 \pm 8\%$, $n = 8$) and 5-HT ($32 \pm 8\%$, $n = 6$), but did not affect the BC induced by substance P ($-5 \pm 9\%$, $n = 4$).

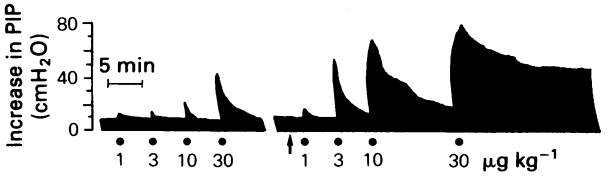

Figure 5 Representative tracing showing the effects of enalaprilic acid, administered at the arrow ($200\,\mu g\,kg^{-1}$), on the bronchoconstriction induced by increasing doses of substance P, administered at the dots, in the anaesthetized guinea-pig. About one hour after completion of the first dose-response curve, the second curve was constructed, 2 min after the administration of enalaprilic acid.

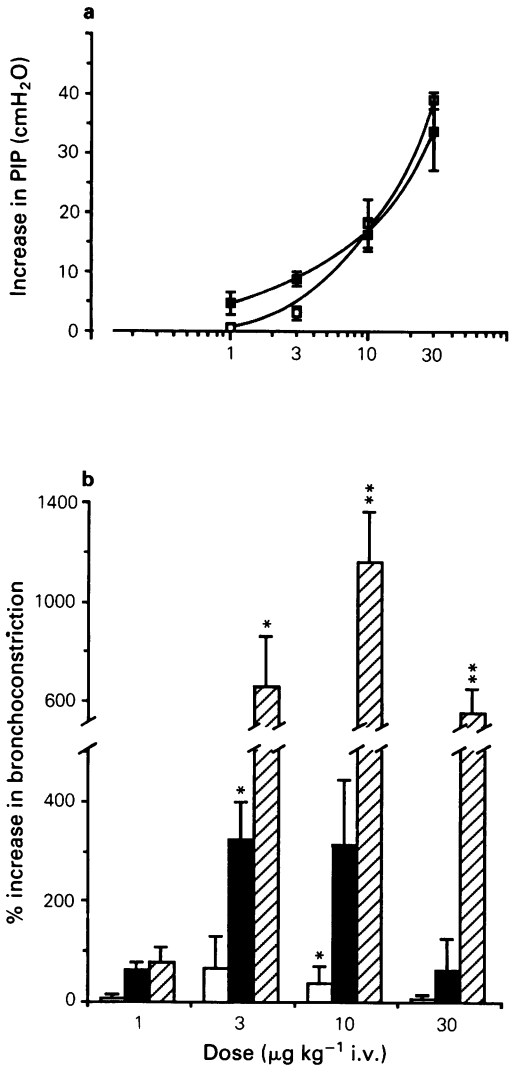

Figure 6 Bronchoconstriction induced by 5-hydroxytryptamine and substance P and effects of enalaprilic acid (200 $\mu g kg^{-1}$). (a) Dose-response curves for 5-hydroxytryptamine (□) and substance P (■). (b) Open and closed columns represent enalaprilic acid-induced increases of maximal amplitude of bronchoconstriction at different doses of 5-hydroxytryptamine and substance P, respectively. Hatched columns represent increases of time-integrated (between 0 and 5 min) changes in pulmonary inflation pressure induced by substance P. Results are shown as % changes over response observed in vehicle-treated animals, shown in (a). Mean values and s.e.mean (vertical lines) are presented. * $P < 0.05$; ** $P < 0.01$ by Student's unpaired t test.

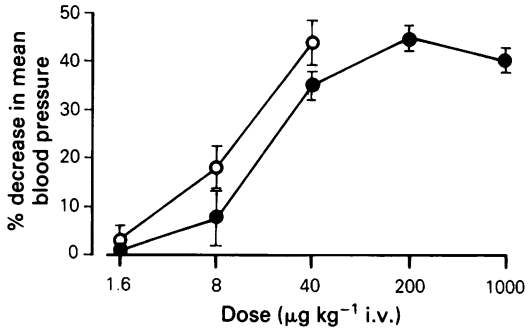

Figure 7 Hypotensive effects of captopril (●) and enalaprilic acid (○) in anaesthetized guinea-pigs. Each animal received a single dose of one agent. Results are expressed as % decrease of basal mean blood pressure.

Mean values and s.e.mean (vertical lines) are presented (n = 3–5). Basal mean blood pressure was 36 ± 1.3 mmHg (n = 30).

Discussion

The present experiments were designed to permit a direct comparison of the effects of ACE inhibitors on the BC induced by different agonists. The doses of substance P, capsaicin and BK used in the main experiments were broadly equipotent in producing BC, thus allowing the comparison of potentiation expressed as % increase over control response. On the other hand, with 5-HT we obtained a BC which was more intense than that observed with the other agents, but still lying on the lower part of the dose-response curve. Thus, a comparison of the potentiation of 5-HT with those of the other agonists may not be appropriate. However, we have shown in separate experiments that potentiation of 5-HT by enalaprilic acid is similarly slight along the whole dose-response curve of the agonist (Figure 6b).

Our results show that ACE inhibitors potentiate the bronchomotor response induced by the four agents tested, but the potency and efficacy of this enhancing action appears different in each case and BK is potentiated only in the presence of propranolol. Moreover, ACE inhibitors cause a profound hypotension in the anaesthetized guinea-pig, as already demonstrated with captopril by Greenberg *et al.* (1979), but in contrast with the effects observed in other normotensive animals, such as rats, rabbits, cats and monkeys (Rubin *et al.*, 1978).

In order to check the possible role of hypotension in the enhancement of BC, we examined the effects of prazosin: this drug slightly potentiated capsaicin- and 5-HT-induced BC, but did not appear to affect substance P-induced BC.

Therefore hypotension *per se* is likely to have a slight potentiating effect on BC, although we do not have an explanation for this finding at the moment. As a consequence, the potentiating effects of ACE inhibitors may have been slightly overestimated and it is possible that potentiation of 5-HT, which was always less than 40%, is due mainly to the hypotensive action of these drugs. However, the same explanation cannot be valid for capsaicin, which was potentiated by captopril to a significantly larger extent ($P < 0.05$ vs 5-HT), and for substance P, which was not potentiated by prazosin in our experiments, while a striking potentiation was produced by ACE inhibitors. The latter effect appears well correlated to serum and lung ACE inhibition. In fact, substance P can be metabolized by ACE (Cascieri *et al.*, 1984), as well as by other enzymes from different sources, such as serine proteinases (Hanson & Lovenberg, 1980), acetylcholinesterase (Chubb *et al.*, 1980) and enkephalinase (Skidgel *et al.*, 1984). The results of these experiments indicate that ACE plays a prominent role in the degradation of substance P itself and/or of an another mediator subserving the actions of substance P, since an inhibition of 50% or less of serum and lung ACE is sufficient to produce a massive increase of substance P-induced BC. A similar mechanism, e.g. inhibition of the metabolism of substance P released from sensory nerves, can also explain the potentiation of capsaicin, but in this case the effects of captopril are inferior both in potency and efficacy. We can think of two possible explanations for this difference: (a) capsaicin can release a number of neuropeptides (including calcitonin gene-related peptide and neurokinin A, which have been identified in the airways and contract airway smooth muscle), as well as substance P (Barnes, 1987). These neuropeptides are not metabolized by ACE, and consequently are not potentiated by captopril; (b) Substance P is probably released in extravasal sites where not only ACE, but also other enzymes, e.g. enkephalinase, play a physiological role (Borson *et al.*, 1987). ACE located in these sites may be less readily accessible to inhibitors than the enzyme located at the endothelial luminal surface, where exogenous substance P is most probably degraded. The first hypothesis may provide an explanation for the lower efficacy of captopril against capsaicin, while the second hypothesis might also explain the lower potency of the drug, due to limited availability at the active site.

Since ACE is identical with kininase II, potentiation of BK

was obviously expected: it is thus a paradox that BK is the only bronchoconstrictor which was not potentiated to any extent in our experiments. The doses of captopril we have tested (200–1000 $\mu g\,kg^{-1}$) are well above those showing ACE-inhibitory activity, thus definitely inhibiting the degradation of BK. However, our results are in agreement with those of Greenberg *et al.* (1979), who showed that captopril is capable of enhancing the bronchomotor effects of BK in the presence but not in the absence of propranolol. These authors suggest that captopril causes an incresae of both BC and release of adrenomedullary catecholamines induced by BK, with the latter effect antagonizing the former one. Thus, the overall effect of captopril on BK-induced BC is null, unless the animals are β-blocked. In this context, it is well known that an adrenergic bronchodilator reflex mechanism also occurs with other agonists (McCulloch *et al.*, 1967) and therefore it would be interesting to investigate the effects of β-blockade on the interaction between substance P or capsaicin and ACE inhibition. Anyhow, this adrenergic reflex is likely to be less important in these cases, since the latter agonists also appear potentiated in the absence of β-blockade.

In conclusion, ACE inhibitors appear capable of enhancing the bronchomotor response to substance P and capsaicin. These results suggest that ACE plays an important role in modulating the *in vivo* response to exogenously administered or endogenously released substance P, and may offer a mechanistic explanation of the cough and bronchial hyperreactivity observed in patients receiving treatment with ACE inhibitors.

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Effects of tetraethylammonium and 4-aminopyridine on outward currents and excitability in canine tracheal smooth muscle cells

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1 The effects of tetraethylammonium (TEA) and 4-aminopyridine (4-AP) on membrane currents and on single channel K currents in smooth muscle cells isolated from canine trachea were examined by use of tight seal whole cell- and patch-clamp techniques.

2 Depolarizing current applied through a recording pipette did not elicit an action potential under current clamp. A strong outward rectification was observed.

3 In most cells under voltage-clamp, only an outward current was observed upon depolarization from −60 mV when a pipette solution contained mainly KCl. The outward current consisted of three components; a large initial transient, a following sustained component and an additional component of irregular small transients on the sustained one. The two transient components were almost abolished when extracellular and pipette solutions contained 2.2 mM Cd^{2+} (0 mM Ca^{2+}) and 10 mM EGTA, respectively. The sustained component was well maintained under these conditions.

4 TEA at low concentrations (< 1 mM) effectively decreased the transient components and made the outward current smooth; it also suppressed the sustained component at higher concentrations. In outside-out patches, external 1 mM TEA reduced the single channel conductance of Ca-activated K channels by about 87% whereas 3 mM 4-AP did not. 4-AP at low concentrations (< 3 mM) selectively reduced the sustained component of the outward current.

5 A Ca current recorded after the suppression of outward current by internal Cs^+ had a peak of approximately 200 pA at +10 mV (holding potential: −60 mV). The half inactivation voltage in the steady-state was approximately −30 mV.

6 Simultaneous application of 1 mM TEA and 4-AP reduced the outward current and unmasked a Ca current. Under these conditions, an action potential with overshoot was easily elicited under current clamp.

7 It is concluded that the low excitability of canine tracheal smooth muscle cell upon depolarization is due to a large outward K current which consists of Ca-dependent and Ca-independent components. The peak amplitude of the Ca current is similar to that in highly excitable smooth muscle cells such as those of the ureter.

Introduction

The smooth muscle of canine (Stephens & Kroeger, 1970; Suzuki *et al.*, 1976) and bovine trachealis (Kirkpatrick, 1975) shows low excitability. No action potential is elicited even when the cell is depolarized by stimulation with electrical pulse(s) or agonists such as acetylcholine, histamine, 5-hydroxytryptamine etc. (see Kirkpatrick, 1980). Therefore a significant contribution of pharmaco-mechanical coupling to the agonist-induced contraction has been suggested in these preparations (Farley & Miles, 1977; Coburn & Yamaguchi, 1977). In guinea-pig trachea, the smooth muscle cells often exhibit spontaneous electrical slow waves (Small, 1982).

It has been found that a K channel blocker, tetraethylammonium (TEA), increases the membrane excitability in canine and bovine tracheal smooth muscle cells and even elicits repetitive action potentials and concomitant rhythmic contractions similar to the spontaneous contractions of intestinal smooth muscle (Stephens *et al.*, 1975; Kroeger & Stephens, 1975; Kirkpatrick, 1975). Subsequently, it was reported that other K channel blockers such as 4-aminopyridine (4-AP) (Imaizumi & Watanabe, 1983; Kannan *et al.*, 1983) and procaine (Imaizumi & Watanabe, 1982) also have similar but not identical effects to those of TEA on the muscle. The decrease in outward rectification upon depolarization by these K channel blockers was observed in quiescent smooth muscles by use of conventional microelectrodes and the partition method (Casteels *et al.*, 1977; Kirkpatrick, 1975; Suzuki *et al.*, 1976; Hara *et al.*, 1980), and appeared to be responsible for the increase in excitability. Moreover, ^{42}K- and ^{86}Rb-efflux experiments suggest that these compounds decrease K^+ permeability perhaps, by suppressing different K channels (Imaizumi & Watanabe, 1981; 1982; 1983).

The application of the patch-clamp technique to single smooth muscle cells from canine trachea reveals the existence of a Ca-activated K channel on the cell membrane (McCann & Welsh, 1986) as well as in other smooth muscle cells (Inoue *et al.*, 1985; Benham *et al.*, 1985; 1986; Singer & Walsh, 1987; Bregestovski *et al.*, 1988). Recently, a small voltage-dependent Ca channel current has been recorded in single canine tracheal smooth muscle cells (Kotlikoff, 1988).

The present study using whole cell- and patch-clamp techniques was undertaken to elucidate the mechanisms involved in the increase of membrane excitability of canine tracheal smooth muscle cells by TEA and 4-AP. The objective was to determine whether TEA and 4-AP block different K currents and whether the Ca current in tracheal smooth muscle really is much smaller than in other highly excitable smooth muscle cells.

Methods

Cell isolation

Circular muscle strips were obtained from the cervical trachea of dogs weighing 8–14 kg. The mucosa, adventitial, areolar

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and connective tissues were carefully removed from the smooth muscle layer in normal Krebs solution under a binocular microscope (× 30). The procedure of cell isolation was the same as that described by Imaizumi *et al.* (1989a). The tissue was immersed in Ca-free Krebs solution (Ca omitted, no EGTA) for 40–60 min in a test-tube at 37°C. Thereafter, the solution was replaced with Ca-free Krebs solution containing 0.1% albumin and 0.1% collagenase (490 u ml^{-1}, YAKULT, Tokyo) for 30 min. The solution was then replaced with Ca-free and collagenase-free Krebs solution. The tissue was gently agitated with a glass pipette with a short shank and a fire-polished tip. After approximately 10 min agitation, the solution became turbid due to the presence of isolated smooth muscle cells. A few drops of cell suspension were placed in a recording bath (0.3 ml) mounted on the stage of a phase-contrast microscope (Nikon TMD) for electrical measurements.

Solutions

The normal Krebs solution contained (mM): Na^+ 137, K^+ 5.9, Ca^{2+} 2.2, Mg^{2+} 1.2, Cl^- 123.5, HCO_3^- 25, $H_2PO_4^-$ 1.2, glucose 14 and was gassed with a mixture of 95% O_2 and 5% CO_2. For electrical recordings, a HEPES buffered solution of the following composition was used (mM): Na^+ 137, K^+ 5.9, Ca^{2+} 2.2, Mg^{2+} 1.2, Cl^- 149.7, glucose 14, HEPES 10. The solution was gassed with 100% O_2 and the pH was adjusted to 7.2 with NaOH. The pipette solution contained (mM): K^+ 140.0, Na^+ 10.0, Mg^{2+} 4.0, Cl^- 148.0, adenosine-triphosphate (ATP) 5.0, ethyleneglycol-bis-(β' amino-ethyl ether) N,N,N', N'-tetraacetic acid (EGTA) 0.5, the pH of which was adjusted to 7.2 with KOH. When measuring Ca currents, the 140 mM KCl in the pipette solution was replaced with equimolar CsCl and the concentration of EGTA was increased to 10 mM. The pCa of the solutions for measuring Ca activated K channel current activity in excised patches was adjusted with a Ca-EGTA buffer (Benham *et al.*, 1986).

Electrical recordings

Whole cell voltage and current clamp and patch clamp commands were applied to single cells or patches by the method introduced by Hamill *et al.* (1981). The details were similar to those described previously (Imaizumi *et al.*, 1989a,b). The resistance of the pipette was between 2 and 5 MΩ for the whole cell-clamp and between 7 and 15 MΩ for the patch-clamp. A hydraulic micromanipulator (MO-102 or MO-203 Narishige Scientific Instruments, Tokyo, Japan) was used to position the microelectrode near the cell. All procedures for approaching the cell, making a GΩ seal and rupturing the cell membrane were done under × 800 magnification. Inside- or outside-out patch-clamp procedures (Hamill *et al.*, 1981) were performed to examine the effects of TEA and 4-AP on single K channel currents by use of a List EPC-7 amplifier. Single channel current recordings were filtered at 1 kHz by a 4 pole Bessel filter and sampled at 250 μs (see below). The seal resistance formed between cell membrane and tip before impalement was between 10 and 50 GΩ. All measurements were performed at 25 ± 1°C.

Data storage and analysis

Membrane potential and current were monitored on a storage oscilloscope (VC-10, Nihon-koden, Tokyo, Japan or VC-6041, Hitachi, Tokyo, Japan) and stored on video-tape after digitizing by use of a PCM-recording system (PCM 501ES, SONY, Tokyo, Japan; modified to obtain a frequency-response from d.c. to 20 kHz). The data on the tape were replayed later and loaded into a computer (IBM-AT) through an A-D converter (Datatranslation; DT2801A) using data-acquisition software (AQ). Data analysis was performed on an IBM-AT using software, DAS, developed in Dr Wayne Giles' laboratory (University of Calgary, Canada; also AQ). Part of the analysis of single channel current was performed by use of a programme developed by Dr John Dempster (University of Strathclyde, Great Britain). Selected records were printed out with a laser printer (HP Laser Jet Series II) or an X-Y plotter (Roland, DXY-1300).

Statistics and drugs

Summarized data are shown as mean values with s.e.mean. Statistical differences were tested by Student's *t* test. The following drugs were used; tetraethylammonium chloride, 4-aminopyridine, EGTA (Wako Pure Chemical Industries, Ltd., Osaka). Nicardipine and Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) were kindly supplied by Yamanouchi Pharmaceutical Comp. and Bayer (Japan), respectively.

Results

Only relaxed cells having lengths of between 100 and 180 μm were used for electrical recordings (approximately 150 cells). These cells showed contractile responses to acetylcholine, 10 μM, and also to KCl leaked from recording pipettes. Figure 1a shows typical recordings of membrane potential changes elicited by depolarizing or hyperpolarizing current applied through the recording pipette to a single smooth muscle cell under the current clamp mode. The resting membrane potential of this cell was −44 mV and the average of 19 cells

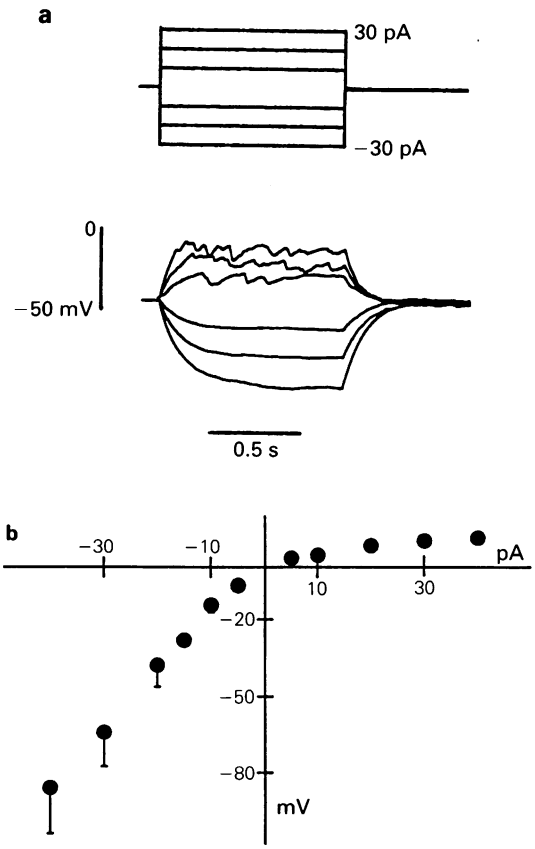

Figure 1 (a) Membrane potential changes (lower traces) recorded under the current clamp mode from a single smooth muscle cell isolated from canine trachea. Six traces were superimposed. The resting membrane potential of this cell was −44 mV. Depolarizing or hyperpolarizing current (upper traces) was applied through the recording pipette. (b) Summarized data showing the relationship between the applied current amplitude (abscissa scale) and resulting changes in membrane potential (ordinate scale) ($n = 6$). Note the strong outward rectification.

derived from 8 trachealis was -41.8 ± 1.5 mV. Neither action potentials nor active depolarizing responses were observed upon depolarization in any cell examined ($n = 19$), while hyperpolarizing humps were often observed when a strong depolarizing current was applied. As illustrated in Figure 1b, the cell membrane showed quite strong outward rectification. The membrane resistance determined between approximately -60 and -100 mV assuming a linear current-voltage relationship was 2.6 ± 0.5 GΩ ($n = 6$). The total membrane capacitance calculated from the membrane time constant (121.8 ± 12 ms, $n = 8$) and the membrane resistance was 47 pF.

Figure 2a shows the outward current evoked upon depolarization from a holding potential of -60 mV under voltage clamp conditions. Without subtraction of leakage current, no inward current was observed in 25 cells out of 30. In five cells, a small and very transient inward current was detected between the capacitive transient and the activation of outward current (not shown). The outward current upon depolarization was very noisy in all cells examined. At potentials positive to 0 mV, a large transient outward current was activated during the initial part of a depolarizing pulse. The current then decreased to a sustained level (sustained component) on which small transient outward currents were superimposed. This differentiation of the depolarization-evoked outward current has previously been observed in the longitudinal smooth muscle cells from the rabbit small intestine (Ohya *et al.*, 1987).

To examine whether the occurrence of transient outward currents was random or not, five recordings obtained at the same potential were averaged (Figure 2a(ii)). It is clear that they only occurred irregularly except at the initial part of the depolarizing pulse. Figure 2b shows the outward currents recorded after extracellular Ca^{2+} was replaced with equimolar Cd^{2+}. The transient components disappeared and the current was activated more slowly and smoothly at all potentials. The peak outward current (Figure 2c) was decreased by about 55% at +10 mV. Figure 2d compares the minimum outward current in normal solution with the current evoked at the end of a 500 ms pulse in Cd^{2+} solution. The steady component was decreased by about 25% at +10 mV. These observations suggest that the transient components of outward current depend quite strongly upon Ca influx during depolarization whereas the sustained component is relatively independent of extracellular Ca.

Exposure to 4-AP (0.3 and 3 mM) had little effect on the transient component of outward current but significantly decreased the sustained phase (Figure 3a(i) and (ii)). After Ca^{2+} was replaced with Cd^{2+}, 4-AP (1 mM) markedly reduced the smooth outward current (Figure 3b). The effect of 4-AP was more apparent in the initial part of depolarization under these conditions. Such results mean that the activation of the outward current was slowed down by 4-AP or, alternatively, the blocking effect of 4-AP was reduced during a long depolarizing pulse (500 ms). The decrease in the sustained component produced by 3 mM 4-AP was about 60% at +10 mV whereas the initial transient current was reduced by about 25% (Figure 3c; see also Figure 6). If the effect of 1 mM 4-AP on the outward current was measured at 50 ms from the start of the depolarization in Cd^{2+} solution, there was a greater reduction at 0 mV (by about 60%) than at more positive potentials (by about 40% at +30 mV, Figure 3d).

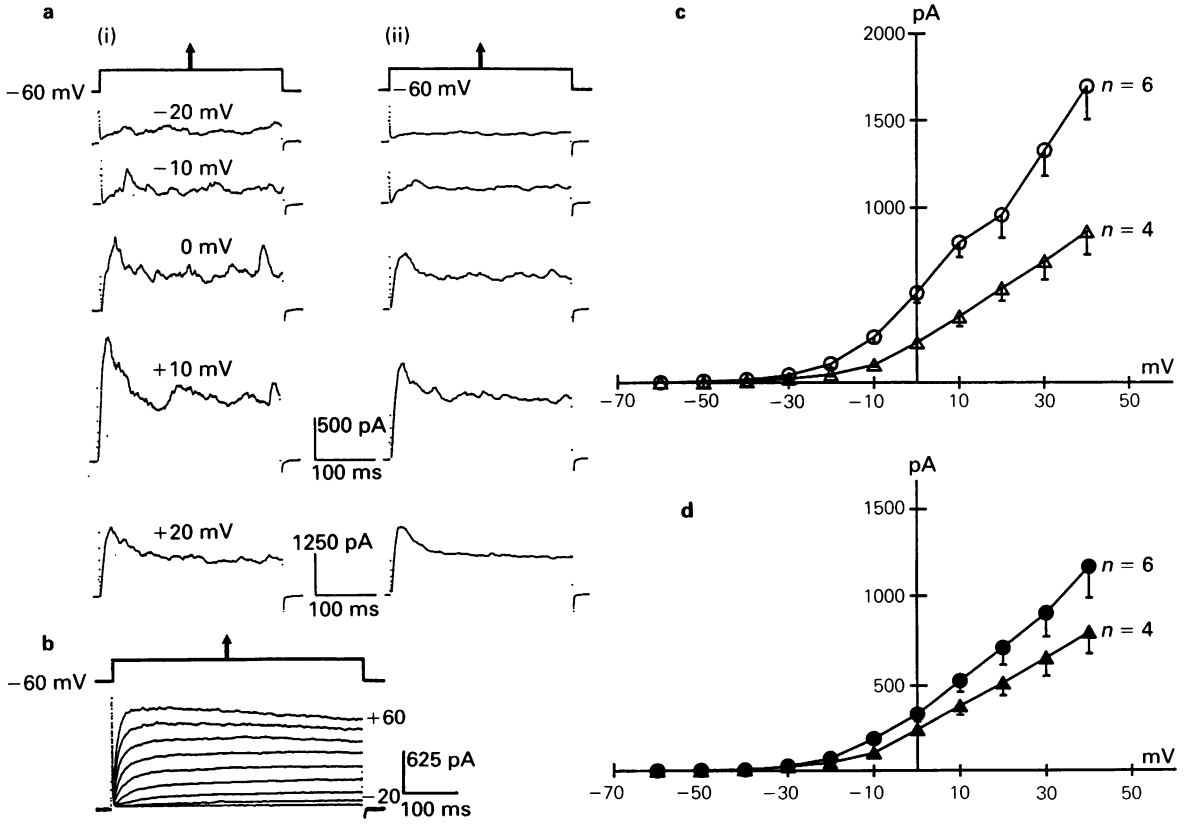

Figure 2 (a) (i) Membrane current elicited by depolarization from a holding potential of -60 mV to the potentials indicated. (ii) Average of five traces at potentials shown in corresponding traces in a(i). Note that the calibration for the trace at +20 mV is different from that for less positive potentials. Leakage current was not subtracted. (b) Outward currents elicited by depolarization from -60 to the potentials between -20 and +60 mV by 10 mV steps in a solution containing 2.2 mM Cd^{2+} instead of 2.2 mM Ca^{2+}. Nine current traces were superimposed. Note that the outward currents were rather smooth. (c) Summarized data of peak outward currents activated at potentials shown on abscissa scale. Circles (○) and triangles (△) indicate the amplitude of peak outward current in normal and Ca-free Cd solutions, respectively. (d) Circles (●) denote the summarized data of least outward current amplitude after the initial large transient outward current in normal solution. These approximate to the amplitude of the steady component: Triangles (▲) illustrate the outward current amplitude measured at the end of a 500 ms pulse in Ca-free Cd solution.

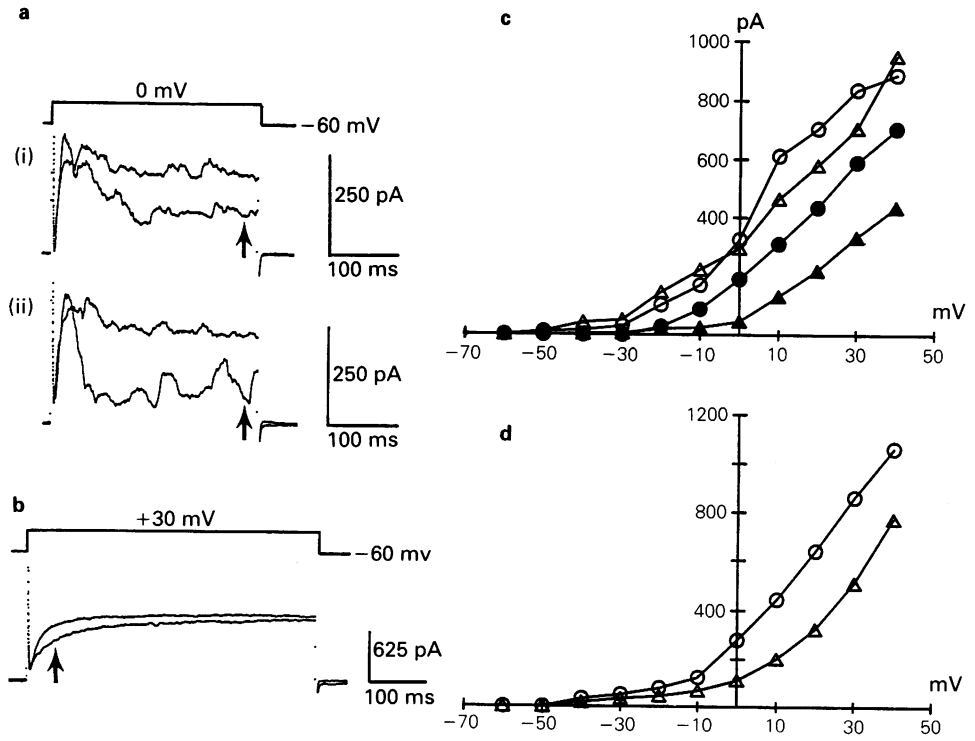

Figure 3 (a and b) Effects of 4-aminopyridine (4-AP) on the outward current elicited by depolarization from −60 to 0 and +30 mV in normal (a(i), (ii)) and Ca-free Cd solutions (b), respectively. Note that the initial transient outward current was not affected by 0.3 (a(i)) and 3 mM (a(ii)) 4-AP, while later part of current was markedly decreased. In the Cd solution (b), the outward current was apparently decreased by 1 mM 4-AP at the initial but not the later part of depolarization. (c) Open and closed symbols indicate the maximum and minimum amplitude of outward currents, respectively, at potentials on the abscissa scale. Circles and triangles denote the amplitude in the absence and presence of 3 mM 4-AP, respectively. (d) Effects of 1 mM 4-AP on the outward current measured 50 ms after the start of depolarization in Ca-free Cd solution. Circles and triangles indicate the current in the absence and presence of 1 mM 4-AP, respectively.

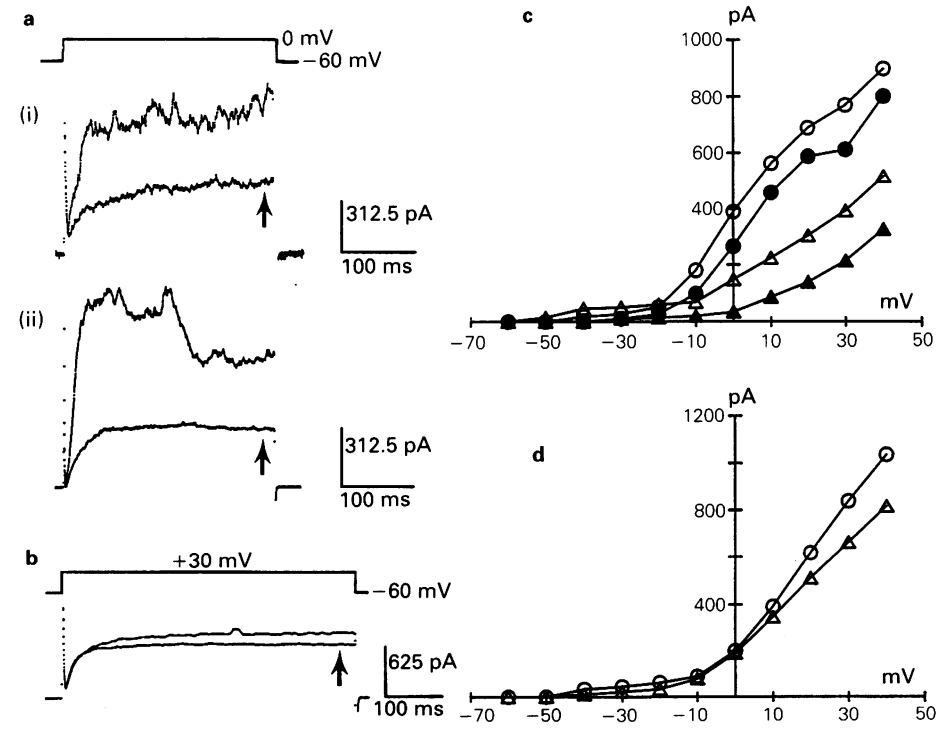

Figure 4 (a and b) Effects of tetraethylammonium (TEA) on the outward currents in normal (a(i), (ii)) and Ca-free Cd solutions (b), respectively. Both transient and sustained components were decreased by 0.3 (i) and 3 mM (ii) TEA. Note that the TEA-resistant current is smooth as that in the Ca-free Cd solution. In the Ca-free Cd solution, 1 mM TEA decreased the later part of outward current (b). (c) Summarized data of outward current amplitude in the absence (circles) or presence (triangles) of 3 mM TEA in the normal solution. Open and closed symbols denote the maximum and minimum amplitude, respectively. (d) The outward currents measured at the end of a 500 ms pulse before (circles) and after (triangles) the application of 1 mM TEA in the Ca-free Cd solution. Note that the effect of TEA is more apparent at positive potentials.

In contrast to 4-AP, TEA (0.3 and 3 mM) affected the transient components of evoked outward current (Figure 4a(i) and (ii)). The current traces became smooth and similar to those observed when extracellular Ca^{2+} was replaced with Cd^{2+}. In a Cd^{2+} solution (Figure 4b), the inhibitory effect of 1 mM TEA on the smooth current appeared gradually during a long depolarizing pulse. The data obtained in normal solutions are summarized in Figure 4c. It is clear that 3 mM TEA inhibits both transient and sustained components. In Cd^{2+} solution, the block by 1 mM TEA is more apparent at positive potentials (Figure 4d).

Data on the effects of TEA and 4-AP on the transient and steady components of outward current elicited by depolarization from −60 to 0 mV at 0.05 Hz are plotted as concentration-response relationships in Figure 5. The amplitude of both components in each cell was determined by averaging the values obtained from three or four traces (not from averaged traces) after the effect of the drug had reached a steady level. Current amplitude was normalized as the value relative to the peak or the minimum outward current in each cell before drug application. The approximate IC_{50} values of TEA for the inhibition of transient and sustained components were 0.3 and 3 mM, respectively. The IC_{50} values of 4-AP were >10 mM and about 0.3 mM, respectively. The results strongly indicate that 4-AP selectively decreases the steady component whereas TEA inhibits both the transient and steady components, with the former more susceptible to TEA than the latter.

The experiments in which Cd^{2+} solution was used suggested that the initial and subsequent repetitive transient components were due to the activation of Ca^{2+}-dependent K^+ current. In order to confirm the implication that TEA, in contrast to 4-AP blocks Ca^{2+}-activated K^+ channels, single channel recordings were performed in which an outside-out patch configuration was used. In preliminary experiments, the relationship between the channel open probability and the pCa of the internal solution was determined from inside-out patches (data not shown). The number of channels included in each patch was quantified at pCa 3.7. There was an average of 3.5 channels per patch ($n = 10$; pipette resistance: ≃10 MΩ). The open probability of the channel was calculated at a certain pCa and potential using current amplitude histograms similar to those shown in Figure 6a(iii) and b(iii). At 0 mV, the approximate open probabilities at pCa 7.0 and 6.5 were 0.1

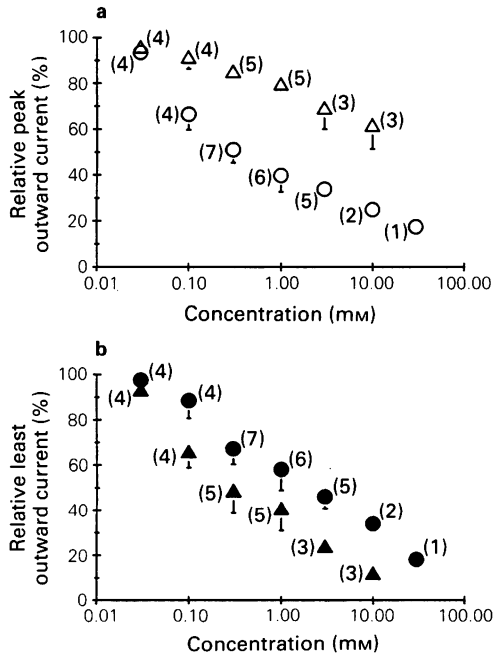

Figure 5 The relationships between the concentration of tetraethylammonium (TEA) (circles) or 4-aminopyridine (4-AP) (triangles) and the effects on the outward current elicited by depolarization from −60 to 0 mV in normal solution. The maximum (a) and the minimum (b) amplitudes of outward current were normalized against those before the application of drugs, respectively, and plotted on the ordinate scale. The numbers in the parentheses are the numbers of cells used.

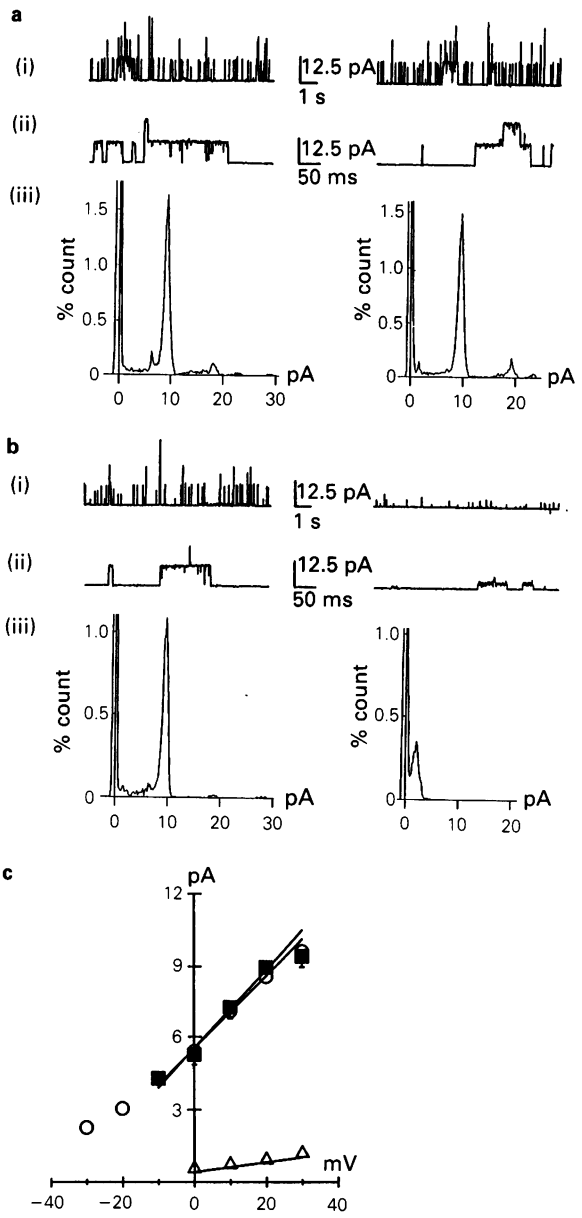

Figure 6 (a) Effect of 1 mM 4-aminopyridine (4-AP) on the single channel current of Ca-activated K channel under outside-out configuration. The pCa of the pipette solution was set at 7.5 to keep the open probability low. The bathing and pipette solutions include 5.9 and 140 mM K^+ respectively. The patch was held at +20 mV. The unitary current of 9.4 pA was little affected by 1 mM 4-AP (a(i) and (ii)). The histograms (iii) indicate that the open probability was also unaffected by 1 mM 4-AP (left: control; right: 1 mM 4-AP). The ordinate scale of the histogram is the percentage of total sample count in each bin (0.2 pA step) relative to the count during the recording period (8.2 s). The ratio of total open time (integration of bins distributed around the peaks at about 9.4) vs. recording period was 0.135 and 0.121 in the control and in the presence of 1 mM 4-AP, respectively. (b) Both the unitary current and the open probability of the K-channel at +20 mV were decreased markedly by the presence of 1 mM tetraethylammonium (TEA) ((i), (ii) and (iii)). Note that the current at open state is very noisy (ii). (c) The effects of 3 mM 4-AP (closed squares) and 2 mM TEA (triangles) on the conductance of the channel. The channel conductance in the control was 154 ± 10 pS (open circle, $n = 5$). The conductance was markedly decreased by 2 mM TEA (19.9 ± 2.3 pA, $n = 3$, $P < 0.001$ vs. control) but not by 3 mM 4-AP (166 ± 5.5 pA, $n = 3$, $P > 0.05$ vs. control). The solid lines were fitted by eye.

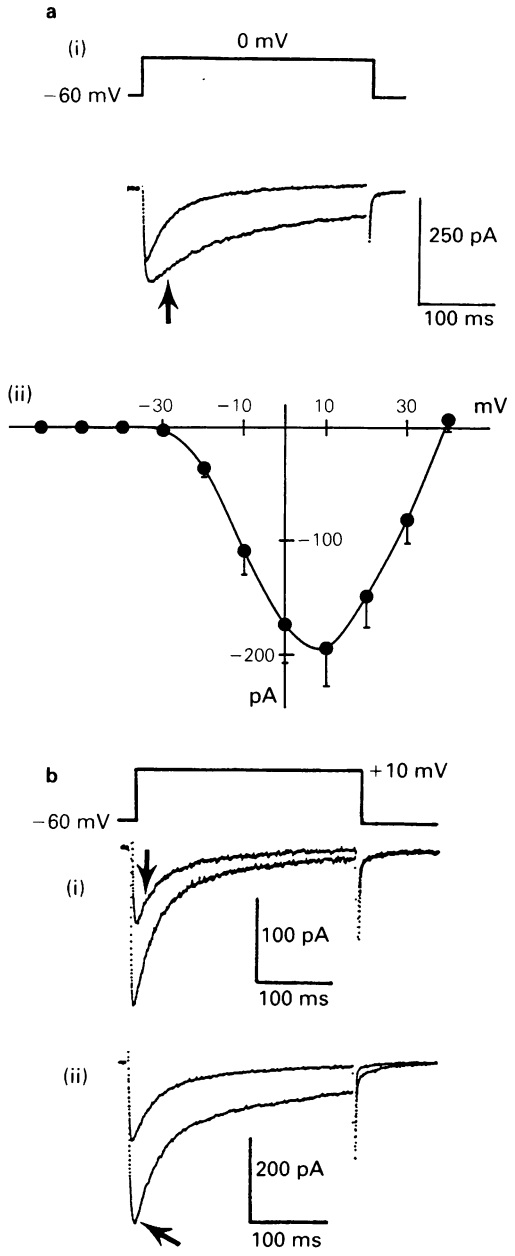

Figure 7 (a) Ca current activated by depolarization from −60 to 0 mV (i) and the summarized data of current-voltage relationship of I_{Ca} ((ii), $n = 6$). Outward currents were blocked by 140 mM Cs^+ in the pipette solution. When extracellular 2.2 mM Ca^{2+} was replaced with equimolar Ba^{2+}, the inward current increased and became sustained (a(i), indicated by an arrow). (b) Ca current elicited by depolarization from −60 to +10 mV. I_{Ca} was decreased by the exposure to 0.1 μM nicardipine ((i), arrow). In the presence of 1 μM Bay K 8644, the inward current was markedly enhanced ((ii), arrow).

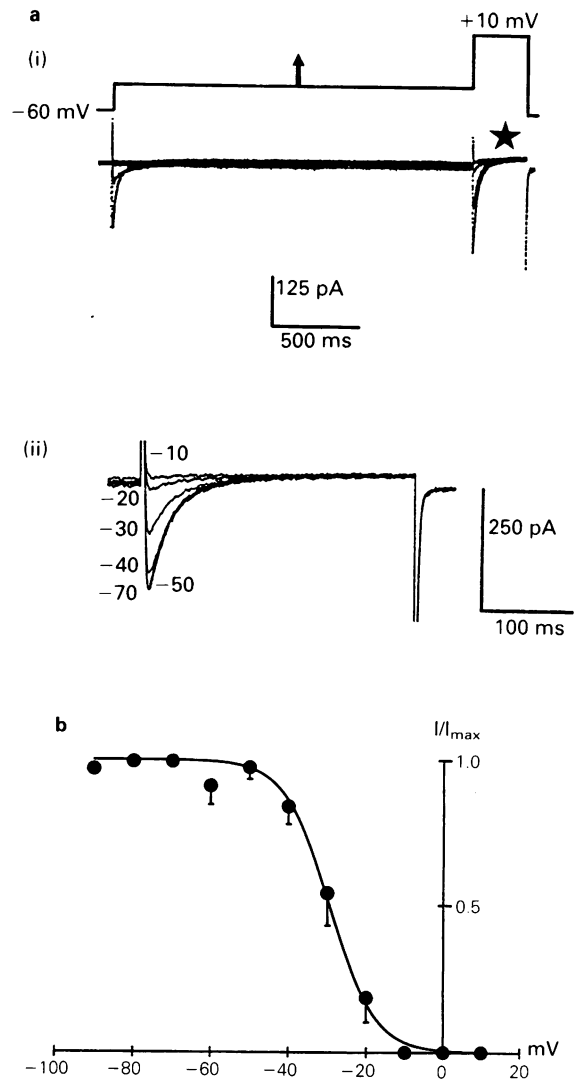

Figure 8 The voltage-dependence of steady-state inactivation of Ca channel current in canine tracheal smooth muscle cells. (a) The conventional double pulse protocol (upper trace in (i)). The duration of P_1 and P_2 were 2 s and 300 ms, respectively. The P_2 potential was +10 mV. The P_1 potential was changed by 10 mV steps at 0.05 Hz. In the lower panel in a(i), five traces at different P_1 potentials are superimposed. The Ca currents at P_2 pulse (indicated by a star) are shown on a faster time scale in a(ii). (b) The summarized data of the steady-state inactivation obtained in the manner shown in (a) ($n = 5$). The data for each cell were fitted by the Boltzmann function (see Text). The values for the half inactivation voltage and the slope factor are −29.9 ± 3.8 mV and −5.8 ± 0.9 mV ($n = 5$), respectively and were used to plot the line shown.

and 0.8, respectively and, therefore, highly dependent upon the pCa ($n = 3$, data not shown). In these preliminary experiments, the pipette and bath solutions contained 140 and 5.9 mM K^+, respectively. Figure 6a shows the effect of 1 mM 4-AP on the single channel events of a large conductance Ca^{2+}-activated K^+ channel (see also Figure 6c) using an outside-out configuration. The concentrations of Ca^{2+} in the pipette and bath solutions were pCa 7.5, and 2.2 mM, respectively. Under these conditions, neither the unit current size nor the open probability was changed by 1 mM 4-AP (Figure 6(a)). Similar results were obtained in a further four cells. In contrast to 4-AP, 1 mM TEA decreased the unitary current from 8.5 to 2.5 pA and increased the noise at the open state (Figure 6b(i) and (ii)). The open probability also appeared to be decreased by 1 mM TEA (Figure 6b(iii)). Data on the effects of 2 mM TEA and 3 mM 4-AP on the single channel conductance are summarized in Figure 6(c). The conductance was significantly decreased by 2 mM TEA from 154 ± 10 pS ($n = 5$) to 19.9 ± 2.3 pS ($n = 3$) but not by 3 mM 4-AP (166 ± 5.5 pS, $n = 3$).

Although the large outward current observed upon depolarization must be at least partially responsible for the strong rectification and for the lack of excitability under current clamp conditions, it is also possible that the low excitability is attributable to a small Ca inward current. Figure 7a(i) shows recordings of the inward current activated by depolarization from −60 to 0 mV. The pipette solution contained 140 mM Cs^+ instead of K^+ (Klöckner & Isenberg, 1985b). In almost all cells examined (>30 cells), a substantial inward current (>100 pA at the peak) was recorded in the region of +10 mV. The inactivation of this current was fast but was markedly slowed by the substitution of extracellular 2.2 mM Ca^{2+} with equimolar Ba^{2+} (Figure 7a(i)). The peak current amplitude

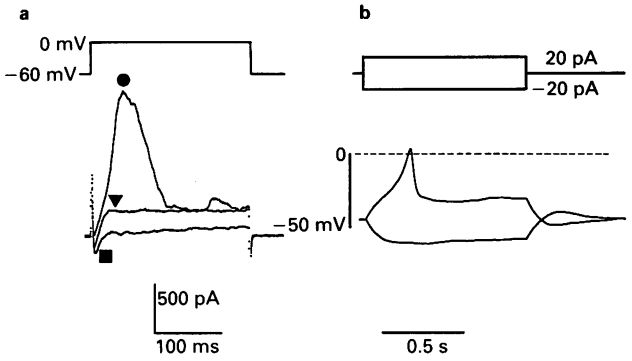

Figure 9 The effects of simultaneous application of 1 mM tetraethylammonium (TEA) and 4-aminopyridine (4-AP) on membrane currents (a) and membrane excitability (b). The initial transient outward current elicited by depolarization from −60 to 0 mV (a, indicated by a circle) was effectively suppressed by 1 mM TEA (a, indicated by a triangle). Additional application of 1 mM 4-AP decreased the sustained component (a, indicated by a square) and unmasked the Ca current. In the presence of 1 mM TEA and 4-AP, a clear action potential with overshoot (5 mV) was elicited (b) in the same cell. The resting membrane potential of this cell in the presence of 1 mM TEA and 4-AP was −45 mV.

was increased by about 40% ($n = 3$) after the substitution. A summary of data on I_{Ca} is illustrated in Figure 7a(ii). Peak current (194.7 ± 33.2 pA, $n = 6$) was obtained near +10 mV in normal solution containing 2.2 mM Ca^{2+}. The current was markedly decreased by 0.1 μM nicardipine to about 40% of the control (Figure 7b(i)) and blocked completely by the addition of 500 μM Cd^{2+} (data not shown, $n = 3$). Exposure to 1 μM Bay K 8644 doubled the peak amplitude of I_{Ca} (Figure 7b(ii); $n = 3$). The voltage-dependence of I_{Ca} inactivation was examined by use of a conventional double pulse protocol (Figure 8a) in normal solution. The inactivation occurred at potentials positive to −60 mV and was complete in the region of −10 mV (Figure 8b). The data obtained from each cell were well described by the Boltzmann function.

$$I/I_{max} = 1/[1 + \exp\{(V_{1/2} - V_m)/S\}]$$

where $V_{1/2}$ is the membrane potential at which one-half inactivation occurs and S is the slope factor at $V_m = V_{1/2}$. The averaged $V_{1/2}$ and S were −29.9 ± 3.8 mV and −5.8 ± 0.9 mV ($n = 5$), respectively.

From the approximate IC_{50} values of TEA and 4-AP for the inhibition of the transient and sustained components of outward current, respectively, the simultaneous application of TEA and 4-AP (each 1 mM) should inhibit a large part of the outward current and increase membrane excitability. The results of such an experiment are shown in Figure 9. The application of 1 mM TEA significantly reduced the transient outward current upon depolarization from −60 to 0 mV under voltage clamp. The addition of 1 mM 4-AP produced a further decrease in the remaining sustained component of the outward current, and revealed a greater inward current. Under these conditions, a clear action potential was elicited by depolarizing current (Figure 9b; the same cell). A single application of 1 mM TEA or 4-AP did not allow the cell to elicit an action potential in response to depolarizing stimulation, whereas small active depolarizing responses of about 10 mV were occasionally observed. Similar observations were obtained from 5 separate cells.

Discussion

The pharmacological approach using TEA and 4-AP in the present study clearly shows that at least two distinct K^+ currents are activated upon depolarization in canine tracheal smooth muscle cells. Such a result was expected from a previous study in canine trachea in which the inhibitory effects of TEA and 4-AP on ^{86}Rb-efflux were additive (Imaizumi & Watanabe, 1983). The existence of two distinct K currents has also been identified by use of TEA and 4-AP as pharmacological tools in rabbit portal vein (Beech & Bolton, 1987). The transient component of outward current is apparently a K current which flows through a Ca activated K channel and which can be blocked by extracellular TEA at relatively low concentrations (see Inoue *et al.*, 1985). Although three kinds of Ca activated K channel have been reported in smooth muscle cells (Inoue *et al.*, 1985; 1986), the K channel with a large conductance of approximately 200 pS (140 mM K^+ symmetry; $K_{L\text{-}Ca}$ channel) and with a high sensitivity to extracellular TEA appears to be the major K^+ channel responsible for the outward current in several types of smooth muscle cell (urinary bladder: Klöckner & Isenberg, 1985a; stomach: Mitra & Morad, 1985; Walsh & Singer, 1987; ureter: Imaizumi *et al.*, 1989a; taenia caeci: Yamamoto & Kao, 1989). The IC_{50} of TEA for the inhibition of the transient outward current was 0.3 mM in the present study. Although the measurement of inhibition might be an underestimate due to the lack of inward current subtraction, the value appears to be reasonable since 1 mM TEA reduced the charge movement per unit time through a single $K_{L\text{-}Ca}$ channel by over 70% (see Figure 6b(iii)). It is, therefore, clear that the transient outward current is more susceptible to TEA ($IC_{50} = 0.3$ mM) than the sustained component ($IC_{50} = 3$ mM). The present results also showed that low concentrations of 4-AP (<3 mM) did not block the $K_{L\text{-}Ca}$ channel. At higher concentrations, however, 4-AP (>3 mM) appeared to decrease the open probability of the $K_{L\text{-}Ca}$ channel. This contrasts with the data obtained from rabbit portal vein (Beech & Bolton, 1987) and suggests that the action of 4-AP on $K_{L\text{-}Ca}$ could vary depending on the type of smooth muscle cell.

In tracheal smooth muscle, the outward current at 0 mV was decreased only by about 50% in Ca-free Cd solution or on exposure to 3 mM TEA which probably suppresses the $K_{L\text{-}Ca}$ channel almost completely. The remaining sustained component of outward current was a delayed rectifier K current (I_K) which was activated voltage-dependently and was quite sensitive to 4-AP ($IC_{50} = 0.3$ mM). TEA also inhibited this component ($IC_{50} = 3$ mM). These two IC_{50} values are rather accurate since the inactivation of I_{Ca} is fast enough to disregard its negative contribution to the sustained component of outward current. The possibility that I_K is modulated by intra- and/or extracellular Ca^{2+} was not examined in the present study. Moreover, it is also quite possible that I_K may have been partly suppressed by the replacement of Ca^{2+} with Cd^{2+}, since some voltage- and time-dependent K currents are sensitive to divalent cations such as Mn^{2+}, Cd^{2+} and Co^{2+} (Mayer & Sugiyama, 1988; Beech & Bolton, 1989; Yamamoto & Kao, 1989). This type of K current which is voltage-dependent, Ca-independent, highly sensitive to 4-AP and exhibits relatively slow activation and inactivation, has been observed in nerves (Meeves & Pichon, 1977; Hermann & Gorman, 1981; as single channel current: Quandt, 1988) and muscles (Fink & Wettwer, 1978) including the smooth muscle cells from rabbit pulmonary (Okabe *et al.*, 1987), rabbit femoral, bovine coronary arteries (Muraki & Takeda, unpublished observation), rat aorta (Toro & Stefani, 1987), rabbit portal vein (Beech & Bolton, 1987) and rabbit iris sphincter (Furuichi, unpublished observation). However, cells from the taenia caeci (Imaizumi *et al.*, 1989a), urinary bladder (Klöckner & Isenberg, 1985a), and ureter (Imaizumi *et al.*, 1989a) of the guinea-pig do not possess a delayed rectifier K current which is highly sensitive to 4-AP. This might suggest that in general, smooth muscle cells which show low excitability possess a substantial 4-AP-sensitive I_K, although a similar current has been described in rabbit portal vein which is spontaneously excitable (Beech & Bolton, 1989).

The I_K in canine tracheal smooth muscle can be easily distinguished from the Ca^{2+}-independent transient outward current, the so called A-current, I_A, which is also highly sensitive to 4-AP (Thompson, 1982; Rogawski, 1985). The I_A has a

much faster inactivation, is less sensitive to TEA than I_K, and has been found in smooth muscle cells from portal vein (Beech & Bolton, 1989), ureter (Imaizumi *et al.*, 1989a; Lang, 1989) and vas deferens (Muraki & Takeda, unpublished observation). In the present study, the single channel currents underlying the I_K were not identified and are the subject of further study.

The blockade of I_K by TEA and 4-AP exhibited different characteristics (see Figure 3c and Figure 4c). In Cd^{2+} solution the effect of TEA developed gradually during a long depolarizing pulse, whereas those of 4-AP were apparent as a slow-down of I_K activation. TEA may act as an open channel blocker from the inner surface in a manner similar to that described for squid axon (reviewed by Armstrong, 1975) or alternatively it may act from the outside in other neurones (reviewed by Stanfield, 1983). It has been reported that the affinity of 4-AP for K channels is decreased by depolarization (Ulbricht & Wagner, 1976; Yeh *et al.*, 1976). This may explain one observed feature of 4-AP-induced blockade of I_K in the tracheal cells; a decrease in blockade during depolarization rather than a slow-down of I_K activation.

Under the conditions of the present study, it was confirmed that the inward current upon depolarization in tracheal smooth muscle was an almost pure Ca current as has already been reported in several smooth muscle cells (Klöckner & Isenberg, 1985b; Mitra & Morad, 1985; Ganitkevich *et al.*, 1986; Ohya *et al.*, 1986; Nakazawa *et al.*, 1987; Kotlikoff, 1988). Although a small I_{Ca} was previously reported in the same preparation (< 50 pA; Kotlikoff, 1988), we found a relatively large I_{Ca} (> 100 pA, $n > 30$) in most well-shaped cells in the normal solution containing 2.2 mM Ca^{2+}. The amplitude of the I_{Ca} (average 200 pA in 2.2 mM Ca solution) corresponds to that in cells from guinea-pig ureter (Imaizumi *et al.*, 1989a) and is greater than that in aorta (Toro & Stefani, 1987) and pulmonary (Okabe *et al.*, 1987), ear (Arronson, *et al.*, 1989), coronary and femoral arteries (Muraki & Imaizumi, unpublished observation). The reason for the difference in the amplitude of I_{Ca} in canine tracheal smooth muscle cells in the two studies (Kotlikoff, 1988; present investigation) is not clear. However, it may be related to the different types of dog employed and to modification of channel properties during cell isolation.

In the present study, the types of Ca channel were not examined carefully (Benham *et al.*, 1987; Yatani *et al.*, 1987; Yoshino *et al.*, 1988; Loirand *et al.*, 1989; Arronson *et al.*, 1989). The I_{Ca} in our preparation showed high sensitivity to nicardipine and also to Bay K 8644, whereas the latter was not effective in a previous study (Kotlikoff, 1988). The present observations favour the presence of an L type channel (Yatani *et al.*, 1987). The half inactivation voltage of −30 mV obtained in normal solution (2.2 mM Ca^{2+}) is not really consistent with the presence of a T type channel as has been suggested by Kotlikoff (1988). The relatively fast inactivation of I_{Ca} in the present study could be explained by a large degree of Ca^{2+}-dependent inactivation of I_{Ca} since Ba^{2+} currents exhibit much slower inactivation rates (Ganitkevich *et al.*, 1987; Nakazawa *et al.*, 1987; Ohya *et al.*, 1988). The contribution of high threshold (L-) type Ca channel current may be significant in canine tracheal smooth muscle as in other several smooth muscle cells (ureter: Imaizumi *et al.*, 1989a; vas deferens: Nakazawa *et al.*, 1988; portal vein: Loirand *et al.*, 1989; saphenous vein: Yatani *et al.*, 1987; taenia caecum: Yoshino *et al.*, 1988).

The simultaneous application of relatively low concentrations of TEA and 4-AP markedly increased membrane excitability (Figure 9). This was due to the additive inhibitory effects of these drugs on outward current. In vascular smooth muscle it has been suggested that TEA may also directly increase the Ca permeability (Haeusler *et al.*, 1980). However, our preliminary experiments with canine tracheal cells suggest that TEA and 4-AP (3 mM) do not markedly affect the amplitude of I_{Ca} under conditions in which K currents were blocked by internal 140 mM and external 10 mM Cs^+ (Muraki, unpublished observation). In guinea-pig ureter, spike potentials are observed under physiological conditions and I_{Ca} is very similar in amplitude to I_{Ca} in dog trachea (Imaizumi *et al.*, 1989a; present study). Although the inactivation of I_{Ca} in trachea is much faster than in ureter, it is similar to that observed in other spike-generating smooth muscles such as taenia caeci (Ganitkevich *et al.*, 1986), portal vein (Arronson *et al.*, 1988) and urinary bladder (Klöckner & Isenberg, 1985b). Thus the presence of 4-AP-sensitive delayed rectifier K current (I_K) in dog trachea and its absence from guinea-pig ureter may be a critical feature in determining the excitability of these two types of smooth muscle.

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The effects of epithelium removal on the actions of cholinomimetic drugs in opened segments and perfused tubular preparations of guinea-pig trachea

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1 Isolated segments of guinea-pig trachea or perfused tracheal tubes were arranged for the recording of trachealis tension changes in Krebs solution containing indomethacin (2.8 μM).

2 In opened tracheal segments, epithelium removal caused modest (2–3 fold) potentiation of the effects of acetylcholine (ACh) and methacholine (MeCh) but failed to potentiate carbachol (CCh), bethanechol (BeCh), oxotremorine or KCl.

3 Pretreatment with ecothiopate potentiated effects of ACh and MeCh but not of CCh or BeCh. Removal of epithelium in ecothiopate-treated tissue potentiated effects of ACh and MeCh but not of CCh or BeCh.

4 Guinea-pig ileum challenged with ACh was used as a bioassay system for cholinesterase activity. Scrapings of tracheal epithelium did not hydrolyse ACh.

5 Histochemical staining revealed no fibres positive for acetylcholinesterase or pseudocholinesterase in the tracheal epithelium. However, the underlying tissues contained acetylcholinesterase-positive nerve fibres and the trachealis muscle itself stained positively for pseudocholinesterase activity.

6 Neither tetrodotoxin (3 μM) nor hexamethonium (500 μM) modified the ability of epithelium removal to potentiate ACh.

7 In perfused tracheal tubes where spasmogens were added to the luminal perfusate, epithelium removal potentiated effects of ACh (31 fold), CCh (10 fold), oxotremorine (2 fold) and KCl.

8 In perfused tracheal tubes where spasmogens were added to the Krebs solution superfusing the adventitial surface of the tissue, epithelium removal significantly reduced the potency of CCh, oxotremorine and KCl.

9 It is concluded that the selectivity and magnitude of the potentiation of cholinomimetics caused by epithelium removal depends on the route by which the cholinomimetic agent gains access to the trachealis muscle. The potentiation of acetic acid esters of choline seen in opened tracheal segments does not reflect the loss of epithelial cholinesterase activity and does not depend on the activity of nervous reflex arcs in the tracheal wall. The reduced potency of adventitially-applied cholinomimetics and KCl seen in epithelium-denuded tissue strongly suggests that the epithelium can moderate trachealis sensitivity to cholinomimetic agents not only by releasing epithelium-derived relaxing factor but also by acting as a barrier to drug diffusion.

Introduction

Investigations of the role of the airways epithelium in modulating the sensitivity and responsiveness of the underlying smooth muscle to drugs and neurotransmitters have recently been the subject of review (Goldie *et al.*, 1988; Fedan *et al.*, 1988). It seems clear that epithelium removal can potentiate some, though not all, spasmogenic drugs acting on trachealis muscle. Possible mechanisms underlying this potentiation include removal of the source of an epithelium-derived relaxant factor (EpDRF), removal of an epithelial site of metabolic loss or removal of an epithelial barrier to drug diffusion.

When the action of cholinomimetic drugs in guinea-pig trachea is considered, epithelium removal has been reported to potentiate the effects of acetylcholine (ACh) (Finnen *et al.*, 1986; Holroyde, 1986; Murlas, 1986; Flavahan *et al.*, 1988; Fine *et al.*, 1989) and of methacholine (MeCh) (Hay *et al.*, 1986) but not of carbachol (CCh) (Goldie *et al.*, 1986; Lennart-Lundblad & Persson, 1988). It has been suggested (Goldie *et al.*, 1988) that the selective potentiation of acetic- as opposed to carbamic acid esters of choline might be explained in terms of the acetic esters, but not the carbamic esters, having the ability to liberate EpDRF from epithelial cells. However, whereas acetic esters of choline are susceptible to hydrolysis by cholinesterases the corresponding carbamic acid esters are resistant to hydrolysis (Koelle, 1965). Accordingly, selective potentiation of the acetic esters of choline could also represent removal of an epithelial source of cholinesterase activity. Some of the present experiments were designed to test this latter hypothesis.

Other experiments in the present study were designed to determine whether the potentiation of ACh effects induced by epithelial removal is dependent on the functioning of tracheal intramural nerves and whether the magnitude and selectivity of the potentiation of cholinomimetics is dependent on the route by which these agents diffuse towards the trachealis muscle. Preliminary accounts of our findings have been communicated to the British Pharmacological Society (Dixon *et al.*, 1989; Good & Small, 1989).

Methods

Tissue bath experiments with opened tracheal segments

Tricoloured guinea-pigs (400–550 g) of either sex were killed by stunning and bleeding. Tracheae were excised, cleaned of adhering adipose and connective tissue and opened by cutting longitudinally through the cartilage rings diametrically opposite the trachealis. The opened trachea was cut into small segments each containing 3–4 cartilage rings.

Tracheal segments from each animal were allocated randomly to one of two groups, namely epithelium-intact (Ep+)

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and epithelium-denuded (Ep−) tissues. The Ep− tissues were prepared by rubbing away the epithelial cells with a pair of forceps (Flavahan *et al.*, 1985). Ep+ and Ep− tissues were set up for the isometric recording of tension changes essentially as described by Foster *et al.* (1983). Tissues were mounted in Krebs solution containing indomethacin (2.8 μM) and were subjected to an initial imposed tension of 1 g. Thereafter, a period of 30 min was allowed for the dissipation of spontaneous tone, a process facilitated by repeated changes of the bath fluid.

A single, cumulative concentration-effect curve was then constructed with ACh (10 nM–10 mM, 10 fold increments), MeCh (10 nM–1 mM, 10 fold increments), CCh (1 nM–10 μM, 3 fold increments), bethanechol (BeCh, 10 nM–1 mM, 10 fold increments), oxotremorine (1 nM–10 μM, 3 fold increments) or KCl (5 mM–40 mM, 2 fold increments).

Tissue contact times for each applied concentration of these agonists were such as to allow full development of the attainable tension increase. The generation of a true maximal response to KCl was avoided in order to minimise osmotic damage to the tissue. Following the application of 40 mM KCl, the maximal spasmogenic response of the tissue was induced by the administration of 10 mM ACh. Responses to KCl were calculated in terms of the maximal response to ACh.

In experiments involving inhibition of cholinesterase activity, test tissues were exposed to 100 nM ecothiopate for 15 min and were washed with ecothiopate-free Krebs solution for 30 min before challenge with a cholinomimetic agent. Time-matched control tissues were treated identically but were not exposed to ecothiopate.

In experiments where the effects of hexamethonium (500 μM) or tetrodotoxin (3 μM) were examined, these agents were incubated with the tissues for 30 min before construction of the concentration-effect curve for ACh.

Experiments with perfused tracheal tubes

Lengths (2 cm) of unopened trachea were used in the perfusion experiments. These tracheal tubes were allocated randomly to Ep+ and Ep− experimental groups. In the latter case the epithelium was removed by drawing a 10 × 0.5 cm strip of gauze through the tracheal lumen as described by Munakata *et al.* (1988). The tracheal tubes were cannulated at each end and mounted in a tissue bath (200 ml capacity) as shown in Figure 1.

Krebs solution (at 37°C and equilibrated with 95% O_2, 5% CO_2) perfused the tracheal lumen at a flow rate of 5 ml min^{-1}. The Krebs solution in the tissue bath was recirculated from a 600 ml reservoir at a flow rate of 150 ml min^{-1}. Tension changes occurring in the trachealis muscle were recorded isometrically by inserting fine wire hooks into the wall of the trachea at each extremity of the trachealis muscle. The lower hook was anchored whilst the upper hook was connected by thread to an Ether UF1 transducer. Checks made by use of a dye (Basilen Blue) showed that the recording hooks did not puncture the tracheal wall in a way which allowed mixing of the solutions bathing the luminal and adventitial surface of the tissue.

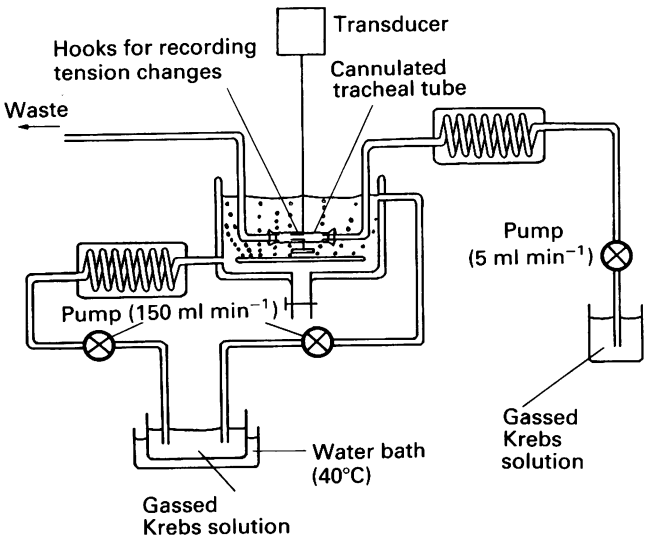

Figure 1 The apparatus used to record trachealis muscle tension changes in perfused tubular preparations of guinea-pig trachea.

Drugs were applied to the luminal surface of the trachea by their addition to the luminal perfusate. Drug application to the adventitial surface of the trachea was achieved by their addition to the Krebs solution perfusing the tissue bath. In order to obtain rapid mixing within the 800 ml of recirculating solution, drug was added both to the reservoir of Krebs solution and to the solution in the tissue bath.

Following a 30 min equilibration period a single, cumulative concentration-effect curve to a luminally- or adventitially-applied spasmogen was constructed. Spasmogens examined in this way were ACh (10 nM–10 mM, 10 fold increments), CCh and oxotremorine (1 nM–100 μM, 3 fold increments) and KCl (5 mM–40 mM, 2 fold increments).

Detection of cholinesterase activity by bioassay

Lengths (2.5 cm) of guinea-pig ileum were set up for the isotonic recording of length changes under a resting load of 1 g. A sequential concentration-effect curve for ACh was constructed with 4 fold concentration increments and a 30 s contact time. The EC_{80} for ACh was determined from this curve and was applied to the ileum at 4 min intervals by use of aliquots ($\simeq$100 μl) taken from an ACh stock solution (165 μM) incubated at 37°C in a shaking water bath. When responses to the ACh challenge became constant, the tissues were used for the bioassay of cholinesterase activity.

ACh hydrolysis in a 3 ml sample of the stock solution was studied following the addition of (a) nothing or (b) 0.1 ml 2 mM EGTA solution or (c) 0.1 ml of a mixture of 0.3 ml guinea-pig whole blood and 0.3 ml 2 mM EGTA solution or (d) epithelial scrapings ($\simeq$17 mg) from a whole trachea. In some experiments the epithelial scrapings from 2 or more tracheae were combined and added to the stock solution of ACh.

Three min after addition of these materials to the ACh stock solution, an aliquot equivalent to the EC_{80} for ACh was removed from the solution and applied to the ileum. Similar challenges were made at 6 min intervals thereafter. The hydrolysis of ACh was assessed in terms of the reduction in the response to the ACh challenge.

Histochemical localization of cholinesterase activity

The distribution of acetylcholinesterase and pseudocholinesterase was investigated in transverse sections of trachea by a method based on the Gomori (1952) modification of the Koelle (1951) thiocholine technique as summarised by Pearse (1972). Transverse sections (10 μm) of trachea were fixed in calcium formol (10% w/v $CaCl_2$ in 10% formyl saline) at 4°C for 20 min. Sections were then preincubated for 30 min at room temperature in phosphate buffer (pH 7.0) containing either 10 μM BW284C51 or 10 μM iso-OMPA (see below). Sections were then transferred to Gomori solution (pH 6.0) at 37°C for 15 h. The Gomori solution contained either acetylthiocholine iodide (7 mM) or a mixture of butyrylthiocholine iodide (6 mM) and 10 μM BW284C51. Sections were immersed in 0.1% w/v ammonium sulphide solution for 45 s before washing and counter-staining (15 s) with Mayer's haematoxylin. Sections were washed, dehydrated (serial alcohols and tetrachloroethylene) and mounted in Canada balsam.

Histological assessment of epithelium removal

At the end of the experiments involving opened tracheal segments or perfused tracheal tubes, tissues were fixed (24 h) in

Table 1 Effects of epithelium removal on the actions of cholinomimetic drugs and KCl in guinea-pig isolated trachealis treated with 2.8 μM indomethacin

Agonist	*Epithelium-intact*	*Epithelium-denuded*	n
ACh			
$-\log EC_{50}$	5.28 ± 0.07	5.80 ± 0.06*	24
maximum (g)	1.80 ± 0.10	1.50 ± 0.20	24
MeCh			
$-\log EC_{50}$	5.99 ± 0.05	6.31 ± 0.06*	12
maximum (g)	2.20 ± 0.20	2.60 ± 0.20	12
CCh			
$-\log EC_{50}$	7.03 ± 0.04	7.14 ± 0.04	18
maximum (g)	2.20 ± 0.20	2.10 ± 0.20	18
BeCh			
$-\log EC_{50}$	5.63 ± 0.04	5.67 ± 0.03	18
maximum (g)	2.10 ± 0.10	1.70 ± 0.20	18
Oxotremorine			
$-\log EC_{50}$	7.31 ± 0.07	7.44 ± 0.09	6
maximum (g)	1.50 ± 0.20	1.30 ± 0.20	6
KCl			
$-\log EC_{50}$†	1.86 ± 0.07	1.85 ± 0.06	6
maximum (g)†	1.40 ± 0.20	1.20 ± 0.20	6

Data are means (± s.e.mean) of values from the tabulated (*n*) number of tissues.
† The use of high (>40 mM) concentrations of KCl was avoided in these experiments and the maximal response was defined as that induced by 10 mM ACh.
* Indicates a value significantly different from the corresponding value obtained for epithelium-intact tissue.

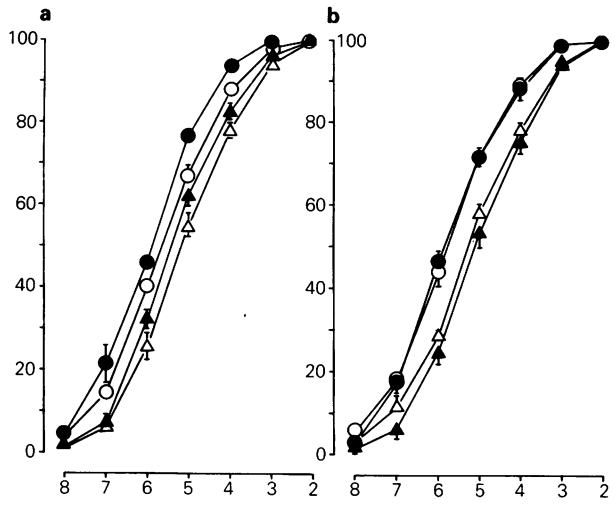

Figure 2 Opened segments of guinea-pig trachea treated with indomethacin (2.8 μM): the influence of hexamethonium (500 μM; a) or tetrodotoxin (3 μM; b) on changes in the potency of acetylcholine (ACh) caused by removal of the epithelium. Abscissae: $-\log$ molar concentration of ACh. Ordinates: tension expressed as a percentage of the maximum tension change evoked by ACh. (●) Responses of epithelium-denuded (Ep−) tissues observed in the presence of hexamethonium or tetrodotoxin; (▲) responses of epithelium-intact (Ep+) tissues observed in the presence of hexamethonium or tetrodotoxin. The equivalent open symbols represent responses of Ep− and Ep+ time-matched control tissues exposed neither to hexamethonium nor tetrodotoxin respectively. Data represent the means of values derived from 6 tissues; s.e.means shown as vertical bars.

10% formyl saline (0.1 M phosphate-buffered). Following tissue dehydration in serial alcohols and clearing with chloroform, 5 μm transverse sections of trachea were cut. These sections were re-hydrated and stained with haematoxylin and eosin. The sections were again dehydrated prior to mounting in Practamount: xylene (50:50). The integrity of the epithelium in these sections was assessed by light microscopy.

Drugs, solutions and statistical analysis of results

Drug concentrations are expressed in terms of the molar concentration of the active species. Where KCl was used as a spasmogen, the stated concentration excludes the K^+ provided by the formulation of the Krebs solution.

The following drugs were used: acetylcholine chloride; acetyl-β-methylcholine chloride (methacholine); acetylthiocholine iodide; butyrylthiocholine iodide; BW284C51 (1, 5-*bis*(4-allyl dimethylammonium phenyl-pentane-3-one); carbamylcholine chloride (carbachol); ecothiopate iodide; EGTA (ethylene glycol-*bis*-(β-amino-ethylether)N,N′-tetraacetic acid); hexamethonium bromide; indomethacin; *iso*-OMPA (tetramonoisopropyl pyrophosphortetramide); oxotremorine sesquifumarate; potassium chloride; tetrodotoxin. With the exceptions of ecothiopate (Ayerst) and potassium chloride (BDH) all drugs were obtained from the Sigma Chemical Company. Stock solutions of ACh and indomethacin were prepared in absolute ethanol, those of other drugs in distilled water.

The Krebs solution used in all the experiments contained 2.8 μM indomethacin and had the following composition (mM): Na^+ 143.5, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 127.6, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11.1. The significance of differences between means was assessed by a two-tailed, unpaired *t* test, by analysis of variance or by Studentized range tests. The null hypothesis was rejected when $P < 0.05$.

Results

Light microscopic examination of sections taken from rubbed (Ep−) tracheal segments revealed that epithelial cells were virtually absent. In approximately 10% of preparations, small patches of epithelium were retained. Such patches were often located towards the cut ends of the cartilage rings. Tissue rubbing did not appear to damage submucosal tissue or the underlying trachealis muscle. In unrubbed (Ep+) tissues the epithelium was well-maintained even after experiments of several hours duration.

Table 2 Guinea-pig trachealis treated with indomethacin (2.8 μM): effects of pretreatment of epithelium-intact and epithelium-denuded tissue with ecothiopate (100 nM for 15 min) on the $-\log EC_{50}$ values for cholinester cholinomimetic agents

	Ecothiopate-treated tissues		*Time matched controls*	
Agonist	*Epithelium-intact*	*Epithelium-denuded*	*Epithelium-intact*	*Epithelium-denuded*
ACh	5.72 ± 0.09*	6.34 ± 0.12*†	4.67 ± 0.12	5.20 ± 0.14†
MeCh	6.28 ± 0.03*	6.63 ± 0.05*†	5.74 ± 0.08	6.15 ± 0.06†
CCh	6.89 ± 0.05	6.93 ± 0.03	6.80 ± 0.03	6.87 ± 0.02
BeCh	5.41 ± 0.03	5.48 ± 0.07	5.38 ± 0.04	5.48 ± 0.03

Data are mean (± s.e.mean) values of $-\log EC_{50}$ derived from experiments on six tissues.
* Significantly different from corresponding time-matched control preparations.
† Significantly different from corresponding epithelium-intact tissues.

ACh, MeCh, CCh, BeCh, oxotremorine and KCl each produced concentration-dependent increases in tension in both Ep+ and Ep− tracheal segments treated with indomethacin. In both Ep+ and Ep− tissues, the rank order of potency for the cholinomimetic agents was oxotremorine > CCh > MeCh > BeCh and ACh. Removal of the epithelium did not alter the maximal response to any of the spasmogens. However, epithelium removal caused 2–3 fold potentiation of the effects of ACh and MeCh but did not potentiate CCh, BeCh, oxotremorine or KCl (Table 1).

Pretreatment of Ep+ or Ep− tissues with ecothiopate potentiated the actions of ACh and MeCh but not those of CCh or BeCh. Furthermore, removal of epithelium from ecothiopate-treated tissues potentiated ACh and MeCh but did not potentiate CCh or BeCh (Table 2). The extent of the latter potentiation (2–4 fold) was similar to that observed on removal of the epithelium from tissues not exposed to ecothiopate.

In Ep+ and Ep− tissues treated with hexamethonium (500 μM), log concentration-effect curves for ACh were shifted slightly but significantly to the left of the corresponding curves for tissues not exposed to hexamethonium. Epithelial removal potentiated ACh effects both in tissue treated with hexamethonium and in tissue not exposed to this agent (Figure 2). The degree of potentiation observed in the hexamethonium-treated tissue did not differ significantly from that observed in the time-matched control tissues.

Treatment of Ep+ and Ep− tissues with tetrodotoxin (3 μM) had no significant effect on the position of log concentration-effect curves for ACh relative to the corresponding curves in tissues not exposed to the toxin. Epithelial removal potentiated ACh both in tissue treated with tetrodotoxin and in tissue not exposed to this agent (Figure 2). The degree of potentiation observed in the toxin-treated tissues did not differ significantly from that observed in the time-matched control tissues.

Experiments with perfused tracheal tubes

Light microscopic examination of transverse sections of tracheal tubes through which the gauze strip had been pulled (Ep− tissues) revealed that epithelial cells had been almost completely removed. Small patches of epithelium sometimes remained in the region of the cartilage rings. The pull-through with the gauze caused no visible damage to the submucosa or trachealis muscle. In unrubbed tracheal tubes (Ep+ tissues) the integrity of the epithelium was generally well-maintained despite the period of luminal perfusion with Krebs solution. Occasionally, however, small, focal patches of epithelial damage were seen.

ACh (10 nM–10 mM), CCh (1 nM–100 μM), oxotremorine (1 nM–100 μM) and KCl (5–40 mM) each caused concentration-dependent tension development in the indomethacin (2.8 μM)-treated tracheal tubes. This held true for both Ep− and Ep+ tissues and for both adventitial and luminal routes of administration. In the case of KCl, tissue responses did not exceed 67% of the carbachol maximum both for adventitial or luminal application (Figure 3).

When drugs were administered by the luminal route, epithelium removal potentiated effects of ACh (31 fold), carbachol (10 fold), oxotremorine (2 fold) and KCl (Table 3, Figure 3). In contrast, when drugs were applied to the adventitial surface of

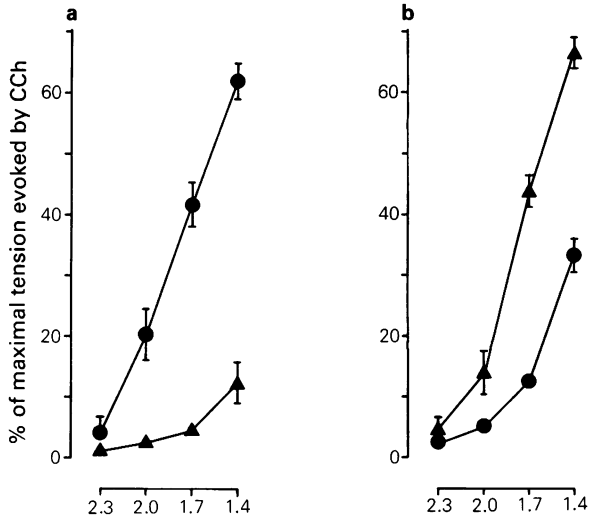

Figure 3 Indomethacin (2.8 μM)-treated, perfused guinea-pig tracheal tubes: the effects of epithelium removal on luminally- (a) or adventitially-applied (b) KCl. Abscissae: −log molar concentration of KCl. Ordinate scale: tension developed as a percentage of the maximal spasm induced by carbachol. (▲) Indicates epithelium-intact (Ep+) tissues; (●) indicates epithelium-denuded (Ep−) tissues. Data points represent the means of values from 6 tissues. Vertical bars indicate s.e.mean. Note that epithelium removal increases the potency of luminally-applied KCl but reduces the potency of adventitially-applied KCl.

Table 3 Effects of epithelium removal on the actions of cholinomimetic drugs and KCl in perfused guinea-pig tracheal tubes treated with 2.8 μM indomethacin

	Luminal application		*Adventitial application*		
	Epithelium-intact	*Epithelium-denuded*	*Epithelium-intact*	*Epithelium-denuded*	n
ACh†					
−log EC_{50}	3.86 ± 0.10	5.35 ± 0.12*	5.20 ± 0.07	4.88 ± 0.07	5
maximum (g)	1.0 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.2	5
CCh					
−log EC_{50}	5.67 ± 0.08	6.67 ± 0.08*	6.84 ± 0.03	6.49 ± 0.07*	6
maximum (g)	2.2 ± 0.2	2.1 ± 0.2	1.4 ± 0.3	1.2 ± 0.1	6
Oxotremorine					
−log EC_{50}	6.98 ± 0.03	7.28 ± 0.07*	7.08 ± 0.10	6.66 ± 0.08*	6
maximum (g)	1.7 ± 0.02	1.8 ± 0.2	1.7 ± 0.3	1.5 ± 0.1	6
KCl†					
−log EC_{25}	ND	1.87 ± 0.08	1.90 ± 0.04	1.51 ± 0.02*	6
maximum (g)	1.5 ± 0.1	1.4 ± 0.2	1.7 ± 0.1	1.7 ± 0.2	6

Data are means (± s.e.mean) of values from the tabulated (*n*) number of tissues.
* Indicates a value significantly different from the corresponding value in epithelium-intact tissues.
† These agonists were relatively impotent and therefore maximal responses were evoked by carbachol. EC_n values for these agents were calculated by reference to the carbachol-induced maximum response.
ND not determined.

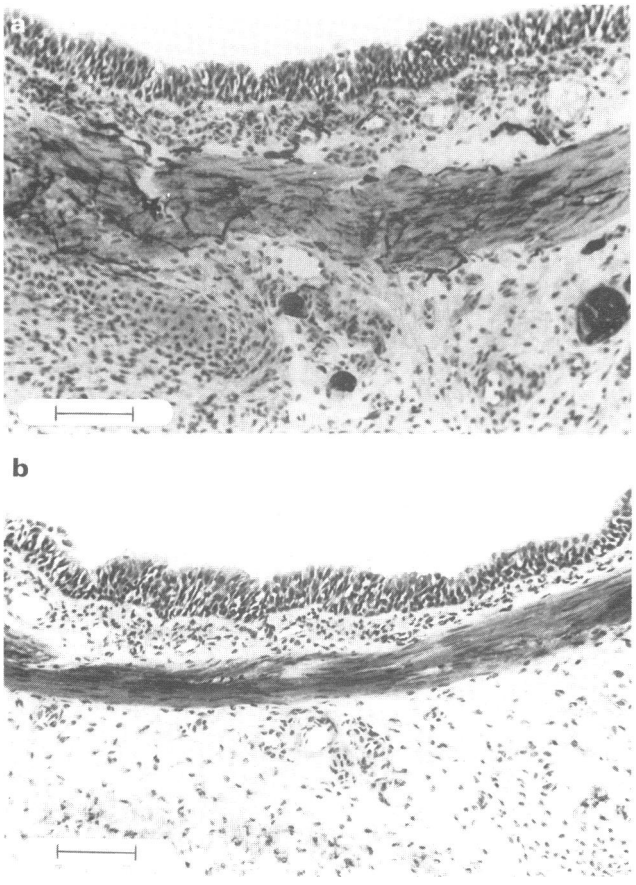

Figure 4 Transverse sections through the dorsal region of guinea-pig trachea stained for cholinesterase activity. The tracheal lumen appears towards the top of the field, the adventitial surface of the organ is towards the bottom of the field. (a) Section stained for acetylcholinesterase. Note the acetylcholinesterase-positive fibre running through the trachealis muscle. Note similar fibres in the submucosa. Ganglia located in the adventitia also stain intensely for acetylcholinesterase. Little or no acetylcholinesterase activity is present in the tracheal epithelium. (b) Section stained for pseudocholinesterase. Note that the trachealis muscle itself stains for pseudocholinesterase activity. Little or no pseudocholinesterase activity appears in the submucosa or the tracheal epithelium. Calibration bar = 50 μm.

the trachea, epithelial removal reduced the potency of CCh, oxotremorine and KCl (Table 3, Figure 3). There was also an apparent reduction in the potency of ACh though this just failed to achieve statistical significance. Epithelium removal did not alter the maximal responses of the perfused tracheal tubes to carbachol or oxotremorine. This held true both for advential or luminal administration (Table 3).

Detection of cholinesterase activity by bioassay

Aliquots from an untreated stock solution of ACh or from a solution to which 0.1 ml 2 mM EGTA solution had been added, evoked ileal contractions which remained constant in amplitude over a 45 min period. This suggests that EGTA did not induce the hydrolysis of ACh and that little or no spontaneous hydrolysis occurred.

Ileal responses to aliquots from an ACh stock solution treated with epithelial scrapings from a whole trachea also remained constant over a 45 min period. A similar observation was made for stock solutions of ACh treated with epithelial scrapings pooled from several tracheae. In contrast, ileal contractions evoked by aliquots removed from an ACh stock solution treated with 0.1 ml of a mixture of 0.3 ml guinea-pig blood and 0.3 ml 2 mM EGTA solution, progressively decreased in amplitude and were abolished within 45 min. This suggests that whole blood was able to hydrolyse ACh but that epithelial scrapings from the trachea were devoid of hydrolytic activity.

Histochemical localisation of cholinesterase activity

Acetylcholinesterase-positive nerve fibres were observed running between and within the trachealis smooth muscle bundles. Similar fibres were observed in the adventitia and acetylcholinesterase-positive ganglia were also observed in this region. While some acetylcholinesterase-positive fibres were observed in the submucosa, no acetylcholinesterase-positive structures were observed in the tracheal epithelium (Figure 4a).

The trachealis muscle itself stained for pseudocholinesterase activity. Some staining for this enzyme was also observed in the adventitia and the perichondrium (Figure 4b). Little or no pseudocholinesterase activity was detected in the submucosa or the tracheal epithelium.

Discussion

Epithelium removal from opened segments of trachea: its effects on the actions of cholinomimetic drugs

In contrast to the present study, previous reports concerning the effects of epithelium removal on responses of guinea-pig trachea to cholinomimetic agents have generally involved a single muscarinic agonist. ACh has been tested in the absence of (Finnen *et al.*, 1986; Holroyde, 1986; Murlas, 1986) or presence (Flavahan *et al.*, 1988; Fine *et al.*, 1989) of indomethacin, and epithelium removal has been reported to cause 2–13 fold potentiation of this cholinester. MeCh and CCh have been examined in the absence of indomethacin and while epithelium removal caused 2 fold potentiation of MeCh (Hay *et al.*, 1986) it failed to potentiate CCh (Goldie *et al.*, 1986; Lennart-Lundblad & Persson, 1988).

The results of the present study have confirmed in indomethacin-treated, opened tracheal segments that epithelium removal potentiates effects of ACh and MeCh but not of CCh. They have also revealed that epithelium removal fails to potentiate either BeCh or oxotremorine. It is therefore apparent that epithelium removal from opened segments of guinea-pig trachea potentiates cholinomimetics (ACh and MeCh) which are susceptible to hydrolysis by cholinesterase enzymes but not those (CCh, BeCh, oxotremorine) which are resistant to esterase attack. This observation prompted us to investigate whether the tracheal epithelium might possess cholinesterase activity and might therefore act as a site of metabolic loss for the acetic acid esters (ACh and MeCh) of choline.

The tracheal epithelium as a site of metabolic loss for cholinesters

While removal of the epithelium from opened tracheal segments potentiated only those cholinomimetics which are susceptible to esterase attack, several findings suggested that the tracheal epithelium did not act as a source of esterase activity and did not therefore represent a selective site of metabolic loss.

Our pretreatment of tracheal segments with ecothiopate potentiated effects of ACh and MeCh but not of CCh or BeCh. This strongly suggests that the ecothiopate pretreatment had inhibited tracheal cholinesterase activity. Despite this, epithelium removal in ecothiopate-treated tissues provided potentiation of ACh and MeCh but did not potentiate CCh or BeCh. Furthermore ACh and MeCh were each potentiated to an extent similar to that observed in control tissues. We must conclude, therefore, that the effects of epithelial removal cannot be equated with the loss of cholinesterase activity. Our experiments with ecothiopate were prompted by

the earlier work of Flavahan *et al.* (1985). These authors showed that ecothiopate did not abolish the ability of epithelium removal to potentiate ACh acting on canine bronchus.

That the epithelium of guinea-pig trachea does not represent a significant source of cholinesterase activity was confirmed by the present experiments involving both the bioassay of cholinesterase and its localization by histochemistry. Some property other than susceptibility to esterase attack clearly must determine why, in opened segments of guinea-pig trachea, acetic cholinesters are potentiated by epithelium removal whereas other cholinomimetic agents are not.

Potentiation of cholinomimetics caused by epithelium removal: influence of the route of cholinomimetic administration

The present experiments with indomethacin-treated perfused tracheal tubes revealed that, when cholinomimetics were applied to the luminal surface of the organ, epithelium removal potentiated not only ACh but also CCh and oxotremorine. Furthermore, the potentiation of ACh was of greater magnitude ($\simeq$30 fold) than that observed in the experiments with opened tracheal segments ($\simeq$3 fold). Clearly, when cholinomimetics are applied only to the luminal surface of the trachea, epithelium removal potentiates both esterase-susceptible and esterase-resistant derivatives. That the mechanism of this potentiation is not specific for agents which directly activate muscarinic cholinoceptors is suggested by our observation that KCl, too, was potentiated under these circumstances (Table 3).

When the cholinomimetics were applied to the adventitial surface of the tracheal tubes the potencies of CCh and oxotremorine were significantly reduced by epithelial removal. An apparent reduction in the potency of ACh was also observed. These findings clearly show that, in such preparations, the effects of epithelium removal on the actions of cholinomimetics are very dependent on the route by which the cholinomimetic diffuses towards the trachealis muscle. It is evident that epithelium removal can potentiate both esterase-susceptible and esterase-resistant cholinomimetics providing that these agents are diffusing towards the trachealis muscle only from the luminal surface of the trachea.

Marked (>100 fold) potentiation of luminally-applied ACh was evoked by epithelium removal in the experiments of Munakata *et al.* (1989). These authors performed intraluminal pressure recording in guinea-pig trachea and, in contrast to the present study, did not find that epithelium removal could significantly reduce the potency of agonists applied to the adventitial surface of the tissue.

Our observation that epithelium removal can reduce the potency of spasmogens applied to the adventitial surface of the trachea cannot be explained in terms of removal of an epithelial source of EpDRF. Instead, it may reflect the activity of an epithelial barrier to drug diffusion. Such a barrier could hinder drug passage across the tracheal wall in either direction.

In the present study the luminal or the adventitial surface of the trachea was superfused by drug-free Krebs solution, thereby providing an infinite sink for drug penetrating from the opposite side of the tracheal wall. If the epithelium were acting as a significant, two-way barrier to drug diffusion, then, in the case of drug application to the luminal surface, epithelium removal would facilitate drug penetration to the remainder of the tracheal wall and would increase the drug concentration gradient therein. Potentiation of the drug would then result. In the case of drug application to the adventitial surface, epithelium removal would promote drug loss into the luminal perfusate and thereby increase the drug concentration gradient across the remainder of the tracheal wall. In this circumstance reduced potency of the drug would result.

In the present study dispositional mechanisms could therefore contribute to the ability of epithelial removal to potentiate cholinomimetics applied luminally to perfused tracheal tubes but to reduce the potency of the same drugs applied to the adventitial surface. It is tempting to suggest that the differing degrees of potentiation observed amongst the luminally-applied cholinomimetics simply reflect their different abilities to permeate an epithelial barrier to drug diffusion. However, the explanation cannot be so simple because, in epithelium-denuded tracheal tubes, the rank order of potentiation of the luminally-applied cholinomimetics (ACh > CCh > oxotremorine) differs from the rank order of potency reduction (CCh $\simeq$ oxotremorine > ACh) observed when these compounds are applied to the adventitial surface of epithelium denuded tracheal tubes (Table 3). It may therefore be that the cholinomimetics not only differ in their abilities to permeate an epithelial barrier to drug diffusion but also, as suggested by Goldie *et al.* (1988), in their abilities to release EpDRF from the tracheal epithelium. Measurements of the rate at which cholinomimetics penetrate the tracheal wall and measurements of their abilities to release EpDRF are needed to clarify the situation.

Role of tracheal intramural nerves in the potentiation of ACh caused by removal of epithelium from opened tracheal segments

Epithelial cell loss or damage has been associated with airway hyperreactivity in asthmatic patients (Laitinen *et al.*, 1985). Furthermore, it has been suggested (Barnes *et al.*, 1988) that epithelium cell loss might provoke bronchial hyperreactivity by making sensory nerve terminals more accessible to inhaled noxious stimuli. If this hypothesis is correct, it implies that bronchial hyperreactivity in asthma must (at least in part) have an underlying neurogenic basis. Accordingly, it seemed appropriate to examine whether the potentiation of ACh caused by epithelial removal in guinea-pig trachea was dependent on the activity of intramural nerves.

We have previously shown (Boyle *et al.*, 1987) that tetrodotoxin (3 μM) can virtually abolish the effects of intramural nerve stimulation in guinea-pig trachea and that hexamethonium (500 μM) virtually ablates neurogenic responses induced by nicotine. The present failure of hexamethonium and tetrodotoxin to prevent potentiation of ACh caused by epithelium removal suggests that such potentiation does not depend on neural transmission across cholinergic synapses involving postsynaptic nicotinic cholinoceptors and does not depend on neuronal action potential discharge. Accordingly it may be that epithelium removal from the trachea *in vitro* does not provide a model of airways hyperreactivity which has a neurogenic basis.

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Effect of capsaicin-sensitive sensory nerves on plasma glucose and catecholamine levels during 2-deoxyglucose-induced stress in conscious rats

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1 Sensory fibres innervate the adrenal medulla but their function is not known. In this paper we have studied the effect of capsaicin-sensitive sensory fibres on the adrenal catecholamine (CA) response and blood glucose response to 2-deoxyglucose (2-DG)-induced glucopenic stress in conscious rats.

2 2-DG at 500 mg kg^{-1} (i.v.) induced a 2.5 fold increase in plasma glucose levels, a 3.5 fold increase in inferior vena caval (i.v.c.) plasma noradrenaline (NA) levels and a 7 fold increase in i.v.c. plasma adrenaline (Ad) levels over 60 min. The hyperglycaemia in response to 2-DG was attenuated by pentolinium and by left splanchnicotomy plus right adrenalectomy. These procedures also caused a complete inhibition of the increase in plasma CA.

3 The hyperglycaemia in response to 2-DG was attenuated by pretreatment of rats with capsaicin as neonates, suggesting that capsaicin-sensitive sensory fibres are required for regulation of plasma glucose in response to glucopenic stress.

4 The increase in i.v.c. plasma CA levels in response to 2-DG during the early phase of glucopenia (first 30 min) in the conscious rats pretreated with capsaicin was the same as in the rats pretreated with vehicle alone. During the later phase of glucopenia (after 45 min), the increase in plasma CA levels in rats pretreated with capsaicin was higher than in the rats pretreated with vehicle alone.

5 In vehicle-pretreated rats and capsaicin-pretreated rats the tissue NA and Ad levels in the adrenal medulla after 8 h of stress were depleted to the same extent. However, tissue CA levels in the capsaicin group recovered faster over 24 h than in the vehicle group.

6 These results indicate that capsaicin-sensitive sensory fibres are not required to maintain adrenal CA secretion during glucopenic stress in the conscious rat but are required for maintenance of blood glucose levels.

Introduction

Sensory neurones in the dorsal root ganglia and nodose ganglia innervate the adrenal medulla via the splanchnic nerve and vagal nerve (Mohamed *et al.*, 1988; Coupland *et al.*, 1989). Substance P (SP) is present in sensory nerves including those in the splanchnic nerve that innervates the adrenal medulla (Linnoila *et al.*, 1980; Gamse *et al.*, 1981; Pfister & Gorne, 1983), and can be released *in vivo* from the adrenal medulla in response to stress (Vaupel *et al.*, 1988). The function of SP and sensory innervation in the adrenal medulla, however, is not known. Studies *in vitro* in bovine chromaffin cells in culture and rat isolated perfused adrenal glands indicate that SP modulates catecholamine (CA) secretion (Boksa & Livett, 1984b; Zhou & Livett, 1987; 1988) so as to maintain catecholamine (CA) output during stress.

Capsaicin is a selective neurotoxin for sensory nerves. When injected into rats as neonates, it causes sensory C-fibres to degenerate, and therefore causes a depletion of SP and other mammalian tachykinins in the sensory nervous system (for review, see Buck & Burks, 1986). Therefore, capsaicin has been widely used as a pharmacological tool to investigate SP function in sensory neurones (for review, see Holzer, 1988). We showed previously that neonatal pretreatment with capsaicin abolished the neuronal component of adrenal CA secretion in response to insulin-induced hypoglycaemia or histamine stress in the anaesthetized rat (Khalil *et al.*, 1986; 1987). These results on anaesthetized rats support our hypothesis that SP-containing sensory fibres innervating the adrenal medulla are required to maintain CA secretion during stress (Khalil *et al.*, 1986; 1987). However, this hypothesis has been challenged by two recent studies.

Firstly, Amann & Lembeck (1986) and Donnerer (1988) proposed that the attenuation of CA secretion in capsaicin-pretreated rats was caused by a failure of peripheral communication to the central nervous system due to the damage of sensory afferent C-fibres. This conflict may be resolved by use of 2-deoxyglucose (2-DG) to produce a state of neuroglucopenia with subsequent adrenal CA secretion (Niijima, 1982; Storlien 1985). 2-DG stimulates glucose-sensitive neurones in the hypothalamus directly, so bypassing the peripheral sensory afferents. However, it still shares the same efferent pathway as insulin-induced hypoglycaemia (Hokfelt & Bydgeman, 1961; Brown, 1962; Niijima, 1982) for the release of adrenal CAs.

Secondly, anaesthetics such as pentobarbitone inhibit CA outflow during 2-DG-induced stress (Taborsky *et al.*, 1984a,b; Havel *et al.*, 1987) or hypovolemic stress (Howe *et al.*, 1986). It is possible that the failure of capsaicin-pretreated animals to secrete CAs in response to stress (Khalil *et al.*, 1986; 1987) was a function of the pentobarbitone anaesthesia. In the present study, therefore, we have studied the role of capsaicin-sensitive sensory nerves in the regulation of adrenal CA secretion induced by 2-DG in conscious freely-moving rats fitted with indwelling venous cannulae.

Methods

Animals and surgical preparation

Buffalo rats, male, 10 weeks old (290–380 g), were raised under conditions of constant temperature (21°C) and had free access to standard rat pellets and water. For placement of indwelling cannulae the rats were anaesthetized i.p. with a mixture (0.2 ml

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per 100 g) containing 1% of sodium brietal and 1.5% of sodium amytal (2 ml kg^{-1}). The operation area on the neck and catheter outlet area on the back were shaved and sterilized with 70% ethanol. A longitudinal incision 1.5 cm long was made in the skin on the ventral surface of the neck on the right side. The right jugular vein was exposed by blunt dissection and cannulated with a piece of silastic tubing (i.d. 0.025 × o.d. 0.045 in.). A 4 cm length of cannula was inserted into the vein and extended so that the tip of the cannula rested in the inferior vena cava, approximately 1 cm below the right atrium. The cannula was secured into the jugular vein with a cotton tie. The patency of the cannula was tested by withdrawing blood. The free end of the cannula was led subcutaneously to the dorsal surface of the head and externalized through a small incision. The catheter was filled with heparin saline (200 iu ml^{-1}) and plugged with a short stainless steel pin. Finally, the neck incision was sutured and painted with 70% ethanol or Hexifoam. The total operation time was 15–20 min and the animals regained consciousness by 45 min. After operation the animals were housed individually. Thereafter, on a daily basis, the catheters were flushed with heparin saline to maintain catheter patency. The experiments were performed in a quiet laboratory on the 4th day after operation on rats fasted overnight. Before sampling, the rats were heparinized by flushing the catheter with 0.5 ml of heparin saline (200 iu ml^{-1}). A polythene tube (PE-28, i.d. 0.40 × o.d. 0.80 mm), which had been connected to a reversible peristaltic pump (Pharmacia P-1) and filled with saline, was connected to the indwelling cannulae. After 10 min (the rat having become quiet and freely moving) sampling was begun by turning on the pump at a rate of 0.5 ml min^{-1} and blood was collected into an ice-cold Eppendorf tube. After each sampling, an equivalent volume of saline was reinfused.

Capsaicin pretreatment Buffalo rat pups of either sex were injected s.c. with either capsaicin (50 mg kg^{-1}) or vehicle under analgesia (Innovar. Vet, Smith Kline and French Laboratories, N.S.W., Australia; dilution 1:100, 10 ml kg^{-1}, s.c.) on the 3rd day after birth as previously described in detail (Khalil *et al.*, 1986). The analgesia was assessed effective as the rat pups remained calm and by the absence of vocalization upon the injection of capsaicin. The solution was prepared as follows; capsaicin 10 mg was dissolved in 0.2 ml ethanol and 0.2 ml Tween-80 and then mixed with 1.6 ml 0.9% NaCl. The survival rate of capsaicin-injected pups was the same as for vehicle-injected pups and non-injected pups. When the rats were 10 weeks old the males were selected for the studies described below.

Left splanchnicotomy and right adrenalectomy Complete denervation of the splanchnic nerve was performed in adult male Buffalo rats (290–380 g) as described previously in detail (Khalil *et al.*, 1987). Briefly, under anaesthesia with Equithesin (0.2 ml 100 g^{-1} body weight; Equithesin is a pentobarbitone sodium/chloral hydrate mixture in $MgSO_4$, propylene glycol and ethanol, see Tagerud & Cuello, 1979), an L-shaped incision was made on the left side of the abdomen. The left splanchnic nerve trunk under the diaphragm was cut and adipose tissue surrounding the left adrenal gland was dissected from the gland. The abdomen was closed and the rats allowed to recover in a warm environment and monitored for 4 h after the operation by which time they had fully recovered from anaesthesia. They were then transferred to separate cages in an air-conditioned room with free access to food and water. The site of incision healed without infection in all cases. These operated rats were used for experiments after two weeks. Right adrenalectomy was carried out on these rats with left splanchnicotomy at the same time as the preparation of the indwelling catheter (see above). A 2-cm-long incision was made on the right abdomen and the right adrenal gland removed. The operation for removal of the right adrenal gland was completed in 5 min.

Assay of catecholamines and plasma glucose

Blood samples were centrifuged at 4000 *g* for 15 min, 2°C. The plasma was separated and transferred into Eppendorf tubes containing 30 μl sodium metabisulphate (10%) and stored at −70°C until assay. For assay of adrenaline (Ad) and noradrenaline (NA), CAs were extracted from the plasma on acid-washed alumina and eluted with 0.2 M perchloric acid (PCA). The extracted samples were injected onto a high performance liquid chromatography (h.p.l.c.) system with electrochemical detection as described by Livett *et al.* (1987). The recovery was corrected by adding an internal standard dihydroxybenzylamine (DHBA) to the extraction buffer. The content of CA in plasma was calculated as pmol ml^{-1}.

Plasma glucose was estimated by the hexokinase method (Glucoquant, Boehringer, Mannheim, FRG). 2-DG could not be detected in concentrations lower than 27 mmol l^{-1} with this method.

Adrenal medullary catecholamine determination

Adult male Buffalo rats (10 weeks old, 290–380 g) from the vehicle- and capsaicin-pretreatment groups were killed by stunning and decapitation and the adrenal glands were removed and homogenized in 2 ml 0.4 M PCA. The supernatant after centrifugation was injected into the h.p.l.c. system directly.

Experimental procedures

Experiments in vivo *on conscious freely-moving rats* Rats were fasted overnight before the experiments. After the experiments, the rats were given free access to food and water, until killed. Each animal was used only for a single blood sampling experiment.

Effects of 2-DG on CA secretion Four groups of rats ($n = 5$ in each group) were used. *Group 1:* After withdrawal of a resting blood sample, 2-DG (500 mg kg^{-1}) was infused i.v. over a 5 s period. Blood samples were withdrawn 4 times thereafter, at 15 min intervals. *Group 2:* After withdrawal of a basal sample, hexamethonium (2 mg kg^{-1}) and atropine (1 mg kg^{-1}) in normal saline were infused i.v. before and 30 min after 2-DG. Blood sampling was as in group 1. *Group 3:* After withdrawal of a basal sample, pentolinium (2 mg kg^{-1}) was infused i.v. before and 30 min after 2-DG. Blood sampling was as in Group 1. *Group 4:* Rats with right adrenal glands removed and left splanchnic nerve cut were given 2-DG and samples taken for plasma glucose and plasma CAs as described for Group 1. Animals in Group 4 were subjected to a separate series of experiments on the next day in which the left denervated adrenal glands were isolated and stimulated *in vitro* to confirm that the denervation was successful (see below).

Experiments in vitro *on isolated perfused denervated adrenal glands* On the day following blood sampling *in vivo*, rats from group 4 above were anaesthetized with Equithesin (0.2 ml 100 g^{-1}, i.p.), the left adrenal vein cannulated with PE-10 tubing and the adrenal gland was isolated and perfused with Krebs bicarbonate solution. After 20 min equilibration the adrenal gland was subjected to electrical stimulation with a Grass S48 stimulator (60 V, 10 Hz and 3 ms) for 2 min and the perfusate collected for CA determination. After a 10 min interval, the gland was perfused with 0.1 mM dimethylphenylpiperazinium iodide (DMPP) for 2 min and the perfusate collected for CA determination. As controls, adrenal glands from 5 non-denervated rats were prepared for *in vitro* perfusion.

Experiments on capsaicin pretreated rats Two sampling procedures were used *in vivo* on capsaicin-pretreated rats and

vehicle-pretreated rats. In one protocol blood samples were withdrawn at 0, 15, 30, 45, and 60 min after 2-DG and adrenal glands were removed for CA assay after 24 h. With the other protocol, blood samples were withdrawn at 0, 5, 60, 90, and 120 min after 2-DG and the adrenal glands were removed for CA assay after 8 h. Data from two protocols were plotted together. To study the effect of 2-DG on tissue levels of adrenal CA, five capsaicin- and five vehicle-pretreated rats without blood sampling and without using 2-DG treatment were operated on as for insertion of the venous cannula and served as sham-operated controls.

Materials

Hexamethonium, atropine sulphate, capsaicin and pentolinium were obtained from Sigma Chemical Co, U.S.A.; 2-deoxyglucose was obtained from Aldrich Chemical Co, U.S.A.; Silastic tubing was from Dow Corning Corporation, U.S.A.

Statistical analysis

Data are expressed by means ± s.e.mean. Significant differences were assessed by analysis of variance and Student's *t* test.

Results

Plasma glucose levels during 2-deoxyglucose-induced stress

The resting plasma glucose level in rats after an overnight fast was 102 ± 5 mg%. After 2-DG (500 mg kg^{-1}, i.v.), the plasma glucose level increased to 189 ± 13 mg% at 15 min and to 250 ± 37 mg% at 30 min and then levelled off. Ganglionic blockade with hexamethonium and atropine had no significant effects on plasma glucose levels after 2-DG (Figure 1a), whereas pentolinium significantly attenuated the increase in plasma glucose by 50% ($P < 0.01$) at 30, 45, and 60 min after 2-DG. Plasma glucose levels following 2-DG in rats with their right adrenal glands removed and left splanchnic nerves cut were significantly lower than in the control animals at 30 ($P < 0.05$), 45 ($P < 0.01$), and 60 ($P < 0.05$) min.

Plasma catecholamine levels in 2-deoxyglucose-induced stress

The resting i.v.c. plasma levels of NA and Ad before 2-DG were 6.8 ± 1.1 and 5.7 ± 0.8 pmol ml^{-1}. Plasma NA levels after 2-DG increased gradually up to 24.1 ± 2.8 pmol ml^{-1} at 60 min ($P < 0.01$ at all the points compared to resting level). Ad levels increased to 39.7 ± 8.1 pmol ml^{-1} at 30 min and then levelled off at 45 and 60 min after 2-DG ($P < 0.01$ at all time points compared to the resting level). As Figure 1 shows, the CA response to 2-DG was significantly suppressed but not blocked by the treatment with hexamethonium and atropine ($P < 0.05$ at 45 and 60 min for NA and $P < 0.01$ at 45 and 60 min for Ad, compared with 2-DG alone). However, pentolinium at 2 mg kg^{-1} completely blocked the increase in plasma CA in response to 2-DG (Figure 1). The Ad response to 2-DG was completely abolished and NA response to 2-DG was substantially inhibited in the rats with their right adrenal gland removed and left splanchnic nerve denervated (Figure 1).

Catecholamine secretion from denervated glands in vitro

CA secretion evoked by electrical field stimulation of isolated perfused glands taken from rats which had undergone surgical adrenal denervation was almost completely abolished (Figure 2). However, CA secretion from these glands in response to the nicotinic agonist DMPP was unaffected (Figure 2). Glands from control animals responded well to both nerve and drug stimulation.

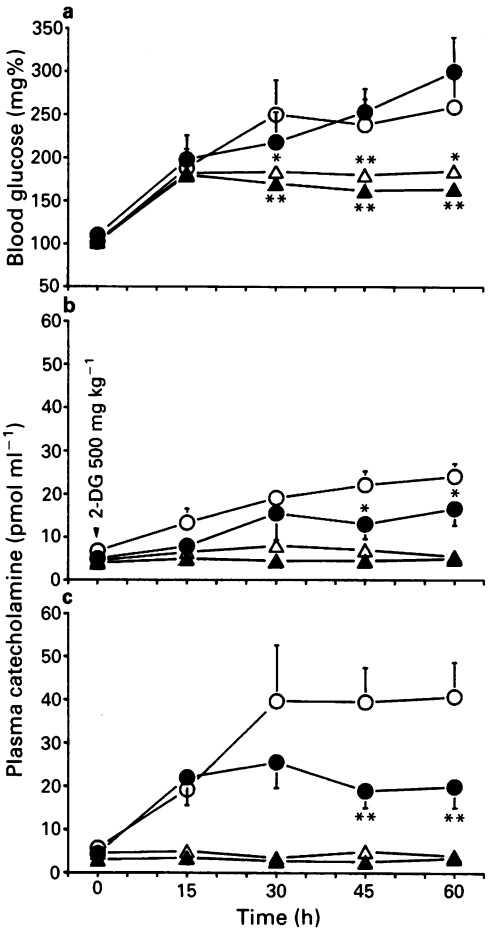

Figure 1 Effect of 2-deoxyglucose (2-DG) on plasma glucose and catecholamine (CA) levels in conscious rats. After withdrawal of basal blood samples, 2-DG (500 mg kg^{-1}) was infused i.v. and blood was sampled every 15 min from the inferior vena cava for CA and plasma glucose assay. (a) Plasma glucose; (b) noradrenaline; (c) adrenaline. (○) 2-DG only; (●) 2-DG + hexamethonium (2 mg kg^{-1}) + atropine (1 mg kg^{-1}); (△) 2-DG experiments in the animals with right adrenal glands removed and left splanchnic nerves denervated; (▲) 2-DG + pentolinium (2 mg kg^{-1}). * $P < 0.05$; ** $P < 0.01$ compared with 2-DG only (○). Data are plotted as means with s.e.mean shown by vertical bars ($n = 5$). Where error bars are not visible they lay within the dimensions of the symbol.

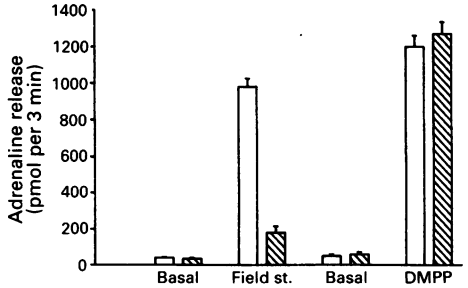

Figure 2 Effect of surgical adrenal denervation on adrenaline secretion evoked by field stimulation of isolated perfused adrenal gland. Two weeks after left splanchnicotomy and one day after the 2-deoxyglucose experiment, the left adrenal glands were isolated, perfused and stimulated at 10 Hz with 60 V and 3 ms pulse as described in the text. Open columns: control glands from non-denervated rats; hatched columns: denervated glands. DMPP: dimethylphenylpiperazinium iodide. Data are plotted as means with s.e.mean shown by vertical bars ($n = 4$).

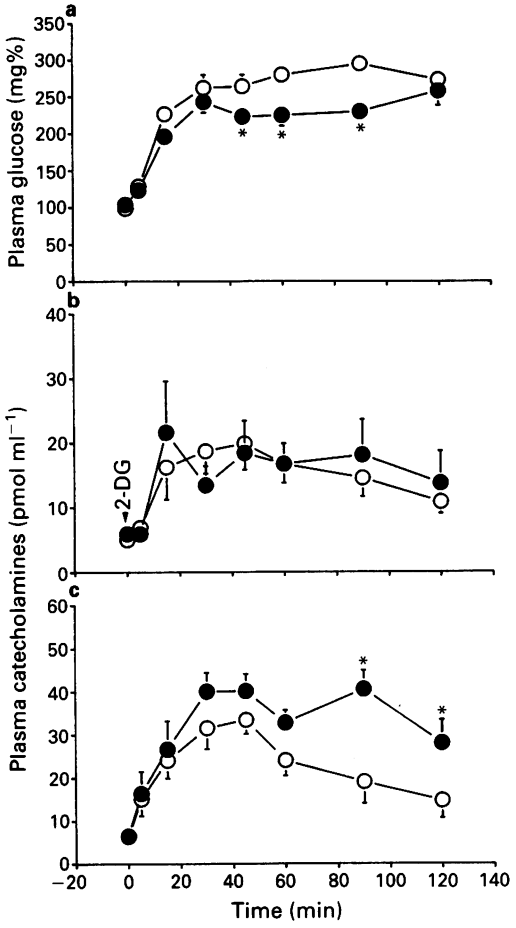

Figure 3 Effect of capsaicin pretreatment on plasma glucose and catecholamine levels in response to 2-deoxyglucose (2-DG) in conscious rats. The rats were treated with either 50 mg kg^{-1} capsaicin (s.c.) or with vehicle on the third day after birth. Stress experiments were performed when rats were 8–12 weeks old. After withdrawal of the basal blood samples, 2-DG 500 mg kg^{-1} was infused i.v. and blood samples were withdrawn at the times shown in the figure. (a) Plasma glucose; (b) noradrenaline; (c) adrenaline. (○) Vehicle control rats; (●) capsaicin-pretreated rats. * $P < 0.05$ compared with vehicle-pretreated rats. Data are plotted as means with s.e.mean shown by vertical bars ($n = 10$ at 0 and 60 min and $n = 5$ at 5, 15, 45, 90 and 120 min).

Effect of capsaicin pretreatment on plasma glucose levels after 2-deoxyglucose

There was no significant difference in the resting plasma glucose levels between capsaicin- and vehicle-pretreated rats (Figure 3a, $t = 0$). In the first 30 min after 2-DG, plasma glucose levels in capsaicin-pretreated rats increased to the same levels as in vehicle-pretreated rats, whereas at 45, 60, and 90 min, the plasma glucose levels in capsaicin-pretreated rats were significantly attenuated ($P < 0.05$ compared with the levels in vehicle-pretreated rats). By 120 min, there was again no significant difference in the plasma glucose levels between the two groups (Figure 3a).

Effect of capsaicin pretreatment on plasma catecholamine levels after 2-deoxyglucose

Plasma CA levels in response to 2-DG in both capsaicin- and vehicle-pretreated rats exhibited substantial individual variation. Plasma Ad levels in some rats increased up to 150 pmol ml^{-1}, whereas the increase was much less in other rats. The time course of plasma CA changes after 2-DG also exhibited large variation between individuals of same treatment group. The increment in plasma CA levels in some rats peaked earlier, some later. On average, plasma NA and Ad

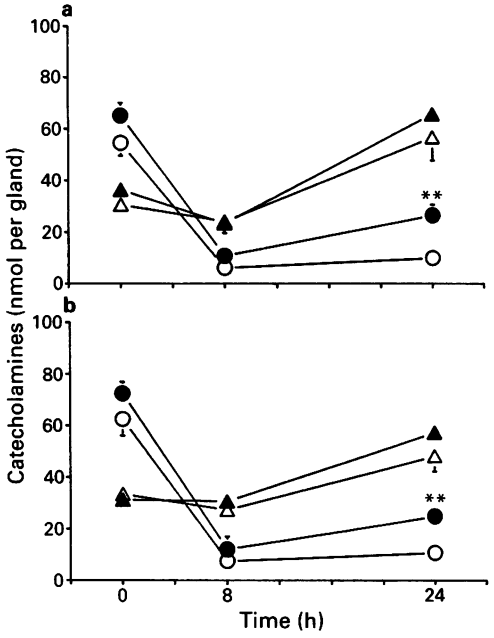

Figure 4 Effect of 2-deoxyglucose (2-DG) on tissue catecholamine (CA) levels in the adrenal gland. 2-DG (500 mg kg^{-1}) was infused i.v. to conscious rats pretreated with capsaicin or vehicle as neonates. The rats were killed at 0 (sham-operated group, without 2-DG), 8 and 24 h after 2-DG and CA levels in the adrenal medulla were measured. (a) Left adrenal glands; (b) right adrenal glands. (○) Adrenaline (Ad) vehicle; (●) Ad capsaicin; (△) noradrenaline (NA) vehicle; (▲) NA capsaicin. ** $P < 0.01$ compared with vehicle-pretreated rats. Data are plotted as means with s.e.mean shown by vertical bars ($n = 5$ at each point).

levels started to increase at 5 min in both groups and peaked at 30–45 min, and declined thereafter. After 60 min, plasma Ad levels in the capsaicin-pretreated rats were still high and at 90 and 120 min approximately twice that in the vehicle pretreated rats ($P < 0.05$ at 90 and 120 min) (Figure 3c). There was no significant effect of capsaicin pretreatment on plasma NA levels (Figure 3b).

Effect of capsaicin pretreatment on adrenal catecholamine concentrations

Eight hours after treatment with 2-DG, Ad concentrations in the left adrenal glands of vehicle-pretreated rats were depleted by 88.4% and in the capsaicin-pretreated rats by 84.6% (Figure 4). NA concentrations in the left adrenal glands of capsaicin- and vehicle-pretreated rats were depleted by only 21.3% and 33.4% respectively. There were no significant differences between capsaicin and vehicle pretreated groups 8 h after 2-DG.

The tissue CA levels recovered faster over 24 h in the capsaicin group (from 10.8 to 26.5 nmol per gland) than in the vehicle group (from 6.2 to 10.0 nmol per gland) (Figure 4). The tissue levels of Ad in the adrenals of the capsaicin group after 24 h were significantly higher than those in the vehicle group ($P < 0.01$). The adrenal NA levels in both groups after 24 h not only recovered but also increased above the fasting control levels. The changes in CA levels in the right adrenal gland after 2-DG were similar to those in the left adrenal gland.

Discussion

Effect of 2-deoxyglucose on plasma glucose levels

The results of the present study confirmed that 2-DG administration induces hyperglycaemia and secretion of Ad from the

adrenal medulla. We found that the ganglion blocker pentolinium partially blocked the hyperglycaemia but completely blocked the increase in adrenal CA secretion. Similarly, right adrenalectomy plus left splanchnicotomy partially blocked the hyperglycaemia but completely blocked the increase in adrenal CA secretion (Figure 1).

The mechanism of the hyperglycaemic response to 2-DG is not fully understood. The compound 2-deoxyglucose (2-DG), is a non-metabolizable D-glucose analogue. When administered systemically or intraventricularly, it causes depletion of glucose within cells (glucopenia) (Wick *et al.*, 1955) and brings about a neurogenic activation of glucose-sensitive neurones in the hypothalamus with increased sympathetic outflow and consequent hyperglycaemia (Muller *et al.*, 1971), and CA secretion (Hokfelt & Bydgeman, 1961; Himsworth, 1979; Taborsky *et al.*, 1984b; Donnerer, 1988) from the adrenal medulla. It is believed that the hypothalamus (Muller *et al.*, 1971; Storlien, 1985), and central (Niijima, 1982) and peripheral sympathetic pathways (Niijima, 1975) and the adrenal medulla but not the sensory afferents (Storlien, 1985; Amann & Lembeck, 1986) are involved in this pathway. Earlier studies demonstrate that adrenalectomy and adrenal denervation inhibit the hyperglycaemic response to 2-DG (Brown, 1962; Frohman *et al.*, 1983), indicating that it is mediated by the adrenal medulla. Studies by Storlien *et al.* (1985) show that the 2-DG-induced hyperglycaemia is blocked by pretreatment with guanethidine, an adrenergic nerve blocker, suggesting that the hyperglycaemic response to 2-DG is mediated by sympathetic nerves innervating the liver rather than by adrenaline released from the adrenal gland. Our results in the present study indicate that cholinergic secretion of Ad from the adrenal medulla is not solely responsible for the 2-DG-induced hyperglycaemia. In this regard, it is known that 2-DG increases glucagon (Frohman & Bernadis, 1971) and pancreatic polypeptide (PP) (Taborsky *et al.*, 1984a) secretion and inhibits insulin secretion. These pancreatic endocrine hormones may therefore contribute to the non-cholinergic component of 2-DG-induced hyperglycaemia.

Effect of capsaicin pretreatment on plasma glucose levels

In the present study, we found that the hyperglycaemia in response to 2-DG was attenuated by capsaicin pretreatment. This result is in accordance with results obtained with insulin-induced hypoglycaemia in which the recovery of blood glucose levels after insulin was impaired in capsaicin pretreated rats (Zhou *et al.*, 1990). The reason for this dysfunction in glucose homeostasis is not clear. The secretion of the counter-regulatory hormones, adrenaline and glucagon in response to 2-DG, might be impaired in the absence of sensory fibres. However, Ad secretion in response to 2-DG in capsaicin-pretreated rats was not impaired (Figure 3c), being even higher than in vehicle-pretreated rats. This indicates that the attenuation in hyperglycaemia in capsaicin-pretreated rats is not caused by impairment of CA secretion. Thus the attenuation of hyperglycaemia may have been caused by dysfunction of glucagon secretion. Glucagon is the primary counter-regulatory hormone (Gerich *et al.*, 1979) and its secretion is increased in response to 2-DG administration (Hedo *et al.*, 1978; Frohman *et al.*, 1983). SP infusion *in vivo* induces hyperglucagonaemia (Brown & Vale, 1976; Lundquist *et al.*, 1979), suggesting depletion of SP by capsaicin pretreatment may cause dysfunction of glucagon secretion. In addition, the impairment of adrenal cortical hormone secretion in capsaicin-pretreated animals (Lembeck & Amann, 1986; Donnerer & Lembeck, 1988) may also contribute to the attenuation of the blood glucose response to 2-DG.

Effect of 2-deoxyglucose on plasma catecholamine levels

The basal levels of plasma CAs were higher in the present study than those reported by others (Taborsky *et al.*, 1984a; Howe *et al.*, 1986). The difference may be explained by the fact that we sampled blood from the inferior vena cava close to the point of venous output from the adrenal gland. In fact one of the advantages of our sampling technique is that it overcomes the problem of high clearance of blood-borne CAs that occurs on passing through tissue vascular beds. Despite widespread use of plasma CAs in the clinical literature, peripheral venous plasma Ad concentrations are a very poor substitute for central venous or arterial concentrations.

2-DG induced a marked increase in plasma CA levels (Figures 1 and 3) with an approximately 90% depletion in tissue Ad levels in the adrenal glands (Figure 4). To investigate the mechanism involved in the initial CA secretion induced by 2-DG, we used hexamethonium (C_6) and atropine to see whether the CA response to 2-DG was under cholinergic control. The results showed that C_6 and atropine did not completely block the CA response. Two possible explanations for these results were: (1) that there were non-cholinergic splanchnic nerve components contributing to the control of adrenal CA secretion after 2-DG; and that (2) C_6 and atropine at all concentrations used were not effective in blocking ganglionic transmission in the conscious rat. Consequently, pentolinium was used to produce a potent ganglion blockade in the conscious rat (Howe *et al.*, 1986). Pentolinium completely blocked CA secretion after 2-DG, indicating that the CA response to 2-DG was controlled by the cholinergic nervous system and that C_6 at $2\,mg\,kg^{-1}$ and atropine at $1\,mg\,kg^{-1}$ were not effective blockers of the cholinergic response in conscious rats.

To study the source of CA secretion in response to 2-DG, we cut the left splanchnic nerve and removed the right adrenal gland before the release experiment. The results showed that CA secretion was completely blocked in the rats with the left splanchnic nerves denervated and the right adrenal glands removed (Figure 1), indicating that the increase in plasma CA in response to 2-DG came almost entirely from the adrenal medulla rather than from sympathetic nerve terminals.

The results in Figure 2 indicate that the procedure used to denervate the adrenal medulla by cutting the splanchnic nerves was successful as shown by an almost complete lack of CA secretion in response to electrical field stimulation of such glands *in vitro*. The secretory function of the adrenal chromaffin cells remained intact, however, because a full CA secretory response was elicited by the nicotinic agonist DMPP.

Effect of capsaicin pretreatment on catecholamine secretion

Numerous studies on a variety of systems have shown that CA secretion in response to nicotinic stimulation is very quickly desensitized *in vitro* (Boksa & Livett, 1984a). That desensitization does not occur *in vivo* even during severe stress suggests that there are physiological mechanisms preventing desensitization of the nicotinic response. Our previous work with bovine adrenal chromaffin cells (Boksa & Livett, 1984b) has shown that SP functions to protect against desensitization of the nicotinic response induced by either acetylcholine or nicotine. More recently, we have shown that SP at low concentrations ($<10^{-6}$ M) facilitates CA secretion evoked by short term (6 min) or long term (2 h) electrical field stimulation of the isolated perfused adrenal glands of the rat (Zhou & Livett, 1987; 1988). These *in vitro* studies suggest that SP may act to prevent desensitization of the nicotinic response during stress *in vivo*. In addition, capsaicin-pretreatment, which depletes SP from the splanchnic nerve innervating the adrenal medulla, abolished CA secretion in response to insulin-induced hypoglycaemic stress, cold stress (Khalil *et al.*, 1986) and the neuronal component of histamine-induced stress (Khalil *et al.*, 1987) in anaesthetized rats. However, in the present study, CA secretion in response to 2-DG-induced glucopenia occurred at the same initial rate in capsaicin-pretreated rats as in the vehicle-pretreated rats. At later times, this response in the capsaicin-pretreated rats was even higher than in the vehicle-pretreated rats (Figure 3b,c). CA depletion

in the adrenal medulla 8 h after 2-DG in capsaicin pretreated rats was not significantly different from that in vehicle-pretreated rats. There are three possible explanations for the difference in results between the present study and our previous studies: (1) Sensory afferents of reflex arcs which can be damaged by capsaicin pretreatment are involved in the stimuli used in the previous studies; (2) Pentobarbitone used in the previous studies may suppress CA output during stress (Taborsky *et al.*, 1984a,b; Howe *et al.*, 1986; Havel *et al.*, 1987); (3) Greater blood sample withdrawal in the previous studies (7.5 ml vs 5 ml in the present study) may have resulted in hypovolemia which added to the three stimuli employed (insulin stress, cold stress and histamine stress).

By using 2-DG directly to stimulate central sympathetic outflow in the present study in freely moving conscious rats, we excluded any possible involvement of sensory afferents in the activation of CA secretion from the adrenal medulla by a reflex pathway and effects of anaesthetics on CA secretion. We also reduced the blood sample volume to avoid hypovolemic stress. The results of the present study therefore, demonstrate that in the freely-moving conscious rats, SP-containing capsaicin-sensitive sensory fibres innervating the adrenal medulla are not required to maintain CA secretion during prolonged glucopenic stress. They are, however, involved in the glucose homeostasis in conscious animals. A similar conclusion was reached from studies on the effects of insulin/somatostatin-induced hypoglycaemia on CA release from capsaicin-pretreated and vehicle-pretreated conscious rats sampled *in vivo* via indwelling cannulae (Zhou *et al.*, 1990).

In conclusion, capsaicin-sensitive sensory fibres innervating the adrenal gland appear not to be required for activation and maintenance of CA secretion during prolonged glucopenic stress in the conscious freely-moving rat. The mechanism demonstrated in isolated chromaffin cells (Boksa & Livett, 1984b), in the rat perfused adrenals *in vitro* (Zhou & Livett, 1987; 1988) and in the anaesthetized rat (Khalil *et al.*, 1986; 1987) in which endogenous substance P serves to modulate the nicotinic secretion of catecholamines in response to acetylcholine, nicotinic agonists, electrical stimulation and a number of metabolic and physical stimuli appears to be subservient to control mechanisms operating at higher levels in the conscious animal. Nevertheless, CA secretion in the conscious animal does not exhibit desensitization in response to physiological stress. The mechanisms responsible for preventing desensitization of the CA secretory response during stress *in vivo* remain to be determined.

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Pharmacological analysis of established ventricular fibrillation

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1 The effects of anti-arrhythmic drugs on the power spectrum of established ventricular fibrillation induced by endocardial electrical stimulation, have been studied in greyhounds anaesthetized with sodium pentobarbitone (35 mg kg^{-1}, i.v.).

2 In dogs receiving no drug, initial recording of ventricular fibrillation showed a dominant frequency of 9.9 ± 0.7 Hz (lead II) and 10.0 ± 0.6 Hz (endocardium). After 3.3 min the frequency had fallen to 4.0 ± 0.4 Hz in lead II, but remained high in the endocardium (10.7 ± 0.5 Hz).

3 Lignocaine significantly reduced the dominant frequency for fibrillation recorded from lead II at (0–80 s), and for endocardial fibrillation at (0–200 s).

4 Pretreatment with propranolol or bretylium had little effect on the time course of the dominant frequency of fibrillation in lead II or the endocardium.

5 Verapamil prevented the fall in frequency seen in lead II after 80 s in the no drug group. A significantly higher frequency was maintained in both lead II (14.7 ± 0.9 Hz) and the endocardium (14.8 ± 0.9 Hz) for 3.3 min, compared with the no drug group ($P < 0.01$).

6 Activation of fast sodium channels may determine the rapid frequency of the initial stages of ventricular fibrillation. The rapid fall in dominant frequency in lead II after fibrillation for 80 s can be prevented by calcium channel blockade and may be due to intracellular accumulation of calcium.

Introduction

Ventricular fibrillation develops in some 18% of patients treated within the first hour after acute myocardial infarction (Pantridge *et al.*, 1974). Antiarrhythmic agents are often administered to prevent ventricular fibrillation in patients with acute infarction, or with arrhythmias associated with chronic ischaemic heart disease. For example, class I and class III drugs are often given to prevent ventricular arrhythmias such as ventricular fibrillation, and early intervention with class II drugs is reported to reduce mortality after acute myocardial infarction (First International Study, 1986). Many studies have been made of the effects of such drugs on the electrical activity in normal and diseased myocardium, and the drugs have been classified on this basis (Singh & Vaughan Williams, 1972; Vaughan Williams, 1984). However the effects of such drugs on established fibrillation are largely unknown.

It is now recognized that ventricular fibrillation is not a totally random arrhythmia, and that the ECG signal shows a degree of electrical regularity. The frequency content of ventricular fibrillation changes with time in a characteristic fashion (Worley *et al.*, 1985; Martin *et al.*, 1986), with a dissociation in endocardial and epicardial activity (Carlisle *et al.*, 1990). The reasons for this deterioration in the waveform are uncertain. To investigate further the electrophysiological changes occurring during fibrillation, we have studied the effects of antiarrhythmic drugs on the frequency content of electrically-induced ventricular fibrillation in the dog. Some of these results have been presented to the Physiological Society (Adgey *et al.*, 1987).

Methods

Greyhound dogs (weight range 20–36 kg) were anaesthetized after intravenous sodium pentobarbitone (Sagatal, May & Baker, 35 mg kg^{-1}). After endotracheal intubation, ventilation with room air was started (Palmer Ideal pump; tidal volume 12 ml kg^{-1}, ventilation rate 18 min^{-1}). A cannula was inserted into the femoral artery and connected to a transducer (Statham) for blood pressure monitoring, with the ECG (Devices). Under X-ray control, 2 endocardial pacing catheters (U.S.C.I. 6F) were positioned transvenously in the apex of the right ventricle via the external jugular vein, one for inducing ventricular fibrillation electrically and the other for recording endocardial ventricular fibrillation. A foreleg vein was cannulated for the administration of drugs.

A blood sample was taken from the femoral artery for assay of plasma potassium during the control period and just after the onset of ventricular fibrillation. Oesophageal temperature was monitored by an electronic thermometer (Comark 1604-2).

Drugs

Five groups, each with 5 dogs, were studied. There was a control period of 1 h after the surgical stress. The selected drug was then administered intravenously over 5 min. *Group 0* No drug: ventricular fibrillation was induced without administering any antiarrhythmic drug. *Group 1* Lignocaine hydrochloride (Antigen Ltd.; 10 mg kg^{-1}). *Group 2* Propranolol hydrochloride (Inderal, ICI; 0.4 mg kg^{-1}). *Group 3* Bretylium tosylate (Bretylate, Wellcome; 10 mg kg^{-1}). *Group 4* Verapamil hydrochloride (Cordilox, Abbott; 1 mg kg^{-1}).

In Groups 2 and 4, ventricular fibrillation was induced 15 min after the drug was given. In Group 1, ventricular fibrillation was induced 5 min after drug administration, as lignocaine has a particularly short half-life. In the bretylium group (3), ventricular fibrillation was not induced until 2 h later, due to the long delay in onset of its maximum electrophysiological (Anderson *et al.*, 1980) and antiarrhythmic effects.

In Groups 1 and 2, the plasma concentrations of lignocaine and propranolol were measured in arterial blood samples taken before injection of the drug (control samples), and just after the onset of ventricular fibrillation (effective concentrations).

Ventricular fibrillation was induced by stimulating the right ventricle with a short train of electrical pulses (100 Hz; 10 V; 5 ms pulse duration) delivered by an isolated stimulator

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(Devices 2533) via one of the endocardial catheters. At the onset of ventricular fibrillation, ventilation was stopped to reduce low frequency variation in lung volume. Fibrillation was recorded on tape from both lead II and the endocardial lead for at least 15 min. At the end of each experiment the heart was excised and weighed.

After amplification (Hellige Multiskriptor EK22) the lead II and right ventricular endocardial ECG signals were converted independently to digital form and stored on dual-channel cassette-tape (Sony Stereo Cassette Corder TCS-350). An 8 bit A-D converter was used at a sampling rate of 430 Hz. The frequency response of the recording and play-back system was determined to be 0.35–55 Hz (Carlisle *et al.*, 1990).

Fast Fourier transform analysis of the reconstituted ventricular fibrillation signal was performed by use of a Bruel & Kjaer Spectrum Analyzer Type 2031. This microprocessor-based system transforms data from the time domain to the frequency domain, giving a 400 channel, constant bandwidth, logarithmic plot of the root mean square power against frequency. The reference level for amplitude was always 1 microvolt rms.

Individual time windows of 4 s duration were analysed. The resulting power spectra were then averaged linearly to give analyses of 40 s periods of ventricular fibrillation. Discontinuity between the windows was reduced by the use of a Hanning weighting. When the power spectrum was obtained, the frequency with the largest amplitude was found with the screen cursor and alphanumeric display. A hard copy of the spectrum was taken for future reference (Bruel & Kjaer X-Y Recorder Type 2308). During the analysis, the signal was monitored on an oscilloscope (Gould 0S4020) to avoid artefact.

Statistical methods

The null hypothesis, that there was no significant difference between the values observed in separate groups, or between the values at different times, was tested. The significance level for the rejection of this hypothesis was $P < 0.05$. Analysis of variance was performed with Duncan's multiple range test for multiple comparisons between means (ONEWAY, SPSS Inc, ICL 2900 computer). Paired or unpaired t tests were used if only 2 groups were being compared. All results are reported as mean ± standard error of the mean (s.e.mean).

Results

Body weight and heart weight did not differ significantly between the groups (Table 1). Both plasma potassium values (Table 1) and body temperature (not shown) did not change significantly during the experiments and were similar between groups.

Haemodynamics

There were no significant changes in heart rate and mean arterial blood pressure during the control period in the dogs given no drug (Table 1).

The intravenous administration of lignocaine caused an increase in heart rate ($P < 0.02$), and a transient fall in blood pressure ($P < 0.005$) which recovered before ventricular fibrillation was induced 5 min later (Table 1).

Propranolol caused an immediate and persistent fall in heart rate ($P < 0.05$), with no significant effect on blood pressure (Table 1).

Bretylium had powerful haemodynamic effects, causing a sharp increase in heart rate ($P < 0.02$) and mean arterial pressure. At 2 h after injection, blood pressure was still significantly elevated (Table 1).

Verapamil caused transient atrio-ventricular conduction disturbances, with second degree block in 3 of the 5 dogs, followed by complete block in 2 of these 3. A pronounced fall in blood pressure followed administration of the drug in all of the dogs ($P < 0.005$), with some recovery before induction of fibrillation 15 min later (Table 1).

Lead II ECG

In the no drug group, the dominant frequency of ventricular fibrillation recorded from lead II was initially 9.9 ± 0.7 Hz. There was a slight increase at 80 s, and then it fell rapidly to 5.2 ± 0.4 Hz (Figure 1).

In the lignocaine-treated animals (mean plasma concentration 5.0 ± 0.5 $\mu g\,ml^{-1}$; Figure 1), the dominant frequency was significantly lower than that in the no drug group for the first 80 s ($P < 0.05$), beginning at 7.25 ± 0.54 Hz. Subsequent frequencies of fibrillation were not significantly lower than in the no drug group.

Propranolol had no significant effect on the time course of the frequency of ventricular fibrillation in lead II (mean plasma concentration 117.8 ± 22 $ng\,ml^{-1}$; Figure 1).

Pretreatment for 2 h with bretylium was also without effect on the initial dominant frequency of fibrillation in lead II, but prevented the subsequent rise in frequency at 80 s ($P < 0.05$; Figure 1). It had no apparent effect after this time.

While the dominant frequency of the verapamil-treated group was similar to the no drug group for the first 40 s, verapamil prevented the rapid fall in frequency seen in these control dogs after this time. Later there was a significant rise in frequency to 14.7 ± 0.9 Hz ($P < 0.01$; Figure 1).

Endocardial ECG

The dominant frequency of fibrillation recorded from the right ventricular endocardium in the no drug dogs was similar to that in lead II for the first 80 s. However, after this time the frequency did not fall rapidly as in lead II, and remained above 9 Hz (Figure 2).

Lignocaine significantly reduced the dominant frequency of endocardial fibrillation at 40–200 s ($P < 0.05$; Figure 2). Moreover, there was a fall in frequency with time which was not present in the no drug group.

Propranolol had no significant effect on endocardially-recorded ventricular fibrillation (Figure 2). As in the no drug group, the dominant frequency was well maintained.

Table 1 Body and heart weights, heart rates and arterial blood pressures before and after drug, and plasma potassium concentrations at the onset of ventricular fibrillation in the 5 groups of dogs

	Body weight (kg)	*Heart weight* (g)	*Heart rate* (min^{-1}) *Pre-drug*	*Pre-VF*	*Arterial BP* (mmHg) *Pre-drug*	*Pre-VF*	*Plasma* K^+ (mM)
No drug	25.3 ± 1.0	272 ± 12	131 ± 18	131 ± 18	122 ± 12	122 ± 12	3.8 ± 0.2
Lignocaine	28.1 ± 1.4	331 ± 25	107 ± 7	129 ± 10*	114 ± 8	110 ± 9	4.2 ± 0.3
Propranolol	27.0 ± 0.9	292 ± 22	124 ± 9	110 ± 6*	123 ± 7	123 ± 8	3.9 ± 0.1
Bretylium	27.1 ± 1.5	315 ± 21	109 ± 13	123 ± 21	125 ± 8	166 ± 8**	3.7 ± 0.1
Verapamil	28.8 ± 2.7	318 ± 27	102 ± 17	91 ± 8	120 ± 11	90 ± 9*	3.7 ± 0.2

The arterial blood pressures after bretylium ($P < 0.01$) and verapamil ($P < 0.05$) were the only significant differences from values for the control No Drug group. Significant within-group differences between pre- and post-drug values are indicated (* $P < 0.05$; ** $P < 0.01$).

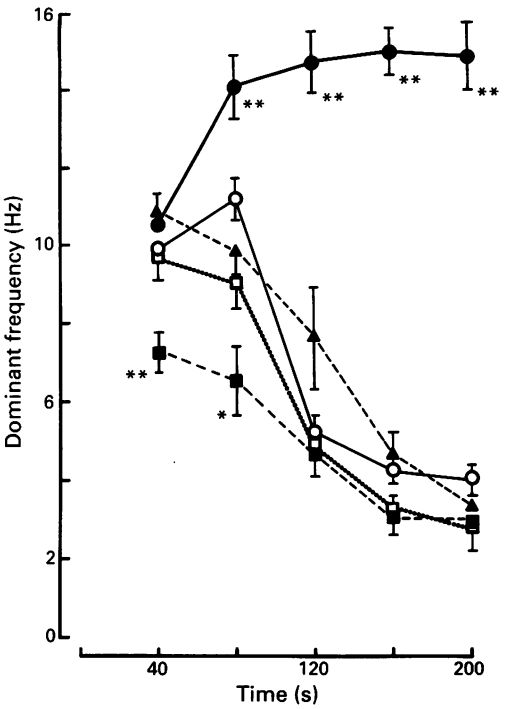

Figure 1 The change with time of the dominant frequency of electrically-induced ventricular fibrillation, recorded from lead II, in dogs receiving no drug (○) and in dogs pretreated with lignocaine (10 mg kg^{-1}, ■), propranolol (0.4 mg kg^{-1}, ▲), bretylium (10 mg kg^{-1}, □) or verapamil (1 mg kg^{-1}, ●). The significances of differences from values in the no drug group is shown: * $P < 0.05$; ** $P < 0.01$.

Bretylium reduced the dominant frequency recorded from the endocardium after fibrillation for 160 s ($P < 0.01$). Before this time it had no apparent effect (Figure 2).

Verapamil had no effect on the initial frequency of fibrillation in the endocardium, but significantly increased the frequencies at all times at 80–200 s ($P < 0.02$; Figure 2). Although endocardial and lead II fibrillation in the dogs given no drugs had quite different time courses, this difference was not seen in the animals treated with verapamil.

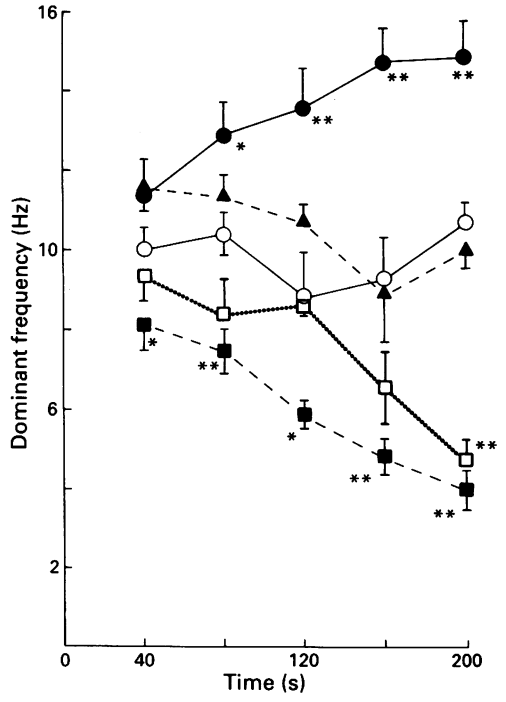

Figure 2 The change with time in dominant frequency of electrically-induced ventricular fibrillation, recorded on a right ventricular endocardial lead in Groups 0–4. (○) No drug; (●) verapamil; (■) lignocaine; (▲) propranolol; (□) bretylium. Note that with no drug the frequency was maintained.

The ratio of the peak frequency in lead II as a % of the peak frequency in the simultaneous endocardial recording was used to express the degree of heterogeneity in the myocardium. In the dogs given no drugs the initial value of 104 ± 2% fell to 61 ± 14% after 3.3 min ($P < 0.01$). Similar values were obtained in the dogs receiving propranolol (initial 95 ± 3, final 34 ± 7: $P < 0.01$) and bretylium (initial 99 ± 3, final 37 ± 3: $P < 0.01$). These data contrast with those in the dogs given verapamil, where the initial ratio (92 ± 4%) did not change significantly with time (100 ± 4% after 3.3 min). Since lignocaine depressed frequencies in both lead II and the endocardium, these ratios did not change significantly (90 ± 3 initially, 85 ± 26 after 3.3 min).

Discussion

In this study, it was found that the frequency characteristics of ventricular fibrillation were greatly altered by lignocaine and verapamil, but changed little by propranolol and bretylium. The effects of each drug on endocardial fibrillation were generally similar to the effects on fibrillation recorded from lead II. Large doses of drugs were deliberately used, and this was reflected in their haemodynamic effects. However, the plasma concentrations of lignocaine and propranolol just after the onset of fibrillation were in the therapeutic range (Rosen *et al.*, 1975; Coltart & Shand, 1970).

Lignocaine

In the present study, lignocaine reduced the dominant frequency of ventricular fibrillation recorded at the body surface for the first 80 s, but had no significant effect thereafter. This suggests that the electrical activity in ventricular fibrillation depends initially on mechanisms which can be depressed by lignocaine, namely the conductance of the fast sodium channels in the cardiac cell membrane (Singh & Vaughan Williams, 1971). Prolongation of the effective refractory period and slowing of conduction by lignocaine are the likely physiological mechanisms. Its lack of effect after 80 s, when the frequency is lower in the untreated animals, suggests that other mechanisms associated with global myocardial ischaemia have caused depression of fast channel activity by this time. It is unlikely that the effect of lignocaine was inhibited, as it exerts greater effects in the presence of hyperkalaemia (Singh & Vaughan Williams, 1971), and ischaemia (Kupersmith, 1979).

In the endocardium, lignocaine significantly reduced the dominant frequency throughout the 3 min period of analysis. Thus during ventricular fibrillation, fast sodium channels remain of importance in the endocardium for at least 3 min, longer than in the myocardium. Fast channel activity may continue in the Purkinje fibre network of the endocardium due to slower metabolic deterioration, from a smaller metabolic rate, large glycogen stores, or perhaps direct oxygenation from the ventricular blood (Worley *et al.*, 1985). Further studies are required to confirm that these effects are possessed by other Class 1 drugs.

Propranolol

Circulating catecholamine levels are some 4–9 times higher at the onset of ventricular fibrillation than after myocardial infarction (Little *et al.*, 1985), giving some basis for expectations that β-adrenoceptor blocking drugs would slow the dominant frequency of fibrillation. Furthermore pretreatment with propranolol is known to reduce myocardial oxygen consumption, and myocardial necrosis after coronary artery ligation (Reimer *et al.*, 1973). However, propranolol had little effect on the time course of fibrillation in these experiments.

Both the dose of propranolol and the plasma concentrations ($118 \pm 22\,ng\,ml^{-1}$) are sufficient to cause β-adrenoceptor blockade (Coltart & Shand, 1970; Taggart *et al.*, 1984). Inhibition of fast sodium channels by β-adrenoceptor blocking drugs (class I effects) occurs at concentrations some 30 times those used in the present study (Davis & Temte, 1968), and propranolol has no class III action after acute administration (Taggart *et al.*, 1984).

Bretylium

The benzyl quaternary ammonium compound, bretylium tosylate has a number of actions on the heart. Class III antiarrhythmic properties (Vaughan Williams, 1984) increase the action potential duration and the effective refractory period of canine ventricular muscle and Purkinje fibres in the dog heart (Bigger & Jaffe, 1971). After administration noradrenaline is released from peripheral adrenergic nerve endings (Gilmore & Siegel, 1962), with a marked rise in heart-rate and blood-pressure. In the present study pretreatment with bretylium prevented the increase in the dominant frequency of ventricular fibrillation occurring at 80 s, but otherwise had no significant effect on ventricular fibrillation recorded from lead II. Bretylium, like propranolol, had little effect on endocardial fibrillation, and demonstrated no effects attributable to a Class 3 drug. Since bretylium ($4–6\,mg\,kg^{-1}$, i.v.) is one of the few drugs reported to be capable of chemical defibrillation in man (Sanna & Arcidiacono, 1973), its lack of effect in our study was surprising.

Verapamil

Verapamil, a calcium channel blocker, had striking effects on the dominant frequency of ventricular fibrillation, preventing the rapid fall in frequency seen in the untreated dogs after 80 s of fibrillation. Indeed, there was a rise in dominant frequency to almost 14 Hz at 3 min in recordings from both Lead II and the endocardium.

What is the explanation for these effects? Low concentrations of verapamil ($0.5\,mg\,l^{-1}$) reduced the duration of the action potential during rapid stimulation in the canine papillary muscle (Hirata *et al.*, 1979). A reduction in action potential duration induced by verapamil could lead to a shorter refractory period, faster conduction and an increase in the dominant frequency of ventricular fibrillation. In open chest dogs, verapamil increased the conduction velocity and reduced the delay in activation time caused by myocardial ischaemia (Elharrar *et al.*, 1977). In the ischaemic pig heart, verapamil also reduced the release of K^+, delayed the onset of slow conduction, and often prevented conduction block (Fleet *et al.*, 1986).

It may be proposed that these electrophysiological changes determine the effect of verapamil on the dominant frequency of ventricular fibrillation. However, this is unlikely to be the important mechanism. An effect on the dominant frequency of ventricular fibrillation resulting from action potential shortening due to pretreatment with verapamil should be evident at the very beginning of ventricular fibrillation, and not just after 80 s. Furthermore, nifedipine, which is virtually devoid of electrophysiological effects on the heart (Singh & Nadamanee, 1982) also increases the dominant frequency of ventricular fibrillation in a fashion similar to verapamil (Martin *et al.*, 1986).

This suggests that the main action of verapamil is to prevent calcium accumulation. After verapamil ($2\,\mu M$) Vaughan Williams (1984) has observed a faster rate of rise at the onset of the action potential, indicative of improved cell-to-cell coupling. It is well established that cardiac intercellular nexuses are uncoupled by a raised intracellular calcium concentration (Dahl & Isenberg, 1980), and that this may follow reduced activity of the Na^+-K^+ pump (De Mello, 1976). Calcium overload may also be an important primary or secondary agent in ischaemic myocardial damage (Cheung *et al.*, 1986), as calcium-channel blocking drugs reduce the extent of myocardial damage from acute regional (Reimer *et al.*, 1977) and global ischaemia (Clark *et al.*, 1977).

What are the clinical implications if intracellular calcium overload is responsible for the rapid fall in the dominant frequency of ventricular fibrillation after 80 s? This time has already been noted as critical for recovery from fibrillation. In dogs, if the heart was defibrillated before 60–90 s recovery was usual, but after 90 s of fibrillation the animals invariably died (Geuze & DeVente, 1983; Martin *et al.*, 1986). If the chance of defibrillation success is indeed related to the dominant frequency of ventricular fibrillation, then frequency analysis of fibrillation may be useful in future research on resuscitation. However, the negative inotropic effects of verapamil may militate against widespread use of this drug in clinical cardiac arrest.

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Cigarette smoke, nicotine and capsaicin aerosol-induced vasodilatation in pig respiratory mucosa

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1 Anaesthetized pigs were used to study vascular responses in the sphenopalatine artery (SPA), superior laryngeal artery (SLA) and bronchial artery (BA) upon exposure to cigarette smoke or aerosol of nicotine and capsaicin. Direct blood flow recordings were made with ultrasonic probes around the vessels.

2 Smoke from one cigarette was administered as inhalation for 2 min with or without a Cambridge filter which removes the particulate matter including nicotine from the smoke. Aerosols of nicotine (2.5 mg) or capsaicin (10 mg) were administered to the nose or the lower airways for 3 min.

3 Cigarette smoke exposure caused a reproducible reduction of the vascular resistance (VR) suggesting vasodilatation in the SPA, SLA, and especially the BA. The vasodilatation was not modified by the Cambridge filter, suggesting that it was caused by vapour phase components rather than nicotine.

4 The smoke effect was not changed after pretreatment with the cyclo-oxygenase inhibitor, diclofenac, or with atropine, guanethidine, H_1- or H_2-histamine receptor antagonists, nedocromil, or by vagotomy. The smoke-evoked decrease in VR was not modified by the nicotinic receptor antagonist chlorisondamine in the SLA or BA.

5 In pigs pretreated with increasing doses of capsaicin two days earlier, the decrease in VR upon smoke exposure in both the BA and SLA was unaffected while the change in VR was attenuated in the SPA.

6 Nicotine aerosol had no effect on VR in the peripheral airways supplied by the BA while a decrease in VR was observed in the SLA and SPA. The nicotine response was reduced after capsaicin pretreatment in the nasal and upper tracheal circulation.

7 Capsaicin aerosol reduced VR in the vascular beds supplied by the SPA, SLA and BA and this response was markedly reduced after capsaicin pretreatment.

8 The mechanisms underlying vasodilatation upon cigarette smoke exposure in the bronchial mucosa are at the moment unclear while both non-cholinergic parasympathetic and sensory components may be involved in the nose. Capsaicin induced a vasodilatation at all levels via sensory mechanisms, whereas nicotine-evoked vasodilatation is restricted to the upper airway mucosa and is at least partly dependent on parasympathetic reflexes involving capsaicin-sensitive sensory nerves.

Introduction

Inhaled irritants in the upper airway provide triggering responses to defence reflexes that include coughing and sneezing. These effects are potentiated when irritants reach the lower airways (Widdicombe, 1977; Coleridge & Coleridge, 1984). Furthermore, responses to different stimuli vary at diverse levels of the respiratory tract (Sheppard, 1988). Thus, rapid adaptating receptors in the larynx and trachea are particularly sensitive to particle irritants such as cigarette smoke rather than to chemicals (Widdicombe, 1986). Intrapulmonary irritant receptors are stimulated by gases such as sulphur dioxide and ozone and also by several inflammatory mediators (Sampson & Vidruk, 1975; Widdicombe, 1988). Stimuli that excite the irritant receptors also cause bronchoconstriction and tracheal mucous secretion (Coleridge & Coleridge, 1986). These effects are mediated by the vagus nerve (Widdicombe, 1988), as well as local endings of C fibre afferents which are present in the respiratory tract (see Lundberg *et al.*, 1988a). The C fibres are sensitive to capsaicin and activated by almost the same group of stimuli as those that affect irritant receptors (Coleridge & Coleridge, 1986). They are also stimulated by inhaled irritants such as the vapour phase of the cigarette smoke in the nasal mucosa (Lundblad & Lundberg, 1984) as well as the rat tracheal mucosa (Lundberg & Saria, 1983). Local nicotine administration also induces protein extravasation in the trachea most likely by the stimulation of nicotinic receptors on sensory nerves (Lundberg *et al.*, 1983, see Saria *et al.*, 1988). Nicotine-evoked sneezing (Lundblad *et al.*, 1984b) or coughing (Forsberg *et al.*, 1988) is not, however, mediated via activation of capsaicin sensitive afferents. Nevertheless, the vascular effects of cigarette smoke exposure in the airways have not yet been directly studied although a classical sign of smoke exposure in the eyes is conjunctival reddening suggesting hyperaemia.

In the present investigation, the nasal, tracheal and bronchial vascular resistance (VR) responses were therefore compared in the pig upon inhalation of cigarette smoke as well as nicotine or capsaicin aerosol.

Methods

Surgical procedure

Twenty pigs (25–30 kg body weight) were fasted overnight and premedicated with ketamine hydrochloride (20 mg kg^{-1}, i.m.). Anaesthesia was then induced by pentobarbitone (20 mg kg^{-1}, i.v.) and maintained by a continuous i.v. infusion of pentobarbitone (3 mg kg^{-1} h^{-1}). After establishment of anaesthesia as revealed by loss of nociceptive reflexes such as evoked by pinching the nipple or ear, a tracheostomy was made and the pigs were intubated and artificially ventilated with a mixture of air and oxygen using an Engström respirator, model 150. For muscle relaxation, pancuronium bromide (0.25 mg kg^{-1}, i.v.) was given intermittently. The body temperature was kept between 38–39°C by use of a heating pad connected to a thermostat.

Briefly (for details on the surgical approach see Matran *et al.*, 1989a), the right femoral vessels were cannulated for continuous recordings of mean arterial blood pressure (MAP),

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heart rate (HR) and for intravenous administration of Ringer solution and heparin (5000 i.u.). After partial right mandibulectomy, the sphenopalatine artery (SPA) was dissected free, and a probe was placed around the vessel and connected to a T 202S ultasonic blood flowmeter (Transonic system Inc., NY, U.S.A.). The bronchial (BA) and the right superior laryngeal (SLA) arteries were also exposed and similar flow probes as above were placed around the vessels.

Arterial blood gas partial pressures were regularly monitored with an automatic blood gas analyser (IL 1302, Metric AB, Solna, Sweden). Pulmonary air flow was measured with a heated Fleisch pneumotachograph Model No. 1 connected to a Statham PM15 E pressure transducer and tracheal pressure was measured with a Statham PM131·TC pressure transducer and was used as the transpulmonary pressure due to the thoracotomy. The signals were analysed by an E 80 T pulmonary computer (Processdata AB, Uppsala, Sweden).

Experimental procedure

A Walton Horizontal Smoking Machine (Guerin *et al.*, 1979) was used to expose animals continuously to cigarette smoke during 2 min or intermittently for 2 s every 10 s. In the same animal, smoke exposure was continued for an extended period or intermittently repeated with a Cambridge glass fibre filter, inserted into the smoke stream, which removed the particulate portion of the smoke and therapy >99% of the nicotine content (Wartman *et al.*, 1959; Coggins *et al.*, 1980). Smoke exposure was given with Kentucky 2R1 standard cigarettes which deliver smoke with a known composition: tar 42 mg, nicotine 2.5 mg and carbon monoxide 25 mg per cigarette. Smoke was delivered to the nose via a tube inserted into the right nostril or to the lung via the respirator. In some experiments ($n = 5$), the reproducibility of the vascular response in the same animal was tested by repeating the cigarette smoke exposure with and without filter three times with 10 min intervals. To analyse the mechanism of cigarette smoke-induced vasodilatation the experiments ($n = 6$) were also done after pretreatment with guanethidine (3 mg kg^{-1}, i.v.) to reveal a possible influence of sympathetic mechanisms, atropine (1 mg kg^{-1}, i.v.) to block the parasympathetic muscarinic cholinoceptor influence or chlorisondamine (3 mg kg^{-1}, i.v.) to inhibit the nicotinic receptor transmission in autonomic ganglia (Matran *et al.*, 1989a). To block neuronal mechanisms including the stimulation of sensory irritant or nicotinic receptors, smoke exposure was also carried out after aerosol application of the local anaesthetic substance lignocaine (40 mg in 2 ml NaCl) ($n = 6$) or nedocromil (60 mg in 3 ml NaCl) ($n = 6$) given to the lung for 3–4 min with an ultrasonic nebulizer (Engström Medical). Because stimulation of rapidly adapting receptors causes pulmonary effects by a reflex increase in vagal efferent activity (see Widdicombe, 1988), experiments ($n = 5$) were also carried out after sectioning of both cervical vagal nerves. Furthermore, six pigs were pretreated with diclofenac (3 mg kg^{-1}, i.v.) to block a possible release of vasodilator prostaglandins or with a combination of the H_2-histamine antagonist, cimetidine (10 mg kg^{-1}) and the H_1-histamine antagonist, terfenadine (2 mg kg^{-1}) (Alving *et al.*, 1989).

Two days after systemic pretreatment with capsaicin, cigarette smoke exposure was also carried out. After anaesthesia (see above) 10 animals were intubated and artificially ventilated. When they were anaesthetized (see above) increasing doses of capsaicin (total dose 50 mg kg^{-1}) were administered by slow (about 3 ml min^{-1}) injection given subcutaneously (four sites on each side) over 4 h. Then, 2 h after the last injection the animals were allowed to regain spontaneous breathing and the tubes removed. They were regularly monitored during a further 2 h period. No difference in the behaviour of the pig was noted in the capsaicin pretreated animals when they regained consciousness compared to a vehicle pretreated group ($n = 10$) used as control. These experiments were approved by the Ethical Committee for animal research at the Karolinska Institute.

Nicotine aerosol (2.5 mg in 2 ml NaCl) and capsaicin aerosol (10 mg in 1 ml 60% ethanol), were given to the nose and the lung by use of an ultrasonic nebulizer in 9 control pigs and after pretreatment with capsaicin. Ethanol (60%) did not induce any vascular reactions. Furthermore, intravenous injection of nicotine (250 μg kg^{-1}) was performed in five controls and five chlorisondamine pretreated animals.

Apart from the pretreatment with capsaicin, the pigs were killed by an overdose of pentobarbitone at the end of the experiment.

Calculations

Change in blood flow in the vascular beds supplied by the SPA, SLA and BA following injections were defined as both peak changes in vascular resistance (VR) and the total integrated changes in ml from basal values. VR was calculated as the MAP divided by the blood flow in the supplying artery (Daly & Hebb, 1966). Data are given as mean ± s.e.mean and statistical differences were estimated by Student's paired t test or Mann Whitney U-test. Differences were considered to be significant when $P < 0.05$.

Drugs

The following (source in parentheses) were used: ketamine hydrochloride (Ketalar, Parke-Davis, U.S.A.), pentobarbitone (Mebumal) and atropine (ACO, Solna, Sweden), pancuronium bromide (Pavulon, Organon, The Netherlands), guanethidine, chlorisondamine and diclofenac (Ciba-Geigy, AG, Basel, Switzerland), clonidine and terfenadine (Sigma Co., St Louis, U.S.A.), nicotine bitartrate (Swedish Tobacco Company, Sweden), capsaicin (Fluka Chemie AG, Switzerland), lidocaine (Astra, Södertälje, Sweden) and nedocromil (Fisons, Leicestershire, England).

All the substances except capsaicin and terfenadine were dissolved in saline solution. Capsaicin was initially dissolved in 10% ethanol and 20% Tween 80 for subcutaneous injection or in 60% alcohol for aerosol challenge and subsequently diluted in saline solution. Terfenadine was dissolved in 60% ethanol.

Results

Response to cigarette smoke exposure

Continuous cigarette smoke inhalation for 2 min caused a long lasting increase (5 to 7 min) in blood flow in the vascular beds supplied by sphenopalatine (SPA), superior laryngeal (SLA) and bronchial (BA) arteries (Figures 1a, 2a) with the largest decrease in VR (60%) suggesting vasodilatation in BA (Table 1). There was no significant difference in the total integrated blood flow increase (Figure 3) or the peak decrease in VR (Table 1) upon smoke exposure in the presence of the Cambridge filter in any of the vascular beds. Furthermore, MAP and HR were not changed upon cigarette smoke inhalation with filter while MAP and HR were increased by 82% and 25%, respectively, in 2 pigs out of 10 during the inhalation of smoke without filter. The smoke-evoked peak decrease in VR or the integrated blood flow increase was not modified by capsaicin pretreatment in SLA and BA (Figures 1b, 2b, 3 and Table 1). In contrast, although the peak VR change was uninfluenced by capsaicin pretreatment (Table 1), the local integrated blood flow increase was reduced in the SPA both in the presence ($P < 0.01$) and in the absence ($P < 0.05$, Mann Whitney U test) of the Cambridge filter (Figures 1b, 3). Cigarette smoke-induced vasodilatation was reproducible at short intervals in BA (Figure 4). Intermittent exposure to cigarette smoke also provoked a clear cut increase in blood flow in BA which was not reduced by capsaicin pretreatment (Figure 5).

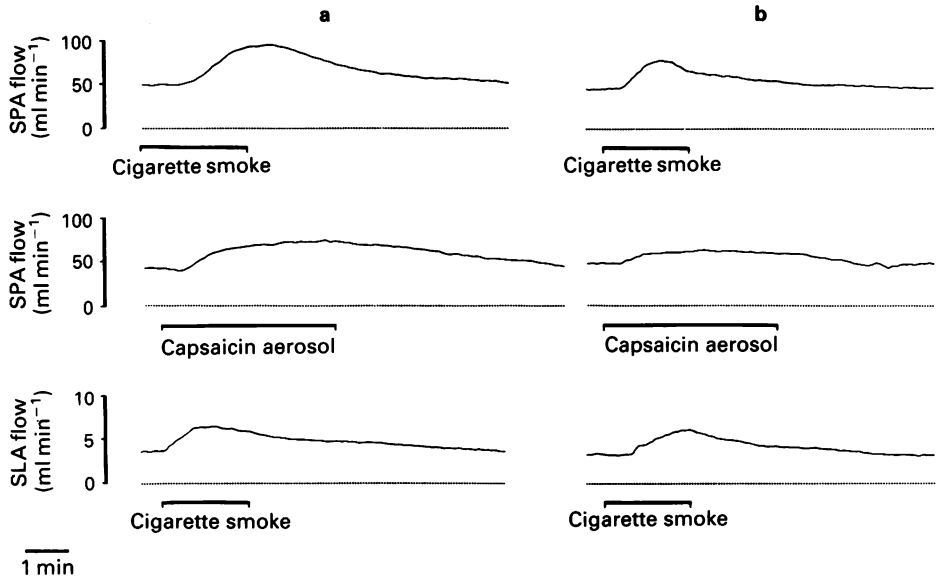

Figure 1 Typical example of changes in nasal (sphenopalatine artery flow, SPA flow) or laryngeal and upper tracheal blood flow (superior laryngeal artery flow, SLA flow) caused by cigarette smoke exposure (without filter) and capsaicin aerosol (10 mg) in controls (a) and after systemic pretreatment with capsaicin (b). Time scale for 1 min is indicated by bar.

Lignocaine or nedocromil aerosol in the lung and bilateral vagotomy had no effect on the smoke induced decrease in VR of BA (Table 2). Intravenous injection of chlorisondamine lowered the basal blood flow in the vascular beds supplied by SLA and BA but this drug also decreased the MAP and consequently the smoke evoked change in VR was not modified (Table 2). On the other hand, chlorisondamine pretreatment increased basal SPA blood flow from 38 ± 9 to 112 ± 23 ml min^{-1} and decreased MAP from 138 ± 18 to 91 ± 10 mmHg. No decrease in the vasodilator response was

Table 1 Peak decrease in vascular resistance in control and systemically capsaicin-pretreated pigs exposed to cigarette smoke without (−) or with (+) Cambridge filter or aerosol of nicotine (2.5 mg) and capsaicin (10 mg)

		Cigarette smoke		*Nicotine aerosol*		*Capsaicin aerosol*	
	Cambridge filter	*Control*	*Capsaicin pretreatment*	*Control*	*Capsaicin pretreatment*	*Control*	*Capsaicin pretreatment*
Sphenopalatine artery	+	35.7 ± 3.5	29.2 ± 3.0				
	−	42.6 ± 4.1	35.8 ± 2.6	17.5 ± 3.6	1.3 ± 1.1**	34 ± 6.6	10.6 ± 4.0***
Superior laryngeal artery	+	34.0 ± 8.2	36.1 ± 6.1				
	−	40.7 ± 6.8	42.8 ± 7.4	22.8 ± 3.9	10.7 ± 4.6*	33 ± 7.7	21 ± 9.8
Bronchial artery	+	51.0 ± 5.4	38.0 ± 5.3				
	−	60.4 ± 4.5	ND	0	0	24 ± 8.4	4.1 ± 2.1**

Data are given as mean ± s.e.mean and expressed as percent change compared to control conditions. $n = 6$–10, $^{**}P < 0.01$, $^{***}P < 0.001$ by Mann Whitney U test. ND = not determined.

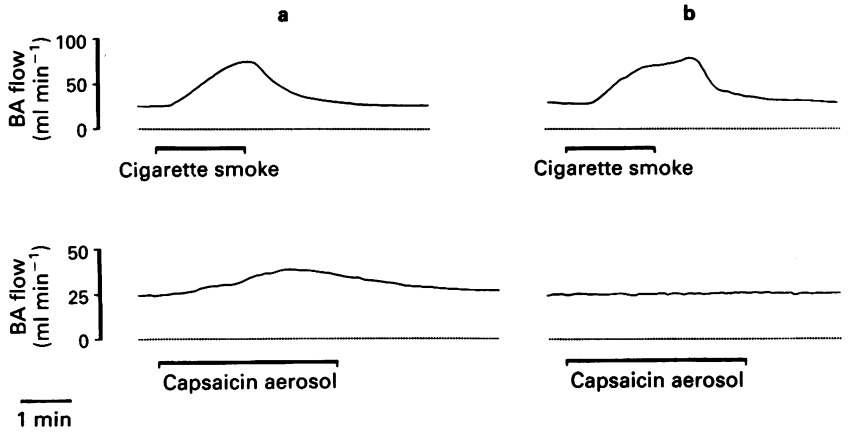

Figure 2 Original tracings showing the effects of cigarette smoke exposure without filter and capsaicin aerosol (10 mg) on bronchial arterial blood flow (BA flow) in controls (a) and after systemic pretreatment with capsaicin (b). Time scale for 1 min is indicated by bar.

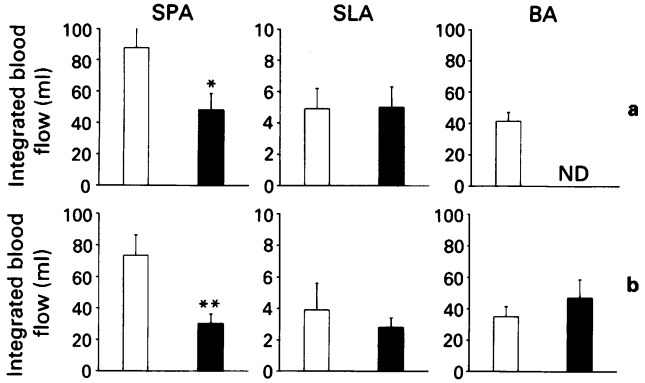

Figure 3 Integrated blood flow changes in ml of the nasal (sphenopalatine artery, SPA), laryngeal and upper tracheal (superior laryngeal artery, SLA) and bronchial (BA) circulation after exposure to cigarette smoke (a) without or (b) with Cambridge filter. Data are given as mean values, open columns indicate control, filled columns systemically capsaicin-pretreated animals; vertical bars show s.e.mean. $*P < 0.05$, $**P < 0.01$ compared to response in controls by Mann Whitney U test. ND = not determined.

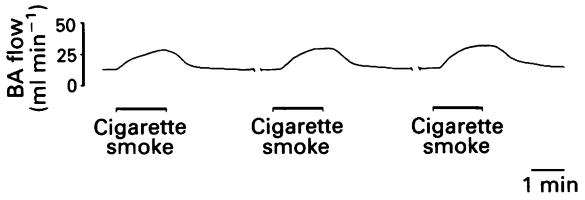

Figure 4 Original tracing showing the effect of smoke exposure on the bronchial blood flow (BA flow) when repeated three times with 10 min intervals in the same animals. Time scale for 1 min is indicated by bar.

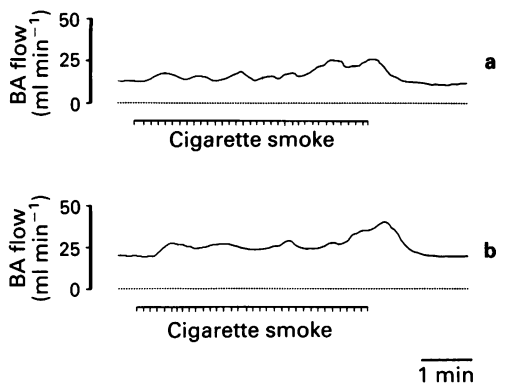

Figure 5 Typical example of changes in bronchial blood flow (BA flow) caused by intermittent exposure to cigarette smoke for 6 min (about 6 puffs per min) as indicated by vertical lines in control (a) ($n = 6$) and capsaicin-treated (b) ($n = 6$) animals. Time scale for 1 min is indicated by bar.

observed upon smoke exposure in SPA after chlorisondamine pretreatment (Table 2). Moreover, pretreatment with diclofenac or antihistamines did not change the smoke response in any of the three vascular beds (Table 2).

Smoke exposure without filter in the lung provoked a bronchodilatation as revealed by reduction of pulmonary resistance by 24.4 ± 5.3% ($P < 0.001$) and increase in dynamic compliance by 15 ± 1.6% ($P < 0.001$ Student's t test, $n = 8$). Although it was absent upon insertion of the Cambridge filter, this bronchomotor response was not modified by pretreatment with capsaicin, chlorisondamine, diclofenac or antihistamines; their respective values were 26 ± 5.3%, 27 ± 9.4%, 21 ± 6.1% and 26 ± 4.0%.

Response to capsaicin

Capsaicin aerosol caused a slowly developing, long lasting increase in blood flow with a peak decrease in VR of 25–30% (Table 1) in SPA, SLA and BA (Figures 1, 2). In the capsaicin pretreated pigs, capsaicin aerosol-evoked vasodilatation was markedly reduced in BA and SPA ($P < 0.01$ and $P < 0.001$, respectively, Mann Whitney U test) while it was lowered in the SLA ($P < 0.05$) (Figure 6, Table 1). Capsaicin aerosol had no effect on MAP or HR in both control and capsaicin pretreated animals. Furthermore, the basal VR in SPA, SLA and BA was not altered by the pretreatment with capsaicin. Pulmonary resistance or dynamic compliance was not changed by capsaicin aerosol.

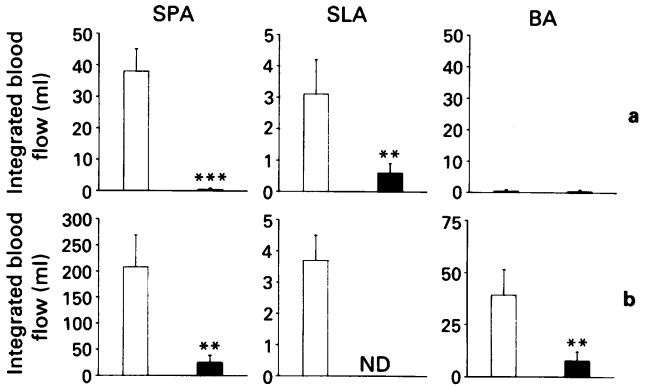

Figure 6 Integrated blood flow changes in ml of the nasal (sphenopalatine artery, SPA), laryngeal and upper tracheal (superior laryngeal artery, SLA) and bronchial (BA) circulation after challenge with aerosol of nicotine (a) or capsaicin (b) in controls (open columns) and systemically capsaicin-treated (filled columns) animals. Data are given as mean with s.e.mean shown by vertical bars. $**P < 0.01$ and $***P < 0.001$ compared to response in controls by Mann Whitney U test. ND = not determinated.

Table 2 Peak decrease in vascular resistance in control and systemically pretreated-pigs with chlorisondamine, diclofenac, antihistamines, nedocromil aerosol, lidocaine aerosol and after bilateral vagotomy (for details see Methods) on the vasodilator effect of cigarette smoke exposure in the presence of Cambridge filter

	Sphenopalatine artery		*Superior laryngeal artery*		*Bronchial artery*	
	Control	*Pretreatment*	*Control*	*Pretreatment*	*Control*	*Pretreatment*
Chlorisondamine	36 ± 10.1	31 ± 8.6	41 ± 7	42.7 ± 7.4	47 ± 8.6	45 ± 6.8
Diclofenac	42 ± 8.6	46 ± 7	48 ± 11.2	40 ± 8.4	51 ± 6.2	60.7 ± 8.8
Antihistamines	44 ± 6.1	38 ± 9.9	36 ± 12.1	42 ± 9.1	49 ± 8.5	49 ± 6.7
Nedocromil	31 ± 11	39 ± 8.1	40 ± 8.7	34 ± 8.1	45 ± 8.4	50 ± 10.1
Lignocaine	—	—	—	—	49 ± 9.2	46 ± 8.5
Bilateral vagotomy	—	—	—	—	50 ± 6.2	54 ± 8.2

Data are given as mean ± s.e.mean and expressed as percent change compared to control conditions. $n = 5$–6. No significant difference by Mann Whitney U test between control and pretreatment.

Response to nicotine

In control pigs, nicotine aerosol also induced a decrease in VR in the nasal and upper tracheal circulation while it had no effect on the peripheral airways supplied by BA (Figure 6, Table 1). In capsaicin pretreated pigs, both the area under the curve of blood flow and the change in VR evoked by nicotine were reduced in SPA ($P < 0.001$) and SLA ($P < 0.01$, Mann Whitney U test) (Figure 6, Table 1). Nicotine aerosol did not influence MAP, HR or the pulmonary calibre. Intravenous injection of nicotine provoked an increase in blood flow in the SPA, SLA and BA (not shown). Since nicotine also increased the MAP, the VR in the airway mucosa as well as pulmonary calibre were not modified by the systemic injection. Pretreatment with chlorisondamine completely blocked the vascular effects of i.v. nicotine (not shown).

The vehicle pretreated group was used as control to establish whether vehicle injection modified vasodilator responses to cigarette smoke, nicotine or capsaicin aerosol. Capsaicin-evoked vasodilatation was not changed in vehicle pretreated pigs compared to non-pretreated pigs. The vasodilator effects of cigarette smoke exposure and nicotine aerosol were not changed compared to control pigs (non-treated pigs). It was clear that vehicle pretreatment did not modify the effects obtained with different irritant substances.

Discussion

The present findings show a clear cut vasodilatation as revealed by decreased VR upon cigarette smoke exposure in the vascular beds supplied by the SPA, SLA and BA. Cigarette smoke can be subdivided into a particulate portion, with tar and nicotine, and a vapour phase containing a variety of gases and irritant chemicals (see Stedman, 1967). It is likely that cigarette smoke-induced vasodilatation is mainly due to components in the vapour phase rather than tar or nicotine considering the inefficiency of the Cambridge filter in modifying the response. Furthermore, nicotine aerosol had no effect on the BA where the smoke-induced vasodilatation was greatest. The effect of nicotine aerosol in the larynx and bronchi is in accordance with results from vagal nerve stimulation in the pig (Matran *et al.*, 1989a) where vasodilator neuronal mechanisms were found to be purely sensory in the bronchi while parasympathetic non-cholinergic and cholinergic mechanisms involving receptor transmission dominated in the larynx. Many foreign irritants such as dust, ammonia vapour, ether and cigarette smoke evoke irritant reflexes from the respiratory tract (Coleridge & Coleridge, 1984). Tracheal insufflation of powdered talc in cats (Widdicombe, 1954) and of carbon dust in rabbits (Sellick & Widdicombe, 1971) stimulate irritant receptors while the effect of inhaled chemical irritants are less consistent. Furthermore, these irritant substances also cause laryngeal constriction, bronchoconstriction and mucus secretion (Widdicombe, 1964). In our model, cigarette smoke exposure was associated with macroscopical signs of nasal and tracheobronchial secretion as revealed by visual inspection. Unexpectedly, cigarette smoke exposure without filter provoked bronchodilatation in the lung. Since smoke with filter did not evoke any bronchial response, either smoke particles or nicotine may be involved in the smoke-induced bronchodilatation. Vagal activation may not only induce bronchoconstriction but also bronchodilatation (see Diamond *et al.*, 1983) presumably via non-adrenergic, non-cholinergic (NANC) mechanisms which are dependent on nicotinic receptor transmission in local airway ganglia. Alternatively nicotine may activate the sympatho-adrenal system and release catecholamines which have bronchodilator actions, Such an effect is also supported by the slight tachycardia and increase in MAP in a few animals upon smoke exposure without, but not with, the filter; however, this sympatho-adrenal response should have been abolished by chlorisondamine. Furthermore, the bronchodilatation induced by cigarette smoke was not abolished by capsaicin pretreatment, suggesting that sensory C-fibres were not involved. Since cigarette smoke in man is generally assumed to provoke bronchoconstriction dependent on smoke particles activating a vagal cholinergic reflex (Sterling, 1967), the present data from anaesthetized pigs, where cigarette smoke-evoked bronchodilatation may have resulted from the vagal reflexes being attenuated by anaesthesia while the local effect was still intact. Since nicotine aerosol did not change bronchial calibre, other components of the smoke may be of relevance for this reaction.

The cigarette smoke-induced vasodilatation in BA was not abolished by the local anaesthetic lignocaine, by bilateral vagotomy or atropine and chlorisondamine, suggesting that irritant receptors via an afferent vagal pathway are less likely to be involved.

Since the C-fibres are activated by almost the same group of stimuli as those that affect myelinated irritant receptors (Coleridge & Coleridge, 1986), the atropine-resistant vasodilatation in the airway mucosa upon cigarette smoke exposure might be mediated via a capsaicin-sensitive mechanism. In a recent study, we have shown that local blood flow in the upper trachea (SLA) is regulated by both cholinergic and non cholinergic parasympathetic, as well as capsaicin-sensitive sensory components, whereas the vagal control of the bronchial circulation (BA) seems to involve exclusively capsaicin-sensitive sensory nerves (Matran *et al.*, 1989a). Activation of C-fibre afferents by irritant chemicals including nicotine induces a release of multiple tachykinins (Saria *et al.*, 1988) and calcitonin gene-related peptide (CGRP) (Martling *et al.*, 1988) from the lung. These peptides are potent vasodilator agents (see Lundberg & Saria, 1987) and both substance P (SP) and CGRP mimic capsaicin-induced vasodilatation in the tracheobronchial circulation of the pig (Matran *et al.*, 1989b). However, in capsaicin-pretreated pigs, the vasodilatation induced by cigarette smoke was not modified in BA and SLA in spite of the fact that the capsaicin aerosol-induced vasodilatation was strongly reduced, suggesting that sensory nerves were not involved in the smoke response.

On the other hand, cigarette smoke-induced vasodilatation was lowered in the SPA in capsaicin-treated animals. These data are in agreement with earlier work on guinea-pigs which demonstrated that smoke-induced irritation is primarily due to activation of capsaicin-sensitive sensory nerves in the nasal mucosa by vapour phase components (Lundbland & Lundberg, 1984). It may therefore be postulated that axon reflex activation by cigarette smoke can evoke local release of neuropeptides such as SP or CGRP in the nasal mucosa. However, our data, which show that systemic pretreatment with capsaicin did not induce a total loss of response to irritant substances in the nasal mucosa, suggest the possibility that cigarette smoke not only activates capsaicin sensitive C-fibres but may also stimulate other types of chemosensitive afferents. Since the decrease in nasal VR upon exposure to smoke was not influenced by the Cambridge filter, however, it is likely that vapour phase components rather than nicotine activate afferent nerves with subsequent parasympathetic reflexes and decrease in nasal VR. Since atropine did not influence the smoke response, parasympathetic, non-cholinergic nasal vasodilator mechanisms (see Lundberg, 1981) may be activated by smoke.

Irritant substances such as cigarette smoke may also result in release of histamine and various prostanoids, which are known to provoke vasodilatation. However, cyclo-oxygenase inhibition with diclofenac, antihistamines, such as cimetidine and terfenadine, or mast cell stabilization by nedocromil did not influence the acute response induced by cigarette exposure. Since diclofenac does not inhibit release of other mediators, such as lipoxin A which also causes arteriolar dilatation (Dahlen *et al.*, 1987), this issue has to be studied further.

The nicotine aerosol-induced vasodilatation in the SPA and SLA was strongly reduced after capsaicin pretreatment, suggesting a mechanism that is dependent on capsaicin-sensitive

neurones in accordance with earlier works on protein extravasation (Lundberg & Saria, 1983; Lundberg *et al.*, 1988b). Nicotine as well as capsaicin aerosol did not induce a nasal hypertensive reflex in the anaesthetized control pigs while intravenous injection of nicotine provoked an increase in MAP and HR. This nasal cardiovascular reflex following chemical irritation is mediated via capsaicin-sensitive fibres in anaesthetized guinea-pigs (Lundblad *et al.*, 1984a), whereas the sneezing response to nicotine is not affected by capsaicin pretreatment in conscious animals (Lundblad *et al.*, 1984b). Thus nicotine should activate both irritant receptors on myelinated nerves and C fibre afferents in the nasal mucosa. However, the absence of hypertensive response may be explained by the anaesthesia with pentobarbitone which blocked the central nervous system transmission of the nasal reflex.

The absence of nicotine-evoked changes in BA may be explained either by differences in the nervous vascular control between the bronchial mucosa on one hand and the upper tracheal and nasal mucosa (see Matran *et al.*, 1989a) or alternatively that much higher local concentrations of nicotine are required to elicit vasodilatation. The nicotine dose used should, however, be comparable with the composition of the standard cigarette (2.5 mg). Nevertheless, the nicotine component in the smoke does not seem to be of importance for the vasodilatation upon cigarette smoke exposure either in the lung or the nose. This finding is in contrast to the pulmonary reflexes elicited by cigarette smoke inhalation in dogs where the nicotine component is essential (Lee & Morton, 1986). The protective cough reflex upon nicotine inhalation in the guinea-pig is not, however, mediated via capsaicin-sensitive sensory nerves (Forsberg *et al.*, 1988).

In conclusion, cigarette smoke exposure provokes marked vasodilatation in the pig airway mucosa. Our results show that the local blood flow increase in the nose produced by cigarette smoke is mediated at least partially by parasympathetic and sensory components. However, in the peripheral airways supplied by BA the mechanisms underlying the vasodilatation upon cigarette exposure are unclear. Other mechanisms including direct actions of vapour phase components in the smoke (Stedman, 1967) should therefore be investigated.

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A study of the pyrogenic actions of interleukin-1α and interleukin-1β: interactions with a steroidal and a non-steroidal anti-inflammatory agent

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1 The pyrogenic effects of intravenously administered human recombinant interleukin-1α (IL-1α) and IL-1β were studied in the rabbit.

2 Both cytokines produced dose-related increases in body temperature. At all doses studied (100–5000 u kg^{-1}) both cytokines elicited a monophasic increase in body temperature, beginning 15 min and reaching a maximum 45 min after administration.

3 A comparison of thermal response index (TRI_2, the magnitude of febrile responses over 2 h obtained by integrating the change in temperature in °C against time in hours) values indicated that IL-1β (500 u kg^{-1}, $TRI_2 = 0{\cdot}69 \pm 0{\cdot}04$, $n = 4$) was approximately 5 fold more potent than IL-1α (2500 u kg^{-1}, $TRI_2 = 0{\cdot}73 \pm 0{\cdot}07$, $n = 4$, all values are means ± s.e.means) in elevating body temperature. ΔT_{max} values for the above doses of IL-1β and IL-1α were 0.60 ± 0.06 and 0.61 ± 0.03 respectively. When IL-1α and IL-1β were heated for 30 min at 60°C prior to administration no biological activity was observed.

4 A cyclo-oxygenase inhibitor, ketoprofen (3 mg kg^{-1}) administered 15 min before either cytokine completely abolished the fever induced by both IL-1α (2500 u kg^{-1}) and IL-1β (500 u kg^{-1}).

5 Intravenous administration of the steroidal anti-inflammatory agent dexamethasone (3 mg kg^{-1}) 1 h before either cytokine attenuated the fever induced by IL-1α (2500 u kg^{-1}) and IL-1β (500 u kg^{-1}).

6 The effects of ketoprofen and dexamethasone on IL-1 pyrogenicity indicate that prostanoids are almost certainly involved in the responses. The different potencies of IL-1α and IL-1β may be related to their relative ability to stimulate prostanoid biosynthesis.

Introduction

Interleukin-1 (IL-1) is a polypeptide with several biological activities regulating host defence and immune responses e.g., stimulation of thymocyte proliferation via induction of IL-2 release, stimulation of B lymphocyte maturation and proliferation, fibroblast growth factor activity and induction of acute phase protein synthesis by hepatocytes. It is synthesized by a number of cell types, particularly monocytes and macrophages upon stimulation with a variety of agents including lipopolysaccharide (LPS), muramyl dipeptide (MDP), phorbol myristate acetate etc. (Dinarello, 1984). It was speculated that these activities did not reside in only one protein, but in a family of proteins. Recently molecular cloning techniques have established that there are two distinct genes in the human genome which encode for two forms of the IL-1 molecule, a form with an isoelectric point (pI) of 5, termed IL-1α and a pI 7 form, termed IL-1β (Auron *et al.*, 1984; March *et al.*, 1985). Both molecules are synthesized as precursors of 31 000 molecular weight, which are post-translationally processed to biologically active proteins of 17 000 molecular weight (Auron *et al.*, 1984; March *et al.*, 1985). Murine (Lomedico *et al.*, 1984), bovine (Maliszewski *et al.*, 1988) and rabbit IL-1 (Furutani *et al.*, 1985) have also been cloned. IL-1α and murine IL-1 appear to be closely related at the level of the amino acid sequence, with 62% homology, whereas IL-1β is only distantly related to either human IL-1α (26%) or murine IL-1 (30%) (March *et al.*, 1985; Auron *et al.*, 1985). Despite the differences in primary structure, the evidence available indicates that murine IL-1, human IL-1α and human IL-1β all bind to the same receptor site (Kilian *et al.*, 1986; Dower *et al.*, 1987) and have similar biological activities (Oppenheim *et al.*, 1986). IL-1 has been shown to stimulate arachidonic acid metabolism, the most consistently observed effect is the increased production of prostaglandin E (PGE) from various cell types including human macrophages (Kunkel *et al.*, 1986) and fibroblasts (Mizel *et al.*, 1981; Newton & Covington 1987). Fever, which is perhaps the most conspicuous component of the acute phase of the immune response, is thought to occur as a result of the actions of PGE_2 on the pre-optic anterior hypothalamus (PO/AH), (Milton 1982; Dascombe 1985). IL-1 is thought to be an endogenous mediator of fever in response to infectious agents and other exogenous pyrogens such as the purified cell-wall components of bacteria (LPS, MDP) and the double-stranded ribonucleic acid, polyinosinic-polycytidylic acid (Dinarello 1984; Rotondo *et al.*, 1987). Recently we have shown that the non-steroidal anti-inflammatory agent ketoprofen and the synthetic glucocorticoid dexamethasone are antipyretic towards LPS, polyinosinic: polycytidylic acid and rabbit endogenous pyrogen/IL-1 (Abul *et al.* 1987; Rotondo *et al.*, 1988).

In this study we investigated the pyrogenicity in rabbits of intravenously administered human recombinant IL-1α and IL-1β and the effects of ketoprofen and dexamethasone on these responses. We have shown that both these agents reduce IL-1α and IL-1β fever.

A preliminary account of this work has appeared in the Journal (Davidson *et al.*, 1989).

Methods

Male Dutch rabbits, weighing 2.0–2.3 kg were lightly restrained in conventional stocks throughout each experiment. To minimize any error in body temperature measurements due to restraint stress, all rabbits were accustomed to the stocks over a period of 5 days before beginning this series of experiments. All experiments were conducted at the same time of day (10 h 00 min–16 h 00 min) and carried out at an ambient temperature of 22 ± 1°C. Body temperature was measured continuously with rectal thermistor probes (Yellow Springs Instruments-401 series), inserted to a depth of 9 cm, which

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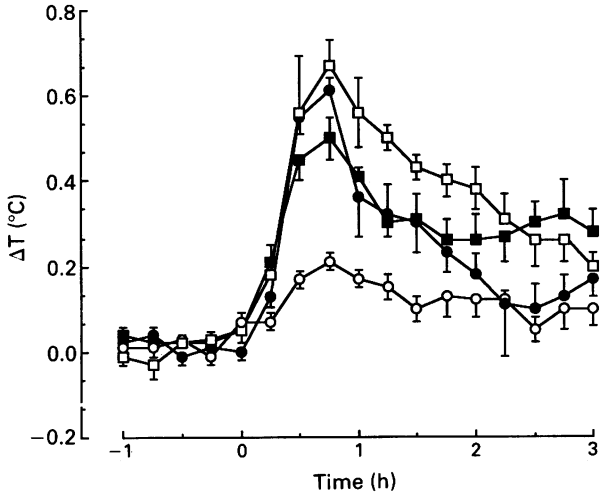

Figure 1 Effect of various doses of interleukin-1α (IL-1α) on body temperature. Rabbits were injected with various doses of IL-1α (u kg^{-1}) at time zero, 500 (○), 1000 (■), 2500 (●) and 5000 (□). Each value represents the mean of the changes in body temperature (°C) from basal for $n = 4$; s.e.mean shown by vertical bars.

were connected to a Jacquet chart recorder. Rabbits were left unhandled after insertion of the probes until body temperature was stable for at least 1 h before any drugs were administered.

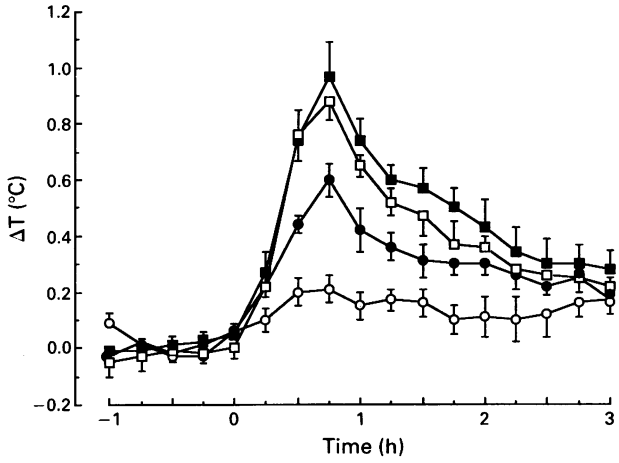

Figure 2 Effect of various doses of interleukin-1β (IL-1β, u kg^{-1}) on body temperature. Details as for Figure 1, except 100 (○), 500 (●), 2500 (□) and 5000 (■) u kg^{-1}.

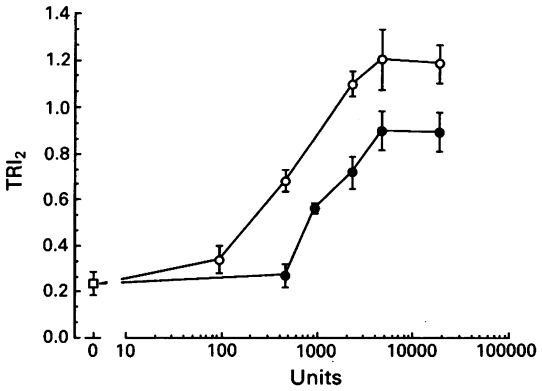

Figure 3 Comparison of the pyrogenicity of interleukin-1α (IL-1α) (●) and IL-1β (○); (□) denotes sterile saline alone. Each point represents the mean of 4 TRI_2 values (calculated as described in the methods section) for 4 rabbits; s.e.mean shown by vertical bars.

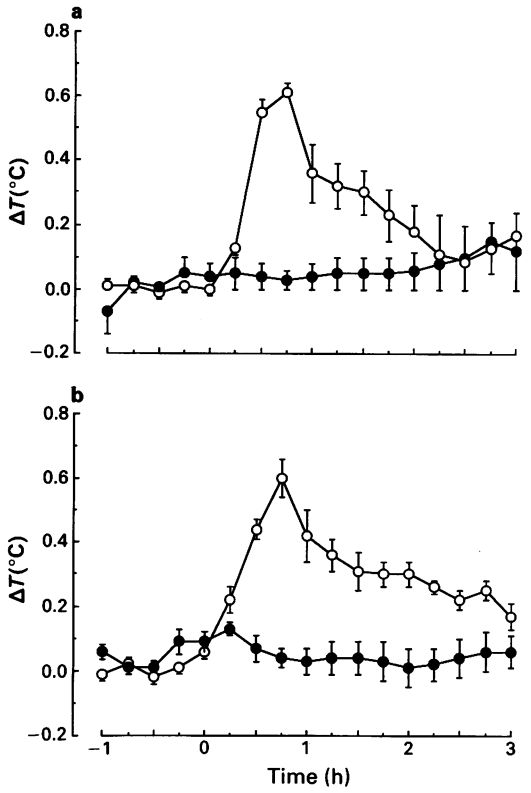

Figure 4 Effect of ketoprofen on interleukin-1 (IL-1) pyrogenicity. Rabbits were injected with 3 mg kg^{-1} ketoprofen (●) or saline (○) s.c. 15 min prior to (a) IL-1α (2500 u kg^{-1} i.v.) or (b) IL-1β (500 u kg^{-1}, i.v.) at time zero. Each value represents the mean of the changes in body temperature (°C) from basal for $n = 4$; s.e.mean shown by vertical bars.

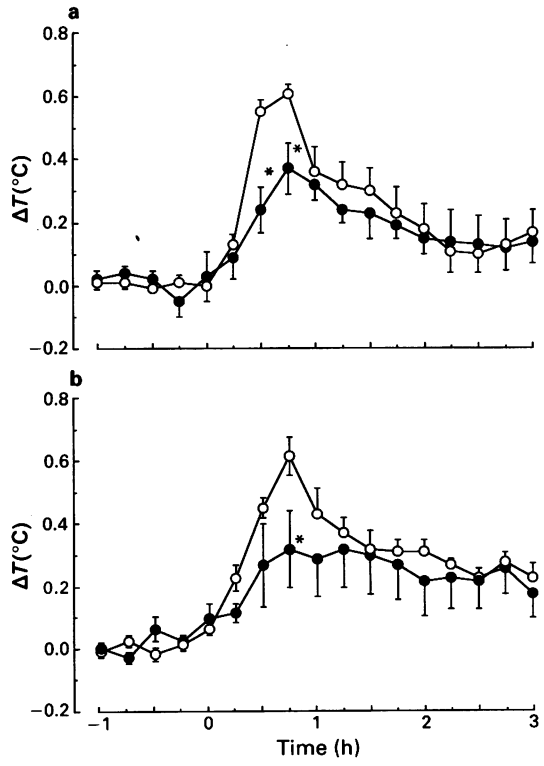

Figure 5 Effect of dexamethasone on interleukin-1 (IL-1) pyrogenicity. Rabbits were injected with 3 mg kg^{-1} dexamethasone (●) or saline (○) i.v. 1 h prior to either (a) IL-1α (2500 u kg^{-1} i.v.) or (b) IL-1β (500 u kg^{-1} i.v.) at time zero. Each value represents the mean of the changes in body temperature (°C) from basal for $n = 4$; s.e.mean shown by vertical bars. *$P < 0.05$, between dexamethasone and saline pretreatments.

Doses of IL-1 were measured in International Reference Units (u) as described by the National Institute for Biological Standards and Control (Hertfordshire, England), from where IL-1α and IL-1β were obtained. One unit of IL-1 is equivalent to 10 pg of protein. Recombinant IL-1, prepared by aseptic techniques, and dexamethasone 21 phosphate disodium (Merck Sharp and Dohme Research Laboratories, Hertfordshire, England) were dissolved in sterile saline (Travenol Laboratories, Norfolk, England). Ketoprofen (May and Baker, Dagenham, England) was prepared by adding warm saline and small amounts of $NaHCO_3$ gradually until it was completely dissolved. In experiments where heated cytokines were administered, the required volume of IL-1 was transferred to sterile Eppendorf tubes and placed in a water bath at 60°C for 30 min. IL-1 and dexamethasone were administered i.v. via the marginal ear vein and ketoprofen was administered s.c. at the nape of the neck. Dexamethasone was administered 1 h and ketoprofen 15 min prior to IL-1. Dexamethasone and ketoprofen were sterilized by passing the solution through Millex-GS 0.22 μm filters immediately before injection. The amount of agent in each dose was adjusted to give an injection volume of 0.5–1.0 ml. The results are expressed as either the change in temperature from basal (ΔT) in °C or as thermal response indexes where the magnitude of the febrile response is determined by integrating the change in temperature (°C) against time (h) to give a thermal response index (TRI) as originally defined by Milton & Wendlant, (1971). Data, expressed as the mean of n experiments $\pm$ the s.e.mean, were analysed by a paired Student's t test. Treatments were randomized, each rabbit acting as its own control and the differences were considered significant when $P < 0.05$.

Results

Various doses of IL-1α and IL-1β were administered i.v. and the febrile response measured for up to 3 h. The response was qualitatively similar with both pyrogens at all doses used, being rapid in onset, typically within 15 min of administration and reaching a maximum after 45 min (Figures 1 and 2). The threshold doses of IL-1α and IL-1β were 500 u kg^{-1} and 100 u kg^{-1} respectively. This produced a maximal rise in rectal temperature (ΔT_{max}) of 0.21 $\pm$ 0.02°C and 0.21 $\pm$ 0.05°C for IL-1α and IL-1β respectively ($n = 4$). Maximal TRI_2 were observed with doses of 5000 u kg^{-1} for both IL-1α and IL-1β (Figure 3) which produced TRI_2 values of 0.916 $\pm$ 0.085 and 1.220 $\pm$ 0.131 respectively showing that IL-1β has greater efficacy than IL-1α. The ΔT_{max} values obtained with 5000 u kg^{-1} were 0.67 $\pm$ 0.06°C and 0.97 $\pm$ 0.12 respectively ($n = 4$, Figures 1, 2 and 3). The magnitude of the febrile response to 20 000 u kg^{-1} of either cytokine was almost identical to that of the response to the same cytokine at the lower dose of 5000 u kg^{-1} and is omitted from Figures 1 and 2 for the sake of clarity but is shown in Figure 3. These data show that IL-1β has a greater pyrogenic action than IL-1α, approximately 5 fold based on the TRI_2 values for 500 u kg^{-1} of IL-1β ($TRI_2 = 0.698 \pm 0.047$) and 2500 u kg^{-1} of IL-1α ($TRI_2 = 0.734 \pm 0.071$). Heating both cytokines to 60°C for 30 min before injection appeared to abolish their pyrogenic actions.

The effect of ketoprofen and dexamethasone on the febrile response to both pyrogens was also studied. Doses of IL-1α (2500 u kg^{-1}) and IL-1β (500 u kg^{-1}) were chosen which gave equivalent increases in body temperature (ΔT_{max} 0.61 $\pm$ 0.03 and 0.60 $\pm$ 0.06 respectively for $n = 4$). Ketoprofen, (3 mg kg^{-1}, s.c.) given 15 min before either cytokine abolished the febrile response to IL-1α and IL-1β (Figure 4). Dexamethasone, (3 mg kg^{-1}) given i.v. 1 h prior to IL-1α and IL-1β attenuated the febrile response to both interleukins and reduced the TRI_2 values to 0.425 $\pm$ 0.098 and 0.450 $\pm$ 0.042 for IL-1α and IL-1β respectively (both $P < 0.02$) and the ΔT_{max} to 0.37 $\pm$ 0.08 and 0.31 $\pm$ 0.12 (both $P < 0.05$) respectively ($n = 4$, Figure 5).

Discussion

Fever is perhaps the most prominent manifestation of the acute phase of the immune response to infection in which the endogenous mediator is thought to be IL-1 (Dinarello, 1984). Many studies of the effects of IL-1 have been carried out with highly purified or recombinant IL-1 from human and other species. However, it still remains unclear why two distinct molecules, IL-1α and IL-1β have evolved which appear to share a broad spectrum of biological activities. Indeed it is not completely resolved whether the activities of IL-1α and IL-1β are the same (Oppenheim *et al.*, 1986).

It has been reported that both recombinant IL-1α and IL-1β are potent pyrogens, however little information is available regarding their relative potencies within the same species. The present study shows that both recombinant human IL-1α and IL-1β are pyrogenic in rabbits when administered i.v., (Figures 1 and 2); however, IL-1β is approximately 5 fold more effective than IL-1α (Figure 3). Dinarello *et al.* (1987) have demonstrated that IL-1α is pyrogenic when injected i.v. into rabbits and Rothwell *et al.*, (1989) have shown that administration of IL-1β into the third cerebral ventricle of rats results in a fever. Work by Stitt & Bernheim (1985) questions the approach of administering IL-1 centrally in that it may produce an artificial situation which may not occur during the normal pathogenesis of fever. It remains unclear whether fever produced by IL-1/EP administered by the intracerebroventricular route is identical in its site and mechanism of action to the fever produced by IL-1/EP administered intravenously and therefore the central administration of IL-1 may not be appropriate for studying its pyrogenic action. Coceani *et al.*, (1988) found that CSF levels of IL-1 were not significantly increased over controls, following i.v. administration, to conscious cats, of a pyrogenic dose of endotoxin or natural human IL-1, indicating blood-borne endotoxin is unable to promote IL-1 generation with the brain and that IL-1 itself cannot cross the blood brain barrier. Also Dinarello *et al.* (1978) injected ^{125}I-labelled IL-1/EP into rabbits which produced a fever but no detectable amounts of radioactivity were found to enter the brain. These observations would suggest that IL-1/EP in the systemic circulation exerts its pyrogenic action outside the CNS.

The increase in body temperature during fever is thought to occur as a result of the actions of prostaglandins of the E series on the PO/AH (Milton & Wendlant, 1970; Milton, 1982; Dascombe, 1985). Non-steroidal anti-inflammatory drugs like aspirin and indomethacin prevent the biosynthesis of prostanoids from arachidonic acid by inhibiting the enzyme cyclo-oxygenase (Flower & Vane, 1974). We report here that the non-steroidal anti-inflammatory agent ketoprofen, which is 12-fold more potent than indomethacin and in the order of 920-fold more potent than aspirin, (Moore, 1985), given s.c. 15 min before either pyrogen, completely abolished the pyrogenic effects of IL-1α and IL-1β. Consequently we propose that IL-1α and IL-1β exert their pyrogenic actions by increasing the level of PGE_2, possibly in the systemic circulation, as peripherally administered agents which inhibit prostanoid biosynthesis such as indomethacin are reported to be unable to cross the blood-brain barrier (Hucker *et al.*, 1966). Rotondo *et al.* (1988), have measured elevations in blood PGE_2 in rabbits, in response to a preparation of rabbit IL-1/EP, which occur simultaneously with increases in body temperature, both of which were completely abolished by treatment with ketoprofen. It is well documented that IL-1 increases the production of prostaglandins of the E series (Newton & Covington, 1987); however, the mechanism by which this occurs remains unknown. In addition we have found that the steroidal anti-inflammatory agent dexamethasone administered i.v. 1 h prior to IL-1α and IL-1β attenuates the febrile response (Figure 5). These results are in agreement with Milton *et al.* (1988) who have shown that, in the rabbit, dexamethasone attenuates both IL-1/EP-induced rises in body temperature and blood levels of PGE_2 and are consistent with the hypoth-

esis that the actions of glucocorticoids are mediated via the induction of lipocortin, a protein inhibitor of PLA_2 (Flower & Blackwell, 1979).

Presumably all the biological effects of IL-1 are receptor-mediated. Human recombinant IL-1α and IL-1β have been reported to bind to the same receptor molecule despite their relatively low homology in primary sequence, suggesting that their tertiary conformation is similar. A number of workers have identified a single high affinity binding protein for both IL-1α and IL-1β with a kd of 10^{-10}–10^{-11} M and a M_r of 80 kDa in human and various other species (see Dower & Urdal, 1987 for review). It is possible that the differences in pyrogenicity of IL-1α and IL-1β described here (Figure 3) may be due to their relative ability to stimulate prostanoid biosynthesis. In a study by Xiao & Levine (1986), recombinant human IL-1α was more effective than recombinant human IL-1β in stimulating arachidonic acid metabolism by primate smooth muscle cells, measured as PGE_2 release, whereas IL-1β was more effective than IL-1α in stimulating arachidonic acid metabolism by rat liver cells. Such diverse activities may be explained by postulating that the IL-1 receptor has different affinities for the two forms of the IL-1 molecule which varies with animal species and/or cell type. Kilian *et al.* (1986) examined the ability of purified recombinant human IL-1α and IL-1β to compete with recombinant [^{125}I]-ILα in receptor binding studies on murine EL-4 thymoma cells. Although human IL-1β was an effective competitor, approximately 2 to 3 fold higher concentrations of human IL-1β were required compared with murine IL-1 or human IL-1α. The dissimilarity between the two IL-1 molecules would suggest that some functions of IL-1 may be mediated by one form and some by the other. One such difference has been reported by Gallagher *et al.* (1987), who found that recombinant human IL-1α but not human recombinant IL-1β supports the growth of Epstein-Barr virus-transformed human B cells, and suggests separate but complementary roles for each molecule as IL-1β was able to synergize with IL-1α to promote cell growth. Recently a receptor for IL-1 has been cloned from murine T cells which binds both IL-1α and IL-1β. Unfortunately no information is available as yet on the signal transduction mechanism of the receptor (Sims *et al.*, 1988). Once available this may shed more light onto the relative functions of IL-1α and IL-1β.

It was also shown in the present study that heating IL-1α and IL-1β resulted in loss of pyrogenicity. This would concur with Xiao & Levine (1986), who have reported loss of PGE_2 stimulating activity when the two cytokines were heated. Heating presumably would lead to loss of the tertiary structure of both molecules, hence they would be unable to bind to their receptor. This together with the fact that no tolerance to IL-1α and IL-1β was observed throughout this series of experiments confirms that the pyrogenic activity shown here of both cytokines, was not due to endotoxin contamination.

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The effects of phosphatidylserine on the decreased cardiovascular response to clonidine microinfused into the nucleus tractus solitarii of old rats

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1 The cardiovascular effects of direct microinfusions of clonidine into the nucleus tractus solitarii (NTS) in rats of different ages were studied.

2 Clonidine microinfused into the NTS produced a dose-dependent hypotensive and bradycardic response, which was reduced by previous microinfusion of yohimbine but not of propranolol or prazosin.

3 Unilateral microinfusion of clonidine into the NTS produced a significantly smaller hypotensive and bradycardic response in 16 month old rats than in 3 month old rats, whereas in older animals (24 month old) the same doses of clonidine were ineffective. In 16 month old rats, yohimbine given into the NTS produced a smaller pressor and tachycardic response than in 3 month old rats whereas in 24 month old animals yohimbine, at the same doses, was unable to change blood pressure and heart rate.

4 Oral administration of phosphatidylserine ($50\,mg\,kg^{-1}\,day^{-1}$ for 30 consecutive days) enhanced the hypotensive and bradycardic response to clonidine microinfused into the NTS in 16 month old rats. The same schedule of treatment with phosphatidylserine did not affect the cardiovascular response to clonidine in young (3 month old) rats and did not restore responsiveness to clonidine in older rats (24 month old).

5 Methoxamine and phentolamine, given intravenously, produced similar pressor and depressor responses, respectively in 3, 16 and 24 month old rats, suggesting that the decreased sensitivity to clonidine in old rats was not due to peripheral cardiovascular changes.

6 In conclusion, the present results indicate that, during aging, there is a decreased sensitivity of α_2-adrenoceptors in the brain. Such an alteration can be reversed by chronic treatment with phosphatidylserine during the initial but not the later stages of aging.

Introduction

The effects of aging on cardiovascular function have been studied in man as well as in several experimental conditions (Fleish, 1981; Walsh, 1987). In particular, the hypertensive state, like other cardiovascular disorders observed in the elderly, has been connected with the prevelance of atherosclerotic damage of the arterial wall and with haemodynamic changes related to thickening of vessels involved in regulation of blood flow (Tuna *et al.*, 1985). Recently, however, data have accumulated showing that during aging there is an impairment of homeostatic mechanisms in the brain (see Agnoli *et al.*, 1983). In addition, it has been shown that, in 12 month old rats, there is a significant decrease in the hypotensive and bradycardic response to clonidine, microinfused directly into the nucleus tractus solitarii (NTS). Furthermore, in older animals (24 month old) clonidine completely failed to affect cardiovascular function (Mollace *et al.*, 1989). In previous experiments it was clearly demonstrated that the NTS plays a crucial role in the relay of peripheral inputs originating from baroreceptors as well as in the central regulation of cardiovascular activity (De Jong & Nijkamp, 1975). The NTS has been shown by autoradiographic studies to possess a high density of α_2-adrenoceptors (Palacios & Wamsley, 1984). In addition, the microinjection of noradrenaline into this structure (De Jong & Nijkamp, 1975) or the intraventricular administration of catecholamines or α_2-adrenoceptor agonists, leads to a reduction in the sympathetic outflow from the CNS accompanied by an increase in parasympathetic tone with a consequent hypotension and bradycardia (Schmitt *et al.*, 1971; Dollery & Reid, 1973; Kobinger, 1975; Sinha *et al.*, 1975; Marmo *et al.*, 1978).

However, the altered sensitivity of α_2-adrenoceptors to the local microinfusion of clonidine is not peculiar to the NTS. It has also been found, recently, in another structure, the locus coeruleus, in which noradrenaline-containing cell bodies are located, that the microinfusion of certain doses of clonidine in 13–15 month old rats, was unable to produce the behavioural and electrocortical sleep response observed in 3 month old rats (De Sarro *et al.*, 1988). In addition, chronic treatment with phosphatidylserine (PS), the most active fraction of brain cortical phospholipids (Bonetti *et al.*, 1983), was able to restore α_2-adrenoceptor responsiveness to clonidine microinfusion into the locus coeruleus (De Sarro *et al.*, 1988). The effects observed after chronic treatment with PS seem to be related to an increase in neuronal membrane fluidity (Gaiti *et al.*, 1981; 1985; Bruni & Toffano, 1982; Toffano, 1985) which in old age is pathologically decreased (Nagy *et al.*, 1983; Schroeder, 1985; Toffano, 1985). Evidence exists that oral or parenteral administration of labelled PS reaches several brain areas including the lower brain-stem (Orlando *et al.*, 1987).

In the present experiments we planned to ascertain whether chronic treatment with phosphatidylserine could restore the responsiveness to clonidine, given into the NTS of rats during aging.

Methods

Adult male Wistar Morini (S. Polo d'Enza, Reggio Emilia, Italy) normotensive rats were divided into three groups: 3, 16 and 24 month old, weighing approx. 250, 500 and 600 g respectively. Intracerebral cannulae were implanted in the rats under sodium pentobarbitone anaesthesia ($55\,mg\,kg^{-1}$ i.p.). Each cannula consisted of two concentric stainless steel tubes, a 1.6 cm long 23 gauge outer tube or guide cannulae which

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was implanted stereotaxically (David Kopf Instrument), into the brain and an inner tube (o.d. 0.2 mm) used for the infusion. *Post-mortem* histological examination of the brain confirmed the exact position of the guide cannula.

Guide cannulae were implanted into the NTS by use of the stereotaxic coordinates according to Paxinos & Watson (1982). Catheters (PE 50), filled with heparinized saline (200 u ml^{-1}) were inserted into the left common carotid artery to record blood pressure.

After surgery a minimum of 48 h was allowed before experiments were carried out.

In animals kept under general anaesthesia (pentobarbitone 55 mg kg^{-1} i.v.) mean arterial blood pressure (MAP) was recorded by attaching an arterial catheter to a Bentley transducer connected to a Gemini polygraph; heart rate (HR) was recorded with a cardiotachographer (Basile, Italy). The old animals were randomly divided into two groups, that received PS (50 mg kg^{-1}) or vehicle orally by gastric gavage, each day for 30 days. The experiments with clonidine or vehicle were carried out 3 days after the last administration of PS, and were performed within the first week after the rats reached their specific age.

To quantify changes of MAP and HR induced by drugs, the area (expressed in mm^2) under the curve of MAP and HR changes during 60 min was calculated according to the 'trapezoidal rule' (Tallarida & Murray, 1981), and the two groups (PS and vehicle) were age-matched.

In order to verify whether clonidine effects were due to an agonist action at the α_2-adrenoceptors, the animals were pretreated with yohimbine, propranolol or prazosin 10 min before the microinjection of clonidine into the NTS and the percentage changes in the cardiovascular effects of clonidine were assessed.

In addition, in animals of different ages the peak response to methoxamine and phentolamine injected i.v. in a bolus within 10 s was assessed.

For statistical evaluation, Student's t test was used. The number of rats (n) used for each dose is shown in parentheses.

Drugs

Clonidine hydrochloride (Boeringher-Ingelheim, Germany), yohimbine hydrochloride (Sigma St. Louis, MO., U.S.A.) and propranolol (Sigma St. Louis, MO., U.S.A.) were easily dissolved in bidistilled pyrogen-free H_2O. Prazosin (Pfizer Inc. New York, U.S.A.) was gently heated until dissolved in pyrogen-free H_2O. All drugs were infused into the NTS with a 1 μl Hamilton syringe, connected by a Teflon tube to an injector cannula. The rate of the infusion was 1 μl min^{-1} and the cannula was left *in situ* for a further 1 min. Each animal was treated only once.

Phosphatidylserine (FIDIA, Abano Terme, Italy) was dissolved in a Tris buffer as previously decribed by Aporti *et al.* (1986).

Methoxamine (a gift from Burroughs Wellcome and Co., U.K.) and phentolamine (Ciba-Geigy, Basel, Switzerland) were injected into the femoral vein in a 0.2 ml volume of saline.

Results

Effects of clonidine in rats of different age

In 3 and 16 month old rats, microinfusion of clonidine (0.25, 0.5 and 1 μg) into the NTS ($n = 6$), produced dose-dependent hypotension and bradycardia. Following microinfusion of 0.25 μg into the NTS of 3 month old rats, a significant ($P < 0.01$) fall in MAP and HR occurred, reaching a maximum 20 min later and lasting at least 60 min (Figures 1 and 2). Microinfusion of the same volume of the vehicle (bidistilled pyrogen-free H_2O) into the same site ($n = 6$) did not modify MAP and HR. Clonidine (0.5 μg) microinfused in areas 2 mm distant from the NTS ($n = 12$) or at a greater distance (caudate nucleus, n. raphe dorsalis, frontal and parietal cortex) did not significantly affect MAP and HR ($n = 4$).

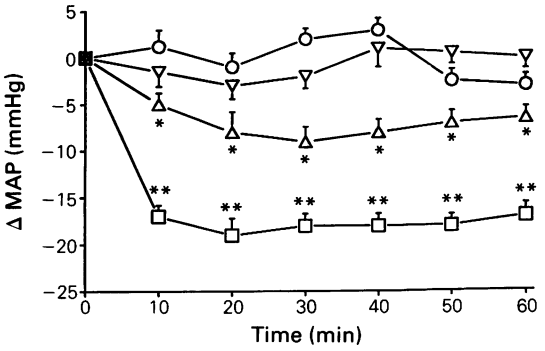

Figure 1 Effects of a single microinjection into the nucleus tractus solitarii of clonidine (0.25 μg) and vehicle (○, same volume) on mean arterial blood pressure (MAP) in 3, (□) 16 (△) and 24 (▽) month old rats. Each point is the mean blood pressure in 6 rats; vertical lines show s.e. * $P < 0.05$, ** $P < 0.01$, between clonidine- and vehicle-treated rats.

In contrast, in 16 month old rats, the microinfusion of clonidine (0.25 μg) into the NTS ($n = 6$) produced a significantly smaller ($P < 0.05$) hypotensive and bradycardic response in comparison to 3 month old animals, the nadir occurring approximately 30 min after microinfusion into the NTS (Figures 1 and 2). In addition, in 16 month old rats the cardiovascular response following 0.5 μg of clonidine into the NTS was significantly ($P < 0.05$) less in comparison to 3 month old animals.

In 24 month old rats ($n = 6$), clonidine (0.25, 0.5 and 1 μg) microinfused into the NTS did not significantly change MAP and HR (Figures 1 and 2).

Effects of clonidine in rats pretreated with yohimbine, propranolol or prazosin

The cardiovascular response to clonidine (0.5 μg) microinfused into the NTS, was dose-dependently reduced by pretreatment with yohimbine (0.5, 1 and 2 μg), microinfused into the same site, 10 min before, in 3 and 16 month old rats ($n = 6$) (Figure 3). In contrast, these doses of yohimbine did not affect the response to subsequent microinfusion of clonidine (0.5 μg) in older rats (24 month) ($n = 6$).

Pretreatment with prazosin (0.1 μmol) or (±)-propranolol (0.25 μmol) did not prevent the cardiovascular response to clonidine (0.5 μg) microinfused into the NTS ($n = 6$).

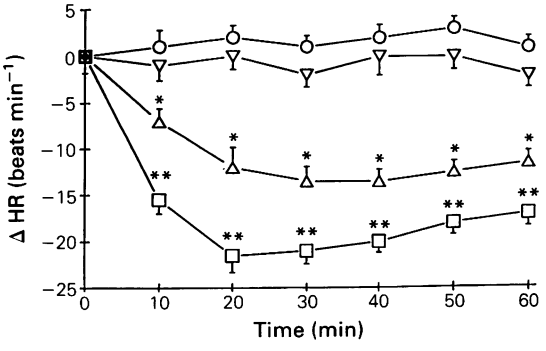

Figure 2 Effects of a single microinjection into the nucleus tractus solitarii of clonidine (0.25 μg) and vehicle (○, same volume) on heart rate (HR) in 3 (□), 16 (△) and 24 (▽) month old rats. Each point is the mean HR in 6 rats; vertical lines show s.e. * $P < 0.05$, ** $P < 0.01$, between clonidine- and vehicle-treated rats.

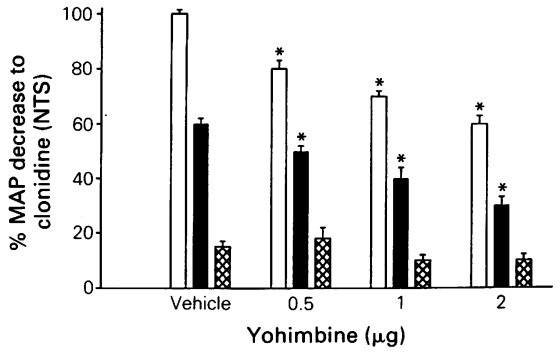

Figure 3 Dose-dependent antagonism by yohimbine given into the nucleus tractus solitarii (NTS) of the mean arterial blood pressure (MAP) decrease induced by subsequent microinfusion into the same site of clonidine (0.5 μg) in 3 (open columns) and 16 (solid columns) month old rats; the MAP response in 24 month old animals (hatched columns) was unaffected. * $P < 0.05$ between yohimbine and vehicle-pretreated rats.

Microinfusion of yohimbine alone (1 and 2 μg) into the NTS, induced a dose-related increase in MAP (Figure 4) and HR in 3 and 16 but not in 24 month old rats ($n = 6$). A lower dose (0.5 μg) of yohimbine ($n = 6$ rats) did not produce cardiovascular changes in any of animals.

Effects of methoxamine and phentolamine on blood pressure and heart rate in rats of different ages

Methoxamine, injected i.v. in a bolus (10 s) of 0.05 mg kg^{-1} induced an immediate rise in blood pressure (in 3 month old rats) reaching its maximum (+42 ± 3.8%) within 10 s of administration and lasting approximately 1 h. No significant differences were observed in the pressor response in 16 (+40 ± 4.4%) and 24 (+41 ± 4.2%) month old rats ($n = 5$) in comparison to 3 month old animals.

In addition, a bolus injection of phentolamine (0.1 mg kg^{-1} i.v.), induced, in 3 month old rats, a fall in blood pressure (−35 ± 4.6%) which started approximately 20 s after the administration, reached a maximum 10 min later and lasted approximately 2 h. The hypotensive response to phentolamine was similar in 16 (−36 ± 5.2%) and 24 (−33 ± 4.8%) month old rats ($n = 6$).

Effects of a pretreatment with phosphatidylserine on clonidine- and yohimbine-evoked cardiovascular responses

Pretreatment with PS (50 mg kg^{-1} orally each day for 30 days) or with the PS vehicle ($n = 6$) was unable to induce any

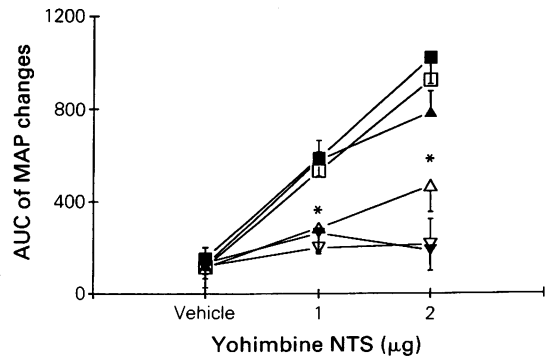

Figure 4 Increase in mean arterial blood pressure (MAP) (AUC, mm^2) evoked by yohimbine (1 and 2 μg) in 3, 16 and 24 month old rats untreated (open symbols) or pretreated (filled symbols) with phosphatidylserine (PS) (50 mg kg^{-1} orally each day for 30 days). Each point is the mean of AUCs for MAP changes in 6 rats and vertical lines represent s.e.mean. * $P < 0.05$ between untreated and PS pretreated rats. (□, ■) Three months old, (△, ▲) 16 month old and (▽, ▼) 24 month old rats.

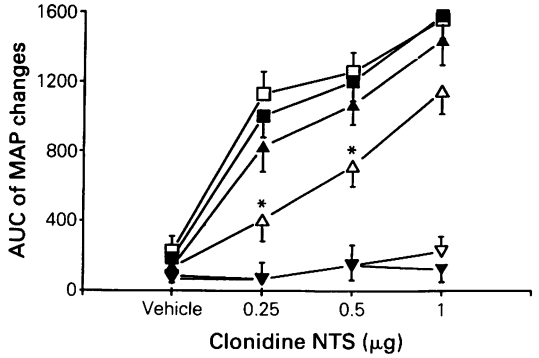

Figure 5 Effects of clonidine (0.25, 0.5, 1 μg) on area under the curves (AUCs) for mean arterial blood pressure (MAP) changes in 3, 16 and 24 month old rats untreated (open symbols) or pretreated (filled symbols) with phosphatidylserine (PS) (50 mg kg^{-1} orally each day for 30 days). Each point is the mean of AUCs for MAP changes in 6 rats and vertical lines represent s.e.mean. * $P < 0.05$ between untreated and PS pretreated rats. (□, ■) Three month old, (△, ▲) 16 month old and (▽, ▼) 24 month old rats.

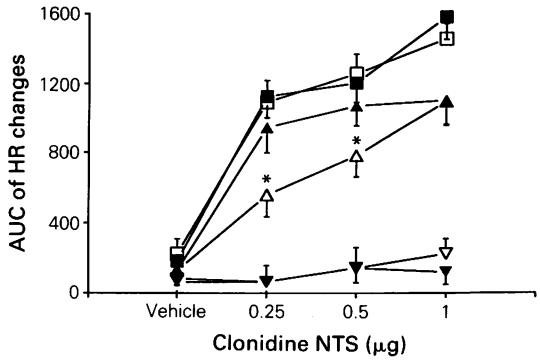

Figure 6 Effects of clonidine (0.25, 0.5 and 1 μg) on AUCs (mm^2) for heart rate (HR) changes in 3, 16 and 24 month old rats untreated (open symbols) or pretreated (filled symbols) with phosphatidylserine (PS) (50 mg kg^{-1} orally each day for 30 days). Each point is the mean of AUCs for HR changes in 6 rats and vertical lines represent s.e.mean. $P < 0.05$ between untreated and PS pretreated rats. (□, ■) Three month old, (△, ▲) 16 month old and (▽, ▼) 24 month old rats.

significant effect on blood pressure and heart rate responses (areas under the curve (AUC)) to clonidine (0.25, 0.5 and 1 μg) and yohimbine (1 and 2 μg) when microinfused into the NTS of 3 and 24 month old rats (Figures 4, 5 and 6).

In contrast, in 16 month old rats, pretreated with PS the cardiovascular responses to clonidine (0.25 μg and 0.5 μg) and yohimbine (1 and 2 μg) ($n = 6$) microinfused into the NTS were significantly increased (Figures 5 and 6).

No effects of PS or PS vehicle pretreatment were observed in the same 16 month old rats on the responses to a higher dose (1 μg) of clonidine microinfused into the NTS (Figures 5 and 6).

Discussion

The present experiments confirm that there is a decrease in the sensitivity of α_2-adrenoceptors in the NTS of rats during aging (Mollace *et al.*, 1989). This structure is involved in the regulation of blood pressure and heart rate. The cardiovascular response to clonidine directly microinfused into the NTS is likely to be central and not peripheral since only a small volume (1 μl) was given, which is unlikely to reach the general circulation in doses sufficient to affect blood pressure and HR. This conclusion was supported by the observation that microinfusion of clonidine into central sites distant from the NTS did not produce cardiovascular changes. The finding that in

young rats, yohimbine alone given into the NTS produced a pressor response suggests that a reduced sensitivity of α_2-adrenoceptors at this level is of physiological importance.

Several results suggest that in old animals, and probably in man, there is an impairment of the basic mechanisms underlying neurotransmission both at the pre- and post-synaptic level in several areas of the brain (see Agnoli *et al.*, 1983; Docherty & Hyland, 1986). It has been suggested that a common feature of the impairment of neurotransmission in old age is attributable to a decrease in neuronal membrane fluidity at presynaptic nerve terminals as well as at post-synaptic membranes (Nagy *et al.*, 1983; Schroeder, 1985; Toffano, 1985). As far as the noradrenergic mechanisms in the brain are concerned, evidence exists for a decreased number of α_1- and α_2-adrenoceptor in rabbit brain (Hamilton *et al.*, 1984) and in the periphery (Docherty & Hyland, 1986).

Previously, we have shown that in the locus coeruleus, a typical area in which noradrenergic cell bodies are contained, there is a decrease in the sensitivity of α_2-adrenoceptors to the local microinfusion of α_2-adrenoceptor agonists and antagonists (De Sarro *et al.*, 1988). Thus the present experiments are consistent with the idea that α_2-adrenoceptors in several areas of the rat brain show an age-dependent decrease in their responsiveness to the topical microinfusion of α_2-adrenoceptor agonists. In particular, the decreased responsiveness to clonidine microinfused into the NTS is illustrated by the significantly smaller hypotensive and bradycardic response in 16 month old rats and by the lack of a response in older animals (24 month old). This indicates that possible biochemical alterations in the response to neurotransmitters at the receptor level (binding site or subsequent transducing coupling mechanisms) deteriorates progressively with aging. It remains to be elucidated whether the decrease in the response to clonidine is due to a decreased number and/or affinity of α_2-adrenoceptors in the NTS or to altered signal transducing mechanisms coupling receptors to effectors. An alteration of membrane fluidity during aging could well explain the decrease in the response to receptor stimulation, since in several models it has been demonstrated that following stimulation of several receptors, for example β-adrenoceptors, there is, as a consequence of an increased phospholipid fluidity, a rotation of β-adrenoceptors and an increased coupling (activation) with adenylate-cyclase catalytic unit (Hirata & Axelrod, 1978). On the other hand, the present experiments show that chronic treatment with PS, a compound able to increase nueronal membrane fluidity (Gaiti *et al.*, 1985; Toffano, 1985), restored the cardiovascular response to clonidine directly applied into the NTS, at least during the initial stages of aging. It is also worth noting that during the early stages of aging (13–15 month old rats) there is a decreased sensitivity to clonidine of α_2-adrenoceptors in the locus coeruleus, an effect that was surmountable at this age but not later (Nisticò G. & De Sarro G. B., unpublished results) by increasing the dose of clonidine (De Sarro *et al.*, 1988).

The reason why in 24 month old rats, PS was unable to restore the α_2-adrenoceptor-mediated response in the NTS is not known, but it seems likely that in the late stages of aging an irreversible lesion occurs. In addition, the present data are consistent with those showing that PS is able to increase the sensitivity of pituitary dopamine receptors to dopamine in aged rats (Di Renzo *et al.*, 1987).

In conclusion, the present experiments confirm that during the aging process there is a decreased responsiveness of α_2-adrenoceptors in the brain to selective α_2-adrenoceptor agonists and that, at least during the early stages of aging, the decrease seems to be reduced by chronic treatment with phosphatidylserine.

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Action of noradrenaline on isolated proximal and distal coronary arteries of rat: selective release of endothelium-derived relaxing factor in proximal arteries

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1 The effect of noradrenaline (NA) on the vascular smooth muscle and endothelial cells in isolated ring segments from the proximal and distal part of the left coronary artery (LCA) in rats was examined.

2 NA had a weak concentration-dependent contractile effect on proximal but relaxed distal segments of the LCA. The maximal NA-induced contraction of the proximal segments was doubled while the relaxation of the distal LCA segments was converted to a contraction after blockade of β-adrenoceptors with propranolol 3×10^{-6} M, thus indicating the presence of both α- and β-adrenoceptors in the arteries, with dominance of α-adrenoceptors and of β-adrenoceptors in the proximal and distal segments of the LCA, respectively.

3 The contractile effect of NA (β-adrenoceptors blocked) was doubled in the proximal LCA segments after the endothelium was removed. Endothelial denudation had, in contrast, no potentiating effect on the contractile response of the distal arteries to NA. Both proximal and distal segments became more sensitive to the contractile action of NA after removal of the endothelium.

4 The spontaneous myogenic tone increased in both proximal and distal LCAs after endothelial removal, indicating spontaneous release of a relaxing endothelial factor in the vessels.

5 Following contraction with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and in the presence of propranolol, 3×10^{-6} M, and prazosin, 10^{-6} M, NA induced an endothelium-dependent relaxation of only proximal but not distal segments of the precontracted LCA. The NA-induced relaxation of the proximal segments of the LCA was not altered by indomethacin 10^{-5} M but was completely abolished after incubation with methylene blue, 3×10^{-6} M, or following endothelium removal. These results are compatible with NA-induced release of EDRF in these arteries.

6 The selective α_2-adrenoceptor agonist, B-HT 933, only induced a weak relaxation of $PGF_{2\alpha}$-precontracted proximal (endothelium intact) LCA segments at a concentration of 10^{-4} M. The NA-induced relaxation of these vessels was unaffected by incubating the vessels with 10^{-5} M B-HT 933. The NA relaxation response curve was shifted ca 1.1 log unit to the right by rauwolscine, 10^{-6} M, giving an estimated pA_2-value of 7.12. The receptor through which NA activates the endothelium appears to be of an atypical α_2-subtype.

Introduction

It is now realized that the endothelium cells are active and regulate vascular smooth muscle tone by releasing relaxing and contracting substances in response to receptor activation or to physiological stimuli, e.g. stretch (Rubanyi, 1988). The factors released constitute a variety of chemical substances: nitric oxide (NO) (Palmer *et al.*, 1987), which causes relaxation via stimulation of the soluble guanyl-cyclase enzyme (Rapoport & Murad, 1983), a polypeptide, endothelin, which induces contraction of vascular smooth muscle (Yanigasawa *et al.*, 1988) and several arachidonic acid derivatives, of which prostacyclin is the most important (Moncada *et al.*, 1976).

Noradrenaline (NA) has previously been shown to induce release of endothelial-derived relaxing factor (EDRF) in epicardial arteries from both dogs and pigs (Cocks & Angus, 1983), but information concerning the action of NA on endothelium in small intramural flow-regulating or resistance arteries in the coronary circulation is lacking, possibly due to the technical problems of studying these vessels.

We have in the present experiments examined the actions of NA on the vascular smooth muscle cells and endothelium in isolated rings of rat coronary arteries taken from the proximal, epicardial, and distal, intramural, segments of the left coronary artery (LCA) of rats.

Methods

Ring segments of the left coronary artery (LCA) were isolated from the proximal and distal parts as previously described (Nyborg & Mikkelsen, 1988). The vessels were mounted on two 40 μm diameter stainless steel wires on an isometric myograph allowing direct determination of the vessel wall force while the internal circumference was controlled (Mulvany & Halpern, 1977). The vessels were kept in oxygenated (5% CO_2 in O_2) physiological saline solution (PSS) of the following composition (mM): NaCl 119, NaH_2CO_3 25, KCl 4.7, $CaCl_2$ 1.5, KH_2PO_4 1.18, $MgSO_4$ 1.17, EDTA 0.026 and glucose 11, and allowed to equilibrate at 37°C, pH 7.4, for 30 min before they were stretched to their optimal lumen diameter for active tension development. This normalized effective lumen diameter, l_0, corresponds to 90% of the diameter the vessels would have had when subjected to a transmural pressure of 13.3 kPa and relaxed (Nyborg *et al.*, 1988).

Each experiment was begun by stimulating the vessels with K-PSS (similar to PSS except that NaCl was exchanged for KCl on an equimolar basis) until reproducible responses were recorded. In the first series of experiments cumulative NA concentration-response experiments were carried out with vessels before and after blockade of β-adrenoceptors with propranolol, 3×10^{-6} M. In the second series of experiments, NA concentration-response curves were obtained with vessels before and after removal of endothelium while the β-adrenoceptors were blocked with propranolol (3×10^{-6} M). In the last series of experiments the vessels were incubated with propranolol (3×10^{-6} M) and prazosin 10^{-6} M before they were contracted with $PGF_{2\alpha}$ 10^{-5} M, and then challenged with NA, a selective α_2-adrenoceptor agonist, B-HT 933 (Timmermans & van Zwieten, 1980) or with NA after incubation with rauwolscine, 10^{-6} M, a selective α_2-adrenoceptor antagonist. In these experiments the pA_2-value ($-\log(K_B(M))$

for rauwolscine was calculated as (log(CR − 1) − log(rauwolscine, M), where CR is the ratio of EC_{50}(M) for the relaxing effect of NA in the presence and absence of rauwolscine (MacKay, 1978).

In order to characterize the factor released from the endothelium by NA, experiments were repeated with proximal LCA segments in the presence of either indomethacin, 10^{-5} M, or methylene blue, 3×10^{-6} M, which inhibits formation of cyclo-oxygenase products (Shen & Winter, 1977) and of guanosine 3':5'-cyclic monophosphate (cyclic GMP) by soluble guanyl-cyclase, respectively (Holzmann, 1982; Martin *et al.*, 1985).

The maximal endothelium-dependent relaxing capacity of the vessels was assessed by examining the effect of acetylcholine, 10^{-5} M, following precontraction with $PGF_{2\alpha}$ (10^{-5} M). The endothelium was removed by inserting a hair from human scalp (distal) or horse tail (proximal) into the vessel lumen which then was gently rubbed by pushing the hair back and forth. Removal of endothelium was considered effective if acetylcholine (10^{-5} M) no longer induced relaxation.

Each NA concentration-response curve was analyzed by iterative nonlinear regression analysis. The regression line was fitted to a sigmoid curve equation; $R/R_{max} = A^n/(A^n + K^n)$, where R_{max} is maximal response developed to NA, A is concentration of agonist and K is the vessel apparent agonist affinity, EC_{50}(M). n is a curve-fitting parameter, the Hill coefficient (Kenakin, 1986).

Responses are given either as active tension, calculated as increase in vessel wall force above resting level divided by twice the vessel segment length, or as relative responses. At the end of each experiment the maximal contractile capacity of the vessels was determined from the difference in wall tension when maximally contracted (with K-PSS to which 10^{-5} M $PGF_{2\alpha}$ and 5-hydroxytryptamine was added), and when the vessel was maximally relaxed in Ca^{2+}-free PSS (similar to PSS except that $CaCl_2$ was replaced with 10^{-4} M EGTA).

Drugs used were (−)-noradrenaline HCl (Sigma), (±)-propranolol (Sigma), prazosin HCl (Pfizer), acetylcholine chloride (Sigma), $PGF_{2\alpha}$ (Dinoprost, Upjohn), 5-hydroxytryptamine HCl (Sigma), methylene blue (DAK), indomethacin (Sigma), B-HT 933 (2-amino-6-ethyl-4,5,7,8-tetrahydro-6H-oxalo-(5,4-d)-azepin dihydrochloride; Boehringer Ingelheim). All drugs were prepared from frozen stock solutions and added to the organ bath in volumes of not more than 0.3% to reach final required concentrations.

Results are given as mean ± s.e.mean (one vessel per rat). Differences between means were analyzed with either Student's *t* test to compare differences between proximal and distal coronary arteries or a two-way analysis of variance (2-way ANOVA) (Sokal & Rohlf, 1969). The latter analysis allowed comparison of differences between means in terms of vessel type, proximal vs. distal, indicated by P_A, or in terms of effect of treatment, here removal of endothelium, indicated by P_E. In cases where the interaction term, P_x, was significant the mean values of the same type of vessels with and without endothelium have been analyzed with Student's *t* test for paired or unpaired data where appropriate. The level of significance was in all tests set at $P < 0.05$. NS indicates no significant difference.

Results

Figure 1 shows the effect of NA on proximal and distal segments of the LCA from rats before and after blockade of β-adrenoceptors with propranolol, 3×10^{-6} M, and after removal of the endothelium of the vessels (propranolol 3×10^{-6} present). Before blockade of β-adrenoceptors, NA relaxed the small distal LCAs over the concentration-range 10^{-8} to 10^{-6} M and partially contracted them at higher concentrations. The large proximal LCAs were variably affected by NA before β-adrenoceptor blockade: NA induced a concentration-dependent relaxation of two vessels but contracted the four others. Incubation of vessels with 3×10^{-6} M propranolol converted the NA-induced relaxation of the distal as well as proximal segments to a contraction, and potentiated the NA-induced contraction of the remaining four proximal segments.

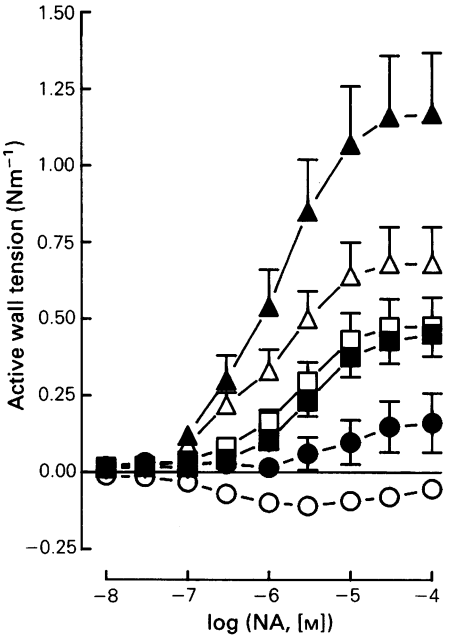

Figure 1 Noradrenaline (NA) concentration-response relations of proximal (closed symbols) and distal (open symbols) segments of the left coronary artery from rats before (circles) and after blockade of β-adrenoceptors with propranolol (3×10^{-6} M) (squares), and after removal of the endothelium (triangles). Responses are given as active wall tension, Nm^{-1}, that is the change in wall force from resting condition divided by twice the vessel segment length (see Methods). Points show mean of responses of 6–14 arteries. Vertical bars show ±s.e.mean. Effective lumen diameter, l_0, of all vessels (see Methods) was 385 ± 20 μm ($n = 14$) and 216 ± 11 μm ($n = 12$) for the proximal and distal arteries, respectively.

The maximal contraction to NA increased by 146 ± 55% ($n = 8$) ($P < 0.01$, paired *t* test) from 0.61 ± 0.22 Nm^{-1} ($n = 8$) to 1.24 ± 0.23 Nm^{-1} ($n = 8$) in the proximal LCA segments after removal of endothelium and in presence of propranolol (3×10^{-6} M), whereas no change was observed in the maximal NA-induced contraction of the distal segments. The maximal active tension to NA was in these vessels 0.57 ± 0.15 Nm^{-1} ($n = 6$) and 0.68 ± 0.12 Nm^{-1} ($n = 6$) before and after removal of endothelium, respectively.

Removal of endothelium caused an increase in the spontaneous myogenic tone from 0.17 ± 0.05 Nm^{-1} ($n = 8$) and 0.16 ± 0.05 ($n = 6$) Nm^{-1} to 1.41 ± 0.25 ($n = 8$) Nm^{-1}, and 0.88 ± 0.28 ($n = 6$) Nm^{-1} in proximal and distal LCA segments, respectively, (P_A NS; $P_E < 0.001$) when kept in standard PSS solution containing 1.5 mM Ca^{2+}. It also caused a significant increase in sensitivity to NA of both proximal and distal segments. The pD_2-values of proximal segments ($n = 8$) were 5.53 ± 0.04 and 5.88 ± 0.09 and pD_2-values of distal segments ($n = 6$) were 5.63 ± 0.16 and 6.00 ± 0.10 before and after removal of endothelium, respectively (P_A NS; $P_E < 0.01$).

Acetylcholine induced a prompt relaxation of $PGF_{2\alpha}$ (10^{-5} M) precontracted proximal and distal rat coronary arteries. After removal of the endothelium, acetylcholine-mediated relaxation was abolished. The mean relaxation induced by acetylcholine (10^{-5} M) was 76 ± 8% ($n = 8$) and 61 ± 9% ($n = 6$) for proximal and distal segments, respectively. The procedure employed to remove the endothelium did not affect the contractile response to $PGF_{2\alpha}$ (10^{-5} M), being 1.75 ± 0.31 Nm^{-1} ($n = 8$) and 1.63 ± 0.27 Nm^{-1} ($n = 8$)

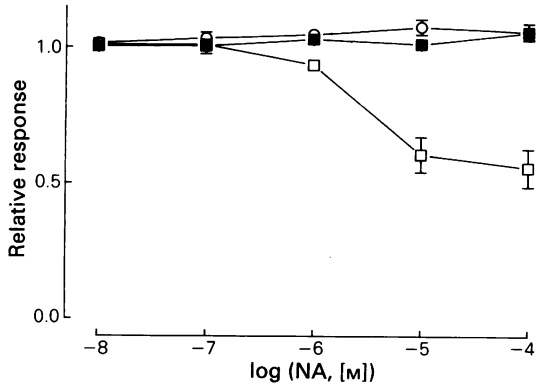

Figure 2 Noradrenaline (NA) concentration-response relationship of 10 proximal endothelium-intact (□), 4 endothelium-denuded (■) and 9 distal endothelium-intact rat LCA segments (○) precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) 10^{-5} M. α_1- and β-adrenoceptors were blocked with prazosin (10^{-6} M) and propranolol (3×10^{-6} M), respectively. Responses are given as a fraction of the initial vessel response to $PGF_{2\alpha}$ just before vessels were challenged with NA. Points show mean of responses and vertical bars ± s.e.mean. Effective lumen diameter, l_0, of vessels (see Methods) was 445 ± 22 μm ($n = 10$) and 499 ± 27 μm ($n = 4$) of endothelium-intact and -denuded proximal segments, respectively, and 227 ± 11 μm ($n = 9$) of distal segments.

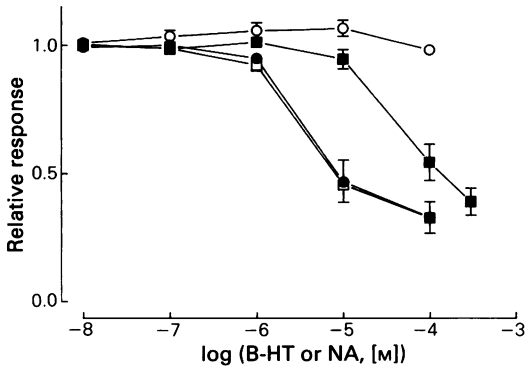

Figure 4 Concentration-response relations of B-HT 933, a selective α_2-agonist (○, $n = 5$), and noradrenaline (NA) in control conditions (□, $n = 12$), NA in presence of B-HT 933 (10^{-5} M) (●, $n = 8$), and NA in presence of rauwolscine (10^{-6} M), a selective α_2-antagonist (■, $n = 11$). α_1- and β-adrenoceptors were blocked with prazosin (10^{-6} M) and propranolol (3×10^{-6} M), respectively. Responses are given as fraction of the initial vessel response to prostaglandin $F_{2\alpha}$ (10^{-5} M) just before they were challenged with B-HT 933 or NA. Points show mean responses and vertical bars ± s.e.mean. Effective lumen diameter of all vessels used was 450 ± 23 μm ($n = 13$).

in the proximal segments and 1.19 ± 0.22 Nm^{-1} ($n = 6$) and 1.18 ± 0.28 Nm^{-1} ($n = 6$) in the distal segments, before and after removal of endothelium, respectively ($P_A < 0.05$; P_E NS). The relative vessel tension induced by $PGF_{2\alpha}$ (10^{-5} M) was similar in both proximal and distal segments, being 40 ± 6% ($n = 8$) and 40 ± 5% ($n = 6$) of E_{max}, respectively.

In the presence of prazosin (10^{-6} M) and propranolol (3×10^{-6} M), NA induced a concentration-dependent relaxation of $PGF_{2\alpha}$ (10^{-5} M) precontracted proximal endothelium-intact segments. No effect of NA was observed in the distal coronary arteries under the same conditions (Figure 2). The maximal relaxation induced by NA in the proximal segments was 46 ± 7% ($n = 10$). The NA-induced relaxation was significantly smaller ($P < 0.05$, paired t test) than the relaxation induced by 10^{-5} M acetylcholine, 61 ± 6% ($n = 10$). The pD_2-value for the NA-induced relaxation was 5.52 ± 0.10 ($n = 10$). The NA-induced relaxation of the proximal segments of the LCA was abolished when the endothelium was removed (Figure 2).

Incubation of vessels with indomethacin (10^{-5} M) did not affect the maximal relaxation induced by NA, being 55 ± 7% in control and 58 ± 5% ($n = 5$) in the presence of indomethacin, nor the NA-sensitivity, pD_2-values being 5.56 ± 0.05 ($n = 5$) in control and 5.56 ± 0.08 ($n = 5$) in the presence of indomethacin, respectively (Figure 3). The 10^{-5} M $PGF_{2\alpha}$-induced response was, however, reduced from 2.68 ± 0.34 Nm^{-1} ($n = 5$) in normal PSS to 1.72 ± 0.32 Nm^{-1} ($n = 5$) ($P < 0.05$, paired t test) in the presence of indomethacin. Methylene blue (10^{-5} M) abolished the NA-induced relaxation of the $PGF_{2\alpha}$-induced contraction (2.39 ± 0.14 Nm^{-1} ($n = 5$)) completely (Figure 3). Methylene blue also increased the spontaneous tone of the proximal segments from 0.26 ± 0.08 Nm^{-1} to 1.62 ± 0.39 Nm^{-1} ($n = 5$) ($P < 0.05$, paired t test).

Experiments with the selective α_2-adrenoceptor agonist, B-HT 933, showed only a small relaxation of the proximal endothelium-intact LCA segments at the highest concentration used, 10^{-4} M (Figure 4). Addition of 10^{-5} M B-HT 933 to the bathing medium did not affect the NA-induced endothelium-dependent relaxation of these vessels (Figure 4). When the selective α_2-adrenoceptor antagonist, rauwolscine 10^{-6} M, was present, the NA-concentration response curve was shifted about 1.1 log unit to the right (Figure 4), giving a pA_2-value of 7.12 ± 0.12 ($n = 11$) for rauwolscine.

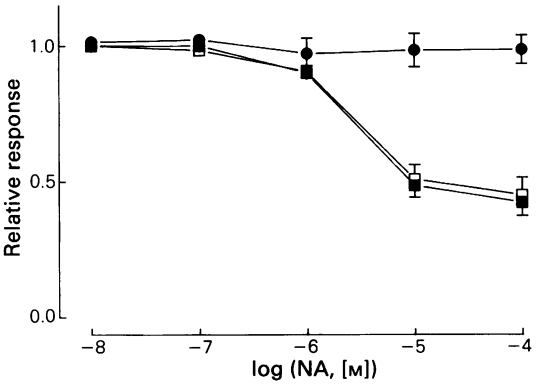

Figure 3 Noradrenaline (NA) concentration-response relationship of 5 proximal endothelium-rat LCA segments precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) 10^{-5} M in control conditions (□), in presence of indomethacin (10^{-5} M) (■), and in the presence of methylene blue (10^{-5} M) (●). α_1- and β-adrenoceptors were blocked with prazosin (10^{-6} M) and propranolol (3×10^{-6} M), respectively. Responses are given as fraction of the initial vessel response to $PGF_{2\alpha}$ just before they were challenged with NA. Points show mean and vertical bars ± s.e.mean. Effective lumen diameter, l_0, of vessels (see Methods) was 462 ± 22 μm (5).

Discussion

It is well known that NA has a different action on coronary arteries depending on their location in the coronary circulation (Malindzak, 1982). The relaxing or contracting action of NA has been attributed to segmental variations in the distribution of α- and β-adrenoceptors. With the recent discovery of endothelium-derived substances it seemed appropriate to re-examine the effects of NA on the coronary circulation.

In the large proximal segments of the LCA, α-adrenoceptor-mediated contraction is opposed both by β-adrenoceptor-mediated relaxation and by endothelium-dependent vasodilatation indicated by the increase in NA-induced contraction of these vessels after blockade of β-adrenoceptors with propranolol alone and removal of endothelium. NA induced only

relaxation in the distal segments of the LCA, indicating that β-adrenoceptor-induced relaxation was dominant in these arteries, as previously shown (Nyborg & Mikkelsen, 1985). These results are thus in agreement with older published data obtained in larger species than rats, showing a gradual shift from NA-induced contraction of large coronary arteries to NA-induced relaxation of small arteries (see Malindzak, 1982).

EDRF is released by NA in coronary arteries of dogs and pigs (Cocks & Angus, 1983). However, only large epicardial arteries, corresponding anatomically to the proximal LCA segments used in the present experiments were used by Cocks & Angus (1983). NA was only able to induce release of relaxing factor(s) from the endothelium in the proximal coronary arteries, indicated by the doubling in maximal contractile response to NA and abolition of NA-induced relaxation of α_1- and β-adrenoceptor blocked vessels after endothelial removal. The contraction to NA of the distal segments of the LCA was in contrast, not potentiated after the endothelium was removed. The lack of potentiation was not due to extensive damage of the vessels by the procedure since the contractile response to $PGF_{2\alpha}$ was unchanged after endothelial removal. It therefore seems to be a differential action of NA on the coronary endothelium depending on size or location of the artery. This may be explained by coronary circulation morphogenesis during which the proximal part of the epicardial arteries is formed from an outgrowth of aortic endothelial mesenchymal cells while the intramural arteries derive from the myocardial mesenchyme (Conte & Pellegrini, 1984). Large proximal coronary artery endothelium function can therefore not be extrapolated to the smaller intramural flow-regulating or resistance arteries, at least in the rat.

The direct NA-induced contraction of the coronary vascular smooth muscle appears not only to be affected by NA-induced release of a relaxing factor, possibly EDRF (NO), in the proximal segments but also by spontaneous release of this substance, since removal of endothelium induced myogenic tone in both proximal and distal segments of the LCA. It is likely that loss of this tonic dilator stimulus is responsible for the augmentation of sensitivity to NA of the coronary arteries after endothelial denudation.

The major factor released from the endothelium by NA appears to be EDRF (NO). Two observations point towards this. Firstly, indomethacin was without effect on NA-induced endothelium-dependent relaxation of the proximal coronary arteries and secondly, this relaxation was completely blocked by methylene blue, which also blocks the acetycholine-induced relaxation (results not shown) in these arteries. Methylene blue inhibits EDRF-mediated vascular relaxation through blockade of the soluble guanyl-cyclase enzyme (Holzmann, 1982; Martin *et al.*, 1985).

The NA-induced endothelium-mediated relaxation of the proximal rat coronary arteries is smaller than that induced by acetylcholine. This difference may be attributed to either less efficient receptor-response coupling of the endothelial α-adrenoceptors compared to the cholinoceptors or/and a lower number of α-adrenoceptors.

The subtype of α-adrenoceptor mediating the endothlium-dependent relaxation by NA was initially characterized as the α_2-subtype (Angus *et al.*, 1986). The present experiments are in partial agreement with this. The NA-induced endothelium-dependent relaxation of the proximal LCA segments was evoked while α_1-adrenoceptors were blocked with prazosin and β-adrenoceptors blocked with propranolol, strongly indicating the involvement of α_2-adrenoceptors. Our attempts to characterize the α-adrenoceptor subtype using selective agonists and antagonists were, however, not straightforward. The selective α_2-adrenoceptor agonist, B-HT 933, had no significant relaxing effect in the concentration range 10^{-8}–10^{-4} M, and it did not affect the NA-induced relaxation which would have been expected if the α_2-adrenoceptor density was too low for a partial agonist to evoke a response by itself (Kenakin, 1986). The NA relaxation curve was shifted to the right by rauwolscine, a selective α_2-adrenoceptor antagonist, with a calculated pA_2-value of 7.12. This value is, however, relatively low compared to previous values, ca. 7.7, reported by Daly *et al.* (1988) for postjunctional α_2-adrenoceptors on smooth muscle cells and too high for pA_2-values for α_1-adrenoceptors (Daly *et al.*, 1988). These results might indicate that the endothelial α-adrenoceptor is different from the postsynaptic smooth muscle α_2-adrenoceptor, but further experiments are needed to clarify this. Clonidine, a selective α_2-adrenoceptor agonist, has been shown to dilate dog coronary arteries *in vivo* by activation of histamine H_2-receptors (Söhngen *et al.*, 1988), but 10^{-5} M cimetidine did not antagonize the NA induced relaxation of the proximal coronary arteries in the present experiments (results not shown).

In conclusion, the present experiments indicate that NA has a complex action on rat isolated coronary arteries consisting of both α- and β-adrenoceptor activation and direct release of EDRF in the proximal part of the coronary vascular bed. The α-adrenoceptor-mediated, NA-induced contraction of the rat coronary arteries is further affected by continuous basal release of an endothelium-derived relaxing factor(s) in the arteries. The results indicate that previous attempts (see Feigl, 1983) to characterize the subtype of coronary artery smooth muscle α- and β-adrenoceptor subtype with selective antagonists, may have been affected by release of EDRF. The endothelial α-adrenoceptor subtype with which NA interacts, could not be classified difinitively but has certain characteristics of the α_2-subtype.

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Systemic and regional haemodynamic interactions between K^+ channel openers and the sympathetic nervous system in the pithed SHR

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1 The interactions between two K^+ channel openers, cromakalim and SR 44866 (infused i.v. at equihypotensive doses), and the sympathetic nervous system at the systemic and regional (mesentery, kidney, hindlimb) vascular levels were investigated in the pithed spontaneously hypertensive rat (SHR) by use of the pulsed Doppler technique.

2 The two K^+ channel openers did not affect postsynaptic α_1- but slightly reduced postsynaptic α_2-adrenoceptor mediated systemic pressor and regional vasoconstrictor responses.

3 Both drugs significantly decreased the systemic pressor and regional vasoconstrictor responses elicited by spinal cord stimulation. These sympathoinhibitory effects were not homogeneously distributed among the different vascular beds, the decreasing rank order being: mesentery > kidney > hindlimb. Simultaneously, the spinal cord stimulation-induced tachycardia remained unaffected.

4 After treatment with K^+ channel openers, restoration of initial blood pressure and vascular tone values by infusion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and vasopressin respectively did not affect and abolished the sympathoinhibitory effects of cromakalim and SR 44866.

5 We conclude that in SHRs the two K^+ channel openers that we investigated exert similar sympathohibitory effects which affect some vascular beds more than others. These effects are not dependent upon the arterial blood pressure level and are most likely prejunctionally located.

Introduction

Activation of K^+ channels has recently been considered as a very attractive mechanism for the design of new cardiovascular drugs. K^+ channel openers increase K^+ conductance towards the extracellular space, promote cellular hyperpolarization and elevate smooth muscle activation threshold. This mechanism is principally involved in the reduction in blood pressure produced by K^+ channel openers already observed in experimental models (Buckingham *et al.*, 1986; Buckingham, 1988; Cook & Hof, 1988; Cook *et al.*, 1988; Cavero *et al.*, 1989; Richer *et al.*, 1989) and in man (Vanden Burg *et al.*, 1986).

Vascular resistance is under the active control of sympathetic nerve fibres and vascular vasomotor tone has been shown to be principally dependent upon activation of α-adrenoceptors. Various vasodilator drugs such as angiotensin converting enzyme inhibitors, calcium antagonists, ketanserin, etc. have previously been reported to interact differentially with the α-adrenergic sympathetic nervous system. All these drugs exert sympathoinhibitory effects vs α_1- (ketanserin, Scalbert *et al.*, 1989) or α_2- (calcium antagonists, Van Zwieten *et al.*, 1986) or α_1- and α_2- (converting enzyme inhibitors, Richer *et al.*, 1984) adrenoceptor-mediated systemic and regional vascular responses. These sympathoinhibitory effects may contribute to their vasodilator and antihypertensive properties.

Regarding K^+ channel openers, *in vitro* experiments have shown that cromakalim inhibits contractile responses to noradrenaline in rat aorta (Weir & Weston, 1986) and portal vein (Hamilton *et al.*, 1986) and in rabbit mesenteric artery (Clapham & Wilson, 1986). Moreover, *in vivo* cromakalim inhibits pressor responses to electrical stimulation of the spinal sympathetic outflow in rats (Buckingham, 1988; Shropshire *et al.*, 1989). In this context, the present study was conducted in order (1) to assess whether the previously described systemic sympathoinhibitory effect of cromakalim also develops at the regional vascular level, (2) if so, to determine whether it affects the different vascular beds homogeneously, (3) to investigate some of the characteristics of this sympathoinhibitory affect, and (4) to assess if the latter is a specific property of cromakalim or if it is also shared by other K^+ channel openers, e.g. SR 44866, a newly developed drug of this group (Richer *et al.*, 1989; Maruyama *et al.*, 1989).

Methods

Animal preparation

Male SHRs (Iffa Credo Laboratories, L'Arbresle, France) (body weight: 370 ± 3 g, systolic blood pressure: 231 ± 2 mmHg) were anaesthetized with sodium pentobarbitone (50 mg kg^{-1} i.p., 0.1 ml 100 g^{-1}). The animals were pithed and placed immediately under artificial ventilation (Harvard respirator, model 680). A femoral and a jugular vein were cannulated respectively for agonist (cirazoline, UK-14,304) bolus injections and for K^+ channel openers or solvent infusions (Harvard perfusor, model 907 A). A carotid artery was cannulated for blood pressure measurement with a transducer (Statham P50) connected to an appropriate preamplifier (Gould Instruments, model 13-4615-10). Heart rate (HR) was derived from the arterial pressure pulse (Gould Instruments Biotach amplifier, model 13-4615-66). Systolic (SAP) and diastolic (DAP) blood pressures as well as HR were continuously recorded (Gould Polygraph, model 6610-06). The animals were bivagotomized and given i.v. atropine (1 mg kg^{-1}) and gallamine (20 mg kg^{-1}). Blood velocity Doppler probes were placed around the upper abdominal aorta, the left renal artery, the superior mesenteric artery and the lower abdominal aorta and were connected to a pulsed Doppler flowmeter (Directional pulsed Doppler, model 545C, University of Iowa, Iowa City, U.S.A.). The velocity values obtained have been demonstrated to be directly and linearly proportional to the corresponding blood flows (BF), respectively cardiac output (CO), renal (RBF), mesenteric (MBF) and hindlimb (HBF)

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blood flows (Hartley & Cole, 1974; Richer et al., 1987). After a 15 min-stabilization period the basal values of all parameters investigated were recorded.

Experimental protocols

The animals were randomly assigned to one of the three following treatment groups: controls, cromakalim and SR 44866. After the end of the stabilization period, an infusion ($0.09\,ml\,kg^{-1}\,min^{-1}$) of solvent (controls), of cromakalim ($1\,\mu g\,kg^{-1}\,min^{-1}$) or of SR 44866 ($0.25\,\mu g\,kg^{-1}\,min^{-1}$) was started and maintained for 15 min. After stopping the solvent or K^+ channel opener infusion, the values of all parameters investigated were again determined.

Experiment A: systemic and regional vascular reactivity to postsynaptic α_1-adrenoceptor stimulation Systemic and regional vascular responses to intravenous injections of cirazoline (0.3, 1, 3, $10\,\mu g\,kg^{-1}$) were assessed in 10–15 animals from each group immediately after the end of the K^+ channel opener or solvent infusion.

Experiment B: systemic and regional vascular reactivity to postsynaptic α_2-adrenoceptor stimulation Systemic and regional vascular responses to intravenous injections of UK-14,304 (3, 10, 30, 100, $300\,\mu g\,kg^{-1}$) were assessed in 10–15 animals from each group immediately after the end of the K^+ channel opener or solvent infusion.

Experiments C and D: systemic and regional vascular reactivity to the electrical stimulation of the whole spinal cord Systemic and regional vascular responses were assessed during electrical stimulation of the spinal cord for 20 s at frequencies of 0.25, 0.5, 1, 2 and 4 Hz with a rectangular pulse of 1 ms at maximal voltage (60 V) with a ST Stimulator (Janssen Scientific Instruments, model 198): either immediately after the end of the K^+ channel opener or solvent infusion in 10–15 animals from each group (Experiment C); or after restoration of blood pressure to its pre K^+ channel opener infusion level (Experiment D). This restoration was achieved after cromakalim or SR 44866 infusion, either by a prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) infusion ($0.008\,ml\,kg^{-1}\,min^{-1}$, $30\,\mu g\,kg^{-1}\,min^{-1}$), or by a vasopressin infusion ($0.09\,ml\,kg^{-1}\,min^{-1}$, $4\,mu\,kg^{-1}\,min^{-1}$) ($n = 8$–10).

Drugs

Drugs used were atropine sulphate (Sigma Chemical Co., St-Louis, MO, U.S.A.), cirazoline hydrochloride (Synthelabo, Paris, France), cromakalim (Sanofi Recherche, Montpellier, France), gallamine triethiodide (Rhône-Poulenc Santé, Vitry-sur-Seine, France), pentobarbitone sodium (Abbott, Saint Remy sur Arve, France), prostaglandin $F_{2\alpha}$ (Upjohn Kalamazoo, Mi, U.S.A.), SR 44866 or 6-cyano-2,2-dimethyl-4-[2-oxo-1,2-dihydro-1-pyridyl]-2H-1-benzopyran) (Sanofi Recherche, Montpellier, France), UK-14,304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline) tartrate, (Pfizer, Orsay, France) and vasopressin (Sigma Chemical Co., St-Louis, MO, U.S.A.). Doses are expressed in terms of the indicated free base or salt.

Solutions of cromakalim ($0.1\,mg\,ml^{-1}$) and SR 44866 ($0.1\,mg\,ml^{-1}$) were prepared in the following solvent: distilled water-PEG 200 (93.75:6.25). These solutions were subsequently diluted with saline (1:10). Control experiments were performed with the active drugs' solvent diluted with saline (1:10).

Calculations and statistical analysis of data

Mean arterial pressure (MAP) was calculated from SAP and DAP as follows:

$$MAP = DAP + \frac{SAP\text{-}DAP}{3}.$$

Total peripheral (TPVR) and regional vascular resistances renal (RVR), mesenteric (MVR), hindlimb (HVR) values were calculated as the ratios of MAP to the value (in kHz) of the corresponding Doppler signals (CO, RBF, MBF and HBF, respectively) and expressed in arbitrary units (AU). Reported values are means ± s.e.mean.

Systemic and regional haemodynamic effects of cromakalim, SR 44866 and solvent Mean baseline values for each investigated parameter (SAP, DAP, MAP, HR, RBF, MBF, HBF, CO, RVR, MVR, HVR, TPVR) were calculated for combined A, B and C experiments in the three treatment groups (controls, cromakalim and SR 44866) and compared by analysis of variance.

For each investigated parameter, percentage variations induced by solvent, cromakalim or SR 44866 were calculated for combined A, B and C experiments in the three treatment groups. Statistical analysis was performed within the control group by Student's paired t test or between the three treatment groups by analysis of variance.

Interactions of cromakalim, SR 44866 and solvent with the sympathetic system In experiments A, B, C and D, absolute changes in blood pressure, heart rate, systemic and regional blood flows and vascular resistances induced by each dose of agonist (Experiments A and B) or by each stimulation frequency (Experiments C and D) were determined from the corresponding values observed just after the end of the solvent or K^+ channel opener infusion (Experiments A, B and C) or just after restoration of blood pressure to its pre-K^+ channel opener level by either $PGF_{2\alpha}$ or vasopressin infusions (Experiment D). Their means were then calculated and compared by analysis of variance.

Results

Table 1 shows the mean basal values of each investigated parameter for combined A, B and C experiments in the three treatment groups. Analysis of variance indicates that for none of these parameters was there a significant difference between the three calculated basal values.

Systemic and regional haemodynamic effects of cromakalim, SR 44866 and solvent infusions in pithed SHR

Table 2 shows the mean percentage changes in SAP, DAP, MAP, HR, RBF, MBF, HBF, CO, RVR, MVR, HVR and TPVR induced by cromakalim, SR 44866 and solvent infusions in combined A, B and C experiments. It appears that the solvent slightly but significantly reduced blood pressure, RBF, MBF, HBF and CO and increased TPVR, MVR and HVR. Cromakalim and SR 44866 also decreased blood pressure significantly and to the same extent, this effect being significantly greater than that of the solvent, but did not affect HR. When compared with the control group, RBF and MBF were significantly lowered by both drugs, CO was significantly decreased by cromakalim but not by SR 44866 and HBF was not significantly affected by either drug. Finally, TPVR and especially HVR were significantly lowered by both drugs whereas RVR and HVR were not significantly modified.

Systemic and regional haemodynamic effects of cirazoline in the pithed SHR and their modifications by cromakalim and SR 44866 (Experiment A)

Figure 1 shows that neither cromakalim nor SR 44866 induced any significant change in cirazoline pressor or vasoconstrictor dose-responses.

Table 1 Baseline values for systolic (SAP), diastolic (DAP) and mean (MAP) arterial blood pressure, heart rate (HR), cardiac output (CO), total peripheral resistance (TPVR), renal (RBF, RVR), mesenteric (MBF, MVR), hindlimb (HBF, HVR) blood flows (BF) and vascular resistances (VR) for combined A, B and C experiments in the three treatment groups (solvent, cromakalim and SR 44866) of pithed SHRs

		Blood pressure (mmHg)			*HR*	*Blood flows* (kHz)				*Vascular resistances* (AU)			
Group	n	*SAP*	*DAP*	*MAP*	(beats min^{-1})	*RBF*	*MBF*	*HBF*	*CO*	*RVR*	*MVR*	*HVR*	*TPVR*
Solvent	38	70.9 ± 1.3	42.5 ± 1.3	52.0 ± 1.3	284.3 ± 5.7	2.35 ± 0.14	3.34 ± 0.16	2.13 ± 0.13	3.11 ± 0.15	25.2 ± 1.6	17.5 ± 1.7	28.1 ± 2.1	18.0 ± 0.8
SR 44866	45	71.3 ± 1.4	44.8 ± 1.1	53.6 ± 1.2	278.8 ± 5.3	2.36 ± 0.15	3.50 ± 0.20	2.11 ± 0.14	2.82 ± 0.16	27.6 ± 2.1	18.5 ± 1.4	28.4 ± 2.1	21.7 ± 1.6
Cromakalim	45	71.0 ± 1.5	43.8 ± 1.4	52.9 ± 1.3	289.0 ± 6.2	2.45 ± 0.14	3.16 ± 0.18	2.14 ± 0.12	2.79 ± 0.14	24.5 ± 1.5	19.8 ± 1.5	27.8 ± 1.5	21.2 ± 1.2
Anova F^2_{127}		0.053 NS	0.744 NS	0.390 NS	0.829 NS	0.061 NS	0.739 NS	0.071 NS	1.309 NS	0.867 NS	0.521 NS	0.024 NS	2.310 NS

Values are means ± s.e.mean. NS: not significant.
AU: arbitrary units.

Systemic and regional haemodynamic effects of UK-14,304 in the pithed SHR and their modifications by cromakalim and SR 44866 (Experiment B)

The two K^+ channel openers induced minor changes in the UK-14,304 pressor or vasoconstrictor responses. Figure 2 shows that only UK-14,304-induced increases in MVR and HVR were significantly reduced by both drugs.

Systemic and regional haemodynamic effects of electrical stimulation of the spinal cord in the pithed SHR and their modifications by cromakalim and SR 44866 (Experiment C)

Figure 3 shows that the hypertensive responses to electrical stimulation were significantly depressed by both K^+ channel openers at all frequencies and to the same extent. The cardiac output increases elicited by electrical stimulation were also reduced by both drugs and significantly so at low stimulation frequencies. In contrast, the positive chronotropic and the systemic vasoconstrictor responses (TPVR increases) elicited were not significantly modified by either drug. Similarly, regional blood flow increases were not modified but regional vasoconstrictor responses in the mesentery, in the kidney and in the hindlimb were significantly decreased by both drugs (Figure 4). This decrease was not homogeneously distributed in the three investigated vascular beds, the drugs preferentially affecting the mesentery (cromakalim: −86%, SR 44866: −91%), followed by the kidney (cromakalim: −67%, SR 44866: −42%) and finally the hindlimb bed (cromakalim: −44%, SR 44866: −37%).

Table 3 shows MAP and HVR absolute values before (basal values) starting K^+ channel openers infusions, after the end of these infusions and after restoration of MAP to its

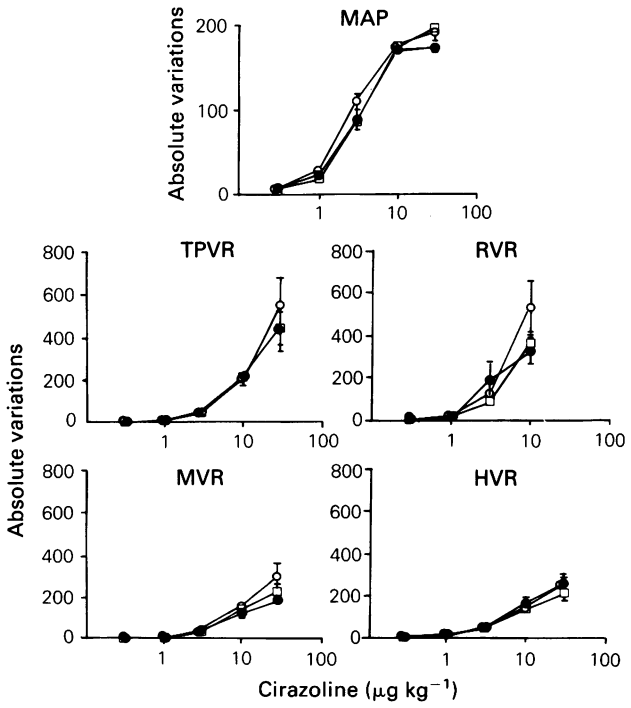

Figure 1 Absolute variations (s.e.mean shown by vertical bars) of mean arterial pressure (MAP, mmHg) and peripheral (TPVR), renal (RVR), mesenteric (MVR) and hindlimb (HVR) vascular resistances (arbitrary units) induced by increasing doses of cirazoline in pithed SHRs pretreated either with cromakalim (□) or with SR 44866 (●) or with the solvent (○).

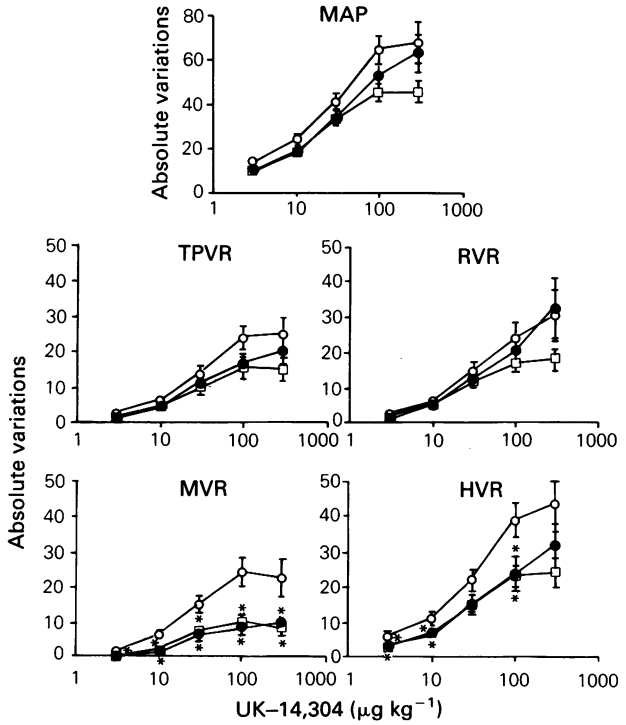

Figure 2 Absolute variations (s.e.mean shown by vertical bars) of mean arterial pressure (MAP, mmHg) and peripheral (TPVR), renal (RVR), mesenteric (MVR) and hindlimb (HVR) vascular resistances (arbitrary units) induced by increasing doses of UK-14,304 in pithed SHRs pretreated either with cromakalim (□) or with SR 44866 (●) or with the solvent (○).
* Value significantly different from corresponding value in the solvent pretreated group: P at least <0.05.

Table 2 Percentage variations of systolic (SAP), diastolic (DAP), mean (MAP) arterial blood pressure, heart rate (HR), cardiac output (CO), total peripheral resistance (TPVR), renal (RBF, RVR), mesenteric (MBF, MVR) and hindlimb (HBF, HVR) blood flows (BF) and vascular resistances (VR) induced by cromakalim, SR 44866 or solvent infusion for combined A, B and C experiments in pithed SHRs

						Blood flows (BF)				*Vascular resistances* (VR)			
Treatment	n	*SAP*	*DAP*	*MAP*	*HR*	*RBF*	*MBF*	*HBF*	*CO*	*RVR*	*MVR*	*HVR*	*TPVR*
Solvent	38	− 5.4*** ± 1.0	− 3.9* ± 1.6	− 4.7*** ± 1.2	0.7 ± 0.7	− 6.9*** ± 1.9	− 10.8*** ± 2.1	− 10.9*** ± 2.0	− 12.8*** ± 1.6	3.9 ± 2.3	8.6*** ± 2.4	9.3** ± 3.0	10.4*** ± 2.1
SR 44866	45	− 15.0[c] ± 0.9	− 20.1[c] ± 1.3	− 17.9[c] ± 1.0	0.5 ± 0.8	− 14.7[a] ± 2.0	− 22.9[c] ± 1.9	− 9.9 ± 2.5	− 14.9 ± 1.4	− 1.6 ± 2.3	9.0 ± 2.7	− 6.0[c] ± 2.9	− 2.5[c] ± 1.7
Cromakalim	45	− 17.5 ± 1.0	− 25.0[c,d] ± 1.5	− 21.7[c,d] ± 1.2	− 0.7 ± 0.8	− 19.0[c] ± 2.2	− 25.6[c] ± 1.9	− 12.9 ± 2.5	− 19.6[b,d] ± 1.4	0.6 ± 3.5	7.1 ± 3.1	− 7.5[c] ± 2.7	− 1.8[c] ± 1.8
Anova F^2_{127}		40.666 S 1‰	53.877 S 1‰	57.303 S 1‰	0.850 NS	9.118 S 1‰	15.181 S 1‰	0.400 NS	5.780 S 1%	0.909 NS	0.138 NS	10.199 S 1‰	14.431 S 1‰

Values are means ± s.e.mean. S: significant, NS: not significant.
Significant % variation (Student's paired t test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).
% variation significantly different from that observed in solvent group (ANOVA): [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.
% variation significantly different from that observed in SR 44866 group (ANOVA): [d] $P < 0.05$.

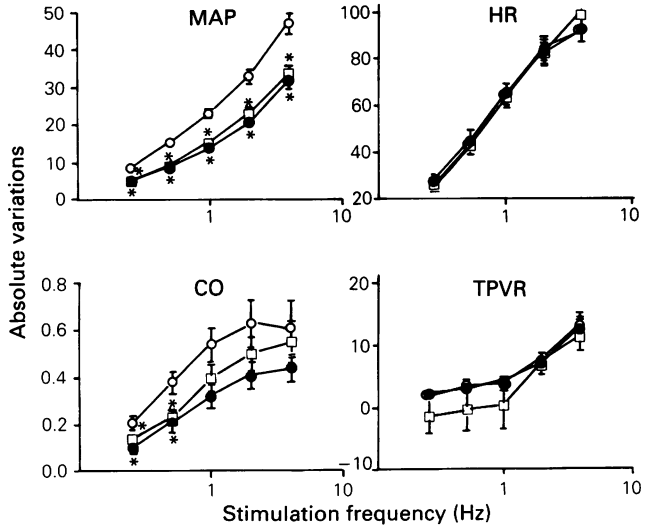

Figure 3 Absolute variations (s.e.mean shown by vertical bars) of mean arterial pressure (MAP, mmHg), heart rate (HR, beats min^{-1}), cardiac output (CO, kHz) and total peripheral vascular resistance (TPVR, arbitrary units) induced by increasing stimulation frequencies of the spinal cord in pithed SHRs pretreated either with cromakalim (□) or with SR 44866 (●) or with the solvent (○).
* Value significantly different from corresponding value in the solvent pretreated group: P at least <0.05.

basal value by either $PGF_{2\alpha}$ or vasopressin infusion, i.e. just before starting electrical stimulation of the spinal cord sympathetic outflow. Table 3 shows that, whatever the treatment group, the vasoconstrictor drug infusion restored blood pressure to within the control range, indicating that the respective doses of $PGF_{2\alpha}$ and vasopressin were properly adjusted. It

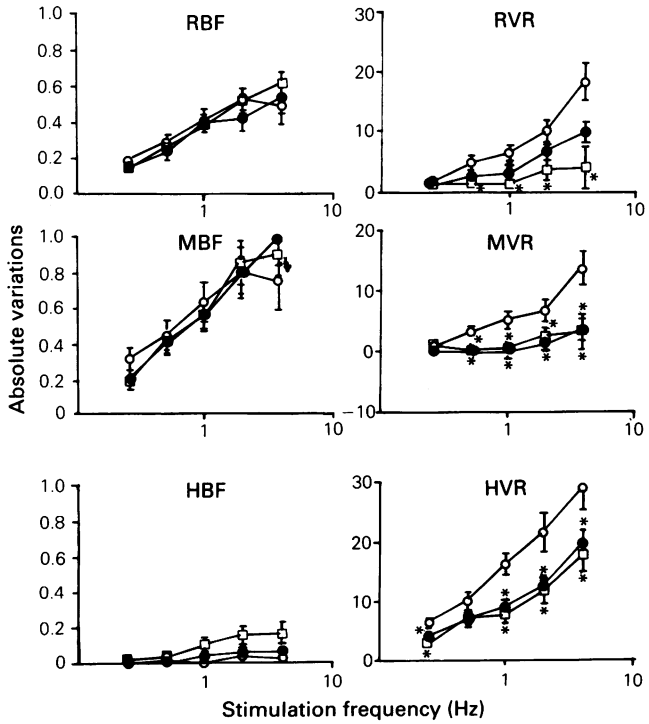

Figure 4 Absolute variations (s.e.mean shown by vertical bars) of renal (RBF, RVR), mesenteric (MBF, MVR) and hindlimb (HBF, HVR) blood flows (BF, kHz) and vascular resistances (VR, arbitrary units) induced by increasing stimulation frequencies of the spinal cord in pithed SHRs pretreated either with cromakalim (□) or with SR 44866 (●) or with the solvent (○).
* Value significantly different from corresponding value in the solvent pretreated group: P at least <0.05.

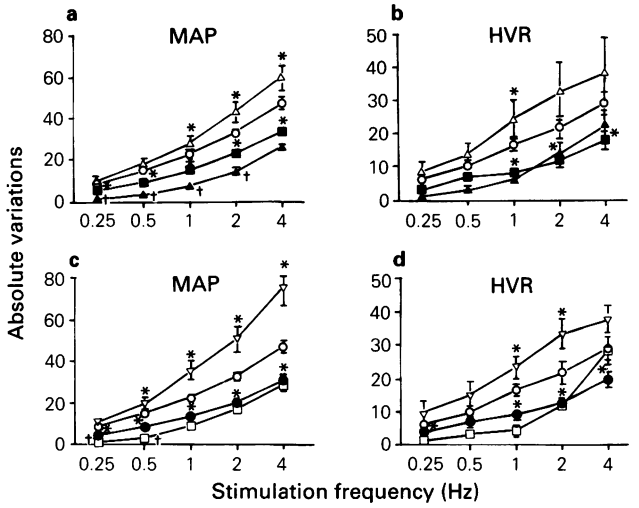

Figure 5 Absolute variations (s.e.mean shown by vertical bars) of mean arterial pressure (MAP, mmHg, a and c) and hindlimb vascular resistance (HVR, arbitrary units, b and d) induced by increasing stimulation frequencies of the spinal cord in pithed SHRs pretreated: (a and b) with cromakalim (■), with cromakalim after prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, ▲), with cromakalim after vasopressin (△) or with the solvent (○), and (c and d) with SR 44866 (●), with SR 44866 after $PGF_{2\alpha}$ (□), with cromakalim after vasopressin (▽) or with the solvent (○).
* Value significantly different from corresponding value in solvent group: P at least <0.05.
† Value significantly different from corresponding value in K^+ channel opener group: P at least <0.05.

also appears that these doses of $PGF_{2\alpha}$ and vasopressin raised hindlimb (Table 3), renal and mesenteric vascular resistances (data not shown) to values higher than those initially observed before K^+ channel opener infusion.

Figure 5 shows that the sympathoinhibitory effects of cromakalim and SR 44866 were abolished after restoration of MAP to its initial value by vasopressin infusion, the pressor and vasoconstrictor responses evoked by electrical stimulation of the spinal cord being even potentiated.

In contrast, Figure 5 shows that the sympathoinhibitory effects of cromakalim and SR 44866 vs the pressor and vasoconstrictor responses evoked by electrical stimulation of the spinal cord were not modified when restoration of MAP to its initial level was achieved by $PGF_{2\alpha}$ infusion, the magnitude of the pressor and vasoconstrictor responses observed after cromakalim or SR 44866 infusion being decreased to the same extent as in the absence of $PGF_{2\alpha}$.

Discussion

Few data are available at present regarding the interactions between K^+ channel openers and the sympathetic nervous system (see Introduction). The main purpose of this study was thus to investigate more thoroughly this problem at the systemic but also at the regional vascular level by use of two different compounds from this group of drugs.

In as much as most of the studies investigating the interactions between antihypertensive drugs of various types and the sympathetic nervous system have been performed previously in SHRs (Antonaccio & Kerwin, 1981; Richer *et al.*, 1984; 1985; Buckingham, 1988; Scalbert *et al.*, 1989), this strain was also used in our experiments in order to allow comparisons. In addition, the respective doses of cromakalim and SR 44866 and the protocol design were chosen according to previous results which showed that in normotensive anaesthetized rats, SR 44866 was about four times more potent than cromakalim in lowering blood pressure and that the

Table 3 Absolute values (± s.e.mean) of mean arterial pressure (MAP) and hindlimb vascular resistance (HVR) before (basal values), immediately after the end of K^+ channel opener infusion and after restoration of MAP to its pre-K^+ channel opener infusion level either by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or vasopressin infusions

Treatment	*Cromakalim*		*SR 44866*	
	MAP	*HVR*	*MAP*	*HVR*
$PGF_{2\alpha}$				
Basal values	50.9 ± 1.3	30.3 ± 1.8	49.8 ± 2.0	30.0 ± 2.9
After K^+ channel opener	40.2 ± 1.3[c]	27.2 ± 2.0[b]	39.4 ± 1.7[a]	28.3 ± 3.5[a]
After K^+ channel opener + $PGF_{2\alpha}$	48.5 ± 1.6	37.9 ± 2.7[a]	51.7 ± 2.2	32.1 ± 2.9
Vasopressin				
Basal values	39.7 ± 1.3	33.7 ± 4.7	42.4 ± 2.2	40.1 ± 5.3
After K^+ channel opener	30.0 ± 1.6[b]	27.8 ± 4.8[b]	34.5 ± 3.0[a]	30.2 ± 1.7[a]
After K^+ channel opener + vasopressin	44.9 ± 3.3	44.3 ± 9.5	41.1 ± 2.6	36.7 ± 2.9

Value significantly different from corresponding basal value (Student's paired *t* test): [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.

duration of the systemic and regional haemodynamic effects of both drugs exceeded 30 min (Richer *et al.*, 1989).

Our present results confirm in pithed SHRs the potency ratio of the two drugs investigated but also show that this experimental model is more sensitive to the K^+ channel openers blood pressure lowering effect than the intact normotensive rat. Thus the doses of cromakalim and SR 44866 required to produce an approximately 20 percent decrease in blood pressure were found to be $1\,\mu g\,kg^{-1}\,min^{-1}$ and $0.25\,\mu g\,kg\,min^{-1}$ respectively in the pithed SHR, the corresponding values in the intact normotensive anaesthetized rat being $2.8\,\mu g\,kg^{-1}\,min^{-1}$ and $0.7\,\mu g\,kg^{-1}\,min^{-1}$ respectively (Richer *et al.*, 1989). It is also noteworthy that in the pithed SHR, HR was, as expected, not affected whereas in conscious animals, with a fully operational autonomic nervous system, tachycardia accompanies the blood pressure lowering effect (Buckingham *et al.*, 1986). Finally, in the pithed SHR, the regional vasodilator profiles of the two drugs were similar, confirming their preferential vasodilator effect in skeletal muscle beds (Cook & Hof, 1988; Cavero *et al.*, 1989).

At equihypotensive doses, cromakalim and SR 44866 exhibited a similar pattern of interactions with cirazoline, UK-14,304 and spinal cord stimulation-induced systemic pressor and regional vasoconstrictor effects. This finding indicates that this pattern is not specific to cromakalim but is shared by other K^+ channel openers, suggesting that it is related to the common effects of these drugs on cell membrane K^+ permeability.

The inability of both drugs to oppose pressor and vascular responses to cirazoline, a selective α_1-postsynaptic adrenoceptor agonist, indicates that they are devoid of any sympathoinhibitory effect versus the systemic and regional vascular responses to postsynaptic α_1-adrenoceptor stimulation.

Systemic and renal vasoconstrictor responses to UK-14,304, a selective postsynaptic α_2-adrenoceptor agonist were not significantly modified by either drug but mesenteric and hindlimb vasoconstrictor responses were significantly reduced and to the same extent by cromakalim and SR 44866. These results demonstrate that these two K^+ channel openers develop only weak sympathoinhibitory effects versus the systemic and regional vascular responses to postsynaptic α_2-adrenoceptor stimulation. Calcium antagonists have previously been shown to reduce the pressor and regional vasoconstrictor effects of selective α_2-agonists but to decrease only slightly those of selective α_1-agonists (Van Zwieten *et al.*, 1986). Our data indicate that K^+ channel openers which exhibit no α_1- and only a weak α_2-sympathoinhibitory effect interact very similarly to calcium antagonists with the sympathetic nervous system. The mechanism of this interaction could be, as it is for calcium antagonists, a reduction of the penetration of Ca^{2+} into the cell, related to the K^+ channel openers-induced hyperpolarization of the cell membrane, rather than the mobilization of intracellular Ca^{2+}.

In anaesthetized pithed SHRs, cromakalim and SR 44866 both exhibited sympathoinhibitory effects against the systemic pressor and regional vasoconstrictor responses induced by spinal cord stimulation. This finding confirms the observation reported by Buckingham (1988) with cromakalim at the systemic level and extends it not only to the three investigated regional vascular beds but also to another K^+ channel opener, namely SR 44866. Furthermore, our data show that the sympathoinhibitory effects of the two K^+ channel openers do not equally affect the three investigated vascular beds, the decreasing rank order being: mesentery > kidney > hindlimb. The mechanism and significance of this heterogeneity in the K^+ channel openers sympathoinhibitory effects are not yet clear and require further investigation. Previously, converting enzyme inhibitors have also been shown to exert non-homogeneous sympathoinhibitory effects, affecting preferentially the renal bed (Richer *et al.*, 1984). On the other hand, nicardipine and ketanserin have been shown to decrease sympathetic mediated responses homogeneously in the different vascular beds investigated (Richer *et al.*, 1985; Scalbert *et al.*, 1989).

In contrast to the vascular responses, the tachycardia induced by electrical stimulation remained unaffected by the two drugs. Despite this unchanged tachycardic response, the increases in cardiac output elicited by electrical stimulation of the spinal cord were significantly reduced by both drugs. This suggests that the inhibitory effects of K^+ channel openers on the pressor responses to sympathetic nerve stimulation result not only from their action on vascular smooth muscle but also from the reduction in cardiac output they induce. This reduction could possibly be linked either to a decreased venous return or to the intrinsic negative inotropic effect of the drugs (Gotanda *et al.*, 1988).

Responses to endogenous catecholamines released by spinal cord stimulation are due mainly to the activation of postsynaptic α_1-adrenoceptors (Langer *et al.*, 1981). In as much as the latter were not affected by the two K^+ channel openers used in our experiments, the mechanism of the sympathoinhibitory effects exhibited by the two drugs versus the vascular responses to spinal cord stimulation appears to be prerather than postjunctional in its location. However, the precise site where sympathoinhibition develops and the specific involvement of K^+ channels in its genesis remain to be demonstrated. Our experiments also clearly show that K^+ channel openers exert their sympathoinhibitory effects only at the vascular level but not at the sinus node since the tachycardic responses to spinal cord stimulation remained unaffected. A similar phenomenon has previously been reported with converting enzyme inhibitors (Antonaccio & Kerwin, 1981).

It also appears from our experiments that the sympathoinhibitory effects of the two K^+ channel openers versus the responses to electrical stimulation of the spinal cord are not

dependent upon the blood pressure level, as previously postulated (Buckingham, 1988). And indeed, if it is true that the sympathoinhibitory effect of cromakalim (Buckingham, 1988) and also of SR 44866 disappears when blood pressure is restored to pre-drug infusion levels by vasopressin, this is not the case when $PGF_{2\alpha}$ is used as a vasoconstrictor agent. The reason for this discrepancy is not clear but might simply be due to an interaction between the vasoconstrictor agent itself and the sympathetic nervous system. In fact, vasopressin has recently been shown to potentiate markedly vasoconstrictor responses to electrical stimulation and to noradrenaline in rabbits (Patel & Schmid, 1988), a phenomenon that could have blunted the sympathoinhibitory effects of K^+ channel openers in our and in Buckingham's (1988) experiments.

Finally, the additional fall in both blood pressure and vascular resistance produced by cromakalim and SR 44866 in pithed SHRs demonstrates that these two K^+ channel openers do not require an intact autonomic nervous system in order to exert their hypotensive action. Thus, the sympathoinhibitory effects of cromakalim and SR 44866 appear not to be involved in the blood pressure decrease. However, the possibility cannot be excluded that they might partly contribute *in vivo* to attenuate some sympathetic vascular reflexes which could limit the vasodilator and/or antihypertensive effects of K^+ channel openers.

In conclusion, it appears that the two K^+ channel openers, cromakalim and SR 44866, exert sympathoinhibitory effects in SHRs. These effects, which selectively affect some vascular beds (mesentery > kidney > hindlimb), are not dependent upon the arterial pressure level and their site of action is most probably prejunctionally located.

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Inhibition by ibudilast of leukotriene D_4-induced formation of inositol phosphates in guinea-pig lung

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1 The effects of a novel anti-asthmatic drug, 3-isobutyryl-2- isopropylpyrazolo [1,5-a]pyridine (ibudilast, KC-404) on leukotriene D_4 (LTD_4)-induced formation of inositol phosphates were studied in slices of guinea-pig lung and hippocampus.

2 In guinea-pig lung, ibudilast inhibited LTD_4 (0.01–1 μM)-induced formation of inositol monophosphate (IP_1) in a concentration-dependent manner (IC_{50} = 10 μM) without affecting LTD_4 receptor binding.

3 Ibudilast (10 μM) inhibited histamine (0.1–1 mM)-induced formation of IP_1 in guinea-pig lung slices but not in hippocampal slices.

4 Inhibition of agonist-induced formation of IP_1 by ibudilast was non-competitive.

5 Ibudilast had no effect on either GTP- or calcium-stimulated phosphatidylinositol specific-phospholipase C activity of lung membranes.

6 These results suggest that ibudilast has no direct effect on LTD_4 receptors, GTP binding proteins (G proteins) or phospholipase C, but inhibits inositol phosphate formation, possibly by interfering with the coupling between receptors and G proteins.

Introduction

Leukotriene D_4 (LTD_4), one of the constituents of slow reacting substance of anaphylaxis, induces prolonged smooth muscle contraction and is believed to play a role in bronchial asthma. Since a rise in the intracellular concentration of free calcium ions is a prerequisite for physiological contraction of smooth muscles, the mechanism of LTD_4-induced smooth muscle contraction is expected to involve intracellular calcium ions. Evidence from the effects of calcium channel blockers (Israel *et al.*, 1987) and a so-called 'intracellular calcium antagonist' (Weichman *et al.*, 1983) on LTD_4-induced smooth muscle contraction, suggests that the calcium ions come from intracellular stores rather than from the extracellular fluid. It is now well known that calcium is released from intracellular stores by inositol 1,4,5-trisphosphate (IP_3). Recently it has been demonstrated that LTD_4 enhances the production of IP_3 and the subsequent release of calcium in guinea-pig lung (Mong *et al.*, 1987a), several smooth muscle cells (Clark *et al.*, 1985; Mong *et al.*, 1988a) and rat basophilic leukaemia cells (Sarau *et al.*, 1987; Mong *et al.*, 1988b). Thus, the effects of LTD_4 in these tissues seem to be related to receptor-mediated hydrolysis of phosphatidylinositol by phospholipase C.

Several different types of compound have been described that are competitive antagonists of LTD_4 receptors (e.g. Mong *et al.*, 1987b; Ahnfelt-Rønne *et al.*, 1988). Ibudilast is a novel, orally active anti-asthmatic that is in clinical use (Nishino *et al.*, 1983). The drug inhibits the increase in vascular permeability induced by antigen in sensitized guinea-pig airway smooth muscle (Eda *et al.*, 1987) and antagonizes LTD_4-induced contraction of guinea-pig lung parenchymal and tracheal strips (Ohasi *et al.*, 1986). To determine the mechanism by which ibudilast antagonizes the pharmacological action of LTD_4, we have examined the effect of ibudilast on LTD_4 receptor binding and the production of inositol phosphates stimulated by LTD_4 in guinea-pig lung preparations.

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Methods

Isolation of membrane preparations from guinea-pig lung

Male Hartley guinea-pigs (300–500 g) were anaesthetized with sodium pentobarbitone (100 mg kg^{-1} i.p.). Lung lobes were dissected from the trachea and homogenized in 10 volumes of 0.32 M sucrose containing 5 mM HEPES (pH 7.4) using a Polytron homogenizer. The crude homogenate was centrifuged (1000 *g* for 10 min) and the resulting pellet discarded. The supernatant was centrifuged (30,000 *g* for 30 min) and the resulting pellet washed, resuspended in ice-cold 50 mM Tris-HCl buffer (pH 7.4) and used as a membrane preparation.

[^{3}H]-LTD_4 binding assay

The [^{3}H]-LTD_4 binding assay was carried out in a 300 μl of 50 mM Tris-HCl buffer (pH 7.4) containing the membrane preparation (0.8 mg ml^{-1}). After incubation for 30 min at 25°C, radioactivity bound to lung membranes was separated from free by vacuum filtration through glass fibre filters (Whatman GF/B) and washed three times with 5 ml of ice-cold Tris-buffer. The filters were placed in glass vials containing 10 ml of scintillant (ACS-II) and radioactivity assessed by liquid scintillation spectrometry.

Specific [^{3}H]-LTD_4 binding, determined from the difference between radioligand bound in the absence and presence of 3 μM LTD_4, was about 80% of the total [^{3}H]-LTD_4 binding. The concentration of [^{3}H]-LTD_4 used for displacement studies was 0.5 nM.

Incorporation of [^{3}H]-myo-inositol into guinea-pig lung slices

The method used was that described by Mong *et al.* (1987a). Guinea-pigs were anaesthetized with sodium pentobarbitone (100 mg kg^{-1} i.p.) and lungs perfused through the pulmonary artery with 50 ml of warm, oxygenated (95% O_2/5% CO_2) Krebs-Ringer-Henseleit (KRH) buffer (composition in mM: NaCl 118, KCl 4.6, $MgSO_4$ 1.1, $CaCl_2$ 1.8, $NaHCO_3$ 24.9, KH_2PO_4 1.0 and glucose 11.1, pH 7.4). Lungs were removed,

rinsed in KRH buffer, and cut into fragments 1–2 mm^3 with a tissue chopper. The tissue was preincubated for 60 min at 37°C in KRH buffer, washed three times and then incubated for 80 min at 37°C in KRH buffer containing [^{3}H]-myo-inositol (6.7 μCi ml^{-1}). The tissue was washed a further three times with fresh KRH buffer, divided into aliquots (10–15 slices per plastic tube) and incubated for 20 min at 37°C in the presence of 10 mM LiCl and various concentrations of drugs (final volume: 300 μl). The tissue was then homogenized briefly with Sonifire (Branson Co.) at 4°C. The homogenate (220 μl) was transferred to other tubes, to which 940 μl of chloroform/methanol (1:2) was added. The protein concentration of the homogenate was determined by the method of Lowry *et al.* (1951).

For the preparation of [^{3}H]-myo-inositol labelled lung membranes, lung slices were incubated with 13.3 μCi ml^{-1} of [^{3}H]-myo-inositol in KRH buffer for 80 min at 37°C. The tissue was washed three times with KRH buffer and homogenized in 10 ml of homogenization buffer (0.25 M sucrose, 10 mM Tris-HCl pH 7.5, 2 mM EGTA, 10 μg ml^{-1} soybean trypsin inhibitor, 25 μM leupeptin). The homogenate was centrifuged twice (1000 *g* for 10 min, and again 30,000 *g* for 20 min). The resulting pellet, defined as the crude membrane fraction, was washed three times with 20 mM Tris-HCl (pH 7.5) and aliquots (200–300 μg protein) were incubated at 37°C for 5 min with drugs in a total volume of 220 μl of 20 mM Tris-HCl buffer (pH 7.5). The reaction was terminated by the addition of 940 μl of chloroform/methanol (1:2).

Incorporation of [^{3}H]-myo-inositol into guinea-pig hippocampal slices

Guinea-pig hippocampal slices (0.25 mm) were prepared and preincubated for 60 min at 37°C in oxygenated (95% O_2/5% CO_2) Krebs-Ringer (KR) buffer (composition in mM: NaCl 122, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.3, $NaHCO_3$ 25, KH_2PO_4 0.4 and glucose 10, pH 7.4). [^{3}H]-myo-inositol incorporation was performed for 60 min and the reaction was carried out as described for lung slices but with KR buffer.

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

Extraction and measurement of [^{3}H]-inositol phosphates were carried out according to the method of Baudry *et al.* (1986). After addition of chloroform/methanol, each tube was vortexed vigorously for 30 s and then sonicated for 5 min at 4°C. Chloroform (310 μl) and H_2O (310 μl) were added and each tube was further vortexed for 10 s, before being centrifuged (1300 *g* for 5 min) to separate the phases. Aliquots (700 μl) of the upper aqueous phase were applied to individual columns of an anion-exchange resin (Bio-Rad AG 1X-8, 100-200 mesh, formate-form). Each column was washed consecutively with 10 ml of ice-cold H_2O, 4 ml of 0.2 M ammonium formate/0.1 M formic acid, 4 ml of 0.4 M ammonium formate/0.1 M formic acid and 4 ml 1.0 M ammonium formate/0.1 M formic acid to elute [^{3}H]-myo-inositol, [^{3}H]-IP_1, [^{3}H]-inositol bisphosphate (IP_2) and [^{3}H]-IP_3, respectively. One ml fractions of eluate were collected, mixed with 5.5 ml of scintillation cocktail (Atomlight), and radioactivity determined by liquid scintillation spectrometry.

Drugs and materials

Ibudilast, LTD_4 and 7-[3-(4-acethyl-3-hydroxy-2-propyl-phenoxy)-2-hydroxyperoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid (FPL 55712) were synthesized in Kyorin Central Research Laboratories. [^{3}H]-myo-inositol (19.0–22.8 Ci $mmol^{-1}$) and ACS-II were obtained from Amersham. [^{3}H]-LTD_4 (38.4 Ci $mmol^{-1}$) and Atomlight was obtained from New England Nuclear. Ethyleneglycol-bis-(β-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA), Tris buffer, leupeptin and soybean trypsin inhibitor were purchased from Sigma Chemical Co.

Results

Effect of ibudilast on LTD_4 binding to guinea-pig isolated lung membranes

The specific binding of [^{3}H]-LTD_4 to lung membranes was saturable and Scatchard analysis of the data yielded a straight line (Figure 1). The dissociation constant (K_D) and maximum binding (B_{max}) were 1.05 ± 0.22 nM and 275.3 ± 16.5 fmol mg^{-1} protein, respectively. The slope factor of the Hill plot was not significantly different from unity, indicating the presence of a single type of binding site ($n_H = 0.95 \pm 0.02$, $n = 3$). Specific binding of [^{3}H]-LTD_4 was completely displaced by the addition of nonradioactive LTD_4 and by FPL 55712, a competitive antagonist for LTD_4 receptors. In contrast, ibudilast did not displace [^{3}H]-LTD_4 from lung membranes (Figure 2).

Effect of ibudilast on IP_1 formation in guinea-pig lung slices

In guinea-pig lung slices, LTD_4 increased the accumulation of [^{3}H]-IP_1 in the presence of 10 mM LiCl (Figure 3). This effect was dependent on the concentration of LTD_4, being nearly maximal at 30 nM LTD_4 and half-maximal (EC_{50}) at 2 nM. Similar concentrations of LTD_4 also increased the formation of [^{3}H]-IP_2 and [^{3}H]-IP_3 but to a lesser extent. The formation of [^{3}H]-IP_1 stimulated by a 10 nM LTD_4 was inhibited in a concentration-dependent manner by ibudilast, with an IC_{50} of 10 μM and a maximal inhibition of about 60% (Figure 4). However, the drug had no effect on the basal formation of [^{3}H]-IP_1. The IC_{50} values for ibudilast were similar whether the concentration of LTD_4 was 10 nM, 100 nM or 1 μM LTD_4 (data not shown). Furthermore, when the tissue was incubated with 10 μM ibudilast, the dose-response curve for LTD_4 was not shifted to the right but the maximal response was decreased by about 40% (Figure 5). This inhibitory effect

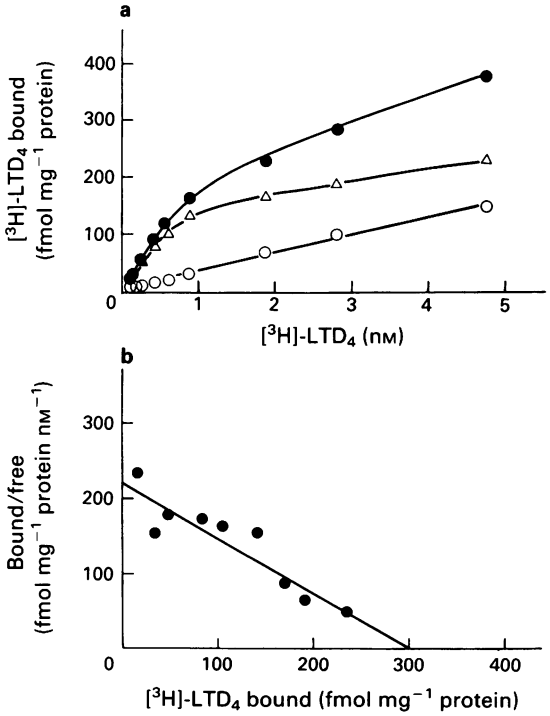

Figure 1 Saturation curve (a) and Scatchard analysis (b) of [^{3}H]-leukotriene D_4 ([^{3}H]-LTD_4) binding in guinea-pig lung membrane preparations. Guinea-pig lung membranes (240 μg) were incubated with various concentrations of [^{3}H]-LTD_4 in 50 mM Tris-HCl buffer (pH 7.4) in a total volume of 300 μl for 30 min at 25°C as described in Methods. (a) Specific [^{3}H]-LTD_4 binding (△) was determined from the difference between radioligand binding in the absence (●) and presence (○) of 3 μM LTD_4.

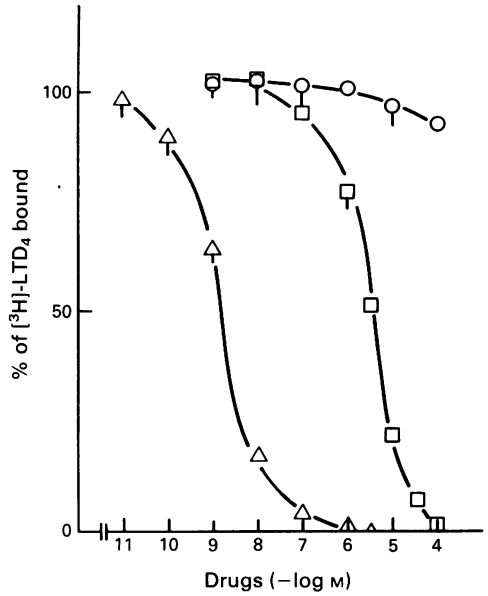

Figure 2 Effect of leukotriene D_4 (LTD_4), ibudilast and FPL 55712 on [^{3}H]-LTD_4 specific binding in guinea-pig lung membrane preparations. Guinea-pig lung membranes (240 μg) were incubated with 0.5 nM [^{3}H]-LTD_4 and various concentrations of nonradioactive LTD_4 (△), ibudilast (○) and FPL 55712 (□) in 50 mM Tris-HCl buffer (pH 7.4) in a total volume 300 μl for 30 min at 25°C, as described in Methods. The results represent the means of 3 experiments and vertical lines show s.e.mean.

remained when the tissue was preincubated with 10 μM ibudilast and then washed before the addition of LTD_4 (data not shown).

Ibudilast also inhibited, non-competitively, histamine-induced formation of [^{3}H]-IP_1 in lung slices (Figure 6a). In

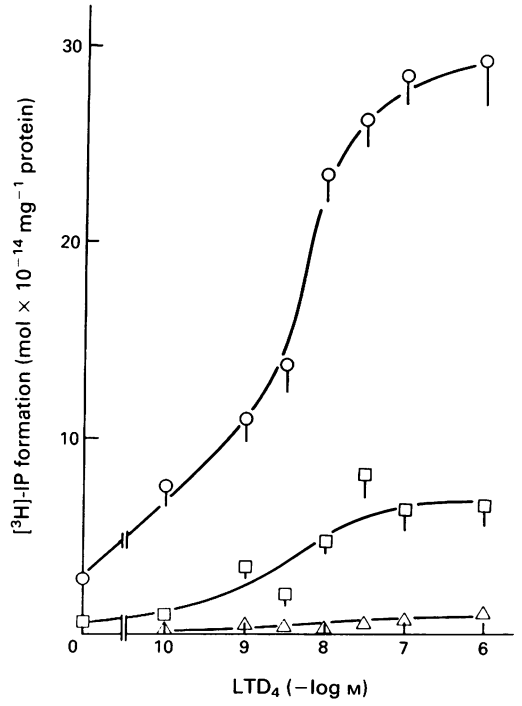

Figure 3 Dose-response curves for leukotriene D_4 (LTD_4)-induced [^{3}H]-inositol phosphate ([^{3}H]-IP) formation in guinea-pig lung. [^{3}H]-inositol-labelled guinea-pig lung slices were incubated with various concentrations of LTD_4 in the presence of 10 mM LiCl for 20 min at 37°C as described in Methods. [^{3}H]-inositol monophosphate (○), [^{3}H]-inositol bisphosphate (□) and [^{3}H]-inositol trisphosphate (△) were extracted and measured separately. The results represent the means of 15 experiments and vertical lines show s.e.mean.

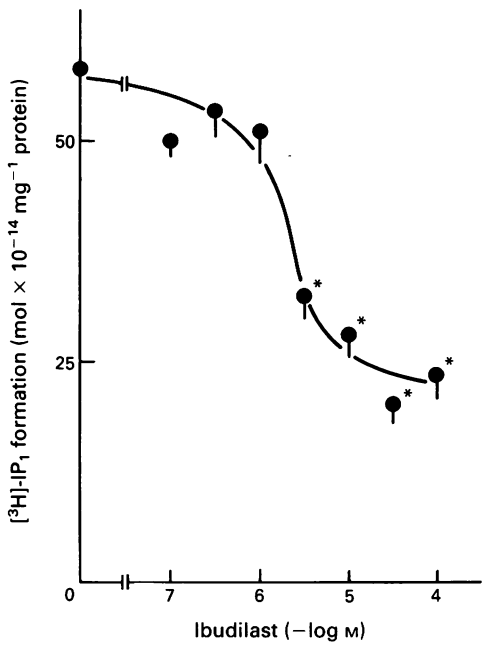

Figure 4 Inhibitory effect of ibudilast on leukotriene D_4 (LTD_4)-induced [^{3}H]-inositol monophosphate ([^{3}H]-IP_1) formation in guinea-pig lung. [^{3}H]-inositol labelled guinea-pig lung slices were incubated with 1 μM LTD_4 and various concentrations of ibudilast in the presence of 10 mM LiCl for 20 min at 37°C as described in Methods. The results represent the means of 3 experiments and vertical lines show s.e.mean. * $P < 0.001$ versus control.

contrast, ibudilast did not inhibit histamine-induced formation of [^{3}H]-IP_1 in guinea-pig hippocampal slices (Figure 6b).

Effect of ibudilast on inositol phosphate formation in guinea-pig lung membrane

Recent evidence suggests that LTD_4 receptor-induced responses are mediated by a G protein (Pong & DeHaven,

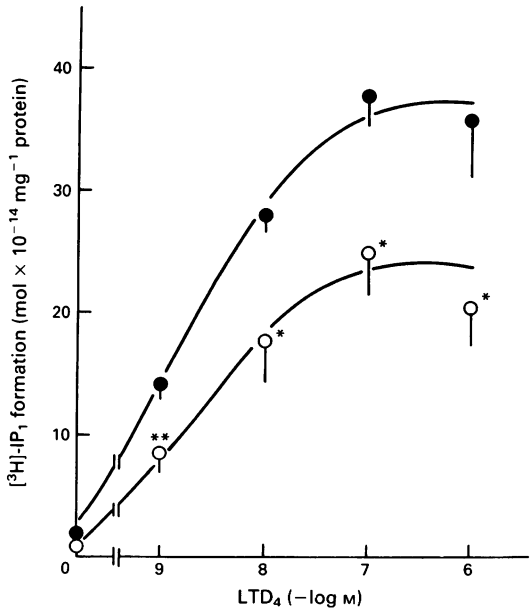

Figure 5 Effect of ibudilast on the dose-response curve of leukotriene D_4 (LTD_4)-induced [^{3}H]-inositol monophosphate ([^{3}H]-IP_1) formation in guinea-pig lung. [^{3}H]-inositol labelled guinea-pig lung slices were incubated with various concentrations of LTD_4 in the presence of 10 mM LiCl for 20 min at 37°C as described in Methods in the presence (○) or absence (●) of 10 μM ibudilast. The results represent the means of 6 experiments and vertical lines shown s.e.mean. * $P < 0.05$; ** $P < 0.01$ versus control.

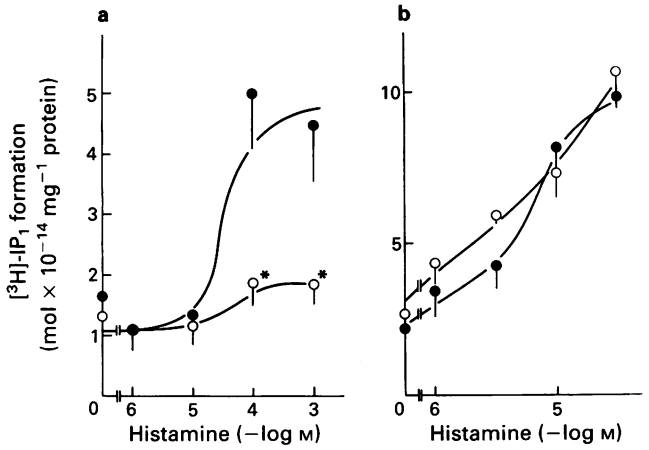

Figure 6 Effects of ibudilast on dose-response curves for histamine-induced [^{3}H]-inositol monosphosphate ([^{3}H]-IP$_1$) formation in guinea-pig lung (a) and hippocampus (b) slices. [^{3}H]-inositol labelled guinea-pig lung and hippocampus slices were incubated with various concentrations of histamine and in the presence of 10 mM or 5 mM LiCl for 20 min at 37°C as described in Methods in the presence (○) or absence (●) of 10 μM ibudilast. The results represent the means of 6 experiments and vertical lines show s.e.mean. * $P < 0.05$ versus control.

1983; Mong *et al.*, 1984). To clarify the inhibitory mechanism of ibudilast on LTD$_4$-induced IP$_1$ formation in lung slices, effects of the drugs on the formation of inositol phosphates were examined in guinea-pig lung membranes. Addition of 100 μM guanosine 5'-triphosphate (GTP) to [^{3}H]-inositol-labelled guinea-pig lung membranes, stimulated the formation of [^{3}H]-IP$_2$ and [^{3}H]-IP$_3$ but decreased the level of [^{3}H]-IP$_1$.

Table 1 Effects of GTP and ibudilast on inositol phosphate (IP) formation by guinea-pig lung membrane

	[^{3}H]-IP formation (mol × 10^{-14} mg^{-1} protein)		
	IP$_1$	*IP$_2$*	*IP$_3$*
Basal	5.60 ± 0.49	2.59 ± 0.46	1.02 ± 0.20
100 μM GTP	3.28 ± 0.26**	7.23 ± 0.79***	4.04 ± 0.32***
10 μM ibudilast	6.36 ± 0.91	3.36 ± 0.68	1.31 ± 0.36
100 μM GTP + 10 μM ibudilast	2.95 ± 0.24**	6.31 ± 0.83*	3.16 ± 0.57*

Guinea-pig lung membrane (200–300 μg) was incubated with 0 or 100 μM GTP in 20 mM Tris-HCl (pH 7.5) in a volume 220 μl at 37°C for 5 min in the presence or absence of 10 μM ibudilast. [^{3}H]-IP$_1$, [^{3}H]-IP$_2$ and [^{3}H]-IP$_3$ were extracted and measured separately. The results represent means ± s.e.mean of 5 experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus basal values

Table 2 Effects of $CaCl_2$ and ibudilast on inositol phosphate (IP) formation by guinea-pig lung membrane

	[^{3}H]-IP formation (mol × 10^{-14} mg^{-1} protein)		
	IP$_1$	*IP$_2$*	*IP$_3$*
Basal	6.89 ± 0.37	4.80 ± 0.10	1.55 ± 0.09
1 mM $CaCl_2$	13.07 ± 0.50*	4.94 ± 0.46	1.97 ± 0.28
10 μM ibudiblast	6.22 ± 0.47	4.93 ± 0.34	1.46 ± 0.25
1 mM $CaCl_2$ + 10 μM ibudilast	13.24 ± 0.22*	6.41 ± 0.83	1.81 ± 0.20

Guinea-pig lung membrane (200–300 μg) was incubated with 0 or 1 mM $CaCl_2$ in 20 mM Tris-HCl (pH 7.5) in a volume of 220 μl at 37°C for 5 min in the presence or absence of 10 μM ibudilast. [^{3}H]-IP$_1$, [^{3}H]-IP$_2$ and [^{3}H]-IP$_3$ were extracted and measured separately. The results represent means ± s.e.mean of 3 experiments. * $P < 0.001$ versus basal values.

Ibudilast (10 μM) did not alter the GTP-stimulated formation of [^{3}H]-IP$_2$ and [^{3}H]-IP$_3$ nor the GTP-induced decrease of [^{3}H]-IP$_1$ (Table 1). Hydrolysis of phosphatidylinositol (PI) but not phosphatidylinositol-4,5-bisphosphate (PIP$_2$) by phospholipase C is activated directly by calcium (Majerus *et al.*, 1985; Deckmyn *et al.*, 1986). Addition of 1 mM $CaCl_2$ markedly increased the formation of [^{3}H]-IP$_1$ without affecting the formation of [^{3}H]-IP$_2$ or [^{3}H]-IP$_3$. Ibudilast (10 μM) did not affect the calcium-stimulated formation of [^{3}H]-IP$_1$ (Table 2).

Discussion

The present results indicate that ibudilast inhibits, non-competitively, the LTD$_4$-induced formation of [^{3}H]-IP$_1$ in guinea-pig lung slices. Ohashi *et al.* (1986) demonstrated that ibudilast inhibited LTD$_4$-induced contraction of guinea-pig lung strips in an apparently competitive manner, when analysed at low concentrations (less than 1.3 μM). However, their data with higher concentrations of ibudilast suggest that the drug may act in a non-competitive manner. The present results from [^{3}H]-LTD$_4$ binding studies indicate that ibudilast has no affinity for LTD$_4$ receptors in guinea-pig lung. In addition, the drug also inhibited the formation of inositol phosphates induced by histamine and platelet-activating factor (PAF) (data not shown) in guinea-pig lung slices. The histamine- and PAF-induced formation of [^{3}H]-IP$_1$ were not mediated by LTD$_4$, because 5-lipoxygenase inhibitors nordihydroguaiaretic acid and esculetin had little effect on the histamine- and PAF-induced formation of IP$_1$. These observations suggest that ibudilast is not a specific antagonist of LTD$_4$ receptors. Histamine also induced formation of IP$_1$ in guinea-pig hippocampal slices. However, ibudilast had no effect on this response, so providing some evidence of tissue selectivity.

The lung membranes have GTP-regulated LTD$_4$ binding sites (Mong *et al.*, 1984), but there are still no results showing the direct coupling of LTD$_4$ receptors to phospholipase C in lung membranes. In this study, we also tried to prepare a guinea-pig lung membrane fraction containing a LTD$_4$ receptor that was functionally coupled to a G protein and phospholipase C, but were unsuccessful. However, in the preparation of guinea-pig lung membranes, $CaCl_2$ stimulated formation of IP$_1$ and GTP stimulated formation of IP$_2$ and IP$_3$. These results indicate that the membrane preparation used in the present study contains a phospholipase C regulated by a G protein. IP$_1$ is produced by dephosphorylation of IP$_3$, a phospholipase C-catlayzed hydrolysis produce of PIP$_2$. IP$_1$ is also directly produced by hydrolysis of PI (Majerus *et al.*, 1985). Phospholipase C is known to be sensitive to GTP and calcium when PIP$_2$ and PI, respectively, are used as the substrate (Deckmyn *et al.*, 1986). Ibudilast had no effect on either the formation of IP$_2$ and IP$_3$ or the decrease in IP$_1$ induced by GTP. Furthermore, it did not affect the calcium-stimulated formation of IP$_1$ or the basal levels of inositol phosphates. These results suggest that ibudilast may not act directly on either G proteins or phospholipase C. However, since the membrane preparation did not respond to LTD$_4$, it is still uncertain whether GTP-stimulation of the enzyme activity in the membranes represents the process activated by GTP in the LTD$_4$ signal transduction pathway. Therefore, the possibility remains that ibudilast inhibits LTD$_4$ signal transduction through an interaction with the process of GTP-stimulation of phospholipase C. Since the dose of ibudilast required for the inhibition of the formation of inositol phosphates is quite high compared to that of the contraction of lung strips, the inhibition of inositol phosphate formation by the drug may not be responsible for its inhibitory effect on the contractile response of guinea-pig lung strips. This may be explained by other possible actions of ibudilast, for example, antagonism of intracellular calcium in the contraction process.

In conclusion, the mechanism by which ibudilast inhibits LTD$_4$-induced PI hydrolysis differs from that of competitive

LTD_4 receptor antagonists such as FPL 55712 and SKF 104353. Also, the compound inhibits responses stimulated by other inflammatory mediators apart from LTD_4. Ibudilast, does not appear to act directly on G protein or phospholipase C, but may interfere with the coupling between G proteins and the receptors for LTD_4, histamine and PAF in lung tissue. If so ibudilast would appear to have a novel mechanism of action and may be a useful tool for studying signal transduction mechanisms in lung.

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Evidence for the involvement of cyclic GMP in adenosine-induced, age-dependent vasodilatation

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1 Adenosine-induced dilatation of rat aorta was present in aorta taken from 4 week-old rats, attenuated with increase in age of rats to 8 weeks, and was virtually absent in the aorta from 12 week-old rats.

2 Removal of the endothelium by mechanical rubbing attenuated adenosine-induced dilatation.

3 Haemoglobin and methylene blue partly reversed the adenosine-induced endothelium-dependent dilatation.

4 The order of potency of adenosine derivatives was 5'-(N-ethylcarboxamido)adenosine (NECA) > 2-phenylaminoadenosine (CV-1808) > 2-chloroadenosine > N^6-([R]-[−]-phenylisopropyl)adenosine (R-PIA) > adenosine > N^6-cyclohexyladenosine (CHA) > N^6-([S]-[+]-phenylisopropyl)adenosine (S-PIA), indicating that adenosine receptors mediating the dilatation are of the A_2 subtype.

5 [^{3}H]-NECA bound to preparations of membranes from rats of 4 weeks old; it was displaced more effectively by NECA and the A_2 ligand CV-1808 than by the A_1 ligands CHA and S-PIA.
ligands CHA and S-PIA.

6 The number but not affinity of specific binding sites for NECA decreased considerably with increase in age of rats to 8 weeks, and binding sites for [^{3}H]-NECA were hardly detected in membrane preparations from rats of 20 weeks old.

7 Adenosine caused a marked increase in cyclic GMP production, but did not induce an increase in the cyclic AMP level.

8 This increase in cyclic GMP production induced by adenosine was abolished by methylene blue or 8-phenyltheophylline, or by removal of the endothelium.

9 The age-associated decrease in adenosine-induced dilatation was found to be associated with a reduction in the formation of cyclic GMP, but not of cyclic AMP.

10 These results suggest that adenosine causes dilatation via A_2 receptors by inducing production of an endothelium-derived relaxing factor (EDRF), which in turn stimulates soluble guanylate cyclase, and so increases production of cyclic GMP. It is also suggested that the main reason for the age-associated decrease in adenosine-induced dilatation is a decrease in the number of A_2-receptors or the ability of the endothelium to produce EDRF, leading to decreased production of cyclic GMP.

Introduction

There are many studies showing that adenosine plays an important role in the regulation of cardiovascular functions (Kukovetz *et al.*, 1978; Berne, 1980; Silver *et al.*, 1984; White & Angus, 1987). Adenosine has been proposed to promote relaxation of vessels by increasing adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation (Kukovetz *et al.*, 1978) and adenosine receptors in several systems have been classified into A_1/R_i or A_2/R_a on the basis of whether adenosine stimulates or inhibits adenylate cyclase (van Calker *et al.*, 1979; Londos *et al.*, 1980). On the other hand, a dissociation between adenosine-induced dilatation and an increase in the cyclic AMP level (Herlihy *et al.*, 1976; Verhaeghe, 1977) has also been demonstrated.

Adenosine has been shown to cause either endothelium-dependent (Gordon & Martin 1983; Kennedy & Burnstock, 1985; Rubanyi & Vanhoutte, 1985) or independent (Collis & Brown, 1983; Kennedy *et al.*, 1985; Mathieson & Burnstock, 1985; White & Angus, 1987) dilatation of various isolated vessels. There are, however, data showing that adenosine caused scarcely any dilatation of rat thoracic aorta (White *et al.*, 1985), or was not as effective as adenosine 5'-triphosphate (ATP) in dilating the coronary artery (Gordon & Martin, 1983; White & Angus, 1987). We have observed that dilatation of rat aorta in response to histamine decreased with an increase in age (Moritoki *et al.*, 1986; 1988), and that the ability of the endothelium to produce endothelium-derived relaxing factor (EDRF) in response to histamine, and to form cyclic GMP decreased with age (Moritoki *et al.*, 1988). These findings suggested that the responsiveness of rat aorta to adenosine might depend on the age of the animals.

In the present study, we examined the mechanism of the dilator effect of adenosine on rat thoracic aorta. We also investigated the effect of the age of rats on adenosine-induced dilatation in relation to guanosine 3':5'-cyclic monophosphate (cyclic GMP) production, and the affinity and number of adenosine receptors.

A preliminary account of some of this work was presented at the International Symposium of Neurotransmitter Receptors, in Hiroshima, in 1987 (Moritoki & Tanioka, 1987), and the International Symposium on Biosignalling in Cardiac and Vascular Systems, in Kyoto, in 1988 (Moritoki *et al.*, 1989).

Methods

Organ bath experiments

Male Wistar rats aged 4 weeks (25–31 days, 127 ± 10 g), 8 weeks (8–10 weeks, 282 ± 6 g), 12 weeks (12–15 weeks, 371 ± 6 g) and 20 weeks (20–22 weeks, 454 ± 8 g) were used. The rats were killed by a blow on the head and cervical dislocation, and bled. Segments of their thoracic aorta, 3 mm in length, were cut with parallel razors and freed from adjacent tissues under a dissecting microscope. These ring segments were set up in a 10 ml organ bath containing Krebs solution of the following composition (mM): NaCl 115.3, KCl 4.9, $CaCl_2$ 1.4, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 11.1. This

solution was maintained at 34°C and bubbled with 95% O_2 and 5% CO_2.

The preparations were maintained at a resting tension of 1.0 g in the bath for 120 min before experiments. Responses were recorded isometrically with a force displacement transducer (Nihon Kohoden SB 1TH). For measurement of dilatation, the arteries were first contracted with noradrenaline at a concentration corresponding to the EC_{80}. Concentration-response curves were constructed by adding adenosine cumulatively to the 10 ml bath in a volume of approximately 20 μl, and dilatations were plotted as a % of the contraction induced by noradrenaline.

The endothelium was removed by rubbing the lumen of the artery with cotton thread, and its removal was confirmed by checking that the dilator response to acetylcholine (ACh) was lost.

Pure haemoglobin was prepared as described by Martin *et al.* (1985). Commercial haemoglobin at a concentration of 1 mM was reacted with 10 mM sodium hydrosulphite and then the reducing agent was removed by dialysis at 4°C.

Adenosine receptor binding assay

Segments of rat thoracic aorta were minced with a pair of scissors in 10 to 20 volumes of 50 mM Tris-Cl buffer (pH 7.4). The mixture was then homogenized 3 times for 30 s periods with 30 s intervals in a Polytron homogenizer at setting 6 in an ice-water bath, and then rehomogenized 5 times with a Potter glass-glass homogenizer. The homogenate was centrifuged at 105,000 *g* for 60 min, and the pellet was resuspended in 50 mM Tris-Cl buffer by 5 strokes of a teflon glass homogenizer for binding assay. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Aliquots (400 μl) of membrane suspension were incubated in triplicate with various concentrations of [^{3}H]-5'-(N-ethylcarboxamido)adenosine ([^{3}H]-NECA), with or without 50 μM NECA, in a total volume of 500 μl at 25°C to determine specific and non-specific binding. After incubation for 60 min, the membrane bound ligand was separated from unbound ligand by vacuum-filtration through a glass fibre filter (GF/C, Whatman). The filter was then washed repeatedly with 10 ml of ice-cold buffer and dried in a vial at room temperature, and the radioactivity trapped on the filter was counted in a liquid scintillation spectrophotometer (Aloka LSC-700). Specific binding was calculated as the difference between total and non-specific bindings.

Competition assays were performed as described above except that a final concentration of 50 nM [^{3}H]-NECA was added to mixture containing membrane and various concentrations of NECA (0.1 nM–1 μM) or N^6-cyclohexyladenosine (CHA) (10 nM–1 μM), and the mixture was incubated for a further 40 min. Data were analysed by the method of nonlinear least-squares by use of a computer programme (Taira & Terada, 1985).

Assay of cyclic nucleotides

Segments of aorta were equilibrated in Krebs solution at 34°C bubbled with 95% O_2 and 5% CO_2 for 2 h before addition of 0.3 μM noradrenaline. Then the preparations were incubated with 10 μM adenosine. After various times of incubation with adenosine, the preparations were quickly frozen in liquid nitrogen and homogenized in ice-cold 6% trichloroacetic acid in a Potter-glass homogenizer in ice. The homogenates were centrifuged at 1700 *g* for 15 min at 4°C, and the supernatants were extracted with 3 volumes of water-saturated ether. Cyclic nucleotides (cyclic AMP and cyclic GMP) were measured by radioimmunoassay (Honma *et al.*, 1977) with commercially available kits. Briefly, cyclic nucleotides in the supernatant were succinylated and incubated with [^{125}I]-succinyl cyclic GMP tyrosine methyl ester or [^{125}I]-succinyl cyclic AMP tyrosine methyl ester and antisera for 18 h at 4°C. Then dextran-coated charcoal was added to the reaction medium to terminate the reaction. The radioactivity in the supernatant was counted in a gamma spectrometer (Aloka ARC-301). Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Each experimental group consisted of 4 to 6 preparations from different rats.

Statistical analysis

Values are expressed as means ± 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.

Materials

The drugs used were adenosine, 5'-(N-ethylcarboxamido)adenosine (NECA), N^6-([R]-[−]-phenylisopropyl) adenosine (R-PIA), N^6-([S]-[+]-phenylisopropyl) adenosine (S-PIA), 2-chloroadenosine, N^6-cyclohexyl-adenosine, 8-phenyltheophylline, acetylcholine chloride, noradrenaline hydrochloride, methylene blue, indomethacin, haemoglobin (all from Sigma Chemical Co., St Louis, MO, U.S.A.), 2-phenylaminoadenosine (CV-1808, Funakoshi Chemical Co., Tokyo, Japan), dipyridamole (Boehringer Ingelheim, FRG), dilazep (Kowa Co., Tokyo, Japan), cilostazole (Otsuka Pharmaceutical Co., Tokushima, Japan) and 5'-N-ethylcarboxamido [8-^{3}H]-adenosine (specific activity, 0.74–1.5 TBeq mmol^{-1}, Amersham Japan). Kits for radioimmunoassay of cyclic GMP and cyclic AMP were from Yamasa Shoyu Co. Ltd. (Choshi, Japan).

Results

Dilatation and aging

Adenosine at concentrations up to 100 μM did not cause dilatation of the thoracic aorta from rats of 12 weeks old (Figure 1). However, in the arteries from rats of 4 weeks old it caused dose-dependent dilatation at concentrations of above 0.3 μM. The maximal dilatation of 79.4 ± 5.5% ($n = 9$) was observed at 300 μM adenosine and the apparent log EC_{50} value was 5.63 ± 0.08.

With an increase in the age of the rats to 8 weeks, about a 30 times higher concentration of adenosine was necessary to produce comparable dilatations to those observed with the arteries from 4 week-old rats, while with aortae from 12 week-old rats, the threshold concentration of adenosine for dila-

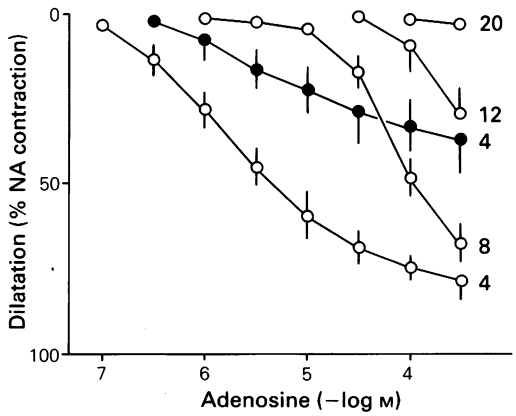

Figure 1 Age-associated change in the dilator response of the rat thoracic aorta to adenosine. The arteries were contracted with noradrenaline at concentrations corresponding to the EC_{80} values. Adenosine was added cumulatively. Dilator responses are expressed as percentages of the noradrenaline-induced contraction. Ages of rats are shown in weeks. (●) Responses of preparations without endothelium from rats of 4 weeks old. Values are means and vertical lines s.e.mean of preparations from 5 to 12 rats.

tation was as high as 100 μM. With a further increase in age to 20 weeks, the aorta no longer responded to adenosine at concentrations of up to 300 μM or even to a single dose of a high concentration of adenosine (300 μM). In addition, the stable adenosine derivatives NECA and 2-phenylaminoadenosine (CV-1808) at concentrations of up to 30 μM caused scarcely any dilatation. Inhibitors of adenosine uptake and adenosine deaminase, such as dipyridamole (Afonso & O'Brien, 1967) and dilazep (Fujita *et al.*, 1980), at concentrations of up to 0.3 μM did not restore the responsiveness to adenosine. Under these conditions, however, arteries dilated in response to histamine and ACh (100 μM, each), although to a somewhat lesser extent than arteries from rats of 8 weeks old; 100 μM histamine and ACh induced dilatations of 63.9 $\pm$ 4.5% (vs 93.6 $\pm$ 5.4% at 4 weeks old) and 67.6 $\pm$ 7.8% (vs 87.2 $\pm$ 7.2% at 4 weeks old), respectively (n = 6).

Characteristics of adenosine-induced dilatation

For characterization of adenosine-induced dilatation, the arteries from rats of 4 weeks old were used. We examined whether the endothelium of the vessels contributes to the dilator response of the artery to adenosine. For this, after confirming the dilator response to ACh of the aortae of 4 week-old rats, the endothelium was removed by rubbing the lumen of the arteries with cotton thread. Then 60 min later, loss of endothelial function was confirmed by checking loss of ACh-induced dilatation. In these aortae without endothelium, the adenosine-induced dilatation was significantly attenuated but not abolished: the concentration-response curve was shifted about 30 times to the right (Figure 1). Dipyridamole and dilazep, at concentrations of up to 0.3 μM, did not enhance or inhibit the endothelium-independent dilator response of the artery to adenosine.

Haemoglobin at concentrations above 10 nM reversed adenosine-induced dilatation of the aorta with endothelium (Figure 2). In contrast, in arteries without endothelium, haemoglobin failed to reverse the adenosine-induced dilatation. In addition, dilatation induced by the cyclic AMP phosphodiesterase inhibitor cilostazole (1 μM) used as a reference was hardly affected by haemoglobin at concentrations of up to 100 μM.

Pretreatment with the guanylate cyclase inhibitor methylene blue (MB, Gruetter *et al.*, 1981) at insufficient concentrations to attenuate cilostazole-induced dilatation, caused a dose-dependent shift of the concentration-response curve for adenosine to the right (Figure 3); in the presence of 0.1, 0.3 and 1 μM MB, the log EC_{50} value of adenosine decreased from the control value of 5.62 $\pm$ 0.18 to 5.31 $\pm$ 0.25, 4.88 $\pm$ 0.19 and 4.21 $\pm$ 0.16 (n = 7), respectively. However, 3 μM MB did not shift the concentration-response curve further, the log EC_{50} value being 4.17 $\pm$ 0.21. In contrast, the adenosine-induced dilatation remaining after removal of the endothelium was not reversed by application of MB at concentrations of up to 3 μM.

In arteries that had been treated with 100 μM indomethacin for 60 min and then washed for 30 min, the dilator effect of adenosine was not affected irrespective of whether the endothelium was intact (apparent log EC_{50} values; 5.50 $\pm$ 0.08 vs 5.58 $\pm$ 0.19, n = 4) or removed (3.93 $\pm$ 0.14 vs 3.90 $\pm$ 0.23).

Next, the adenosine receptor subtype responsible for the dilator response of the arteries from rats of 4 weeks old was characterized by use of several adenosine derivatives (Figure 4). The rank order of the potencies of adenosine derivatives in inducing dilatation was NECA (apparent log EC_{50} value and maximal response: 7.56 $\pm$ 0.18, α = 1.0) > CV-1808

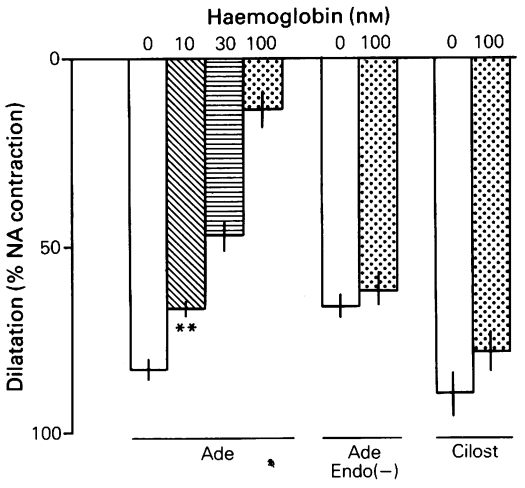

Figure 2 Reversal by haemoglobin of adenosine-induced dilatation of the aorta. To study the reversal by haemoglobin, dilatations were induced either by 30 μM adenosine or 1 μM cilostazole for endothelium intact preparations, and 100 μM adenosine for endothelium removed preparations. Dilatations were expressed as percentages of the contractions induced by the EC_{80} concentration (0.3 μM) of noradrenaline. Concentrations of haemoglobin are shown in nM. Ade, adenosine; Endo (−), endothelium removed preparation; Cilost, cilostazole. Columns show mean values of preparations from 6 rats; vertical lines indicate s.e.mean. $^{**}P < 0.01$, compared with control value.

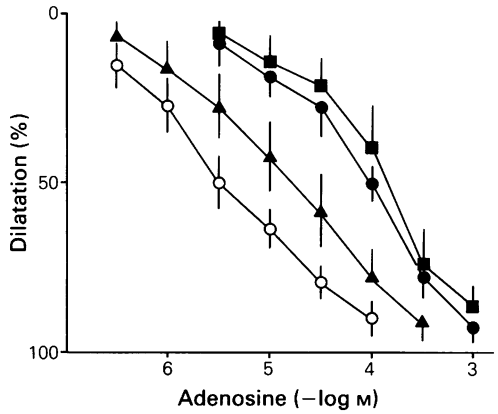

Figure 3 Inhibitory effect of methylene blue on the dilator response of the arteries from rats of 4 weeks old to adenosine. The arteries were treated with the EC_{80} concentration of noradrenaline to produce tone. Methylene blue (MB) was added for 20 min before addition of noradrenaline. Then the dilator response to adenosine was tested. (○), control; (▲), with 0.3 μM MB; (●), with 1 μM MB; (■), with 3 μM MB. Vasodilatations are expressed as percentages of the noradrenaline-induced contractions. Points are means and vertical lines are s.e.mean (n = 7).

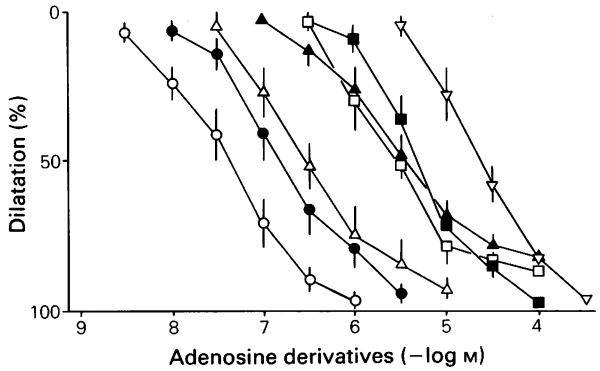

Figure 4 Concentration-response curves for adenosine derivatives showing their order of potency in causing dilatations of thoracic aorta of 4 week-old rats. The ordinate scale shows dilatations of the arteries as percentages of the initial tone induced by the EC_{80} concentration of noradrenaline. (○), 5'-(N-ethylcarboxamido)adenosine; (△), 2-chloroadenosine; (●), CV-1808; (▲), adenosine; (□), N^6-([R]-[−]-phenylisopropyl)adenosine (R-PIA); (▽), S-PIA, (■), N^6-cyclohexyladenosine. Values are means of preparations from 5 to 9 rats, and vertical lines indicate s.e.mean.

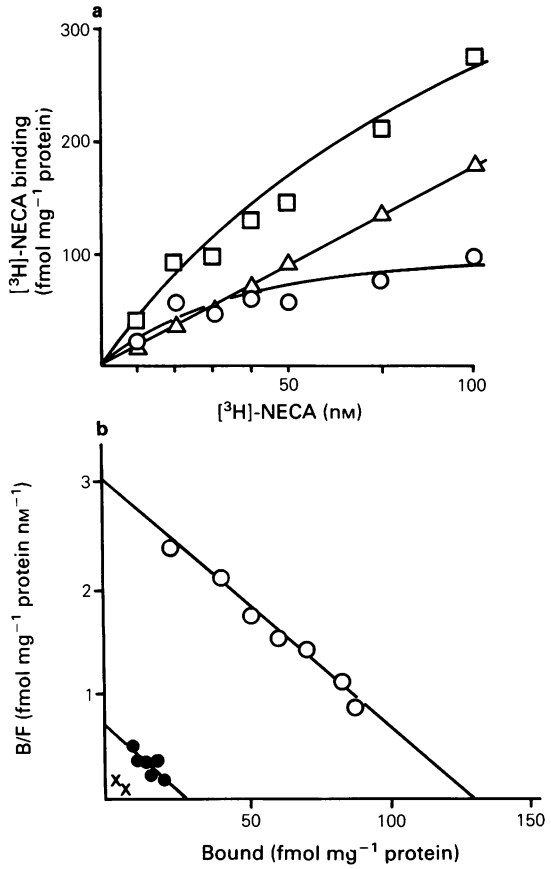

Figure 5 Saturation of [^{3}H]-5'-(N-ethylcarboxamido)adenosine ([^{3}H]-NECA) binding to aortic membranes from rats of 4 weeks old (a). Specific binding (○) was calculated by subtracting non-specific binding (△), determined in the presence of 10 μM NECA, from total binding (□). (b) Scatchard plot of specific binding of [^{3}H]-NECA and effect of aging on the binding. (○), 4 weeks old; (●), 8 weeks old; (×), 20 weeks old. Each point represents the mean of triplicated determination from two experiments.

(6.73 ± 0.11, $\alpha = 1.0$) > 2-chloroadenosine (6.48 ± 0.29, $\alpha = 1.0$) > **R**-PIA (5.57 ± 0.18, $\alpha = 0.87$) > adenosine (5.55 ± 0.09, $\alpha = 0.84$) > CHA (5.36 ± 0.11, $\alpha = 1.0$) > **S**-PIA (4.66 ± 0.12, $\alpha = 1.0$) ($n = 5$–9); NECA was about 800 times more potent than **S**-PIA, and **R**-PIA was about 8 times more potent than **S**-PIA.

Binding assay of adenosine receptors

For further characterization of adenosine receptors, a binding assay for adenosine receptors was carried out with [^{3}H]-NECA as a ligand. [^{3}H]-NECA was found to bind to the membrane preparation of the aorta from rats of 4 weeks old, with a dissociation constant (K_d) of 43.1 nM and a receptor number (B_{max}) of 129.2 fmol mg^{-1} protein (Figure 5).

NECA, CV-1808, **R**-PIA, **S**-PIA and CHA inhibited the specific binding of [^{3}H]-NECA (Figure 6), with apparent K_i values of 0.21, 1.51, 758.5, 446.7 and 467.7 μM, respectively ($n = 4$). The non-selective A_1, A_2 agonist NECA was about 2200 times more effective, and the A_2-selective ligand CV-1808 was about 300 times more effective than the A_1-selective ligand CHA in displacing [^{3}H]-NECA from its binding sites. In addition, the non-selective antagonist 8-phenyltheophylline (8-PT) also inhibited the binding of [^{3}H]-NECA with a K_i value of 2.09 μM.

The specific binding of [^{3}H]-NECA was less in the membrane preparation from 8 week-old rats (Figure 5). Scatchard analysis showed a marked reduction in the number of binding sites for [^{3}H]-NECA to 30.0 fmol mg^{-1} protein. However, the K_d value of the binding sites was 41.4 nM, which was not significantly different from that in preparations from 4 week-old rats (43.1 nM). With a further increase in age of the rats to 20 weeks, the number of binding sites decreased to an undetectable level.

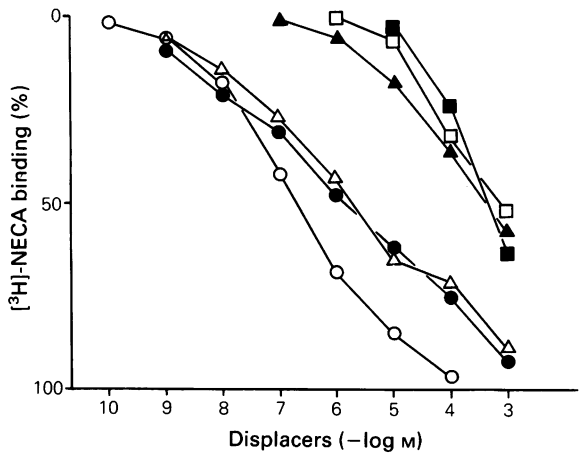

Figure 6 Inhibition by adenosine-related compounds of [^{3}H]-5'-(N-ethylcarboxamide)adenosine ([^{3}H]-NECA) binding to aorta membranes from rats of 4 weeks old. Membrane preparations were incubated with 50 μM [^{3}H]-NECA in the presence of increasing concentrations of unlabelled compounds. (○), NECA; (●), CV-1808; (△), 8-phenyltheophylline; (▲), N^6-cyclohexyladenosine; (□), N^6-([R]-[−]-phenylisopropyl)adenosine (**R**-PIA); (■), **S**-PIA. Data are representative of triplicate determinations in two experiments.

Effect of adenosine on the production of cyclic nucleotides

We examined whether the dilatation was due to an increase in cyclic nucleotides. As shown in Figure 7, adenosine at a concentration of 10 μM, which caused 60–70% dilatation of the artery, increased the cyclic GMP level in 15 s from the basal level of 1.04 ± 0.33 to 3.73 ± 0.37 pmol mg^{-1} protein ($n = 9$).

After removal of the endothelium the basal level of cyclic GMP decreased to 0.16 pmol mg^{-1} protein, and was not increased by incubation with 10 μM adenosine (0.09 ± 0.02 pmol mg^{-1} protein in 15 s, $n = 4$).

In the presence of 0.3 μM MB, which caused slight, but not significant, reduction of basal cyclic GMP production (1.04 ± 0.33 to 0.55 ± 0.20 pmol mg^{-1} protein in 15 s, $n = 7$), the adenosine-stimulated cyclic GMP production decreased to nearly the basal level (0.73 ± 0.10 pmol mg^{-1} protein, $n = 7$).

8-PT itself did not alter the basal level of cyclic GMP. However, in the presence of 10 μM 8-PT, 10 μM adenosine did not stimulate cyclic GMP production (0.82 ± 0.08 in its presence compared to 0.83 ± 0.19 in its absence, $n = 5$).

With an increase in the age of the rats to 8 weeks, the basal level of cyclic GMP decreased to 0.73 ± 0.14 pmol mg^{-1} protein, and the adenosine-stimulated cyclic GMP production decreased to 2.09 ± 0.15 pmol mg^{-1} protein ($n = 8$). In arteries from rats of 20 weeks old, adenosine-stimulated production of cyclic GMP decreased to 0.75 ± 0.25 pmol mg^{-1} protein with a reduction in the basal level (0.35 ± 0.04 pmol mg^{-1} protein, $n = 6$).

In contrast to the marked increase in the cyclic GMP level, the cyclic AMP level was not significantly affected by 10 μM adenosine, irrespective of the age of the rats (Figure 7).

Discussion

In the present study, adenosine was found to induce little discernible dilatation of thoracic aorta of rats of above 20 weeks old, as has been previously observed (White *et al.*, 1985). The inability of adenosine to dilate the arteries was not due to its dislocation from its site of action through its uptake mechanism, or to its degradation by adenosine deaminase, because

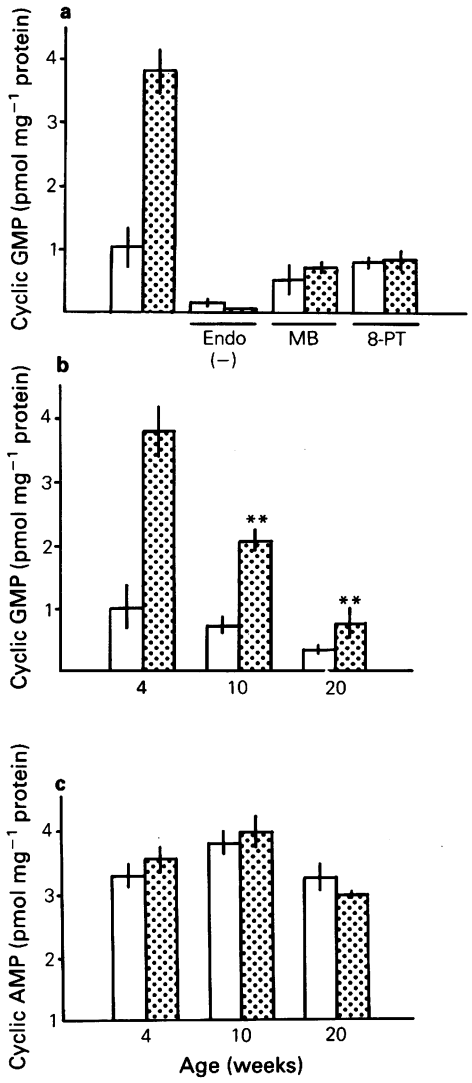

Figure 7 Effects of adenosine on the production of cyclic nucleotides in thoracic aorta from rats of 4 weeks old (a), and effect of aging on the production of cyclic GMP (b) and cyclic AMP (c). Amounts of cyclic nucleotides were measured after incubation with the EC_{80} concentration of noradrenaline (0.3 μM) for 2 min and then with and without 10 μM adenosine for 15 s for cyclic GMP and for 2 min for cyclic AMP. Open columns, control; stippled columns, with 10 μM adenosine. For study of the effect of methylene blue or 8-PT, these agents were added 20 min before and during incubation with adenosine. MB, with 1 μM methylene blue; 8-PT, with 10 μM 8-phenyltheophylline; Endo (−), without endothelium. Ages of rats are shown in weeks. Columns represent the mean values ($n = 4$–9) of preparations from 5 to 7 rats. Vertical lines indicate s.e.mean. $^{**}P < 0.01$, compared with value of the preceding age (unpaired t test).

even in the presence of the inhibitors of adenosine uptake and adenosine deaminase, dipyridamole (Afonso & O'Brien, 1967) and dilazep (Fujita *et al.*, 1980), adenosine had no effect on these preparations, and also the stable adenosine derivatives NECA and CV-1808 caused little dilatation of the arteries. Moreover, the inability of adenosine to cause dilatation of the arteries was not due to rapid development of desensitization due to its cumulative application, because even a single dose of a high concentration of adenosine had no effect on these preparations.

In previous studies, we found that aging decreased the dilatation of rat arteries induced by histamine (Moritoki *et al.*, 1986; 1988). Therefore, in the present study we examined whether the inability of adenosine to dilate the thoracic aorta of rats of 20 weeks old could be due to the age of the rats (Figure 1). We found that adenosine did cause dose-dependent dilatation of the arteries from rats of 4 weeks old, but that with an increase in age of the rats to 8 weeks, the dilator response to adenosine decreased considerably, and that in preparations from rats of over 12 weeks of age a response was no longer detectable. Rats are usually used for experiments on their vasculature at 8 to 15 weeks old. Twelve weeks of age may be borderline for a response of the aorta to adenosine. At this age, the responsiveness to adenosine varied greatly: some preparations did not respond to adenosine, whereas others showed comparable responses to those of the aorta of 4 week-old rats.

The A_2 agonist NECA was more potent than the A_1 agonists **R**-PIA and CHA in inducing dilatation of rat thoracic aorta, and the order of potency of the adenosine derivatives (NECA > CV-1808 > 2-chloroadenosine > **R**-PIA > adenosine > CHA > **S**-PIA) was almost similar to their order of potency in dilating guinea-pig aorta (Collis & Brown, 1983) and in accumulating cyclic AMP in cultured brain cells (van Calker *et al.*, 1979). On the basis of the structure-activity relationship, the adenosine receptors mediating the dilatation of arteries with intact endothelium were judged to be of the A_2 subtype. However, it is unknown whether the maximal dilatations caused by adenosine and **R**-PIA were less than those induced by NECA and S-PIA.

For further characterization of the adenosine receptor subtype responsible for dilatation, we carried out binding studies of adenosine receptors with [^{3}H]-NECA as a ligand. We demonstrated the presence of specific binding sites for [^{3}H]-NECA in the arteries from rats of 4 weeks old (Figure 5). [^{3}H]-NECA was used as an A_2 ligand, but it has also been shown to bind to A_1 receptors and to lack binding specificity (Schütz *et al.*, 1982; Lohse *et al.*, 1988). However, its binding to rat aortic membranes was displaced more effectively by the so-called A_2 ligand CV-1808 (Daly *et al.*, 1987) and NECA than by the A_1 ligand CHA (Figure 6), suggesting that the binding sites are probably of the A_2 subtype.

With a decrease in the dilator response of the aorta to adenosine with age, the B_{max} decreased with no change in the K_d value (Figure 5). These results indicate that during aging the number of binding sites for [^{3}H]-NECA (A_2 receptors) decreased without a change in their affinity for the agonist. However, it is still not known whether the loss of [^{3}H]-NECA binding sites reflects a reduction in the absolute number (density) of binding sites or is secondary to a reduction of endothelial cells. Furthermore, because the membrane preparations used in the present experiments contained membranes of both the endothelium and smooth muscle, it is unknown whether A_2 receptors are localized in the vascular endothelium or smooth muscle, and which of the receptors (in the endothelium or in smooth muscle) contribute to the adenosine-induced dilator effect which disappears during aging. Nevertheless, our finding that the adenosine-induced dilator response decreased after removal of the endothelium suggests that at least some binding sites are present in the endothelium.

The effects of adenosine have been shown to be caused by mechanisms linked to the adenylate cyclase system (Kukovetz *et al.*, 1978; Rosiers & Nees, 1987), and thus to the 2nd messenger cyclic AMP (Londos *et al.*, 1980) and cyclic AMP-dependent protein kinase (Silver *et al.*, 1984). Subtypes of adenosine receptor in the vasculature have been classified as A_1 and A_2; a classification which was originally proposed on the basis of whether they stimulated or inhibited adenylate cyclase (Van Calker *et al.*, 1979). Functional evidence for the presence of A_2-receptors that stimulate cyclic AMP production in cultured coronary endothelial cells has been presented (Rosiers & Nees, 1987). Therefore, the dilator action of adenosine in the rat thoracic aorta could be mediated through an increase in cyclic AMP accumulation. But this is unlikely, because we found that adenosine did not increase the cyclic AMP level significantly. Furthermore, we found that aging did not alter the cyclic AMP level (Figure 7).

In general, the vasodilator effect of adenosine has been found not to depend on the endothelium (De May & Van-

Kennedy et al., 1985; Mathieson & Burnstock, 1985; White & Angus, 1987), but in some vessels adenosine caused endothelium-dependent dilatation (Frank & Bevan, 1983; Gordon & Martin, 1983; Kennedy & Burnstock, 1985; Rubanyi & Vanhoutte, 1985). We found that in rat aorta, removal of the endothelium considerably attenuated the dilator response to adenosine (Figure 1), as has been shown for the rabbit central ear artery (Kennedy & Burnstock, 1985). In addition, haemoglobin (Martin et al., 1985), which inhibits the effects of endothelium-derived relaxing factor (EDRF), reversed adenosine-induced dilatation without affecting the dilatation caused by the cyclic AMP phosphodiesterase inhibitor cilostazole (Umekawa et al., 1984). From these results and considerations, we suggest that the response of rat thoracic aorta to adenosine is in part mediated by the endothelium and thus EDRF which stimulates soluble guanylate cyclase (Rapoport & Murad, 1983).

In the present study, the endothelium-dependent portion of adenosine-induced dilatation of the aorta from rats of 4 weeks old was found to be abolished or reversed by the soluble guanylate cyclase inhibitor MB at low concentrations that were insufficient to affect cilostazole-induced dilatation, suggesting that soluble guanylate cyclase mediates the adenosine-induced vasodilatation. Furthermore, we found that adenosine increased formation of cyclic GMP, and that removal of the endothelium, besides significantly decreasing the basal level of cyclic GMP, almost completely abolished the adenosine-induced increase in cyclic GMP formation (Figure 7). In addition, the soluble guanylate cyclase inhibitor MB was found to abolish the adenosine-stimulated production of cyclic GMP.

The adenosine antagonist 8-PT (Griffith et al., 1981; Kennedy & Burnstock, 1985) was found to abolish the increase in adenosine-stimulated cyclic GMP production (Figure 7). This observation confirms the idea that cyclic GMP production by adenosine is mediated via adenosine-receptors.

We examined whether the age-associated decrease or loss of dilator response to adenosine was due to a reduction of cyclic GMP formation. In fact, we found that cyclic GMP production decreased with age (Figure 7), and that in the aortae from rats of 20 weeks old, which showed scarcely any dilator response to adenosine, the adenosine-stimulated production of cyclic GMP was less than the resting level in preparations from 4 week-old rats. These results suggest that a reduction in cyclic GMP formation during aging is, in part, responsible for the loss of the dilator response to adenosine.

In addition, as has been shown for vasodilatation induced by nitroprusside (Moritoki et al., 1988), reduction of guanylate cyclase activity itself in the vascular smooth muscle seems to be the cause of the age-associated decrease in adenosine-induced vasodilatation.

In the endothelium-removed preparations, adenosine could activate particulate guanylate cyclase in smooth muscle to produce cyclic GMP, just as has been found for atrial natriuretic peptide (Waldman et al., 1984; Winquist et al., 1984). Recently, adenosine was shown to stimulate particulate guanylate cyclase in cultured vascular smooth muscle cells (Kurtz, 1987). Although our findings in rat aorta differ from those of Kurtz in that cyclic GMP formation was endothelium-dependent and was mediated by soluble guanylate cyclase, his results are consistent with our idea that cyclic GMP, but not cyclic AMP, is responsible for adenosine-induced dilatation. However, this possibility seems unlikely, because adenosine did not elevate the cyclic GMP level in preparations without endothelium.

With respect to an action of adenosine which is independent of cyclic nucleotides, there are data suggesting that adenosine acts at an intracellular site and that its effect appears to be mediated by its metabolite inosine through some metabolic processes (Collis et al., 1986). However, this was not the case in the present study, because dipyridamole (Collis & Brown, 1983) and dilazep (Fujita et al., 1980) which block the access of adenosine to an intracellular site, did not inhibit the dilator effect of adenosine.

Adenosine could dilate the arteries through release of prostanoids, as has been found for some vasodilators (Boeynaems & Galand, 1983; Förstermann et al., 1986; Gryglewski et al., 1986). However, indomethacin did not affect adenosine-induced dilatation, indicating that metabolites of the arachidonic acid cascade such as prostacyclin are unlikely to be candidates for mediating the action of adenosine.

In this work, contrary to the well established concept that effects of adenosine are mediated by cyclic AMP, we found that the vasodilator effect of adenosine is in part mediated by cyclic GMP; adenosine acts on A_2-receptors on the endothelium to produce EDRF, which in turn stimulates soluble guanylate cyclase in vascular smooth muscle, thus producing cyclic GMP and inducing dilatation. In addition, the age-associated loss of dilator response of the rat thoracic aorta to adenosine is suggested to be due to (1) a reduction in the number of A_2-receptors in the endothelium, (2) a decrease in the ability of the endothelium to produce EDRF, and (3) a reduction of guanylate cyclase activity, and thus a decrease in the cyclic GMP level. However, the possibility that a reduction in the number of A_2 receptors in the smooth muscle is likely to contribute partly to the age-associated reduction of the dilator response to adenosine cannot be ruled out. The dilator response to adenosine disappeared earlier in life (during development rather than aging) than the responses to other dilators such as ATP, ACh and histamine.

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Evidence that A_2 purinoceptors are involved in endothelium-dependent relaxation of the rat thoracic aorta

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1 The effect of adenosine and some adenosine analogues on the isolated thoracic aorta from rats was compared with the effect of adenosine 5'-triphosphate (ATP) and adenosine 5'-diphosphate (ADP).

2 Both ATP and adenosine analogues caused relaxation of the noradrenaline (30 nM)-contracted thoracic aorta.

3 The order of potency for adenosine analogues was 5'-(N-ethyl) carboxamidoadenosine (NECA) > L-N^6-phenylisopropyladenosine (L-PIA), adenosine 5'-monophosphate (AMP), adenosine indicating the presence of adenosine A_2 receptors.

4 Removal of the endothelium or prior treatment with haemoglobin (10 μM) attenuated relaxant responses to both ATP and NECA, attenuation being greater for ATP than NECA.

5 8-Phenyltheophylline (10 μM) reduced relaxant responses to NECA but not to ATP in the intact tissue.

6 These results provide evidence that there are two components to relaxation of the rat thoracic aorta induced by purinoceptor agonists. The first is an endothelium-dependent mechanism involving release of endothelium-derived relaxant factor (EDRF) and the second is due to a direct effect on smooth muscle.

Introduction

It has been recognized for many years that the purine nucleoside adenosine and nucleotides adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP) are potent vasoactive substances. More recent work has shown that ATP can act as both a vasodilator or vasoconstrictor in isolated vascular preparations (Kennedy & Burnstock, 1985; White *et al.*, 1985; Houston *et al.*, 1987). The vasodilator activity is generally dependent on the presence of an intact endothelium (Furchgott, 1981; De Mey & Vanhoutte, 1982; 1983; Cocks & Angus, 1983; Angus *et al.*, 1983; Martin *et al.*, 1985; White & Angus, 1987), although relaxation to ATP has been demonstrated in the rabbit mesenteric artery (Mathieson & Burnstock, 1985) and portal vein (Kennedy & Burnstock, 1985) due to stimulation of P_{2Y} receptors located on the smooth muscle. Vasoconstrictor activity is principally accepted to be due to direct activation of a receptor present on vascular smooth muscle (Kennedy & Burnstock, 1985; White *et al.*, 1985; Houston *et al.*, 1987).

In a review of the literature Burnstock & Kennedy (1985) suggested that on the basis of rank order of agonist potency, two receptors for purine nucleotides could be identified in blood vessels. The receptors that mediated vasodilatation were present on the endothelial cells and were designated P_{2Y} while receptors mediating vasoconstrictor actions were on the smooth muscle and designated P_{2X}.

The location of the vascular receptors for adenosine is not so well defined. While it is generally agreed that the vasodilator action of adenosine is mediated by the A_2 subclass of the P_1 purinoceptor (Collis & Brown, 1983; Edvinsson & Fredholm, 1983; Kusachi *et al.*, 1983; Ramagopal *et al.*, 1988) some workers suggest that the receptor is located on vascular smooth muscle (Collis & Brown, 1983; White & Angus, 1987), whilst others have shown that removal of the endothelium attenuates vasodilator responses to adenosine (Gordon & Martin, 1983; Rubanyi & Vanhoutte, 1985). The presence of A_2 adenosine receptors on cultures of coronary endothelial cells has also been reported (Des Rosiers & Nees, 1987; Schmidt & Kukovetz, 1986).

The aim of this study was to compare the vasodilator actions of adenosine and ATP in the rat thoracic aorta and using a stable analogue of adenosine, 5'-(N-ethyl)carboxyamidoadenosine (NECA), investigate the location of the vascular A_2 receptor. The mechanism of A_2 receptor-induced vasodilatation was also studied.

Methods

Male Wistar rats weighing 250–350 g were killed by a blow to the head and bled out. The thoracic aorta was exised and rings of 7 mm length were placed in 10–20 ml organ baths filled with Krebs Henseleit solution (composition mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, glucose 11, $CaCl_2 2H_2O$ 2.5, $MgSO_4 7H_2O$ 0.45, KH_2PO_4 1.2, EDTA 0.03, sodium ascorbate 0.09) kept at 37°C and bubbled with 95% O_2: 5% CO_2. Rings of thoracic aorta were mounted horizontally by inserting two wires into the lumen and maintained at 2 g tension. Care was exercised to preserve the integrity of the endothelium on the preparation. After an equilibration period of 45 min a concentration-response curve to noradrenaline (NA) was established. The preparation was then washed out and allowed to recover. The tissue was then contracted with a submaximal concentration of NA (30 nM) and allowed to reach maximum tension before cumulative additions of purine analogues. The duration of each concentration-response curve to purine analogues did not exceed 12 min. Results are expressed as a percentage of the maximum contraction before the addition of purines.

The endothelium of some thoracic aortae was removed by gently rubbing the luminal surface with cotton soaked in Krebs Henseleit solution. Absence of relaxation to acetylcholine was used to indicate the complete removal of endothelial cells. In experiments where haemoglobin, 8-phenyltheophylline (8-PT) or indomethacin were used, the tissue was equilibrated for 30 min before recommencement of purine concentration-response curves.

Drugs

Noradrenaline ((−)-arterenol bitartrate), acetylcholine chloride, adenosine 5'-triphosphate (Na salt), 8-phenyltheophylline, adenosine 5' monophosphate (Na salt), 5'-(N-ethyl) carboxyamidoadenosine, L-phenylisopropyladenosine, adenosine (Na salt), indomethacin, sodium nitroprusside, haemoglobin and 8-phenyltheophylline were obtained from Sigma.

All compounds were made up in aqueous solution with dilutions made in saline, except 8-phenyltheophylline, indomethacin and haemoglobin. 8-Phenyltheophylline was made up to a concentration of 10 mM in 80% v/v methanol containing 0.2 M NaOH and diluted as required in saline and indomethacin was dissolved in 1% sodium carbonate solution. Haemoglobin was prepared as described by Martin *et al.* (1985).

Statistical analysis

The data are expressed as mean ± s.e.mean. Concentration-response curves to noradrenaline were studied by least square regression line analysis (LINUS, Apple IIe computer). Significance of differences was calculated by Student's *t* test. *P* values of less than 0.05 were considered to represent significant differences.

Results

Concentration-response curves to noradrenaline

Noradrenaline elicited concentration-dependent contractions in both intact aortic rings and in rings from which the endothelium had been removed. Concentration-response curves in endothelium-denuded segments were not significantly different ($P > 0.05$) from those in control segments with respect to the slope of the curve (58.5 ± 7.0 intact; 44.6 ± 9.2 denuded; LINUS programme) (Figure 1) though the maximum tension developed to NA at a concentration of 10 μM was significantly greater ($P < 0.05$) in endothelium-denuded tissues (1.16 ± 0.09 g) compared to endothelium-intact tissues (0.81 ± 0.1 g). The results are summarized in Figure 1 (inset histogram). For experiments where the relaxant effects of the purines were studied the submaximal concentration of 30 nM NA was used to precontract the tissue. The mean contraction produced by 30 nM NA was 0.89 ± 0.09 g in intact aortic rings and 0.98 ± 0.11 g in endothelium-denuded rings. At this concentration of NA there was no significant difference in the tension developed ($P > 0.05$).

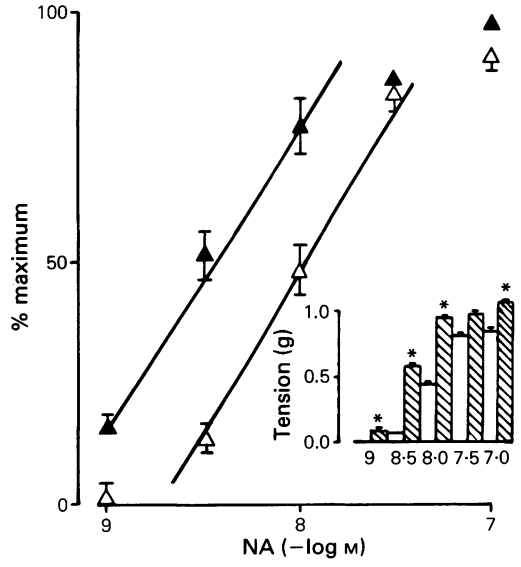

Figure 1 Noradrenaline (NA) concentration-response curve on rat thoracic aorta with the endothelium present (△, $n = 12$) and absent (▲, $n = 12$). Data expressed as mean with s.e.mean shown by vertical bars. The inset histogram represents the grams tension exerted after 2 min exposure at each concentration for endothelium intact (open columns) and denuded (hatched columns) aorta. *Indicates a significant difference between groups: $P < 0.05$, Student's *t* test.

Effects of purine receptor agonists on precontracted aortic rings

ATP induced concentration-dependent relaxation of the precontracted rat thoracic aorta (Figure 2, upper panel). Removal of the endothelial cells almost abolished the relaxant effect of ATP. NECA and L-PIA (0.01–30 μM) caused concentration-dependent relaxations of intact vessel segments. Figure 2 shows traces from an experiment which compares the relaxant actions of NECA (lower panel) to those of the P_2 agonist ATP (upper panel). AMP and adenosine (0.1–300 μM) also produced vessel relaxation (Figure 3a). To correlate the relative potencies of the P_1 receptor agonists the degree of relaxation of the NA (30 nM) contracted tissue to purine agonists at concentration 10 μM were compared. On the contracted rat thoracic aorta the nonselective P_1 receptor agonist NECA had a greater potency than the A_1 receptor agonist L-PIA. Adenosine and AMP were equipotent in relaxing the tissue. In endothelium-denuded rings the relaxant effects of all four agonists were significantly ($P < 0.05$) less than in intact ring segments (Figure 3b). Removal of the endothelium from these tissues did not alter the relaxant responses to the endothelium-independent vasodilator, sodium nitroprusside (data not shown).

Effect of haemoglobin on the relaxant responses to purines

In the endothelium intact NA (30 nM) contracted rat thoracic aorta, haemoglobin (10 μM) induced a further 13.7 ± 4.7% increase in tension (data not shown). Prior treatment with haemoglobin (10 μM) caused a significant ($P < 0.05$) 562 fold rightward shift of the dose-response curve to ATP (Figure 4). There was a significant ($P < 0.05$) 102 fold rightward shift in the concentration-response curve to NECA in the presence of 10 μM haemoglobin (Figure 5). Pretreatment with haemoglobin (10 μM) does not cause a significant shift ($P > 0.05$) in concentration-response curves to sodium nitroprusside (data not shown).

The effect of indomethacin

Prior treatment of intact ring segments with indomethacin (1 μM) did not significantly alter concentration-response curves to either ATP or NECA (data not shown).

The effects of 8-phenyltheophylline

Prior treatment of intact rings with 10 μM 8-PT significantly ($P < 0.05$) inhibited the relaxant response to 10 μM NECA. The relaxant responses of intact aortic rings to 30 μM ATP

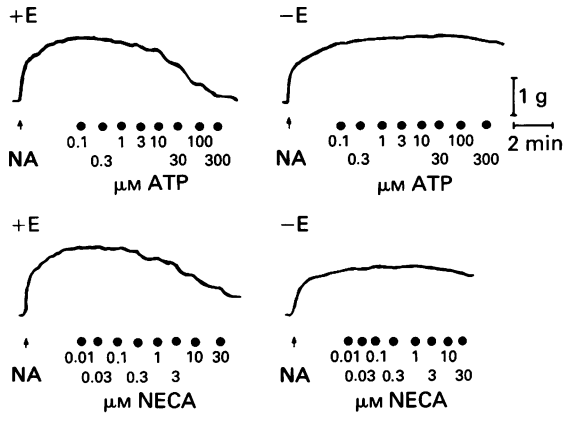

Figure 2 Trace showing the effect of adenosine 5'-triphosphate (ATP, upper panel) and 5'-N-ethyl carboxamidoadenosine (NECA, lower panel) on noradrenaline (NA, 30 nM)-contracted rat thoracic aorta in endothelium intact (+E) and denuded (−E) tissues.

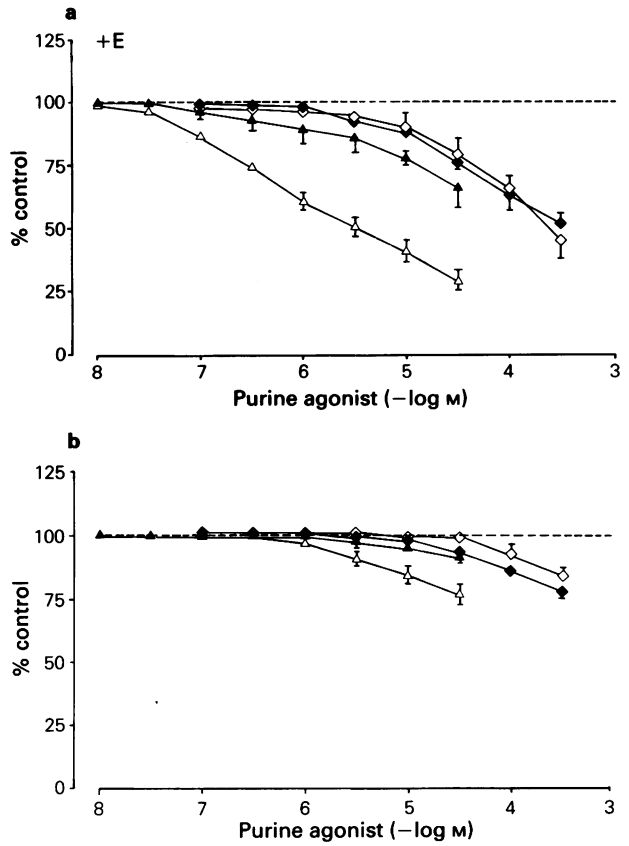

Figure 3 The effect of purine analogues on the noradrenaline (30 nM)-contracted thoracic aorta in endothelium intact (a) and denuded (b) tissues. ($n = 5$–7). (◆) Adenosine; (◇) adenosine 5'-phosphate; (△) 5'-N-ethyl carboxamidoadenosine; (▲) L-N^6-phenylisopropyladenosine. Data are expressed as means with s.e.mean shown by vertical bars.

were enhanced by 10 μM 8-PT (Figure 6) but the change did not reach the level of significance ($P > 0.05$). The addition of 10 μM 8-PT to endothelium-denuded tissues significantly reduced the relaxant response of the thoracic aorta to 10 μM NECA ($P < 0.05$). Any residual effect of ATP on the endothelium-denuded tissue was completely blocked by 8-PT (10 μM, data not shown). The vehicle used for 8-PT stock solutions did not affect relaxant responses to the purines in this tissue.

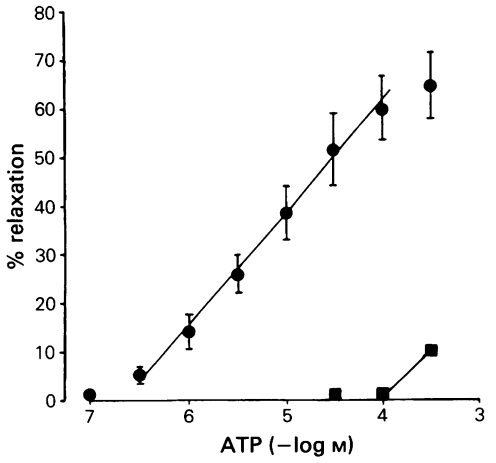

Figure 4 The effect of adenosine 5'-triphosphate (ATP) on noradrenaline (30 nM)-contracted rat thoracic aorta in the presence (■) and absence (●) of haemoglobin 10 μM, $n = 6$ per group. Each point represents mean with s.e.mean shown by vertical bars.

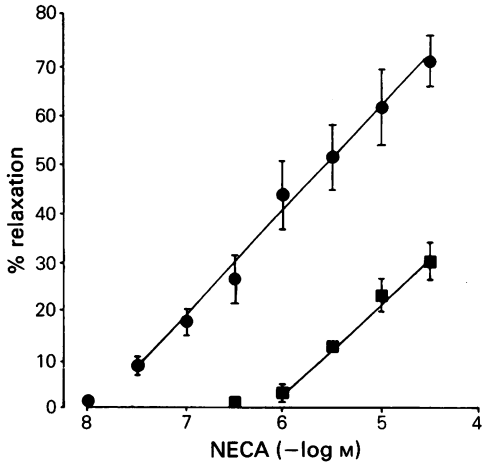

Figure 5 The effect of 5'-N-ethyl carboxamidoadenosine (NECA) on noradrenaline (30 nM)-contracted rat thoracic aorta in the presence (■) and absence (●) of haemoglobin 10 μM, $n = 6$ per group. Each point represents mean with s.e.mean shown by vertical bars.

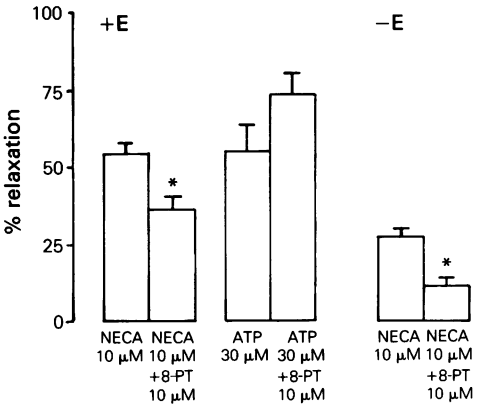

Figure 6 Histogram showing the effects of 8-phenyltheophylline (8-PT, 10 μM) on relaxant responses to 5'-N-ethyl carboxamidoadenosine (NECA, 10 μM) and adenosine 5'-triphosphate (ATP, 30 μM) in noradrenaline-contracted rat thoracic aorta ($n = 5$–6). *Indicates a statistically significant change from control response ($P < 0.05$, two tailed Student's t test).

Discussion

Our results show that the P_2 purinoceptor which mediates relaxation in the thoracic aorta is located on the endothelium since removal of endothelium markedly attenuated relaxation. As pretreatment with haemoglobin, which inhibits the action of endothelium-derived relaxing factor (EDRF) (Martin *et al.*, 1985) also abolished ATP-induced relaxation in this preparation, P_2 receptor-mediated relaxation is due to the release of EDRF. These results are similar to those obtained by Houston *et al.* (1987) for canine blood vessels. Although purine nucleotides have been reported to stimulate prostaglandin synthesis in endothelial cells (Van Coevorden & Boeynaems, 1984) the release of these mediators does not appear to play a role in nucleotide-induced relaxation in this tissue since pretreatment with the cyclo-oxygenase inhibitor indomethacin did not alter the response.

P_1-purinoceptor agonists also relaxed the NA-contracted rat thoracic aorta. The stable analogue NECA is a non-selective P_1 purine receptor agonist but is considered more potent at the A_2 receptor while L-PIA is a selective A_1 receptor agonist (Williams, 1987). NECA was more potent at inducing relaxation of the rat aorta than L-PIA and both adenosine and AMP. This order of potency is consistent with the presence of an A_2 receptor (Van Calker *et al.*, 1979; Londos *et al.*, 1980) and is in agreement with the findings of

other workers (see Introduction for references). Removal of the endothelium attenuated but did not abolish the relaxant responses to all four agonists. This indicates that a component of the response is endothelium-dependent and we suggest that A_2 receptors are present on both endothelial cells and smooth muscle. The endothelial receptors appear, like the P_{2Y} receptors, to mediate the release of EDRF, since haemoglobin caused a significant rightward shift in the concentration-response curve to NECA. Relaxant responses to a single concentration of NECA, once established, were reversed by the addition of haemoglobin to the bath. Cyclo-oxygenase products do not appear to contribute to NECA-induced relaxation in this preparation as indicated by the lack of effect of indomethacin.

The possibility that the endothelium-dependent component of relaxation in responses to NECA was due to an action on P_{2Y} receptors was excluded by the use of the adenosine receptor antagonist 8-PT. 8-PT significantly reduced responses to NECA in both intact and endothelium-denuded tissues, whilst relaxant responses to ATP were enhanced in the intact tissues. The reason for this enhancement is not clear. The very small residual response to ATP in endothelium-denuded tissues was abolished by prior treatment with 8-PT, which suggests that the response was due to adenosine formed by the breakdown of ATP.

The presence of adenosine A_2 receptors on endothelial cells may be of physiological importance in controlling blood flow. It has been suggested that adenosine is a local regulator of blood flow in several vascular systems (see Berne *et al.*, 1979; Burnstock, 1987). Since intravenous administration of adenosine or its stable analogues causes increases in coronary flow in the dog (Raberger *et al.*, 1977) these agonists are either able to cross the endothelium to activate the smooth muscle receptors directly or the vasodilatation is produced by an action on the luminal surface of the vessel. Since the endothelium provides a metabolic barrier to the penetration of a number of vasoactive substances such as 5-hydroxytryptamine and NA (Verbeuren *et al.*, 1988), and adenosine itself in a concentration below the micromolar range is subject to very comprehensive metabolism by the endothelium cells (Nees *et al.*, 1985), it is unlikely that under normal conditions circulating adenosine can cross the endothelium. Von Borstel *et al.* (1983) found that the basal plasma levels of adenosine in the rat were in the order of 1–4 μM and that decreases in blood pressure following intravenous infusion of adenosine occurred at plasma adenosine concentration of 5–6 μM.

The results described here suggest that A_2 receptors are located on both the endothelium and smooth muscle. We propose that the endothelial cell A_2 purine receptor may have a role in rapidly maintaining blood flow in conditions of increased adenosine release such as hypoxia. Increased levels of adenosine may then be able to cross the endothelial barrier to activate smooth muscle receptors directly and thus provide a longer lasting component of vasodilatation as suggested previously by Burnstock (1987).

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Action of cromakalim on potassium membrane conductance in isolated heart myocytes of frog

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1 The effects of cromakalim on membrane currents were studied at 20°C in frog atrial and ventricular cells in patch clamp recording using the whole cell configuration.

2 When cromakalim (1 μM) was applied in the external medium, a time-independent current was activated in a few minutes. Cromakalim induced a weak increase of inward membrane currents recorded during hyperpolarization and a large increase of outward membrane currents recorded during depolarization.

3 The current voltage relationship of the cromakalim-induced current reversed near E_K and rectified in the outward direction.

4 The cromakalim-activated current was inhibited by external application of cesium (20 mM), barium (1.8 mM), tolbutamide (1 mM) and glibenclamide (1 μM).

5 The effects of cromakalim were insensitive to a cytosolic increase in adenosine 5'-triphosphate (ATP, 3–5 mM). Cromakalim had no effects when applied in the cell.

6 Our results confirm that cromakalim activates an $I_{K(ATP)}$-like conductance and suggest that the effects of the drug are due to an action on the external side of the membrane and are independent of the ATP cell content.

Introduction

Cromakalim (BRL 34915, (±)-6-cyano-3,4-dihydro-2,2-dimethyl-trans-4-(2-oxo-1-pyrrolidyl)-2H-benzo(b)-pyran,-3-ol) is a smooth muscle relaxing agent which opens $^{86}Rb^+$ and $^{42}K^+$ permeable K^+ channels resulting in a membrane hyperpolarization in vasculature (Hamilton *et al.*, 1986; Weir & Weston, 1986b; Coldwell & Howlett, 1986; Quast, 1987; Shety & Weiss, 1987; Quast & Baulin, 1988), intestine (Weir & Weston, 1986a), uterus (Hollingsworth *et al.*, 1987), trachea (Allen *et al.*, 1986) and urinary bladder (Fujii, 1987; Foster & Brading, 1987). Numerous studies have tested the way in which cromakalim acts on membrane potential. This drug activates Ca^{2+}-activated K^+ channels in aortic smooth cells (Kusano *et al.*, 1987) but is insensitive to apamin, a blocker of low conductance Ca^{2+}-activated K^+ channels (Weir & Weston, 1986a; Allen *et al.*, 1986; Cook & Hof, 1988). Its action does not require Ca^{2+} entry through dihydropyridine Ca^{2+} channels in the portal vein (Quast, 1987; Southerton & Weston, 1987). Tetraethylammonium chloride, 3,4-dihydropyridine and procaine inhibit the cromakalim-stimulated Rb^+ efflux in the portal vein (Quast, 1987; Southerton & Weston, 1987) and trachea (Allen *et al.*, 1986) as does the venom of the scorpion *Leiurus quinquestriatus Hebraeus* in portal vein (Quast & Cook, 1988). Cromakalim relaxation of the vascular smooth muscle is reversed by the sulphonylurea drug, glibenclamide (Quast & Cook, 1988; Winquist *et al.*, 1988).

In cardiac muscle, cromakalim shortens the action potential duration (Cain & Metzler, 1985; Grosset & Hicks, 1986; Scholtysik, 1987; Osterrieder, 1988; Sanguinetti *et al.*, 1988). Cromakalim increases the K^+ conductance in the ventricular cell membrane of guinea-pig (Osterrieder, 1988; Sanguinetti *et al.*, 1988; Escande *et al.*, 1988). On these cells $BaCl_2$ and tetrahydroaminoacridine block in a potential-dependent manner the cromakalim-induced conductance (Osterrieder, 1988). Recently on guinea-pig ventricular cells Sanguinetti *et al.* (1988) have shown that glibenclamide, an inhibitor of adenosine 5'-triphosphate (ATP)-sensitive K^+ channels (Schmid-Antomarchi *et al.*, 1987) prevented or reversed the shortening of the effective refractory period and action potential duration produced by cromakalim. These results support the hypothesis that cromakalim activates the ATP-sensitive K^+ channels in the heart. The ATP-sensitive K^+ channels have been discovered from single channel recordings by Noma (1983). The physiological significance of these channels in cardiac cells is still under debate. K^+ channel openers like cromakalim and sulphonylurea drugs like glibenclamide are useful tools for studying the role of ATP-sensitive K^+ channels in the heart.

We studied the effects of cromakalim by whole cell voltage clamp techniques in single frog heart myocytes. Our results indicate that, as on mammalian cardiac cells, cromakalim activates potassium conductance. In contrast with mammalian cells, cromakalim had no effects when applied directly to the internal face of the cell. We propose that cromakalim activates an $I_{K(ATP)}$-like current. Some of the results described here have appeared in abstract form (Pilsudski *et al.*, 1989a).

Methods

Preparation of isolated heart cells

Single atrial and ventricular cells from *Rana esculenta* were obtained by an enzymatic dissociation method as described by Bonvallet (1987). The complete auricular tissue and small chunks of ventricular tissue were bathed in nominally calcium-free solution (see the composition of solutions) for 30 min and then transferred to calcium-free solution + collagenase (2 mg ml^{-1}) + protease (0.3 mg ml^{-1}) for 60 min at room temperature. Tissues were kept overnight in calcium-free solution at 6°C. Cells were obtained by shaking the tissues in the superfusing experimental chamber containing calcium-free solution; the latter was then replaced by Ringer modified solution.

Whole cell/experiments/recordings/analysis

Experiments were carried out with the patch clamp technique in whole cell clamp mode at room temperature (20–22°C). Bath solution was continuously perfused and an internal perfusion device allowed fast exchange of the pipette dialysing medium. Patch pipettes were made from borosilicate glass (Clark Electromed., Reading, U.K.). Their resistance was between 2–5 megaohms when filled with 'intracellular-like' medium and the seals ranged from 1 to 10 gigaohms. Whole-cell currents were recorded with a RK 300 patch-clamp amplifier (Biologic, Echirolles France) and stored on a PC

microcomputer using the pCLAMP software (Axon Instr., Burlingame, CH, U.S.A.). Computer programmes for measurements, computations and plots from pCLAMP files were developed in our laboratory.

Pulses protocols

After the establishment of the whole cell recording configuration, a standard double step voltage-clamp protocol was applied at a frequency of 0.1 Hz: from a holding potential of −70 mV, symmetric pulses of ±40 mV were applied to the cell, setting the membrane potential to −30 mV for 100 ms, then to −110 mV for 100 ms. The membrane currents were measured at the end of each step: −70 mV (triangle), −30 mV (lozenges) and 110 mV (squares) (as shown in the figures).

The current voltage relationships were obtained by the application of voltage ramps (1V/s) from −140 to +20 mV every 10 s and smoothed for display.

Drugs and solutions

Chemicals were purchased from Boehringer Mannheim, France (Meylan, France) except pronase (Sigma, St Louis, MO, U.S.A.). Cromakalim was obtained from Beechams Research Laboratories.

The composition of the modified Ringer solution was (mM): NaCl 110, KCl 2.5, $CaCl_2$ 1.8, $MgCl_2$ 2, glucose 10, pyruvic acid 5, HEPES buffer 10, $CoCl_2$ 3 and tetrodotoxin 0.001, pH adjusted to 7.2 with NaOH. CsCl (20 mM), $BaCl_2$ (1.8 mM), tolbutamide (1 mM), glibenclamide (1 μM) and cromakalim (1 μM) were eventually added to this solution. The calcium-free solution was the same as modified Ringer solution with $MgCl_2$ (2 mM) replacing $CaCl_2$ (1.8 mM).

Patch pipettes were filled with an 'intracellular-like' medium containing (in mM): KCl 20, K aspartate 130, KH_2PO_4 1, $MgCl_2$ 1, K-EGTA 5, HEPES buffer 10; pH adjusted to 7.3 with KOH. CsCl (20 mM), K_2ATP (3–5 mM) and cromakalim (1 μM) were eventually added to this solution.

Results

Control experiments

All the experiments were carried out in voltage clamp on frog atrial and ventricular cells in the presence of cobalt and tetrodotoxin to block the inward currents.

Records obtained under control conditions in an atrial cell are shown in Figure 1. The current appeared to be time-independent after the decay of the capacitive transient (Figure 1a). The membrane current was much larger on hyperpolarization than on depolarization, indicating a strong inward rectification. This current could be recorded for more than 30 min without any significant changes in amplitude and kinetics (Figure 1c); 30 min after the beginning of the experiment, the 'intracellular-like' medium was changed by addition of 20 mM CsCl. The inward membrane current recorded on hyperpolarization was then rapidly and completely suppressed (Figure 1b). The amplitude of the membrane current plotted as a function of time indicated the rapid action and reversal of internal perfusion with CsCl and showed the efficiency of the internal perfusion device (Figure 1c). Figure 1d presents the current voltage relationship. The current is maximum outward near −70 mV i.e. 10 to 15 mV positive to the resting potential. A clear negative slope conductance can be noticed for potentials more positive than −70 mV. This control current shares the common properties of the inward rectifying potassium current I_{K1} of amphibians (Hume & Giles, 1983; Simmons & Hartzell, 1987).

Experiments in the presence of cromakalim

The membrane current recorded in the presence of 1 μM cromakalim added to the bath control solution is shown in Figure 2b. The membrane time independent current increased at all membrane potentials. The time course of the effect of external cromakalim indicates a relatively slow action of the molecule (Figure 2c). The membrane current activated by cro-

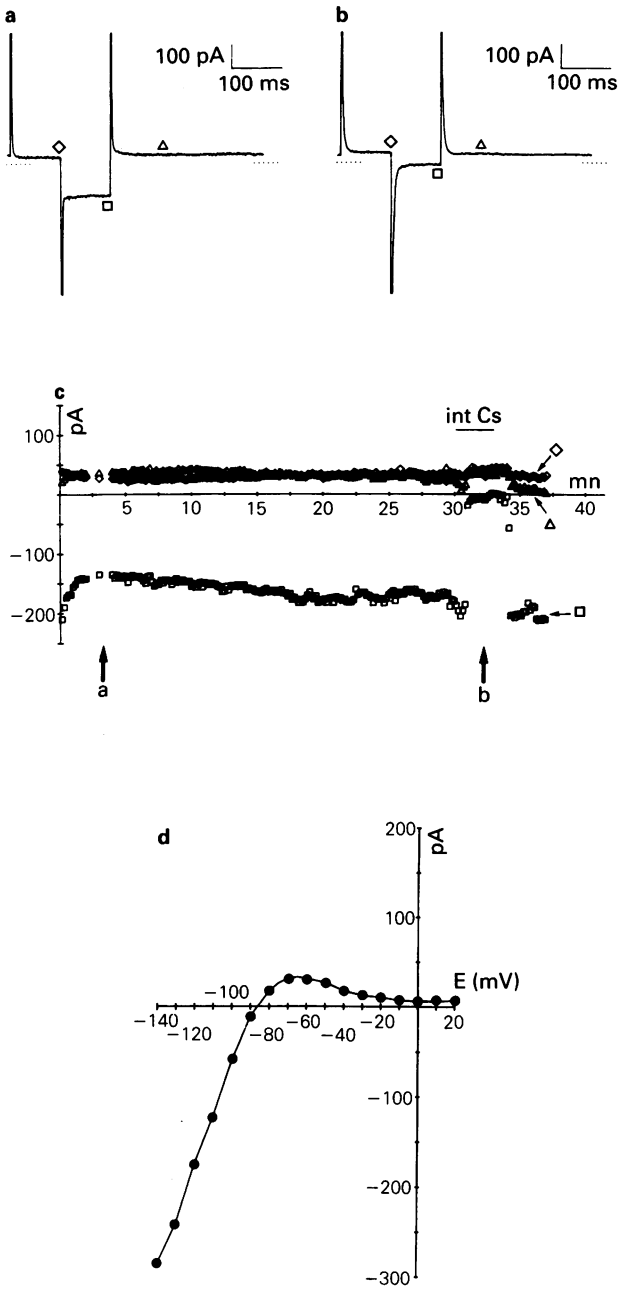

Figure 1 Membrane current recorded from frog atrial myocytes in control conditions. (a) Individual current traces recorded in control solutions with a double voltage step stimulation protocol. The zero current level is indicated by the dots at the right and at the left ends of the traces. The potential was held at −70 mV (△), which is close to the potential at which the current flowing through the inward rectifying potassium channels is maximally outward. The first voltage step was to −30 mV (◇). The following step was to −110 mV (□). The large inward current at this potential reflects the strong inward rectification of the resting cell current. (b) Current recorded from the same cell after 50 s of dialysing with 'intracellular-like' medium with 20 mM CsCl added. (c) Membrane current recorded at −70 mV (△), −30 mV (◇) and −110 mV (□) during 37 min of continuous recording from one frog atrial muscle cell. The current is measured at the end of each step. The bar above the graph (Cs) indicates the period for which the cell was dialysed with CsCl. The arrows below the graph indicate when the traces in (a) and (b) were recorded from this cell. (d) Current voltage relationship from an atrial cell in control conditions. This relationship was obtained by the application of voltage ramps (1V/s) from −140 to +20 mV and has been smoothed for display.

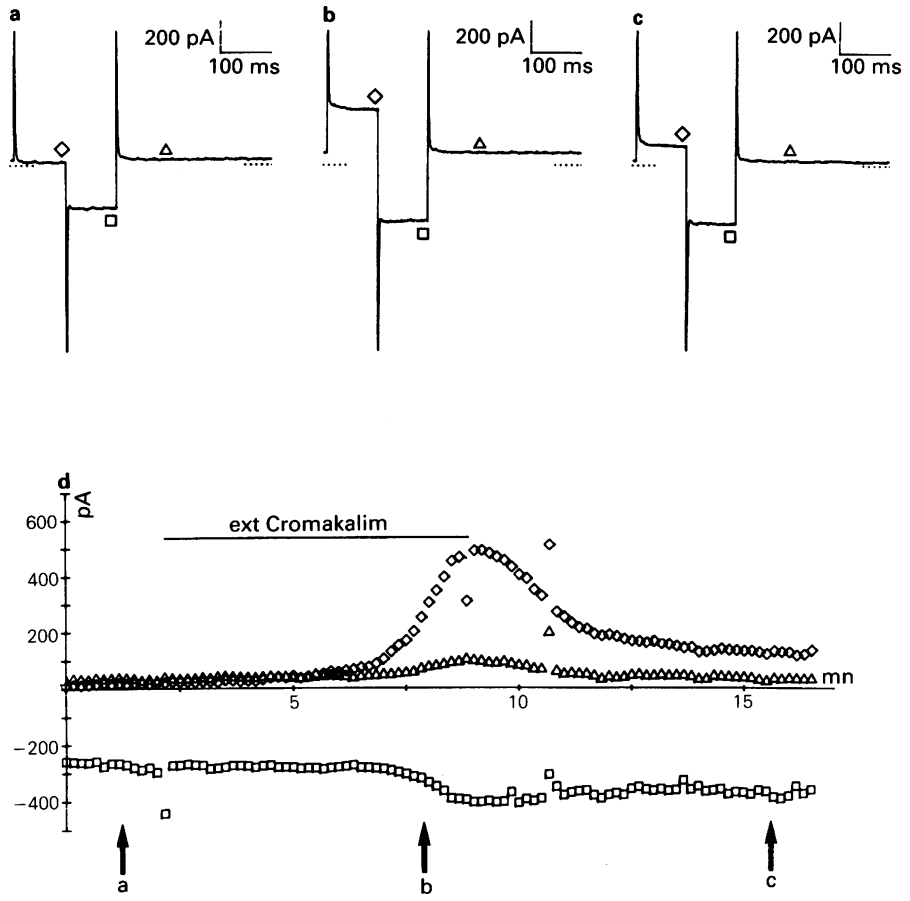

Figure 2 Membrane current recorded from frog atrial myocytes. Effects of cromakalim added in the bath solution upon membrane current. (a) Individual current trace recorded in control conditions with the same protocol as in Figure 1. (b) Current recorded from the same cell when it was superfused with cromakalim 1 μM added in the bath. (c) Current recorded 6 min after the removal of cromakalim from bath solution. (d) Membrane current measured at the three membrane potentials during 17 min of continuous recording from one frog atrial muscle cell. The bar above the graph (Cromakalim) indicates the period for which the bath solution was changed for one which contained 1 μM cromakalim. The arrows beneath the graph indicate when the traces in (a), (b) and (c) were recorded from this cell.

makalim was nearly completely suppressed when returned to the control (Figure 2c,d).

Figure 3a depicts the current-voltage relationships of the current in an atrial cell in control (filled circles) and in the presence of 1 μM cromakalim (open circles).

In the presence of cromakalim, the current was larger than in control in the inward as well as in the outward direction. However, the increase of the outward membrane current was much larger than the increase of the inward membrane current. No negative slope conductance was recorded on this curve. The curves obtained in control and in the presence of cromakalim crossed each other near the potassium equilibrium potential. The difference current (Figure 3b) rectified strongly in the outward direction.

The effect of cromakalim (1 μM) on membrane current was also examined in single isolated ventricular cells (Figures 4 and 5). As described above for atrial cells, the time-independent membrane current was increased at -70 and at -30 mV. A very small increase was seen at -110 mV (Figure 4). In Figure 5a the current voltage relationships obtained in the presence (open circles) and in the absence (filled circles) of cromakalim (1 μM) are superimposed. In the presence of cromakalim the current was largely increased in the outward direction and not modified in the inward direction. The difference current (Figure 5b) rectified very strongly in the outward direction.

The current activated by cromakalim in atrial and ventricular cells was clearly distinct from the inward rectifying potassium current I_{K1}. It appears similar to the current recorded in mammalian ventricular cells (Osterrieder, 1988; Sanguinetti *et al.*, 1988). This current had no tendency to fade for tens of minutes (not shown).

We also checked whether the addition of cromakalim (1 μM) to the internal solution could activate the same membrane currents as the external application to the surface of the cell. When cromakalim was added in the pipette, the membrane current was unchanged for more than 30 min.

Ionic nature of the cromakalim-activated current

The experiments shown in Figure 6 were carried out on atrial cells to determine the effects of two potassium channel blockers on the increase in membrane current induced by cromakalim. Application of 20 mM CsCl (Figure 6a) or 1.8 mM $BaCl_2$ (Figure 6b) in the external medium after the activation of the cromakalim current dramatically reduced both inward and outward membrane currents.

Action of sulphonylurea drugs

The effects of sulphonylurea drugs, glibenclamide and tolbutamide, specific blockers of the ATP-sensitive K^+ current (Schmid-Antomarchi *et al.*, 1987), were tested on the cromakalim-activated membrane current. The effects of glibenclamide on the atrial membrane current activated by cromakalim are shown in Figure 7. In control conditions (Figure 7a) membrane current showed the same characteristics as in Figure 1a. The effects of cromakalim are shown in Figure 7b. The addition of 1 μM glibenclamide to the bath solution suppressed completely both the inward and outward

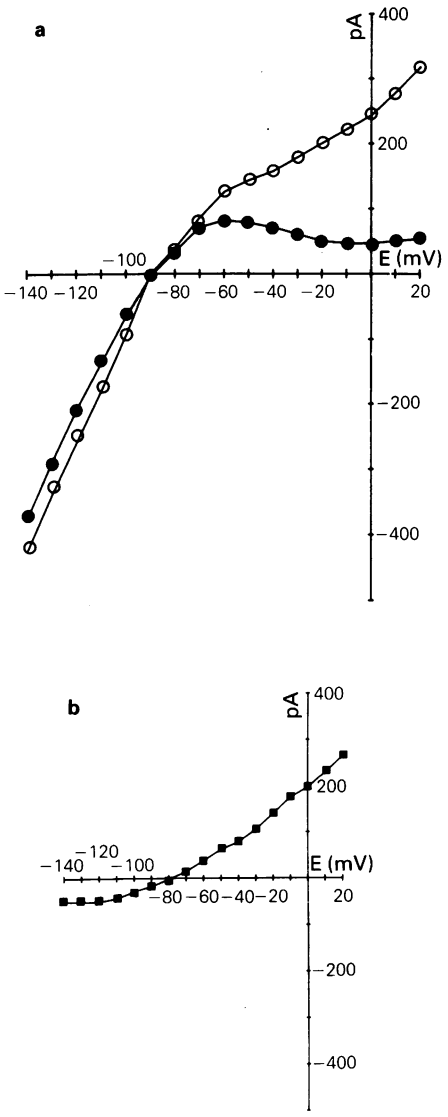

Figure 3 Current-voltage relationships from an atrial cell in control conditions and in the presence of cromakalim. These relationships were obtained as described in Figure 1(d) and measured at different times during the experiments. (a) Superimposed current traces recorded just after the start of whole cell recording (●) and after the superfusion of the cell with 1 μM cromakalim (○). Note the negative slope conductance in the depolarizing direction for the control current and its absence for the cromakalim current. (b) Cromakalim-induced current obtained by difference (membrane currents after addition of cromakalim, ○) − (control membrane currents, ●).

membrane current increase due to cromakalim. The effects of glibenclamide were rapidly reversed when the bath solution containing cromakalim was restored; these effects could be repeated. Similar results were obtained with glibenclamide (1 μM) in the internal medium or tolbutamide (1 mM) in the external solution. These results suggest that in frog heart myocytes cromakalim activates an ATP-sensitive K^+-like current.

Action of intracellular ATP

In the previously described experiments the ATP nucleotide was not present in the 'intracellular-like' medium filling the patch pipette. The effects of internal ATP were examined upon the cromakalim-evoked cell current. Addition of 3 to 5 mM ATP to the intracellular medium, before or during the action of cromakalim did not modify the action of this compound.

Discussion

We describe here the effects of cromakalim on the whole-cell membrane current of atrial and ventricular frog myocytes. In the two types of cells, cromakalim activated a time-independent current; cromakalim had no effect when added to the internal medium. The activation of this current has been reported to be responsible for the decrease in the action potential duration in heart preparations (Osterrieder, 1988; Quast *et al.*, 1988; Sanguinetti *et al.*, 1988).

The reversal potential of the current activated by cromakalim was close to the potassium equilibrium potential, suggesting that this current is carried by potassium ions. We studied the effects of two potassium channel blockers upon the increase in membrane currents induced by cromakalim: 20 mM cesium and 1.8 mM barium rapidly suppressed the effect of cromakalim, confirming that the increase in conductance is due to an increase in potassium conductance as described in mammalian cardiac muscle (Scholtysik, 1987; Osterrieder, 1988; Quast *et al.*, 1988; Sanguinetti *et al.*, 1988; Escande *et al.*, 1988).

Many different types of K channels have been identified in the heart (for review see Carmeliet *et al.*, 1988). The inwardly rectifying K^+ channel, the muscarinic receptor-operated K^+ channel, the Na^+-activated K^+ channel and the delayed rectifier K^+ channel are undoubtedly not activated by cromakalim. Indeed the sulphonylurea drugs tolbutamide and glibenclamide are specific blockers of the ATP-regulated K^+ channel (Schmid-Antomarchi *et al.*, 1987). We have shown that 1 μM glibenclamide fully and selectively blocked the effect of cromakalim. We conclude that the current activated by cromakalim is the ATP-sensitive K^+ current. Such a result was first observed by Sanguinetti *et al.* (1988) and Escande *et al.* (1988) on cardiac myocytes from guinea-pig.

At first it appeared that the effects of cromakalim were not external but internal. Cromakalim could for example depress ATP production and then activate the ATP-K^+ channel. The slow time course of action of cromakalim at 20°C (generally 5 min before any effect) strengthened this idea. In contrast, the rapid and complete reversal when the drug was washed out suggested that cromakalim acted on the external face of the cell membrane. It has been shown that ATP sensitive K^+ channels can be inhibited by intracellular nucleotides such as ADP, GTP, GDP, NAD but preferentially by ATP (for review see Ashcroft, 1988; Stanfield, 1987). The cromakalim-induced current can be recorded in the presence of ATP (3 or 5 mM) in the pipette solution. We observed that addition of ATP after the increase in conductance due to cromakalim did not modify the current. This cannot be due to a poor dialysis of the cell. In frog atrial myocytes we have shown that the exchange of soluble components such as ATP between the pipette and the cytoplasm is fast enough to stop and inhibit an ATP depletion phenomenon in less than 5 min (Pilsudski *et al.*, 1989b). Thus the potassium conductance evoked by cromakalim seems independent of the ATP cell content. The most surprising finding is that cromakalim is completely inactive when applied directly in the cytosol for 30 min. Escande *et al.* (1988) reported that application of cromakalim at the intracellular side of the membrane activated an ATP-sensitive K^+ channel. According to these authors, cromakalim as well as pinacidil or RP 49356 (Escande *et al.*, 1989) must penetrate the cell before acting on K^+-ATP channels. We cannot explain this discrepancy. The difference between the two patch-clamp modes, whole cell in our conditions and inside out in those of Escande *et al.* could be one explanation.

Vascular muscle appears to be more sensitive to the relaxant properties of cromakalim than cardiac tissues (Quast & Cook, 1988; Winquist *et al.*, 1988). Our results showing that cromakalim acts at 1 μM, indicate that frog heart tissues seem to be more sensitive than mammalian (Sanguinetti *et al.*, 1988; Osterrieder, 1988).

We conclude that cromakalim activates a potassium conductance in the cell membrane of isolated cardiac myocytes of

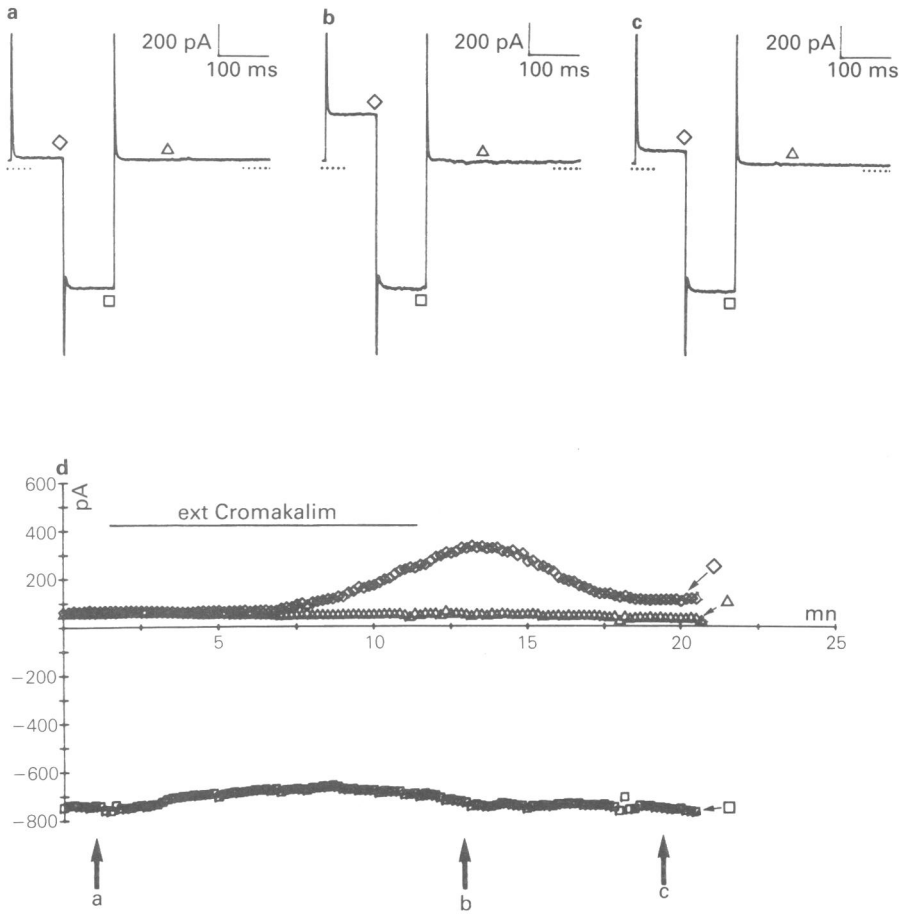

Figure 4 Membrane current recorded in frog ventricular myocytes. Effects of cromakalim added in the bath solution upon membrane currents. (a) Membrane current recorded shortly after establishing the whole cell recording configuration. (b) Membrane current recorded 10 min after superfusing the cell with cromakalim (1 μM). (c) Membrane current recorded 7 min after the removal of cromakalim from bath solution. (d) Membrane current measured at the three membrane potentials during 21 min of continuous recording from one frog ventricular myocyte. Same symbols as in Figure 1.

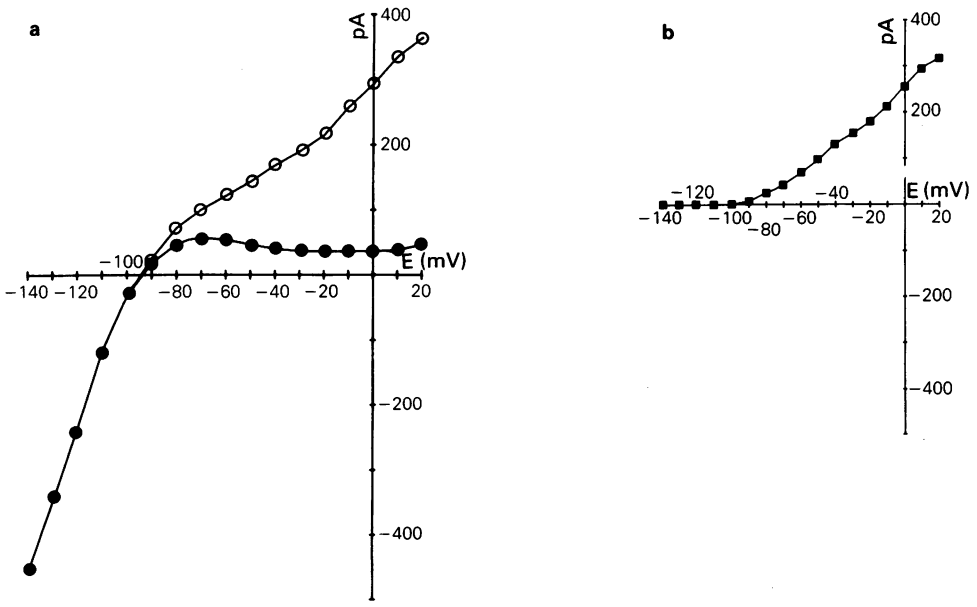

Figure 5 Current voltage relationships from a ventricular cell in control conditions and in the presence of cromakalim. These relationships were obtained as in Figure 1d. (a) Superimposed current traces recorded just after the start of whole cell recording (●) and after the superfusion of the cell with cromakalim 1 μM (○). (b) Cromakalim-induced current obtained by difference (membrane currents after addition of cromakalim, ○) − (control membrane currents, ●).

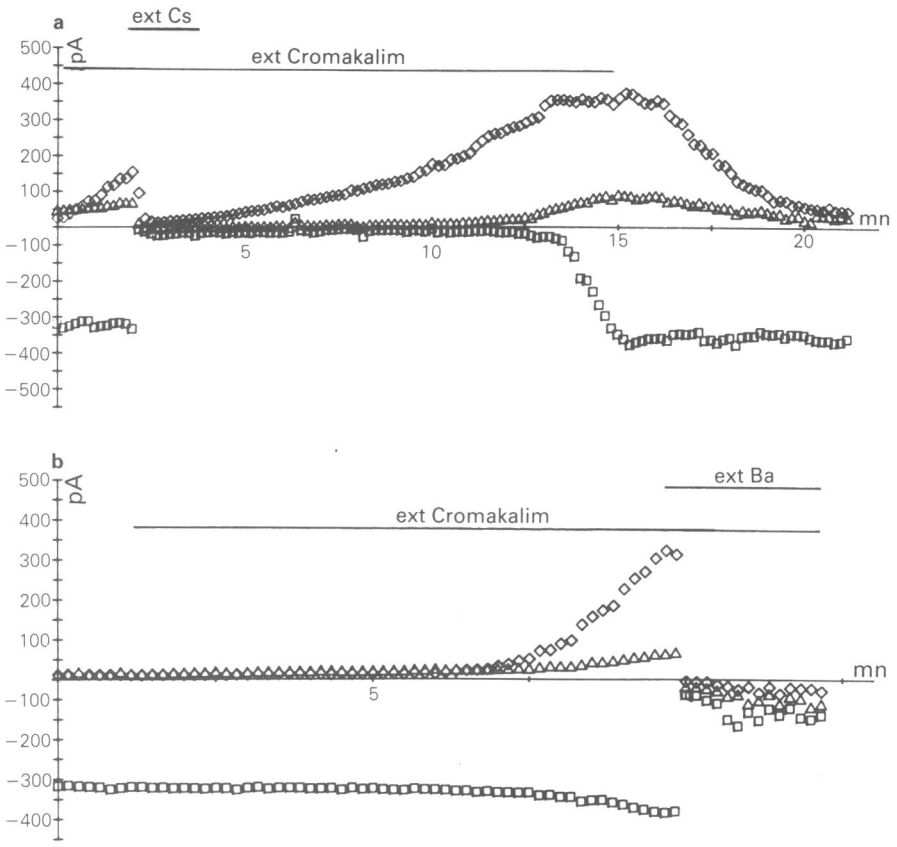

Figure 6 Effects of potassium channel blockers upon membrane current evoked by extracellular application of cromakalim. The format of each graph is the same as in Figure 1c. The bars above the graph indicate periods for which 20 mM cesium (a) and 1.8 mM barium (b) were applied to the external surface of the cell.

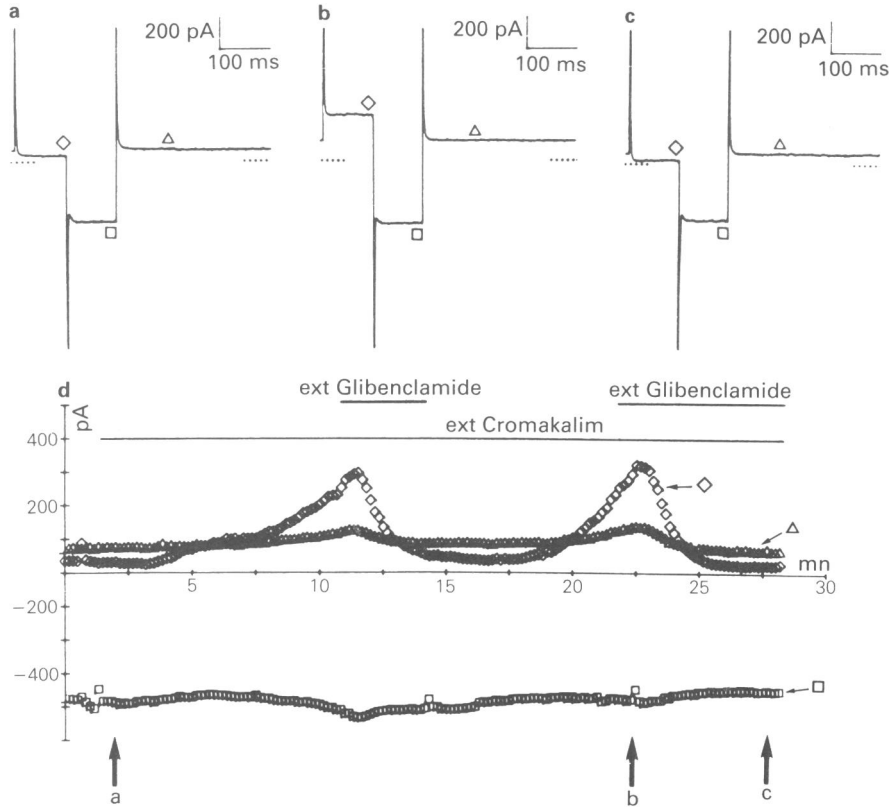

Figure 7 Inhibition of the effect of cromakalim on an atrial cell by addition of glibenclamide (1 μM) to the external solution. (a) Membrane current recorded shortly after establishing the whole cell recording configuration. (b) Membrane current recorded 11 min after the beginning of the superfusion of the cell with cromakalim 1 μM. (c) Membrane current recorded 17 min after the superfusion of the cell and 6 min after the superfusion of the cell with cromakalim and cromakalim + glibenclamide. The effects of cromakalim and glibenclamide can be repeated.

the frog as described previously in mammalian isolated cardiac myocytes. We suggest that cromakalim activates an ATP-sensitive potassium channel. This action is completely independent of the ATP cell content.

Frog isolated cardiac myocytes appear to be very appropriate models for the study of cardiac potassium conductance and new pharmacological compounds acting as blockers or openers of K^+ channels.

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Competitive antagonists discriminate between NK_2 tachykinin receptor subtypes

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1 We have compared the ability of various tachykinins and selective tachykinin receptor agonists to induce contraction of the endothelium-denuded rabbit pulmonary artery (RPA) and hamster trachea (HT) and have estimated the affinity of some newly developed NK_2 selective antagonists in the same tissues.

2 In confirmation of previous findings, experiments with the agonists indicated that NK_2 receptors are the main if not the sole mediators of the response to tachykinins in both RPA and HT. No evidence for significant degradation of neurokinin A (NKA) was found in either tissue when experiments were repeated in the presence of a mixture of peptidase inhibitors (thiorphan, captopril and bestatin, 1 μM each).

3 The peptide antagonists tested were: Peptide I = [Tyr^5, D-Trp^{6,8,9}, Arg^{10}]-NKA(4–10); Peptide II = [Tyr^5, D-Trp^{6,8,9}, Arg^{10}]-NKA(3–10); Peptide III = Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2. The three peptides produced a concentration-dependent rightward shift of the concentration-response curve to NKA in both RPA and HT with no significant depression of the maximal response attainable. The slopes of the Schild plots were not significantly different from unity, indicating a competitive antagonism. Peptides I and II were about 100 times more potent in the RPA than in the HT, while Peptide III was about 100 times more potent in the HT than RPA.

4 The pA_2 values obtained in these two tissues with the three antagonists were not significantly different when tested in the absence or presence of peptidase inhibitors, or when a selective NK_2 receptor agonist, [βAla^8]-NKA(4–10) was used instead of NKA. Similar pA_2 values were obtained after 15 or 90 min of incubation with the antagonists. Peptides I, II and III had no inhibitory effect on contractions produced by noradrenaline in the RPA or by carbachol in the HT.

5 Peptides I, II and III showed weak or no antagonistic activity toward the vasodilatator effect of substance P in the dog carotid artery (NK_1 receptor-mediated) or toward the contractile effect of neurokinin B in the rat portal vein (NK_3 receptor-mediated).

6 These results provide pharmacological evidence for heterogeneity of NK_2 receptors in the RPA and HT. The NK_2 receptors present in these tissues are not discriminated by natural tachykinins or selective agonists, but are recognized with very different affinity by NK_2 receptor antagonists.

Introduction

Tachykinins are a family of peptides which share the common C-terminal sequence Phe-X-Gly-Leu-Met NH_2. In mammals, at least three different tachykinins, namely substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) are the most likely candidates for a transmitter role, both in the central and peripheral nervous system (see Maggio, 1985 and Emson *et al.*, 1987 for reviews).

To date, the available evidence indicates that three distinct tachykinin receptors mediate the biological effects of these peptides in mammalian tissues (Buck *et al.*, 1984; Laufer *et al.*, 1985; Lee *et al.*, 1986; Regoli *et al.*, 1985; 1986; 1987; 1988; 1989; Maggi *et al.*, 1987). These receptors are now termed NK_1, NK_2 and NK_3 and the C-terminal sequence of tachykinins is critical for their activation. Natural tachykinins possess a different affinity for these three receptors, SP, NKA and NKB being more active at NK_1, NK_2 and NK_3 sites, respectively. In the absence of potent and selective tachykinin receptor antagonists, the classification of tachykinin receptors in mammalian tissues has been chiefly based upon the relative order of potency of natural peptides and their fragments in various bioassays or binding experiments. A major problem stems from the fact that natural tachykinins, at high concentrations, stimulate indiscriminately all three receptors. To circumvent this problem much effort has been directed to the development of synthetic agonists capable of selectively stimulating one of the three tachykinin receptors, while having reduced or no activity at the other two sites (Dion *et al.*, 1987; Drapeau *et al.*, 1987; Rovero *et al.*, 1989a,c).

By using receptor-selective synthetic peptides, several tissues such as the endothelium-denuded rabbit pulmonary artery (RPA, D'Orleans-Juste *et al.*, 1986), the human urinary bladder or bronchi (Advenier *et al.*, 1987; Naline *et al.*, 1988; Maggi *et al.*, 1988; 1989b) and the hamster trachea (HT, Maggi *et al.*, 1989a) have been shown to contain an apparently homogeneous class of tachykinin receptor, of the NK_2 type. During the course of a screening program aimed to develop selective antagonists for NK_2 receptors (Rovero *et al.*, 1989a), we found that some peptide antagonists exhibit marked differences in affinity for the NK_2 receptors in the RPA and HT. The present study was undertaken in order to ascertain whether these differences might involve the existence of NK_2 receptor subtypes in these two preparations.

Methods

Male albino New Zealand rabbits (2.5–3 kg), male hamsters (125–150 g) and male albino rats (Wistar strain, 300–350 g) were stunned and bled. Mongrel dogs of either sex (9–15 kg) were killed by administering a high dose (50 mg kg^{-1} i.p.) of pentobarbitone and exsanguination (Regoli *et al.*, 1984).

The endothelium-denuded rabbit pulmonary artery (RPA) and hamster trachea (HT) were prepared for isometric tension recording in 5 ml isolated organ baths containing oxygenated (96% O_2 and 4% CO_2) Krebs solution as described previously (D'Orleans-Juste *et al.*, 1986; Maggi *et al.*, 1989b; Rovero *et al.*, 1989b).

The activity of antagonists at the NK_1 and NK_3 receptors was estimated by use of substance P and neurokinin B as

Table 1 Amino acid sequences of the peptides used in the study

Peptide	*Sequence*
Peptide I	H-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Arg-NH_2
Peptide II	H-Thr-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Arg-NH_2
Peptide III	Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2

agonists in the dog isolated carotid artery (DCA) and rat portal vein (RPV), as described previously (Regoli *et al.*, 1986; 1987; 1988; 1989).

The amino acid sequence of antagonists used is shown in Table 1. For the sake of convenience, these peptides will hereafter be referred to as Peptide I, II and III, respectively.

All experiments commenced after a 120 min equilibration period. The RPA and HT were equilibrated by repeated administration of NKA (0.1 μM) at 15–20 min intervals, until reproducible responses were obtained. The activity of antagonists was estimated after a 15 min incubation period. Preliminary experiments showed that this incubation time was sufficient to observe maximal inhibition toward the effect of NKA, since a similar displacement of the concentration-response curve to the agonist was observed after a 90 min incubation period.

Evaluation of data

Each value in the text, tables and figures is mean ± s.e.mean. Regression analysis was performed by means of the least squares method. EC_{50}s and 95% confidence limits were calculated accordingly. pD_2 values were calculated as the $-\log EC_{50}$. Schild plots (Arunlakshana & Schild, 1959) were constructed from experimental data to calculate pA_2 values by use of a computer programme for Apple IIe (Tallarida & Murray, 1981).

To compare data obtained in different experimental conditions (e.g. in the absence or presence of peptidase inhibitors), pA_2 values were calculated by the constrained plot method (slope constrained to -1) (Tallarida *et al.*, 1979; Tallarida & Murray, 1981).

Peptides

Peptide I ([Tyr^5, D-Trp^{6,8,9}, Arg^{10}]-NKA(4–10) (MEN 10207) and Peptide II ([Tyr^5, D-Trp^{6,8,9}, Arg^{10}]-NKA(3–10) (MEN 10208), SP, NKA, [βAla^8]-NKA(4–10) and NKA(4–10) were synthesized at the Chemistry Department, A. Menarini Pharmaceuticals, Florence, Italy by conventional solid-phase methods. Peptide III (Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2), [Pro^9]-SP sulphone and [MePhe^7]-NKB were synthesized by Dr G. Drapeau at the Department of Physiology and Pharmacology of the University of Sherbrooke, Canada. Neuropeptide gamma (NPγ, γ-preprotachykinin(72–92)-peptide amide) was a kind gift from Dr J.E. Krause, Dept. of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis MO 63110, U.S.A. All other peptides were from Peninsula (St. Helens, U.K.). Other drugs used were noradrenaline HCl (Fluka), carbachol HCl (Merck), thiorphan (Sigma), bestatin (Peninsula), captopril (Squibb).

Results

Effect of agonists

Data in Table 2 show the apparent affinity of natural tachykinins and related peptides in producing contraction of the RPA and HT. Both preparations strongly responded to NKA and NKB as well as to the NKA precursors, NPγ and NPK. SP, although it was a full agonist, was 151 and 279 times less potent than NKA in the RPA and HT, respectively. The heptapeptide, NKA(4–10) which displays limited selectivity for NK_2 receptors as compared to NKA (Regoli *et al.*, 1986) was as potent as the decapeptide in both assays. Evidence for the involvement of NK_2 receptors comes from the use of synthetic, receptor-selective peptides (Drapeau *et al.*, 1987; Dion *et al.*, 1987; Maggi *et al.*, 1989a). In fact, [βAla^8]-NKA(4–10), a selective NK_2 receptor agonist, displayed strong affinity in both tissues, while [Pro^9]-SP sulphone an NK_1-selective agonist, was inactive up to 10 μM and [MePhe^7]-NKB, an NK_3-selective agonist, was active only at μM concentrations in both preparations.

Effect of antagonists

Figure 1 shows the antagonism of the peptides examined on the responses to NKA in the RPA and HT. All peptides produced a concentration-dependent rightward shift of the curve to the agonist; a reduction of the maximal effect of NKA was noted in some experiments on both tissues but this effect was not concentration-related (Figure 1). For the three peptides the slope of the Schild plots (Figure 2) was not significantly different from unity, indicating competitive antagonism. The corresponding pA_2 values indicated that Peptides I and II were more potent than Peptide III in the RPA, while Peptide III was more potent than Peptide I or II in the HT (Table 3).

Because the slopes of the Schild plots were close to unity (Table 3) pA_2 values were also calculated by the constrained plot method as described by Tallarida *et al.* (1979). The analysis indicated that Peptides I and II were about 100 times more potent in the RPA (pA_2 7.89 and 7.64 for Peptide I and II, respectively) than in the HT (pA_2 5.95 and 5.76 for Peptide I and II, respectively) while Peptide III was about 100 times more potent in the HT (pA_2 7.63) than in the RPA (pA_2 5.42).

Since the activity of peptide ligands may be influenced by local metabolism, the activity of test compounds at NK_2 receptors in the RPA and HT was evaluated against NKA in the presence of a mixture of peptidase inhibitors (thiorphan 1 μM, captopril 1 μM, bestatin 1 μM, 15 min before). pA_2 values for each compound (calculated by the constrained plot method) did not differ significantly from those obtained in the absence of enzyme inhibitors. In fact, pA_2 values were 7.60,

Table 2 Contractile response of the endothelium-denuded rabbit pulmonary artery (RPA) and hamster trachea (HT) to natural tachykinins and synthetic receptor-selective agonists

	RPA			*HT*		
Peptides	EC_{50} (nM) (95%c.l.)	pD_2	*RA*	EC_{50} (nM) (95%c.l.)	pD_2	*RA*
NKA	4.9 (4.3–5.5)	8.31	100	24 (17–36)	7.62	100
NKB	35 (12–61)	7.45	14	33 (17–79)	7.48	73
SP	741 (660–890)	6.13	0.7	6700 (3500–9500)	5.17	0.4
NPγ	2.7 (2.0–3.7)	8.57	181	15 (10–23)	7.81	160
NPK	7.6 (5.5–10)	8.12	64	22 (9–29)	7.66	109
NKA (4–10)	3.0 (2.2–3.8)	8.52	163	18 (14–23)	7.74	133
[Pro^9]-SP sulphone	inactive*	—	—	inactive*	—	—
[MePhe^7]-NKB	5750 (2750–8400)	5.24	0.09	1760 (1000–3400)	5.75	1.4
[βAla^8]-NKA (4–10)	4.6 (3.9–5.3)	8.34	106	19 (9–29)	7.72	126

Each value is the mean of 4–6 experiments. * Inactive up to 10 μM. RA = relative affinity, expressed as a fraction of the affinity of NKA(= 100). EC_{50}, concentration of peptide producing 50% of maximal response. pD_2, $-\log EC_{50}$.

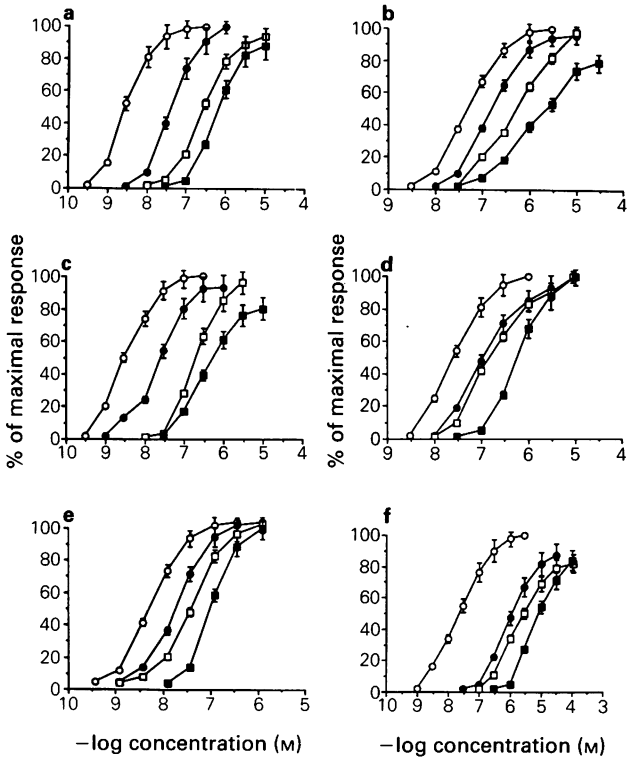

Figure 1 Concentration-response curves showing the rightward displacements of the response to neurokinin A in the endothelium-denuded rabbit pulmonary artery (RPA, a,c and e) or hamster trachea (HT, b,d and f) in presence of increasing concentrations of the antagonists, Peptide I (a,b), Peptide II (c,d) or Peptide III (e,f). In (a): (○) control; ● 0.3 μM; (□) 1 μM; ■ 3 μM Peptide I. In (b): (○) control; (●) 3 μM; (□) 10 μM; (■) 30 μM Peptide I. In (c): (○) control; (●) 0.3 μM; (□) 1 μM; (■) 3 μM Peptide II. In (d): (○) control; (●) 3 μM; (□) 10 μM; (■) 30 μM Peptide II. In (e): (○) control; (●) 10 μM; (□) 30 μM; (■) 50 μM Peptide III. In (f): (○) control; (●) 1 μM; (□) 3 μM; (■) 10 μM Peptide III. Each value is the mean of 4–5 determinations and vertical lines show s.e.mean.

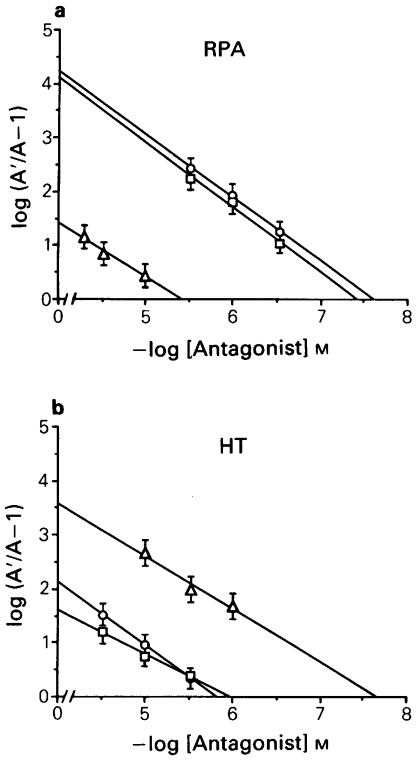

Figure 2 Schild plots relative to the antagonism of the contractile responses to neurokinin A in the endothelium-denuded rabbit pulmonary artery (RPA, a) or hamster trachea (HT, b) by the antagonists, Peptide I (○), II (□) and III (△). Each value is the mean of 4–5 determinations and vertical lines show s.e.mean.

7.48 and 5.47 in the RPA and 5.75, 5.64 and 7.53 in the HT for Peptide I, II and III, respectively. Also the presence of enzyme inhibitors did not modify the activity of NKA in either one of the organ preparations (EC_{50} 4.9 vs 5.4 nM in the RPA and 24 vs. 21 nM in the HT, in the absence and presence of peptidase inhibitors, respectively).

The affinity of test compounds for NK_2 receptors appeared to be agonist-independent, as shown by the closeness of pA_2 values obtained in both preparations with the selective NK_2 agonist, [βAla^8]-NKA(4–10) (Rovero *et al.*, 1989b,c) to those of the natural tachykinin, NKA. In fact, pA_2 values against the selective NK_2 receptor agonist were 7.74, 8.03 and 5.41 in the RPA and 6.15, 5.80 and 7.46 in the HT for Peptide I, II and III respectively.

The problem of equilibration time of antagonists with the receptors located in different tissues, which relates to possible differences in penetration rates, was examined by extending the contact time to 90 min. These experiments ($n = 4$ for each peptide) showed that the pA_2 values obtained after 15 and 90 min contact time were not significantly different from each other. In fact Peptide I and II had a pA_2 of 5.95 and 5.76 in the HT after 15 min contact time and a pA_2 of 5.80 and 5.84 after 90 min contact time, respectively. In the RPA, Peptide III had a pA_2 of 5.42 and 5.60 after 15 and 90 min contact time, respectively.

Possible non-specific smooth muscle relaxant activity of test peptides was assessed by measuring their effect on the response to noradrenaline (RPA) or carbachol (HT). Peptide I (10 μM in the RPA, 30 μM in the HT, 15 min before), Peptide II and Peptide III did not affect the response of RPA and HT to these stimulants ($n = 4$ for each antagonist, data not shown).

Activity of antagonists at NK_1 and NK_3 receptors

Peptides I and II were weak antagonists at both NK_1 (DCA) and NK_3 (RPV) receptors. The corresponding pA_2 values were as follows: Peptide I, 5.2 ± 0.6 (DCA) and 4.9 ± 0.5 (RPV); Peptide II 5.0 ± 0.3 (DCA) and 4.9 ± 0.4 (RPV). Peptide III was inactive as an antagonist in the DCA and

Table 3 pA_2 and slope values calculated from Schild plots (see Figures 1 and 2), for Peptide I, II and III in the rabbit pulmonary artery (RPA) or hamster trachea (HT) against neurokinin A

	RPA		*HT*	
	pA_2 (95% c.l.)	*Slope* (95% c.l.)	*pA_2* (95% c.l.)	*Slope* (95% c.l.)
Peptide I	7.65 (7.41–7.65)	−1.13 (−1.23– −0.94)	5.80 (5.70–5.90)	−1.18 (−1.28– −0.99)
Peptide II	7.47 (7.18–7.87)	−1.14 (−1.30– −0.91)	5.93 (5.65–6.23)	−0.85 (−0.77– −1.01)
Peptide III	5.42 (5.21–5.65)	−0.99 (−0.88– −1.15)	7.67 (7.37–7.99)	−0.98 (−0.87– −1.15)

Each pA_2 value was obtained from 9–12 experiments by means of the Schild plot, as described by Arunlakshana & Schild (1959).

RPV up to 10 μM and behaved as a weak agonist in the DCA with a pD_2 of 5.3 (each value is mean ± s.e. of 6–8 determinations).

Discussion

The three established tachykinin receptors have been identified on the basis of differential affinity displayed by natural or synthetic agonists in various preparations. Development of 'monoreceptorial' preparations, such as the RPA or the HT for NK_2 receptors has been instrumental for the recognition of NK_1, NK_2 and NK_3 receptors as distinct entities mediating the biological action of this family of peptides. By contrast, a large number of the tachykinin antagonists of the first generation have a limited selectivity for the different types of tachykinin receptor and particularly, a poor ability to discriminate between NK_1 and NK_2 receptors (Buck & Shatzer, 1988). More recently, a new generation of antagonists has been developed which displays improved selectivity for NK_1 and NK_2 receptors (McKnight *et al.*, 1988; Williams *et al.*, 1988; Rovero *et al.*, 1990). Peptides I, II and III belong to this new generation of tachykinin antagonists, as they are remarkably selective for NK_2 as compared to NK_1 or NK_3 receptors.

In both the RPA and HT the contractile response to the tachykinins appears to be directly associated with the activation of NK_2 receptors, without any evidence for an indirect component due to the release of other mediators. In fact the responses to NKA were unaffected by blockade of either cyclo-oxygenase or lipoxygenase pathways, removal of the airway epithelium (HT) or antagonists for a variety of peptides and transmitters (D'Orleans-Juste *et al.*, 1986; Maggi *et al.*, 1989a and unpublished data).

Studies with tachykinin agonists indicate that the RPA and HT contain a homogeneous population of NK_2 receptors (D'Orleans-Juste *et al.*, 1986; Dion *et al.*, 1987; Maggi *et al.*, 1989a and present findings). Contrasting with this conclusion are the results obtained in this study with antagonists, which provide pharmacological evidence for heterogeneity of NK_2 receptors.

Peptides I and II emerged from a search for selective NK_2 receptor antagonists based on a screening programme with the RPA as bioassay. Insertion of D-Trp in position 6, 8 and 9 on the backbone of the heptapeptide NKA(4–10) yielded competitive antagonists for the NK_2 receptor and further substitutions with Tyr in position 5 and Arg in position 10 increased both the affinity and selectivity of these antagonists (Rovero *et al.*, 1989a; 1990). Peptide II differs from Peptide I in that the former conserves Thr in position 3, as present in the sequence of NKA. However, the pattern of activity at the tachykinin receptors was not appreciably modified by this change.

Peptide III was synthesized (Regoli *et al.*, unpublished data) on the backbone of the linear antagonist Ac.Leu-Met-Gln-Trp-Phe-Gly NH_2 described by McKnight *et al.* (1988) and Williams *et al.* (1988), by replacing the Met with Asp. The role of Asp in position 4 of neurokinin A has been emphasized in a recent study (Rovero *et al.*, 1989c).

Peptides I, II and III were found to be weak antagonists on the DCA and RPV, two well established tissue bioassays for NK_1 and NK_3 receptors, respectively (Regoli *et al.*, 1987; 1988; 1989). This finding highlights the selective interaction of these peptide antagonists with NK_2 receptors.

Although the three peptides used in this study acted as competitive antagonists in both the RPA and HT, their affinity for NK_2 receptors exhibits a dramatic organ-specific difference in potency. In fact Peptides I and II were significantly more potent in the RPA than in the HT, while Peptide III was significantly more potent in the HT than in the RPA.

A number of factors might have contributed to the different potency of the peptide antagonists observed in the two preparations used in this study. However, control experiments indicate that neither non-specific smooth muscle relaxant activity, peptide degradation by tissue peptidases nor a different penetration rate of the peptides in the two tissues may account for the large differences in affinity measured in this study with these three NK_2 receptor antagonists. In addition, we found similar pA_2 values for the various antagonists with NKA or [βAla^8]-NKA(4–10) as an agonist. This latter peptide is a highly selective NK_2 receptor agonist (Rovero *et al.*, 1989a,c) which appears to be somewhat more resistant to metabolic breakdown than the parent peptide (Patacchini *et al.*, 1989). Therefore, the relative potencies of Peptide I and II on the one hand and of Peptide III on the other in the two NK_2 receptor bioassays were agonist-independent. This finding reinforces the concept that the tachykinin receptors found in these tissues represent NK_2 receptor subtypes rather than a new type of tachykinin receptor.

We interpret our data as pharmacological evidence for the existence of NK_2 receptor subtypes which are not discriminated by the tachykinin agonists currently available, but have sufficient structural differences to be recognized with very different affinity by certain antagonists. Studies are in progress to compare the activity of these and other NK_2 receptor antagonists in various other 'monoreceptorial' assays for NK receptor ligands. Although further experiments and particularly binding data are needed for a definitive conclusion on this point, Peptides I, II and III appear to be useful tools for studying distribution and functional significance of NK_2 receptors and NK_2 receptor subtypes in mammalian tissues.

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Increase in calcium channel current by β-adrenoceptor agonists in single smooth muscle cells isolated from porcine coronary artery

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1 The action of catecholamines (isoprenaline and noradrenaline) and forskolin on membrane currents was studied in single cells freshly dispersed from the pig coronary artery by use of the whole-cell clamp method, usually with electrodes containing CsCl.

2 In normal Krebs solution, with and without 30 mM tetraethylammonium (TEA) and 0.5 mM 4-aminopyridine, isoprenaline (1–5 μM) clearly increased the inward currents elicited by membrane depolarization, without affecting the holding current at −80 mV. The same effect was observed when the external Cl^- was replaced with isethionate. The outward current recorded with K^+-containing electrodes was not significantly affected by isoprenaline.

3 In the presence of 67 mM Ba^{2+} and 30 mM TEA, the maximum inward current recorded with CsCl containing electrodes was 119 ± 7 pA (the mean ± s.e.mean, $n = 90$) in cells where the current was larger than 30 pA. The L-type Ca^{2+} channel was considered to be responsible for these currents, based on the threshold voltage, the slow time course of decay, the large depolarization necessary to produce inactivation, and the high susceptibility to the Ca^{2+} channel antagonist, nicardipine.

4 Isoprenaline and noradrenaline increased the amplitude of inward currents evoked by depolarizing pulses. The maximum inward current was potentiated by 43 ± 7% ($n = 12$) by isoprenaline and 39 ± 10% by noradrenaline ($n = 6$) at a concentration of 1 μM. These effects were strongly inhibited by propranolol, but not phentolamine. Forskolin (10 μM) also potentiated the currents to a similar degree.

5 It is suggested that stimulation of β-adrenoceptors increases the amplitude of inward currents through L-type Ca^{2+} channels in the pig coronary artery and that intracellular cyclic adenosine monophosphate is likely to be inolved in this action.

Introduction

In the pig coronary artery, isoprenaline has been considered to stimulate calcium (Ca^{2+}) uptake into intracellular stores, by increasing adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels (Itoh *et al.*, 1985). This may be partly responsible for the mechanical inhibition mediated through β-adrenoceptors, in addition to the direct action of cyclic AMP on the contractile machinery (Adelstein *et al.*, 1978; Adelstein & Eisenberg, 1980). In the rabbit coronary artery, isoprenaline inhibited the contraction caused by intracellular Ca^{2+} release (the contraction induced by histamine in Ca^{2+}-free medium) (van Eldere *et al.*, 1982). However, when isoprenaline was applied during refilling of the intracellular Ca^{2+} store in excess potassium solution, a subsequent contraction by histamine was potentiated. On the other hand, they also observed that addition of isoprenaline during refilling of the Ca^{2+} store in physiological solution inhibited the subsequent histamine contraction. These results have been interpreted as suggesting that isoprenaline not only potentiates Ca^{2+} uptake into intracellular store, but also inhibits the Ca^{2+}-influx across the plasma membrane.

There are several studies concerning the action of catecholamines on membrane currents in dispersed single cells from blood vessels (guinea-pig pulmonary artery: Byrne & Large, 1987; rabbit portal vein: Byrne & Large, 1988; rat portal vein; Pecaud *et al.*, 1987; rabbit ear artery; Benham & Tsien, 1988; Droogmans *et al.*, 1987). However, no consistent results have been obtained. Many cells are not responsive to catecholamines and even in the same vascular muscle (rabbit ear artery), potentiation (Benham & Tsien, 1988) and inhibition (Droogmans *et al.*, 1987) of inward currents have been reported.

We have studied the effects of catecholamines, mainly isoprenaline, on the membrane currents in single cells freshly dispersed from the pig coronary artery using the whole-cell clamp technique (Hamill *et al.*, 1981). Contrary to expectation, stimulation of β-adrenoceptors clearly increased the inward current flowing through Ca^{2+} channels.

Methods

Pig hearts were collected at a nearby slaughter house and brought immediately to the laboratory; they were kept in cold (about 5°C) physiological solution. A segment of epicardial right coronary artery (3–5 cm from its origin, diameter 1.5–2.5 mm) was carefully dissected. The endothelium was rubbed off with a cotton swab. The muscle was cut into small strips (about 0.5 mm wide) and incubated in physiological solution which contained no Ca^{2+} at 35°C for 30 min. The strips were transferred into the same solution to which was added (mg ml^{-1}) collagenase 2 (Wako), trypsin inhibitor 2 (Sigma), papain 1 (Sigma), bovine albumin 10 (Sigma). After 40 min incubation, the solution was agitated gently for 3 min, filtered through a nylon mesh (0.5 mm), and centrifuged at 1000 r.p.m. for 1 min. The pellet of dispersed muscle cells was resuspended in the Ca^{2+}-free solution. After adhesion of the cells to the bottom of the chamber (0.3 ml) on the stage of a phase-contrast microscope (Diaphot-TMD, Nikon), physiological solution was superfused at a rate of 4 ml min^{-1} at room temperature (20–23°C).

Physiological solution had the following composition (mM): NaCl 127, KCl 6, $CaCl_2$ 2.4, $MgCl_2$ 1.2, glucose 12, HEPES-buffer (N-2-hydroxyethyl-piperazine-2-ethanesulphonic acid) 10; the pH was adjusted to 7.4 with NaOH. Concentrations of

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divalent cations were altered by substituting equiosmolar NaCl. Patch electrodes were usually filled with a solution containing (mM): CsCl 132, $MgCl_2$ 1.6, ATP 5, EGTA (ethyleneglycol-*bis*(β-aminoethyl ether)N,N,N', N'-tetraacetic acid) 5, HEPES 10; the pH was adjusted to 7.0 with NaOH. For the study of the effect of isoprenaline on outward currents, CsCl was replaced with 100 mM K aspartate and 32 mM KCl. Effects of isoprenaline on inward currents were studied in physiological solution containing 2.4 mM Ca^{2+} and also in the presence of 67 mM Ba^{2+} (Ca^{2+}-free). To reduce depolarization-evoked outward currents, NaCl in the external medium was partly replaced with tetraethylammonium (TEA) chloride (30 mM). Cells which showed a maximum inward current of less than 5 pA in the presence of Ca^{2+} and 30 pA in the presence of Ba^{2+} were excluded from the analysis.

Membrane currents were measured using the whole-cell clamp technique (Hamill *et al.*, 1981), with a patch clamp amplifier (L/M EPC-7, List). Data were recorded on a videotape recorder (GX4, National) at a sampling rate of 28.8 kHz with an analogue-digital converter (RP-880, PCM Data Recording System, NF Electronic Instrument) and a low pass filter (3.3 kHz). Analyses were performed on personal computers (7T17 Signal Processor, San-ei and PC 9801, NEC).

The drugs used were: (±)-isoprenaline HCl (Sigma), (±)-noradrenaline HCl (Sigma), phentolamine-methanesulphonate (Ciba-Geigy), (−)-propranolol HCl (Sigma), nicardipine HCl (Yamanouchi), and forskolin (Nippon Kayaku). Forskolin was prepared as 5 mM stock solution in ethanol.

Results

When the membrane was depolarized to more than −40 mV from a holding potential of −80 mV under the whole-cell clamp condition, transient inward currents could be recorded in normal solution containing 2.4 mM Ca^{2+} (Figure 1a). The maximum inward current varied greatly between cells but when currents of less than 5 pA were arbitrarily excluded, the average value was 31 ± 4 pA (mean ± s.e.mean, $n = 13$). There was no discernible contraction associated with the inward current, probably due to a buffering effect of EGTA (5 mM) contained in the electrodes. With repeated depolarizing pulses (400 ms) the amplitude of inward currents tended to decrease slowly with time (run-down phenomenon), particularly when large depolarizing pulses were applied. Although the rate of run-down differed in different cells, more or less constant

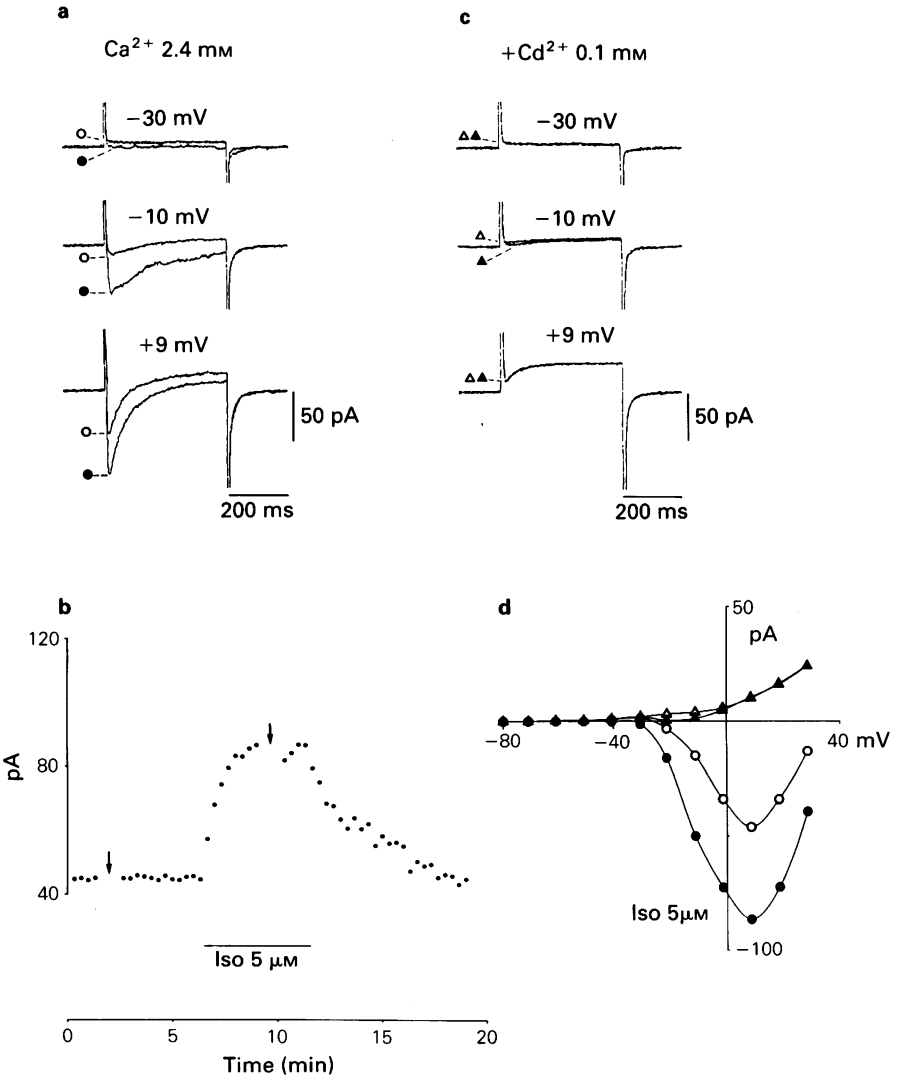

Figure 1 Effects of isoprenaline (5 μM) on membrane currents from a single cell dispersed from pig coronary artery. The currents were recorded with the whole-cell clamp method in normal solution containing 2.4 mM Ca^{2+}, and 400 ms depolarizing pulses were applied from a holding potential of −80 mV at 20 s intervals. (a) Current traces obtained at three different depolarizations, before (○) and after 5 μM isoprenaline application (●). (b) Effect of isoprenaline on the peak inward currents obtained at +9 mV. Isoprenaline (Iso) application is indicated by a horizontal bar. At arrows, membrane potential-current relationships were obtained and peak inward currents are plotted in (d). (c) Current tracings similar to (a), but in the presence of 0.1 mM Cd^{2+}: (△) and (▲) show current records before and after isoprenaline, respectively. Their minimum outward currents are also plotted against membrane potential in (d).

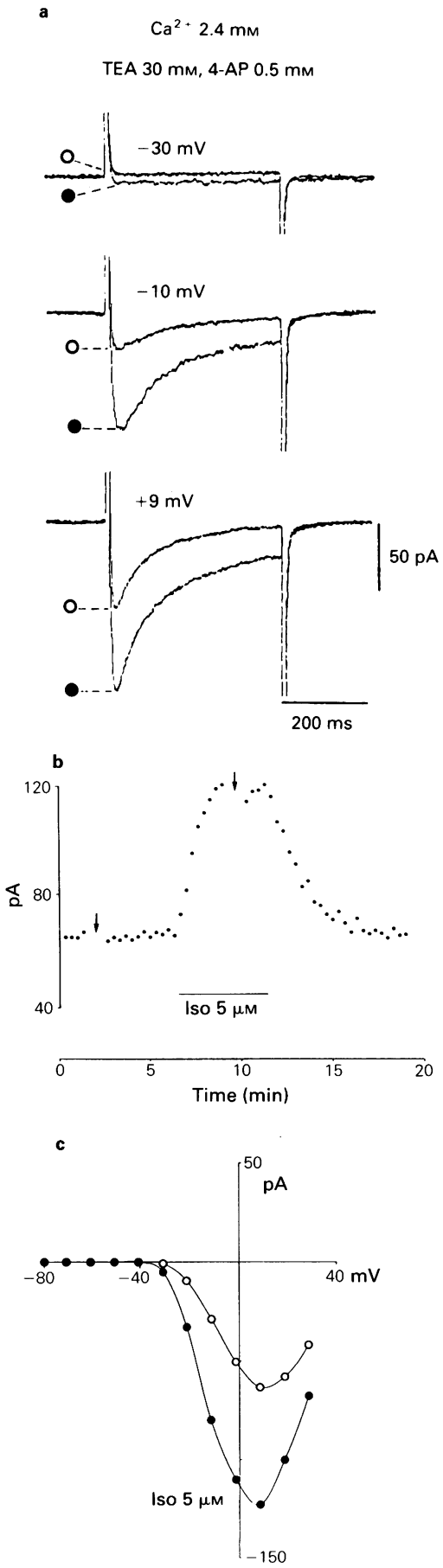

Figure 2 Effects of isoprenaline (5 μM) in the presence of 30 mM tetraethylammonium (TEA) and 0.5 mM 4-aminopyridine (4-AP) applied to suppress the outward currents. Experiments were similar to Figure 1.

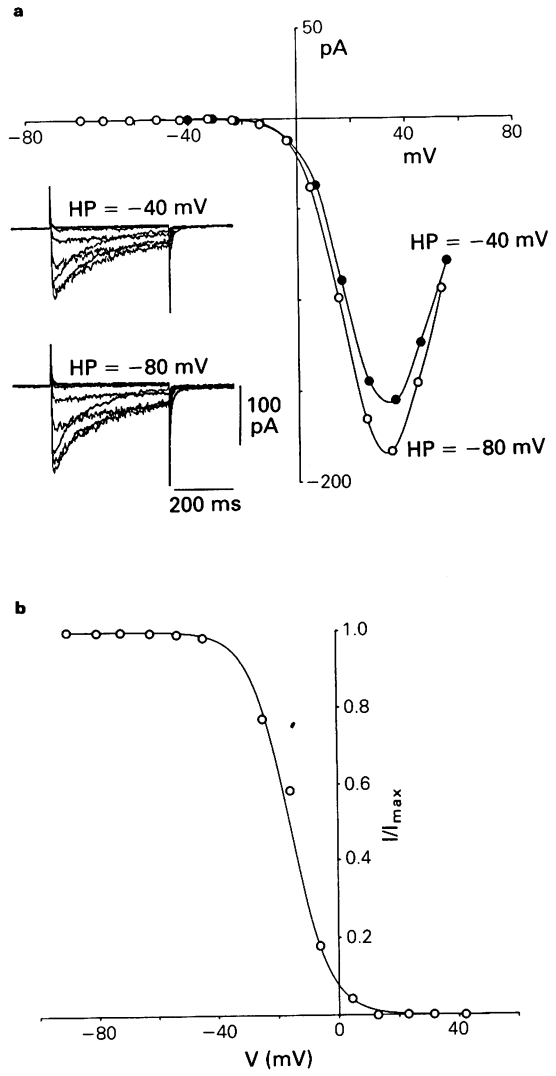

Figure 3 (a) Comparison of voltage-current relationship of peak inward currents evoked by depolarizing pulses at holding potentials of −80 (○) and −40 mV (●) in the same cell in 67 mM Ba^{2+} solution. Recordings shown in the insets were obtained first at holding potential of −40 mV, then at −80 mV. (b) Inactivation curve of peak inward currents obtained with prepulses (V) of 1.6 s and test pulses to +28 mV (the average of three experiments). The curve (relative ratios of observed inward current, I, to the maximum, I_{max}) was drawn from the equation: $I/I_{max} = 1/(1 + \exp[(V - V_h)/k])$, where V_h is the membrane potential that produced half inactivation (−16 mV) and k is the slope factor (6.5 mV).

inward currents could be recorded for a period of 30–90 min in some cells when the pulse interval was longer than 20 s.

Isoprenaline (1–5 μM) clearly increased the inward currents elicited by membrane depolarization, without affecting the holding current at −80 mV. In Figure 1b, the time course of the effects of 5 μM isoprenaline is shown. The peak inward current produced by depolarizing pulses from −80 to +9 mV applied at 20 s intervals was plotted. The effect developed slowly, reaching a steady state in about 3 min and the recovery after washout took about 5 min. The voltage-current (V-I) relationship, obtained after full development of the isoprenaline effect, indicated that the threshold voltage for inward current and the membrane potential at which maximum inward current was recorded were not altered by isoprenaline (Figure 1d).

When 0.1 mM cadmium (Cd^{2+}) was added to the bathing solution, the inward current was suppressed and the effect of isoprenaline was almost completely abolished (Figure 1 c,d).

In the experiment shown in Figure 2, the outward current was inhibited by addition of 30 mM TEA and 0.5 mM 4-

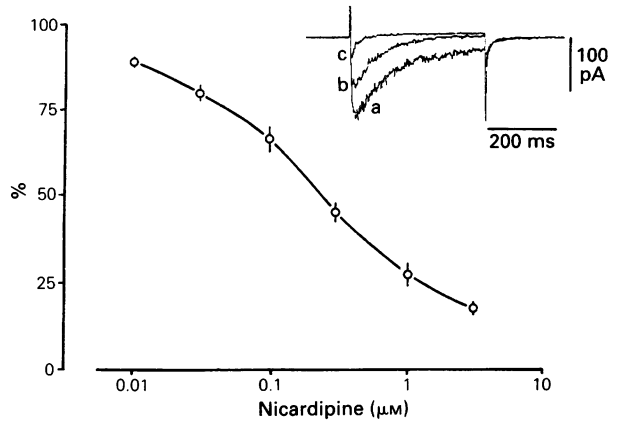

Figure 4 Inhibition of maximum inward currents by nicardipine. Inward currents were produced by depolarization to +28 mV from a holding potential of −80 mV, and the maximum inward current before nicardipine application was taken as 100%. Vertical lines indicate s.e.mean (n = 4–6). Current records in the inset show control (a), in the presence of 0.1 μM (b) and 1 μM nicardipine (c). At each concentration, nicardipine was applied for 3 min.

aminopyridine (4–AP) to the external solution. The potentiating effect of isoprenaline on the inward current was basically the same as the result shown in Figure 1. Isoprenaline (5 μM) increased the maximum inward current from 35 ± 7 pA to 53 ± 5 pA (n = 7).

To investigate the effects of isoprenaline on the outward K^+ current, the whole-cell clamp experiments were carried out in normal external solution and pipettes were filled with 100 mM K aspartate and 32 mM KCl instead of 132 mM CsCl. The average outward current of 339 ± 39 pA (n = 19) was recorded when the membrane was depolarized from a holding potential of −80 to +9 mV. Isoprenaline (5 μM) had negligible effects in 11 out of 19 cells, while it increased slightly (16 ± 2%) in the remaining cells. The V-I curve in a hyperpolarizing direction was not affected by isoprenaline. Substitution of NaCl with equimolar sodium isethionate in the external solution did not modify the membrane current and also the effect of isoprenaline in three cells studied (not shown).

Since the inward current was relatively small in the presence of Ca^{2+}, but increased by replacing with Ba^{2+}, the effect of catecholamines was further studied on inward Ba^{2+} cur-

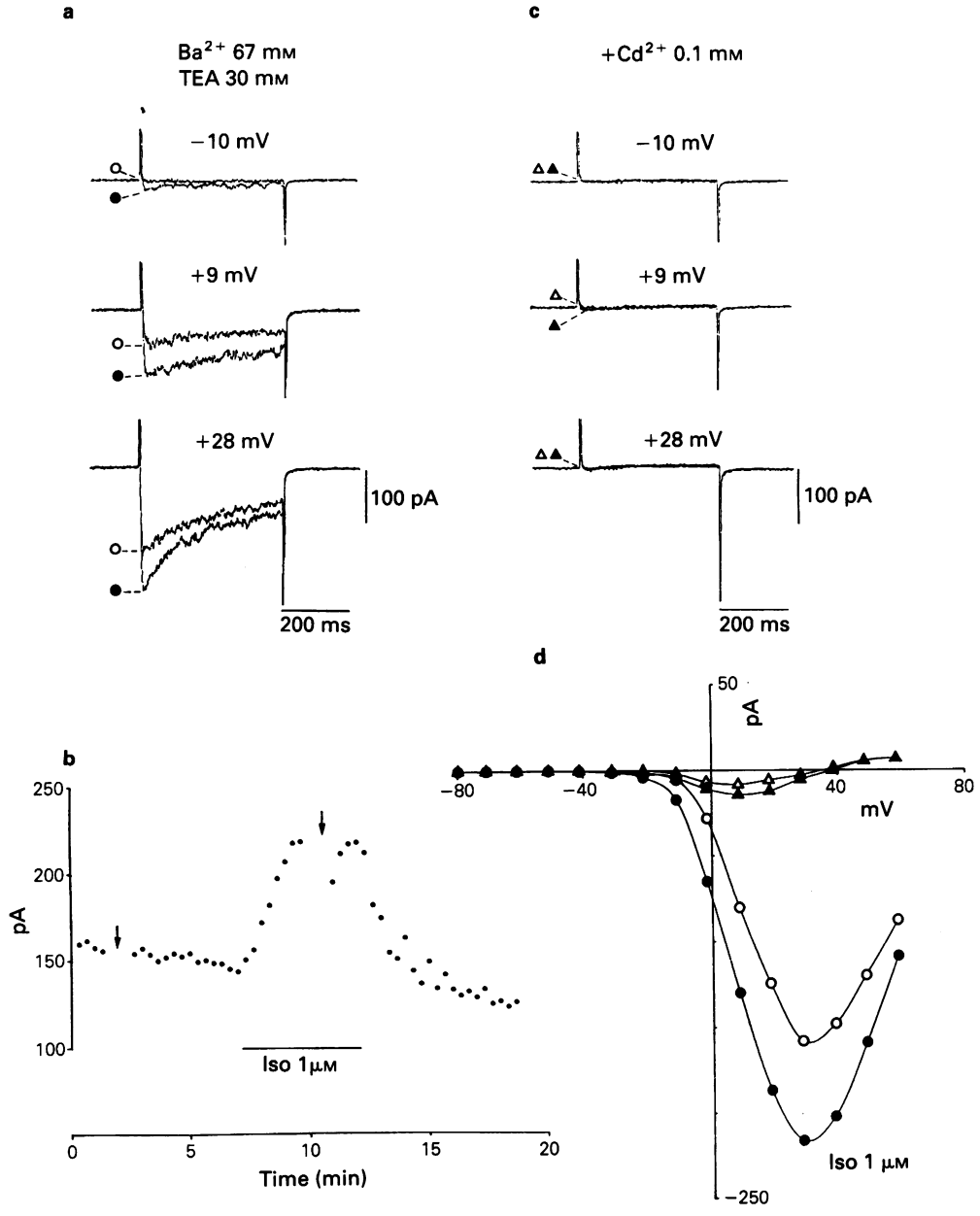

Figure 5 Effects of isoprenaline (1 μM) on inward currents in the presence of 67 mM Ba^{2+} and 30 mM tetraethylammonium (TEA). Experiments were similar to Figure 1. Current tracings (c) and curves (d) with triangles are in the presence of 0.1 mM Cd^{2+}.

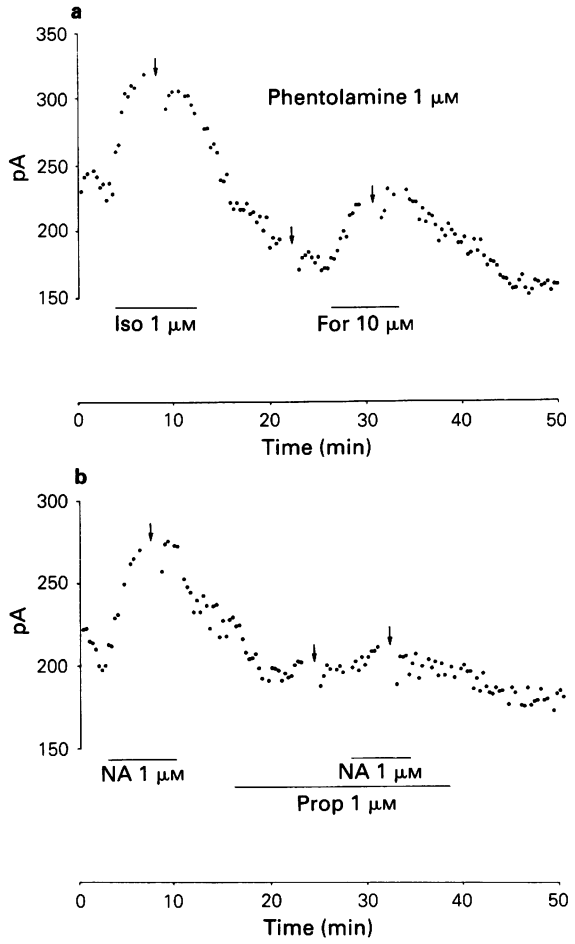

Figure 6 Time course of the effects of isoprenaline (Iso, 1 μM), forskolin (For, 10 μM) (a), and noradrenaline (NA, 1 μM) (b) on maximum inward currents obtained by 400 ms depolarizing pulses to +28 mV from a holding potential of −80 mV every 20 s. At arrows, voltage-current relationships were obtained. In (a) isoprenaline and forskolin were applied in the presence of phentolamine (1 μM), and in (b) the second application of noradrenaline was in the presence of propranolol (Prop, 1 μM).

rents in the following experiments. In the presence of 67 mM Ba^{2+} and 30 mM TEA, the time course of inward current became much slower, and the threshold membrane potential for inward current and the membrane potential at which inward current reached the maximum were shifted by about 20 mV in a depolarizing direction (compare Figures 2c and 3a). The mean value of the maximum inward current in cells where the current exceeded 30 pA was 119 ± 7 pA ($n = 90$) with a holding potential of −80 mV. The V-I relationship and the time course of inward currents were not significantly altered by decreasing the holding potential from −80 to −40 mV.

The steady state inactivation curve obtained with 1.6 s conditioning pulses revealed that inactivation of the inward current started when the membrane was depolarized to less than −40 mV (Figure 3b). The curve could be fitted by an equation, $1/(1 + \exp[(V - V_h)/k])$, where V is the membrane potential, V_h the membrane potential to produce half inactivation and k a slope factor. The average value of V_h and k values were −16 mV and 6.5 mV ($n = 3$), respectively.

Nicardipine, a dihydropyridine Ca^{2+} channel blocker reduced inward currents concentration-dependently between 0.01–3 μM (Figure 4). The reduction of the current was accompanied by a faster decay and decreased noise, as shown in the inset to Figure 4. When a concentration of nicardipine greater than 1 μM was applied, only a very small outward current remained following a transient inward current, suggesting that K^+ current was effectively blocked in the presence of 67 mM Ba^{2+} and 30 mM TEA in the bathing solution and 132 mM CsCl in the pipette solution.

Figure 5 shows effects of isoprenaline (1 μM) on inward currents in the presence of 67 mM Ba^{2+}. Isoprenaline clearly increased inward currents, but both the threshold potential and the membrane potential giving the largest inward current were not significantly affected, as observed in the presence of 2.4 mM Ca^{2+}. External application of 0.1 mM Cd^{2+} almost completely blocked the inward current as well as the effect of isoprenaline (Figure 5c, d).

Figure 6 shows the time course of effects of isoprenaline (1 μM), forskolin, a direct activator of adenylate cyclase (10 μM), and noradrenaline (1 μM) on peak inward currents. Forskolin also increased inward currents, but the effect developed more slowly than with isoprenaline. The steady state was reached in 2–3 min for isoprenaline and 5 min for forskolin. The recovery after washout was also slower for forskolin than for isoprenaline. Ethanol, used as a solvent for forskolin, produced no clear effects. The effect of noradrenaline was very similar to that of isoprenaline.

Comparison of the average of three consecutive inward currents before the first drug application and during its maximum effect gave an average increase of 43 ± 7% ($n = 12$) for isoprenaline (1 μM), 39 ± 10% ($n = 6$) for noradrenaline (1 μM), and 43 ± 10% ($n = 5$) for forskolin (10 μM). There was no clear correlation between the size of the inward current and the degree of potentiation. During the potentiation by catecholamines and forskolin, the rate of early decay of the inward current elicited by depolarization to +28 mV was shortened by about 20%, but that of the late phase of decay remained the same. When isoprenaline application was repeated on the same cell at a 10 min interval, the degree of potentiation became smaller in most cells (on average, the second response being 54 ± 10% of the first response, $n = 5$).

Effects of receptor blocking agents were difficult to study because of the decrease in the response to agonists on repeated application. However, the isoprenaline effect did not seem to be affected by an α-adrenoceptor blocking agent, phentolamine. As shown in Figure 6a, a large of potentiation of inward current with the first application of 1 μM isoprenaline was observed in the presence of phentolamine (1 μM), an average increase being 38 ± 5% ($n = 7$) which is not statistically different from the control value (43 ± 7%, $n = 12$). On the other hand, in the presence of a β-adrenoceptor blocking agent, noradrenaline (1 μM, Figure 6b) and isoprenaline (1 μM, not shown) produced only a marginal or negligible response. The increase in inward current on the first application of isoprenaline and noradrenaline following 10 min pretreatment with 1 μM propranolol was 3 ± 2% ($n = 5$) and 11 ± 4% ($n = 4$), respectively. These results suggest that the increase in inward current by isoprenaline is mediated through β-adrenoceptors.

Discussion

In many vascular smooth muscles, two different types of Ca^{2+} channel are generally found (rat mesenteric artery: Bean *et al.*, 1986; rabbit ear artery: Benham *et al.*, 1987; rat portal vein: Loirand *et al.*, 1986; dog saphenous vein: Yatani *et al.*, 1987). One type is activated by a small depolarization and inactivates relatively quickly at more negative membrane potentials, whereas the other type is activated by a large depolarization and inactivates relatively slowly. The former corresponds to the 'T'-type and the latter to the 'L'-type of Ca^{2+} channel in other tissues (McCleskey *et al.*, 1986). Steady state inactivation studied by changing the holding potentials indicates that the T-type channel is inactivated by 50% between −55 and −40 mV, whereas with the L-type 50% inactivation occurs between −25 and −5 mV (Bean *et al.*, 1986; Yatani *et al.*, 1986; Benham *et al.*, 1987). In the pig coronary artery, however, we failed to demonstrate clearly two different types of inward current in most cells. When the

holding potential was changed from −80 to −40 mV, inward currents were not significantly affected. Since half inactivation was produced at −16 mV, the L-type channel is considered to be dominant in this smooth muscle. Susceptibility of the channel to nicardipine also supports this idea, because the L-type channel is known to be more susceptible to Ca^{2+} channel blockers, such as nitrendipine or nifedipine than is the T-type (Bean *et al.*, 1986; Loirand *et al.*, 1986; Yatani *et al.*, 1987). Since small inward currents with a fast decaying phase were observed near threshold or in the presence of nicardipine in some cells, a small proportion of T-type channel may also exist in the pig coronary artery.

It is well known that catecholamines produce contraction of vascular smooth muscles mediated through α-adrenoceptors. However, electrophysiological results on Ca^{2+} currents in single muscle cells are still confusing. Noradrenaline (1–10 μM) produced no effect on voltage-activated inward currents in dog saphenous vein (Yatani *et al.*, 1987). In some cells of the rabbit ear artery, noradrenaline (1–20 μM) increased the L-type Ca^{2+} current, but this is not mediated by α- or β-adrenoceptors (Benham & Tsien, 1988). Isoprenaline (1–5 μM) was ineffective. In the same artery, inhibition of inward currents by noradrenaline (10 μM) has also been reported (Droogmans *et al.*, 1987). Isoprenaline (10 μM) produced a similar inhibitory effect or no effect. In the rat portal vein (cultured cells), noradrenaline (1 μM) increased inward currents of the T-type channel, but inhibited that of the L-type channel (Pacaud *et al.*, 1987). A major problem in the interpretation of the results is our lack of knowledge of the effects of the enzymatic treatment during cell dispersion and cell perfusion with the pipette solutions on the electrical properties of the membrane.

In the present experiments on the pig coronary artery, both isoprenaline and noradrenaline increased the voltage-dependent inward current. This is considered to be due to an increase of inward Ca^{2+} current through the L-type Ca^{2+} channel, not due to a decrease in overlapping K^+ current, because isoprenaline had a negligible effect on the outward current. Furthermore, under our experimental conditions, the K^+ current has been effectively blocked. The catecholamine action is not blocked by phentolamine, but inhibited by propranolol. Therefore, this is mediated through β-adrenoceptors. Since identical effects can be produced by forskolin which directly activates adenylate cyclase, it is likely that intracellular cyclic AMP is involved in the increase of inward currents.

In cardiac muscle cells, activation of β-adrenoceptors increases inward currents in the L-type Ca^{2+} channel, and this is mediated through phosphorylation of the channel by protein kinase A (Kameyama *et al.*, 1986; Trautwein *et al.*, 1987). Thus, it is possible that the β-receptor-mediated action on the Ca^{2+} channel (L-type) in coronary artery is fundamentally similar to that in cardiac muscle. However, it is interesting that β-receptor stimulation potentiates contraction in cardiac muscle, whereas it produces relaxation of the smooth muscle of the coronary artery.

Isoprenaline may increase inward current by an indirect mechanism. It has been shown in the rabbit portal vein that inward Ca^{2+} (or Ba^{2+}) currents are inhibited by elevated intracellular Ca^{2+} (Ohya *et al.*, 1988). Therefore, if isoprenaline reduces the intracellular Ca^{2+} concentration by activating a Ca^{2+}-pump (Van Eldere *et al.*, 1982; Itoh *et al.*, 1985), this may result in an increase in the amplitude of inward currents. However, this explanation is not supported by the observation that in potassium-depolarized strips of ferret portal vein, either no change or increase in intracellular Ca^{2+} concentration was detected by a Ca^{2+} indicator (aequorin) during relaxation caused by isoprenaline (5 μM) or forskolin (10 μM) (Morgan & Morgan, 1984). In bovine tracheal muscle, it has also been shown with the same method that isoprenaline increases the intracellular Ca^{2+} concentrations by activation of β-receptors (Takuwa *et al.*, 1988). Since intracellular Ca^{2+} was effectively buffered with EGTA, as evident from a lack of contraction associated with inward currents, a change in Ca^{2+} concentration with isoprenaline would be very small. Thus, a direct action of isoprenaline on the Ca^{2+} channel is more likely.

The physiological significance of an increase in inward currents by β-receptor activation is difficult to assess. The main β-receptor-mediated action which produces relaxation is probably due to an effect on the contractile machinery, for example, a reduction of the affinity of myosin light chain kinase for the Ca^{2+}-calmodulin complex (Adelstein *et al.*, 1978; Adelstein & Eisenberg, 1980; Pfitzer *et al.*, 1985). The potentiation of inward currents by isoprenaline is clear when large currents are induced by membrane depolarization to more than −10 mV. This condition does not seem to occur physiologically, but it is interesting that similar Ca^{2+} channels are present in the heart as well as the smooth muscle of the coronary artery.

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Evidence for the involvement of imidazoline receptors in the central hypotensive effect of rilmenidine in the rabbit

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1 Rilmenidine has recently been introduced as a new centrally-acting antihypertensive agent. We examined its cardiovascular effects after intracerebral injection to anaesthetized rabbits. Cumulative doses of rilmenidine injected intracisternally (1 to 300 $\mu g\,kg^{-1}$) led to dose-dependent decreases in arterial blood pressure and heart rate. The effective doses of rilmenidine were lower when injected centrally than when injected intravenously.

2 Pretreatment with the same dose of yohimbine or idazoxan shifted the rilmenidine dose-response curves for its hypotensive and bradycardic effects to the right. Idazoxan, which has an imidazoline structure, proved to be a more active antagonist than yohimbine of rilmenidine centrally-mediated cardiovascular effects.

3 The dose-response curve for the central hypotensive effect of rilmenidine was also shifted to the right after pretreatment with a bovine brain extract. This extract contains the endogenous ligand of the imidazoline-preferring receptors which is not a catecholamine.

4 Rilmenidine, like clonidine, proved to be active when micro-injected into the rabbit nucleus reticularis lateralis region.

5 In conclusion, rilmenidine exhibited in the rabbit a central hypotensive effect which originated in the same area as where clonidine acts. Specific imidazoline-preferring receptors appear to be involved in this hypotensive effect.

Introduction

Rilmenidine is an oxazoline recently developed as a clinical antihypertensive drug (Fillastre *et al.*, 1988). Experiments performed in dogs, cats and rats have suggested the existence of a central component in its hypotensive effect (Laubie *et al.*, 1985; Van Zwieten *et al.*, 1986; Koenig Bérard *et al.*, 1988). In addition, studies performed in both animals and man, have shown that hypotensive doses of the drug do not evoke any significant sedative effect (Fillastre *et al.*, 1988).

Thus the pharmacological profile underlying the hypotensive effect of this drug seemed an interesting study. The aim of the present work was to confirm the central hypotensive effect of rilmenidine and to investigate its mechanism of action in a species suitable for the study of this type of central hypotensive drug, i.e. the rabbit. Therefore, using various techniques, we injected rilmenidine directly into rabbits' brains and recorded cardiovascular parameters in order to determine its site of action. These experiments were performed with reference to previous studies used to locate the site of action of clonidine and clonidine-like molecules (Bousquet *et al.*, 1981; 1984; Wolf & Mohrland, 1984; Head & De Jong, 1986; Ernsberger *et al.*, 1987; Granata & Reis, 1987; McAuley *et al.*, 1989). Experiments using antagonists were carried out to provide some information as to the possible mechanism of action.

Several years ago we demonstrated that, in the cat, the major site for the central hypotensive effect of clonidine was within the nucleus reticularis lateralis (NRL) area of the medulla (Bousquet & Guertzenstein, 1973; Bousquet *et al.*, 1981). More recently, a structure-activity study proved that the imidazolines were able to induce such effects although catecholamines or phenylethylamines could not (Bousquet *et al.*, 1984). These results allowed us to propose the existence of imidazoline-sensitive, catecholamine-insensitive receptors involved both in the hypotensive effect of the imidazolines and in the central regulation of arterial blood pressure (for review see Bousquet *et al.*, 1989). These receptors would seem to be located mainly within the NRL region and imidazoline preferring receptors have recently been characterized in this region both in bovine and human brain (Ernsberger *et al.*, 1987; Bricca *et al.*, 1988; 1989a). An endogenous ligand for this receptor has been partially purified (Atlas & Burnstein, 1984a,b; Meeley *et al.*, 1986; Ernsberger *et al.*, 1988). In fact, a partially purified brain extract contains a substance which is not a catecholamine but displaces [^{3}H]-clonidine bound to brain membrane preparations and increases blood pressure whereas clonidine itself reduces it (Atlas & Burstein, 1984a,b; Bousquet *et al.*, 1986). This substance has been provisionally called 'clonidine displacing substance' (CDS). In addition, we recently observed, through binding tests on human NRL membrane preparations (Bricca *et al.*, 1989b) that rilmenidine, structurally very close to the imidazoline, exhibited a relative selectivity for these receptors. The selectivity of rilmenidine was about 2.5 times higher than that of clonidine for the medullary imidazoline receptors as compared to the cortical α_2-adrenoceptors (Bricca *et al.*, 1989b). In the present work we have investigated further the central site of action as well as the hypotensive mechanism of action of rilmenidine in the rabbit.

Methods

General procedure

Normotensive male rabbits (Fauves de Bourgogne) anaesthetized with pentobarbitone (30 $mg\,kg^{-1}$) injected through the marginal vein of the ear were tracheotomized, immobilized with pancuronium bromide (2 $mg\,kg^{-1}$, i.v.) and artificially ventilated (Bird Mark 8). Arterial blood pressure was recorded with the aid of a catheter placed within the abdominal aorta by way of the right femoral artery. This catheter was connected to a pressure transducer (Statham P23Db) which in turn was connected to a Gould recorder. Instantaneous systolic and diastolic arterial pressures were recorded and mean arterial pressure calculated. Heart rate was calculated from the blood pressure signal.

Intracerebral injections

The animal's head was placed in a stereotaxic frame (La Précision Cinématographique, Française). Drugs were either

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injected intracisternally (100 μl) directly within the cisterna magna using cumulative doses, or micro-injected with one dose per animal into the NRL area (1 μl). In order to inject drugs directly into the NRL region, we placed unilaterally within the brain tissue a needle with an outer diameter of 100 μm. To expose the dorsal surface of the medulla oblongata, the neck muscles were separated from the occipital protuberance and reclined downward. After removing the atlanto-occipital membrane, the occipital bone was resected. By means of a micromanipulator, the needle was positioned at an angle of 30° to the horizontal, 2 mm laterally from the median line and 4 mm above the obex. Then it was inserted for 7.5–8 mm into the medulla oblongata.

At the end of each experiment, Evans blue was injected under the same conditions as the drugs. After the animals were killed, the brain was removed, the brainstem dissected and fixed in 10% formalin. Subsequent histological controls on 50 μm frozen sections confirmed the location of the needle tip and diffusion of the solution in the NRL area, i.e. close to the ventral surface of the medulla oblongata within a region located between the trapezoid bodies rostrally and the pyramids laterally.

At the beginning of each experiment, prior to any drug injection, equal volumes of vehicle were given. Sometimes transient cardiovascular changes were observed after the stereotaxic insertion of the needle within the medulla oblongata, in which case the drug micro-injections were only performed after these parameters had returned to their initial values. Vehicle never produced any significant effect on haemodynamic parameters. In addition, the effects of the drugs were measured only when a steady-state effect was observed, i.e. 5 to 10 min after central or systemic injections. In antagonist experiments, yohimbine, idazoxan or CDS were injected as pretreatment in one dose, 10 min before the injection of cumulative doses of rilmenidine.

Brain extract preparation

Fresh bovine brains were obtained from the local slaughterhouse and rapidly brought, on ice, to the laboratory where dissection took place within one hour.

The chromatographic beds were purchased from Bio-Rad (U.S.A.), the chemicals for Fluka (Switzerland) and the ^{3}H-labelled molecules from New England Nuclear.

The extraction procedure was based upon the protocol described previously by Atlas & Burstein (1984a,b) with some modifications. Instead of being heated, the 50000 *g* supernatant was ultrafiltered through a 1000 daltons cut-off membrane. The resulting dialysate was lyophilised, extracted twice with methanol and the last extract evaporated to dryness under vacuum at 40°C.

The crude extract was submitted to gel filtration chromatography on a BioGel P2 column (length = 100 cm, diameter = 2.6 cm) eluted with a 0.1 M NH_4HCO_3, pH 7.9 buffer at 5°C (flow rate = 30 ml h^{-1}) and monitored at 206 nm. Ion-exchange chromatography using CM-cellulose was performed on the active fraction. The column (length = 10 cm, diameter = 1.6 cm, flow rate = 20 ml h^{-1}) was step eluted with a 0.01 M $HCOONH_4$, pH 4.0 buffer followed by 3 ml of 1 M NH_4OH. After each step, the biological activities of each fraction were tested. The binding assays were performed according to the techniques described elsewhere (Atlas *et al.*, 1987; Bricca *et al.*, 1989a). At each step the fraction selected was the one which preferentially displaced [^{3}H]-clonidine from its binding to human NRL membranes as compared with [^{3}H]-rauwolscine binding to rat cortex membranes, representing the α_2-adrenoceptor model. That extract was named endazoline.

Endazoline was dissolved in 0.9% w/v NaCl solution (saline). One unit of endazoline is defined as the amount needed to displace 50% of [^{3}H]-clonidine specifically bound to human NRL membranes.

Drugs

The following drugs were used: rilmenidine (S3341, Institut de Recherches Servier), clonidine (Sigma), pentobarbitone Na (Nembutal, Abbott), pancuronium bromide (Pavulon, Organon), yohimbine (Sigma) and (±)-idazoxan (RX 781094, Institut de Recherches Servier). Drugs were dissolved in saline. The pH of every solution was adjusted to 7.5.

Statistics and calculations

Data in the text are given as means ± s.e.mean. For each group, the log dose-response curves were used to determine the dose which decreased mean blood pressure by 20% of the initial value (D_{20}). We compared the effects of rilmenidine without and after treatment with the antagonists. Variance analysis of the basal values were performed in order to test the homogeneity of the mean arterial pressure in the different groups. To test the effect of the drug alone, comparisons were made by Student's paired *t* test. Statistical analyses were performed using two-way analyses of variance for repeated measures. The remaining data were compared by Newman-Keuls's test. P values < 0.05 were used as the criterion of significance and n is the number of experiments. The calculations were made by computer assisted analyses with STAT-ITCF programme (I.T.C.F. Boigneville-Maisse, France).

Results

Controls

The five groups of animals were highly homogeneous regarding their average preinjection values of blood pressure. Analyses of variance showed no significant difference in the basal values of the mean arterial pressure (MAP) ($F = 2.14$; $P > 0.05$) and heart rate (HR) ($F = 1.30$; $P > 0.05$) between all the groups of animals.

Cardiovascular effects of intravenous injection of rilmenidine

Cumulative doses of rilmenidine (1 μg to 1 mg kg^{-1}) were injected intravenously to 5 normotensive anaesthetized rabbits. Under such conditions, a dose-dependent decrease of the MAP was observed. The MAP decreased from 92 ± 5 mmHg to 52 ± 5 mmHg ($P < 0.001$) at the maximal dose of 1 mg kg^{-1}. The HR also decreased from 266 ± 12 beats min^{-1} to 188 ± 6 beats min^{-1} ($P < 0.001$) (Figure 1). Thus rilmenidine injected intravenously induced a hypotensive effect which became significant at a dose of 30 μg kg^{-1}. This effect was associated with bradycardia. The dose which

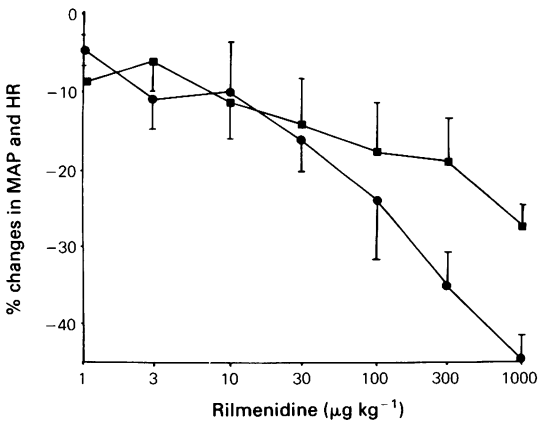

Figure 1 Changes in mean arterial pressure (MAP, ●) and heart rate (HR, ■) with cumulative doses of rilmenidine administered intravenously into pentobarbitone-anaesthetized rabbits ($n = 5$). Vertical bars show s.e.mean.

decreased MAP by 20% (D_{20}) was 70 $\mu g\,kg^{-1}$ in this series. At this dose, the HR decreased by 16%.

Cardiovascular effects of intracisternal injection of rilmenidine

Cumulative doses of rilmenidine (1 to 300 $\mu g\,kg^{-1}$) were injected to 7 anaesthetized rabbits. A dose-dependent decrease of MAP was observed. At the maximal dose of 300 $\mu g\,kg^{-1}$, MAP fell from 99 ± 3 mmHg to 59 ± 2 mmHg ($P < 0.001$). At this dose the heart rate also decreased from 276 ± 10 beats min^{-1} to 190 ± 7 beats min^{-1} ($P < 0.001$) (Figure 2). The D_{20} for fall in MAP was 1.5 $\mu g\,kg^{-1}$ in this series of experiments at which dose the heart rate decreased less than 10%.

Antagonism experiments

Pretreatment with yohimbine Low doses of yohimbine (1.5 $\mu g\,kg^{-1}$ i.e. 5 $nmol\,kg^{-1}$) were given intracisternally to a series of 5 anaesthetized rabbits prior to the injection of cumulative doses of rilmenidine (1 to 300 $\mu g\,kg^{-1}$). Yohimbine itself did not significantly affect the cardiovascular parameters. The pre-rilmenidine value of MAP was 100 ± 2 mmHg in this series of experiments. Yohimbine provoked a moderate shift to the right of the dose-response curve obtained with cumulative doses of intracisternal rilmenidine, the D_{20} of rilmenidine being approximately 30 $\mu g\,kg^{-1}$. The bradycardic effect of rilmenidine was not significantly affected by yohimbine (Figure 2).

Pretreatment with idazoxan (±)-Idazoxan was given intracisternally (1 $\mu g\,kg^{-1}$ i.e. 5 $nmol\,kg^{-1}$) to 5 anaesthetized rabbits prior to rilmenidine injection. Idazoxan itself never influenced the cardiovascular parameters. The dose-effect curves for rilmenidine were displaced toward the right by this pretreatment. At the highest dose of rilmenidine (30 $\mu g\,kg^{-1}$), MAP decreased from 95 ± 5 mmHg to 71 ± 9 mmHg ($P < 0.05$) and the heart rate was reduced from 283 ± 14 beats min^{-1} to 227 ± 14 beats min^{-1} ($P < 0.05$). After pretreatment with idazoxan, the rilmenidine D_{20} was around 180 $\mu g\,kg^{-1}$ for MAP (Figure 2).

Pretreatment with endazoline When given intracisternally to 5 anaesthetized rabbits (500 units in 100 μl), endazoline caused a transient and variable increase in blood pressure. The average values of MAP before endazoline administration were not significantly different (112 ± 6.5 mmHg) 10 min after endazoline pretreatment (116 ± 10 mmHg) ($P > 0.05$). The dose-response curve for rilmenidine was shifted to the right. At the maximal dose of 300 $\mu g\,kg^{-1}$ of rilmenidine, MAP decreased from 116 ± 10 mmHg to 84 ± 6 mmHg ($P < 0.001$). Heart rate decreased from 240 ± 11 beats min^{-1} to 174 ± 14 beats min^{-1} ($P < 0.05$). The rilmenidine D_{20} was around 30 $\mu g\,kg^{-1}$ for MAP and HR after endazoline treatment (Figure 2).

A two way analysis of variance using Newman-Keuls's test showed that pretreatment with the antagonists significantly affected ($P < 0.05$) the effects of rilmenidine on MAP ($F = 25.34$) and HR ($F = 15.08$).

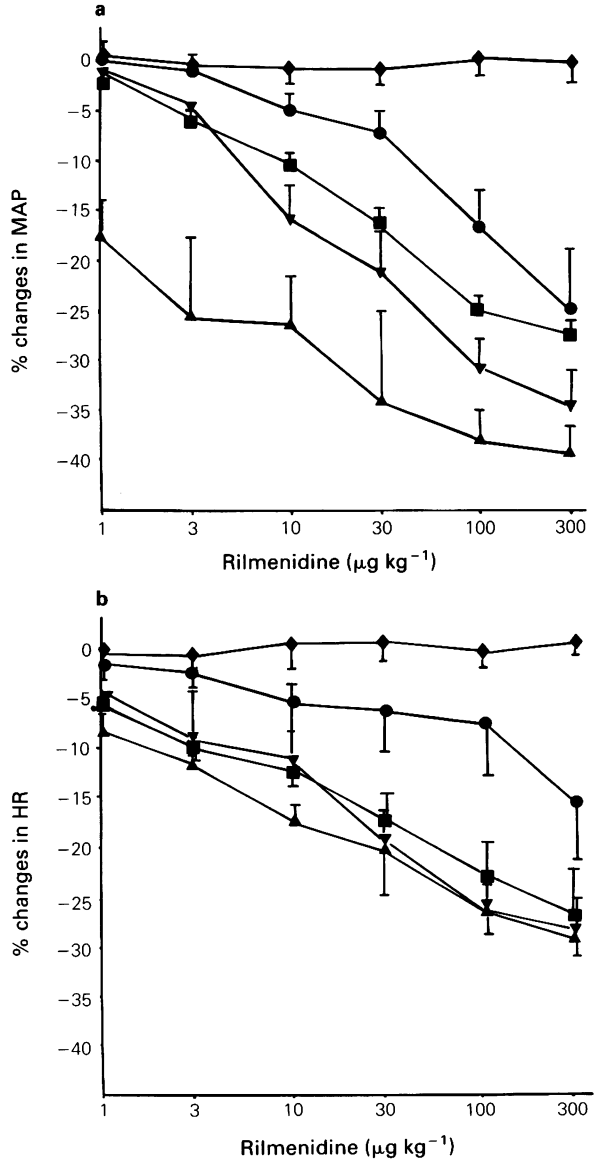

Figure 2 Changes in mean arterial pressure (MAP, a) and heart rate (HR, b) with cumulative doses of rilmenidine injected intracisternally (i. cist) into anaesthetized rabbits: (◆) control ($n = 5$); (▲) rilmenidine ($n = 5$); (▼) pretreatment with yohimbine i. cist. ($n = 5$); (●) pretreatment with idazoxan i. cist ($n = 5$); (■) pretreatment with endazoline i. cist ($n = 5$). Vertical bars show s.e.mean.

Effects of injection of rilmenidine and clonidine into the NRL

Rilmenidine effects Five anaesthetized rabbits were injected with 2 $\mu g\,kg^{-1}$ rilmenidine into the NRL. MAP decreased from 99 ± 4 mmHg to 75 ± 7 mmHg ($P < 0.001$) (i.e. −24 ± 2%) and the heart rate was decreased by 12 ± 3% (266 ± 16 beats min^{-1} to 232 ± 6 beats min^{-1}) ($P < 0.05$) (Figure 3).

Effects of clonidine injection into the rabbit's NRL Under the same experimental conditions, 5 anaesthetized rabbits were injected with 0.06 $\mu g\,kg^{-1}$ clonidine directly into the NRL. A significant drop of 26 ± 2% in the MAP, from 87 ± 6 mmHg to 72 ± 5 mmHg ($P < 0.001$), was observed. The heart rate

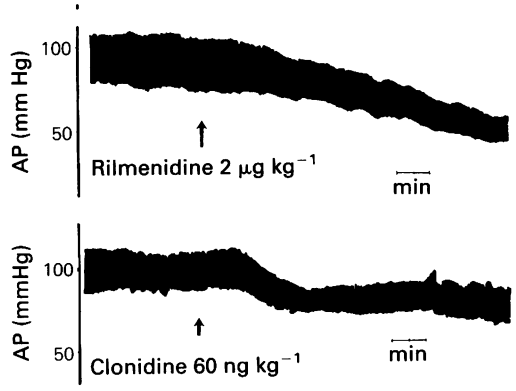

Figure 3 Effects on arterial pressure (AP) of a unilateral microinjection of rilmenidine and clonidine into the nucleus reticularis late-ralis of the anaesthetized rabbit.

also decreased from 242 ± 16 beats min^{-1} to 190 ± 15 beats min^{-1}, i.e. $11 \pm 4\%$ ($P < 0.05$) (Figure 3).

Discussion

In the present study, we compared the cardiovascular effects of cumulative doses of rilmenidine given systemically or intracisternally to anaesthetized rabbits. The intracisternal injection allowed the substance to reach the medullary region very quickly. In fact, the most probable location of the site for the central hypotensive action of rilmenidine was the NRL area of the brainstem, by analogy with the site of action for clonidine (Bousquet *et al.*, 1981; 1984).

We have demonstrated that when injected intracisternally, 40 times lower doses of rilmenidine are able to lower arterial blood pressure than when injected systemically, indeed the D_{20} ratio was of the order of 45.

Rilmenidine proved capable of lowering MAP by about 40% when centrally injected. As this substance was active when injected into the brain at doses which had no effect when given systemically, one can assume the existence of a central hypotensive action of rilmenidine in the rabbit. This observation confirms the results obtained by other authors in cats or dogs (Laubie *et al.*, 1985; Van Zwieten *et al.*, 1986).

It was of importance to locate precisely the site of action of the substance within the medulla oblongata and it was necessary to verify whether this site was the same as that involved in the effects of clonidine. As it has never been proved that clonidine has an effect in the rabbit within the same area as in the cat, namely the NRL, we checked this point first. As we have shown significant decreases in rabbit MAP after microinjections of very low doses of clonidine ($60\,ng\,kg^{-1}$), we can reasonably conclude that in the rabbit at least, a main site for the central hypotensive action of this drug is located within the NRL region. In addition, we have shown that doses of rilmenidine, as low as $2\,\mu g\,kg^{-1}$, when micro-injected in the same area are also able to decrease significantly arterial blood pressure. The blood pressure lowering effect of rilmenidine was always accompanied by a bradycardia.

Van Zwieten *et al.* (1986) reported that yohimbine ($30\,\mu g\,kg^{-1}$), a well-known antagonist of the cardiovascular effects of clonidine totally prevented, when injected into the cat's vertebral artery, the hypotensive effects of a standard dose of rilmenidine. Recently, we studied the ability of very low doses of yohimbine to antagonize the central cardiovascular effects of clonidine in the rat and observed that pretreatment with doses as low as $1\,\mu g\,kg^{-1}$, were able to antagonize the hypotensive effects of clonidine in the SHR (Tibiriça *et al.*, 1988).

If we regard the numerous studies evaluating α-adrenoceptor antagonist potencies of various substances in classical pharmacological tests, it appears from results obtained with catecholamines particularly noradrenaline as agonists, that idazoxan and yohimbine have very similar potencies. In contrast, when imidazolines were used as agonists in the same tests, the results are very similar to those obtained in the present work. Indeed idazoxan proved to be more potent than yohimbine for example, as an antagonist of various effects of clonidine (Chapleo *et al.*, 1981; Doxey *et al.*, 1983; 1984). Thus we have now investigated the effects of yohimbine and idazoxan, given as pretreatment at the same low doses in rabbits, towards the central hypotensive effect of rilmenidine. Yohimbine, at $5\,nmol\,kg^{-1}$ given intracisternally, did not affect resting haemodynamic parameters but partially prevented the blood pressure decrease induced by rilmenidine. Indeed the dose-response curve obtained after intracisternal injection of cumulative doses of rilmenidine was shifted to the right. The D_{20} ratio was around 20. However, when the same dose of idazoxan was given intracisternally, a more marked and significant shift of the dose-effect curve of rilmenidine was observed (D_{20} ratio = 120). This suggests that idazoxan behaves as a more potent antagonist of the hypotensive effects of rilmenidine than yohimbine. Our hypothesis to explain the higher antagonist activity of idazoxan as compared to yohimbine is that the chemical structure (imidazoline) confers to the former a better affinity and selectivity for the imidazoline receptors. Recent binding studies showing that yohimbine cannot compete with [^{3}H]-idazoxan for specific imidazoline binding sites corroborate this hypothesis (Hamilton *et al.*, 1988). The results observed in antagonist experiments are consistent with the view that antagonists with imidazoline structure better prevent the rilmenidine central hypotensive effects than an antagonist with a non-imidazolinic structure, such as yohimbine.

The endogenous ligand for the imidazoline-preferring receptors contained within the bovine brain extracts which we prepared according to a method derived from that of Atlas & Burstein (1984a,b) is a substance corresponding to the following criteria: (1) it preferentially competes with clonidine for the imidazoline preferring binding sites; (2) it has proven hypertensive effects whereas clonidine exhibits a hypotensive effect; (3) it is not a catecholamine.

We propose to name this extract 'endazoline' in order to show that it corresponds to more restrictive and thus more precise criteria than those applied to the former CDS, which was defined only by clonidine binding displacement in rat cortical membrane preparations which contain mainly α_2-adrenoceptors.

In the present work, we observed that pretreatment with endazoline also shifted to the right the dose-effect curves of the central hypotensive action of rilmenidine. These data confirm that the hypotensive effect of rilmenidine originating in the NRL region may involve imidazoline-preferring receptors.

In conclusion, we have shown the existence of a central hypotensive effect of rilmenidine associated with bradycardia in the rabbit. At least in part, the origin of the rilmenidine central hypotensive effect was located within the NRL area, which is the region where clonidine induces its effect. The cardiovascular effect of rilmenidine was well antagonized by a drug with an imidazoline structure: idazoxan. Our results suggest that the hypotensive effect of rilmenidine might involve imidazoline-specific receptors, thus corroborating the rilmenidine selectivity for these receptors already suggested by binding tests (Bricca *et al.*, 1989b).

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The action of diazoxide and minoxidil sulphate on rat blood vessels: a comparison with cromakalim

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1 The actions of diazoxide and minoxidil sulphate have been compared with those of cromakalim in rat aorta and portal vein.

2 Diazoxide and minoxidil sulphate hyperpolarized the rat portal vein in a similar manner to cromakalim.

3 Cromakalim, diazoxide and minoxidil sulphate increased ^{42}K and ^{86}Rb efflux from rat portal vein, although minoxidil sulphate had only a small effect on ^{86}Rb efflux.

4 Cromakalim, diazoxide and minoxidil sulphate increased ^{42}K efflux from rat aorta but only cromakalim and diazoxide increased ^{86}Rb efflux from this tissue.

5 Glibenclamide inhibited the relaxant actions of cromakalim, diazoxide and minoxidil sulphate on rat aorta and the increase in ^{42}K efflux produced by these agents in this tissue.

6 Diazoxide relaxed an 80 mM KCl-induced contraction of rat aorta, whilst cromakalim and minoxidil sulphate were without effect.

7 Cromakalim, diazoxide and minoxidil sulphate had no effect on cyclic AMP or cyclic GMP concentrations in rat aorta.

8 It is concluded that diazoxide and minoxidil sulphate like cromakalim exhibit K^+ channel opening properties in vascular smooth muscle. Diazoxide exerts an additional inhibitory action not related to the production of cyclic AMP or cyclic GMP. The action of minoxidil sulphate may be primarily located at a K^+ channel which is relatively impermeable to ^{86}Rb.

Introduction

Diazoxide is a sulphonamide derivative with antihypertensive properties (Rubin *et al.*, 1962). In addition to its ability to lower blood pressure, diazoxide produces hyperglycaemia, an action associated with decreased insulin secretion from the pancreas (Loubatières *et al.*, 1986). Subsequently, Henquin & Meissner (1982) showed that diazoxide hyperpolarizes mouse pancreatic β cells and increases ^{86}Rb efflux from rat perfused pancreatic islets. These effects are now known to be associated with the opening of a K^+ channel which is modulated by changes in intracellular ATP concentrations (Trube *et al.*, 1986). Minoxidil sulphate is the active metabolite of the antihypertensive drug minoxidil (Johnson *et al.*, 1982). This agent relaxes rabbit isolated mesenteric artery, an action which is inhibited by the K^+ channel blocker tetraethylammonium and is associated with an increase in ^{42}K efflux. From these data, it has been concluded that the inhibitory effects of minoxidil sulphate are produced by the opening of membrane K^+ (Meisheri *et al.*, 1988).

The aim of the present study was to investigate further the mechanism of action of diazoxide and minoxidil sulphate on rat isolated aorta and portal vein. The actions of these agents are compared with those of the benzopyran derivative cromakalim (BRL34915: Ashwood *et al.*, 1986). This potent antihypertensive agent (Buckingham *et al.*, 1986; Buckingham, 1988) opens K^+ channels in several blood vessels and other smooth muscle-containing tissues (Hamilton & Weston, 1989). The consequences of the resulting hyperpolarization are believed to underlie the antihypertensive action of this compound (Hamilton & Weston, 1989). By use of a variety of techniques it was hoped to determine whether the opening of K^+ channels is involved in the relaxant actions of minoxidil sulphate and diazoxide and to detect any similarities or differences between the three antihypertensive agents. Preliminary observations have been presented (Bray *et al.*, 1988).

Methods

Experiments were carried out on portal veins and segments of thoracic aorta isolated from male Sprague-Dawley rats (300–400 g) which were killed by stunning and bleeding. All animals were supplied by the University of Manchester Animal Unit.

Tissue bath experiments

Aorta Each segment of aorta was cut into four rings, approximately 0.5 cm long. Each ring was opened along its longitudinal axis to form a flat sheet and a thread attached via a small bent pin to each longitudinally-cut edge. The endothelium was removed by rubbing the intimal surface with a cotton bud moistened with physiological salt solution (PSS). Strips were mounted for isometric tension recording under a resting tension of 1 g in a tissue bath containing PSS at 37°C, pH 7.4.

After a 75 min equilibration period in PSS, the spasmolytic effects of cromakalim, diazoxide or minoxidil sulphate were evaluated by exposing tissues to KCl (either 20 or 80 mM). When the maximum mechanical effects of these spasmogens had been obtained, the ability of cromakalim, diazoxide or minoxidil sulphate to relax the maintained KCl-induced contraction was investigated by use of a cumulative protocol.

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Where appropriate, tissues were pre-incubated with glibenclamide for 45 min and the relaxant effects of cromakalim, diazoxide or minoxidil sulphate were examined in the continuing presence of glibenclamide. The effect of glibenclamide on the relaxant actions of nifedipine and sodium nitroprusside was also investigated with a similar protocol.

Portal vein Whole portal veins, each approximately 2 cm in length, were mounted for isometric recording under a resting tension of 0.5 g. Mechanical activity was quantified by use of integrators. Veins were equilibrated for 1 h and then exposed to increasing concentrations of cromakalim, diazoxide or minoxidil sulphate, by use of cumulative protocol, until spontaneous mechanical contractions had been abolished.

Determination of cyclic AMP and cyclic GMP levels

These experiments were carried out with segments of rat aorta. The anatomical position of each strip was noted (upper, upper middle, lower middle, lower) before it was assigned, by a balanced design, to the appropriate treatment group. Each tissue was impaled on a needle and equilibrated for 90 min in 80 ml PSS at 37°C, pH 7.4 gassed with 100% O_2. During this time, the PSS was changed once. At the end of this period KCl (20 mM) was added to mimic tissue bath conditions. Cromakalim, diazoxide or minoxidil sulphate in the appropriate concentration was then added and after varying times, the tissues were plunged into liquid nitrogen. Once frozen, the preparations were transferred to individual test tubes containing 1 ml 10% trichloroacetic acid on ice and allowed to thaw. After homogenisation in a Potter glass/glass homogeniser, the homogenate was centrifuged at 3000 *g* for 15 min at 4°C and the supernatant removed for cyclic nucleotide analysis. The remaining precipitate was dissolved in 1 ml 1 M NaOH and a portion was used for protein determination with Pierce Protein Assay Reagent. Bovine serum albumin in 1 M NaOH was used to construct a standard curve.

The supernatant remaining after centrifugation was extracted 4 times with 3 volumes water-saturated ether, the ether phase being discarded each time. Residual ether was removed with nitrogen gas. Samples of the extract were acetylated and assayed for guanosine 3':5'-cyclic monophosphate (cyclic GMP) or adenosine 3':5'-cyclic monophosphate (cyclic AMP) with the appropriate assay kit (NEN).

Measurement of membrane potential

Simultaneous measurement of the electrical and mechanical activity of rat portal vein was carried out as described by Hamilton *et al.* (1986). When recordings had been stable for several minutes tissues were exposed to cromakalim, diazoxide or minoxidil sulphate which were added to the calibrated reservoirs which supplied PSS to the recording chamber at a rate of approximately 2 ml min^{-1}. Microelectrodes of resistance 50–80 MΩ, filled with 3 M KCl were employed and estimates of the magnitude of the resting membrane potential were made by measuring the oscilloscope deflection which occurred following deliberate electrode withdrawal.

^{86}Rb and ^{42}K efflux

Whole portal veins were prepared for use in these experiments. Tissues were attached to a perspex gassing manifold with syringe needles and, after a 10 min equilibration period in PSS at 37°C, were loaded with ^{86}Rb or ^{42}K by incubation with $^{86}RbCl$ (5 μCi ml^{-1} for 90 min) or $^{42}K_2CO_3$ (1.57 μCi ml^{-1} for 180 min), respectively. The efflux of these isotopes from the tissues was assessed by transferring them into 3 ml aliquots of PSS at 2 min intervals. After 8 such 2 min periods, tissues were exposed to PSS alone (controls) or to PSS containing cromakalim, diazoxide or minoxidil sulphate in varying concentrations for the next five collection periods. For the last 4 collection intervals the tubes contained PSS alone. The tissues were blotted and their ^{42}K and ^{86}Rb content was determined, together with that in the collecting tubes, in a manner similar to that described by Smith *et al.* (1986) by gamma counting or β counting with the Cerenkov Method. Endothelium-free segments of rat aorta were prepared and mounted on the gassing manifold as for portal veins. In these experiments ^{42}K and ^{86}Rb efflux were measured simultaneously (dual isotope experiments) using 4 min collection periods with a 20 min exposure to either cromakalim, diazoxide or minoxidil sulphate. The loading solution contained $^{42}K_2CO_3$ (1.57 μCi ml^{-1}) and $^{86}RbCl$ (5 μCi ml^{-1}) and the tissues were incubated in this solution for 3 h. The total radioactivity in the collection tubes was counted in a gamma counter. In some of these experiments, the PSS contained glibenclamide 1 μM throughout the efflux period. The tubes were recounted after 14 half-lives of ^{42}K had elapsed (7 days) and the counts were corrected for ^{86}Rb decay. The values obtained were then subtracted from the counts measured initially to yield the counts due to ^{42}K.

The efflux data were expressed in terms of the rate coefficient (fractional loss of ^{86}Rb and ^{42}K from the tissue standardised for a 1 min period) expressed as a percentage.

Drugs and solutions/statistical analysis

The following substances were used: (±)-cromakalim (BRL34915, Beecham); diazoxide (Glaxo); forskolin (Sigma); glibenclamide (Hoechst); $^{42}K_2CO_3$ (University of Manchester Reactor Facilities, Risley); minoxidil sulphate (see below); nifedipine (Bayer); $^{86}RbCl$ (Amersham); sodium nitroprusside (Sigma). Minoxidil sulphate was prepared as follows: a mixture of 2,4-diamino-6-(1-piperidinyl)pyrimidine-3-oxide (1 g), chlorosulphonic acid (1.11 g, 0.0096 mol) and diisopropylethylamine (2.47 g, 0.191 mol) in chloroform (25 ml) was stirred overnight. The mixture was concentrated and the residue stirred with aqueous sodium bicarbonate, filtered and washed with ether to yield off-white crystals (1.3 g). The crystals were stirred at room temperature with ethanol, filtered and dried to give 1 g minoxidil sulphate as off-white crystals containing 1 mol of ethanol. These were stored at −70°C and a stock solution was prepared freshly each day. The physiological salt solution (PSS) had the following composition (mM): NaCl 129.7, KCl 5.9, $CaCl_2$ 2.54, $MgCl_2$ 1.19, MOPS 10 and glucose 11.1. The solution was gassed with 100% O_2 and adjusted to pH 7.4 at 37°C with NaOH. When KCl was used as a spasmogen, the stated concentration excludes the KCl (5.9 mM) already present in the PSS. When tissues were loaded with ^{42}K, KCl was excluded from the PSS and replaced with $^{42}K_2CO_3$ to give a final concentration of 5.9 mM.

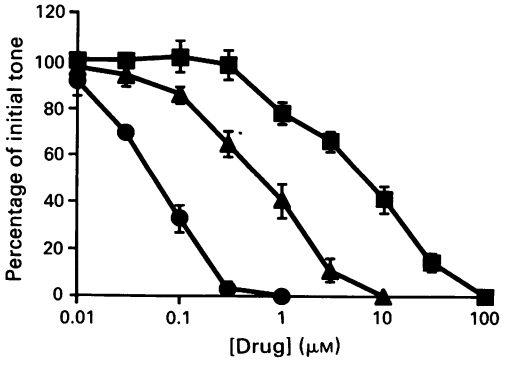

Figure 1 Effect of cumulatively increasing concentrations of cromakalim (●), diazoxide (■) and minoxidil sulphate (▲) on spontaneous mechanical activity of rat portal vein. Points show mean values ($n = 6$) and the vertical bars represent s.e.mean. Ordinate scale: % control integrated mechanical activity in the 2 min period before drug exposure.

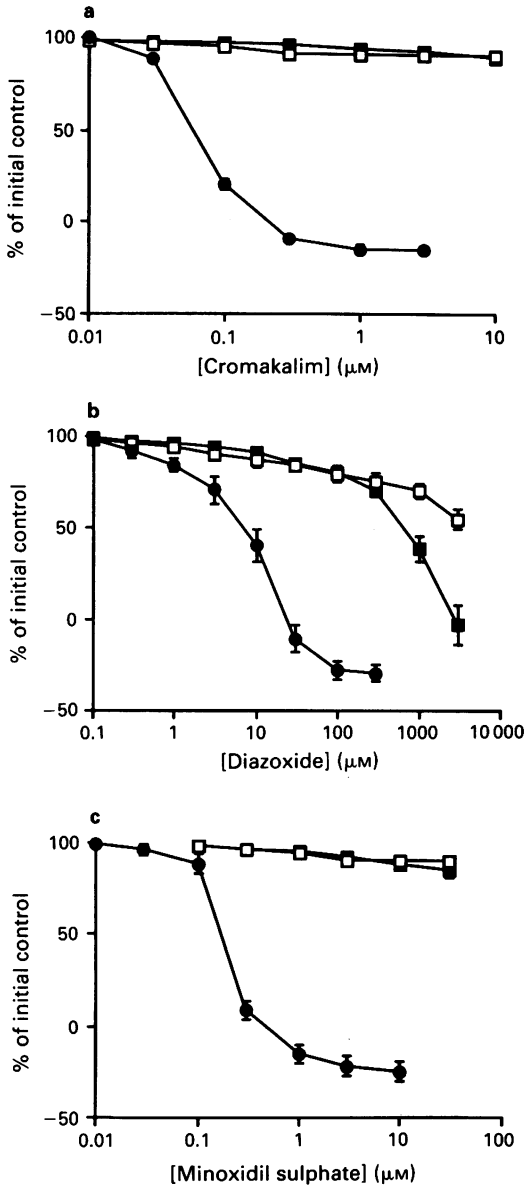

Figure 2 Relaxant effects of cromakalim, diazoxide and minoxidil sulphate against rat aorta precontracted with 20 mM or 80 mM KCl. (a) Cromakalim against 20 mM KCl (●) and 80 mM KCl (■), compared with solvent control (□). (b) Diazoxide against 20 mM KCl (●) and 80 mM KCl (■), compared with solvent control (□). (c) Minoxidil sulphate against 20 mM KCl (●) and 80 mM KCl (■), compared with solvent control (□). The points show mean values ($n = 5$ or 6) and the vertical bars represent s.e.mean. Ordinate scale: percentage of control maximum response to 20 mM or 80 mM KCl as appropriate.

Table 1 Effects of diazoxide, minoxidil sulphate and cromakalim on membrane potential of rat portal vein smooth muscle recorded with intracellular microelectrodes

	Hyperpolarization (mV)
Cromakalim	
1 μM	11.0 ± 1.9 (8) $P \leq 0.01$
10 μM	27.8 ± 2.4 (8) $P \leq 0.01$
Diazoxide	
0.1 mM	2.3 ± 1.3 (4) $P > 0.05$
1 mM	16.0 ± 2.4 (5) $P \leq 0.01$
Minoxidil sulphate	
10 μM	No change (4)
100 μM	6.6 ± 1.0 (5) $P \leq 0.01$

Each value is the mean maximum hyperpolarization ± s.e.mean with the number of observations in parentheses.

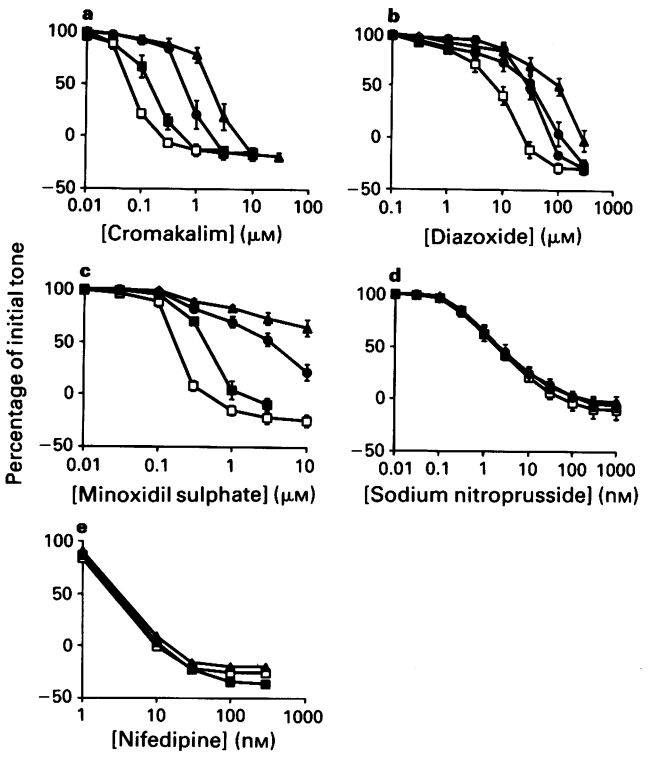

Figure 3 Effect of glibenclamide on the relaxant action of cromakalim, diazoxide, minoxidil sulphate, sodium nitroprusside and nifedipine in rat aorta, precontracted with 20 mM KCl. The effects of glibenclamide 100 nM (■), 300 nM (●) and 1 μM (▲), compared with solvent control (□) on (a) cromakalim, (b) diazoxide, (c) minoxidil sulphate, (d) sodium nitroprusside and (e) nifedipine are shown. The points show mean values ($n = 6$) and the vertical bars represent s.e.mean. Ordinate scale: percentage of control maximum response to 20 mM KCl.

The significance of differences between two means was assessed by a two-tailed Student's t test or analysis of variance, as appropriate.

Results

Tissue bath studies

Rat portal vein Cromakalim (0.01–1 μM), diazoxide (0.5–100 μM) and minoxidil sulphate (0.5–10 μM) each produced complete inhibition of spontaneous activity in the rat portal vein (Figure 1). IC_{50} values for this action were 84 nM, 7.1 μM and 630 nM respectively. Examination of the experimental traces showed that cromakalim, diazoxide and minoxidil sulphate had an identical action on the spontaneous activity of the portal vein. In each case, as the drug concentration was increased, the amplitude of individual tension waves was markedly reduced before any reduction in their frequency was observed.

Rat aorta Cromakalim (0.3–1 μM), diazoxide (0.3–100 μM) and minoxidil sulphate (0.1–3 μM) produced a concentration-dependent relaxation of a 20 mM KCl-induced contraction. The IC_{50} values for this effect were 69 nM, 11 μM and 205 nM, respectively. The time course of the relaxant action of minoxidil sulphate was much slower than that observed with cromakalim or diazoxide.

Diazoxide (0.3–3 mM) produced a concentration-dependent relaxation of an 80 mM KCl-induced contraction, while cromakalim (0.01–10 μM) and minoxidil sulphate (0.1–30 μM) were each without effect (Figure 2).

Glibenclamide (0.1–1 μM) inhibited the ability of cromakalim, diazoxide and minoxidil sulphate to relax a 20 mM KCl-

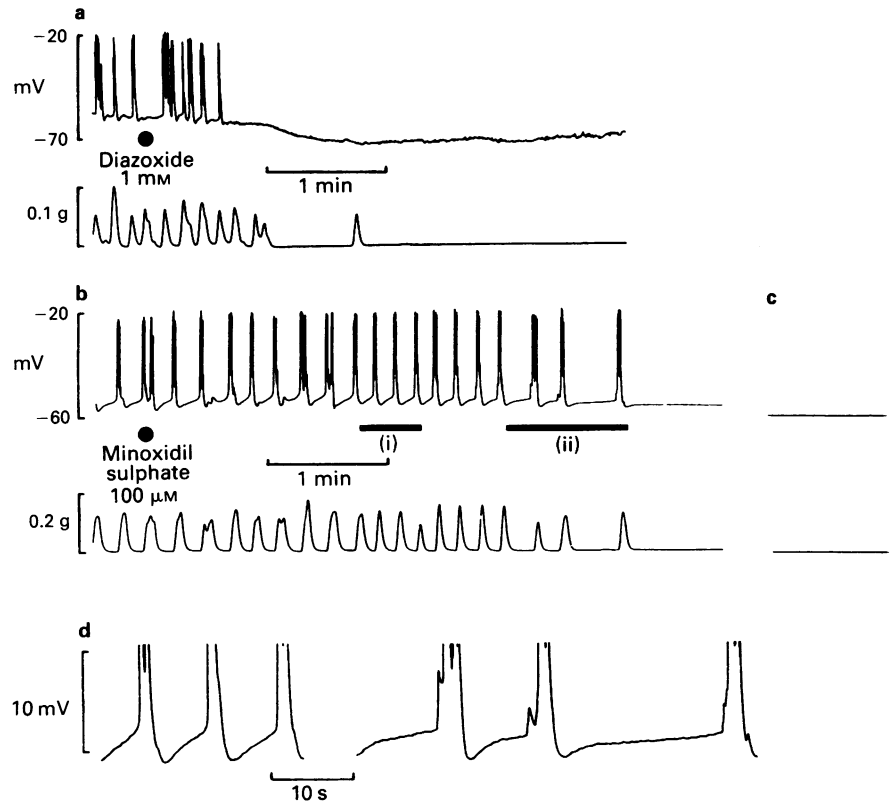

Figure 4 The effects of (a) diazoxide, 1 mM and (b, c) minoxidil sulphate 100 μM on membrane potential (upper trace) and mechanical activity (lower trace), recorded simultaneously in rat portal vein. The filled circles show the points of drug addition. (c) A later, continuous segment of the recording shown in (b) made 10 min after initial exposure to minoxidil sulphate. In (d) segments of the membrane potential trace (continuous lines (i) and (ii)) shown in (b) are presented at greater amplification to illustrate the effects of minoxidil sulphate on pacemaker activity.

induced contraction (Figure 3). At the concentrations of glibenclamide tested, cromakalim and diazoxide appeared to be inhibited in a competitive manner. Schild analysis of the inhibitory action of glibenclamide on cromakalim and diazoxide yielded slopes of 1.21 ± 0.14 and 0.92 ± 0.21 and pA_2 values of 7.2 in both cases. In contrast, minoxidil sulphate was inhibited by glibenclamide in an apparently non-competitive manner. Glibenclamide (0.3–1 μM) had no inhibitory action against nifedipine or sodium nitroprusside (SNP)-induced relaxation of a 20 mM KCl-induced contraction.

Intracellular microelectrode studies

The effects of cromakalim, diazoxide and minoxidil sulphate on membrane potential of rat portal vein are summarized in Table 1. The resting membrane potential of rat portal vein was -56.0 ± 1.4 mV ($n = 16$). Diazoxide (0.1 and 1 mM) produced complete inhibition of spontaneous activity and hyperpolarized the membrane by 2.3 ± 1.3 mV ($n = 4$) and 16.0 ± 2.4 mV ($n = 5$). Diazoxide abolished the spontaneous electrical spiking which is characteristic of electrical activity in rat portal vein within 3 min and produced maximum hyperpolarisation in 5–6 min. Minoxidil sulphate (10 μM) abolished spontaneous electrical spiking without detectable hyperpolarization. The larger (100 μM) concentration of minoxidil sulphate used abolished spontaneous electrical spiking and hyperpolarized the membrane potential by 6.6 ± 1 mV ($n = 5$). At this concentration, minoxidil sulphate took up to 5 min to abolish spontaneous spiking and 10–12 min to produce maximum hyperpolarization. Representative tracings of the effects of diazoxide and minoxidil sulphate on membrane potential and mechanical activity are shown in Figure 4. Cromakalim (1 and 10 mM) abolished spontaneous electrical spiking within 1–2 min and hyperpolarized the membrane potential by 11.0 ± 1.9 mV ($n = 8$) and 27.8 ± 2.4 mV ($n = 8$) respectively within 8 min. Both the electrical and mechanical effects of cromakalim and diazoxide were reversible on washout whereas these effects of minoxidil sulphate were very difficult to reverse.

The effects of minoxidil sulphate (100 μM) on a smooth muscle pacemaker cell in rat portal vein were observed in a single experiment (Figure 4). Minoxidil sulphate slowed the rate of rise of the pacemaker potential, reducing the frequency of the electrical multispike complexes and associated mechanical events. Similar effects were also produced by cromakalim ($n = 3$) and by diazoxide ($n = 1$) in the few experiments in which pacemaker potentials were observed. This action is probably responsible for the decrease in the frequency of spontaneous activity observed with minoxidil sulphate and other K channel opening agents in rat portal vein.

Effects on ^{42}K and ^{86}Rb efflux

Portal vein Cromakalim (1 and 10 μM), diazoxide (0.1 and 1 mM) and minoxidil sulphate (10 and 100 μM) each produced significant increases in ^{42}K efflux (Figure 5). For equivalent relaxant concentrations, the order of potency for this effect was cromakalim > diazoxide > minoxidil sulphate. Cromakalim (1 and 10 μM) and diazoxide (0.1 and 1 mM) each produced large increases in ^{86}Rb efflux, whereas minoxidil sulphate (10 and 100 μM) produced only a small but significant increase (Figure 6). The order of potency for increasing ^{86}Rb efflux was also cromakalim > diazoxide > minoxidil sulphate for equivalent relaxant concentrations. Comparison of the changes in ^{42}K efflux and ^{86}Rb efflux showed that cromakalim, diazoxide and minoxidil sulphate produced larger increases in ^{42}K efflux relative to ^{86}Rb efflux at a given drug concentration.

Rat aorta The effects of cromakalim (1 and 10 μM), diazoxide (0.1 and 1 mM) and minoxidil sulphate (3 and 30 μM) on ^{42}K

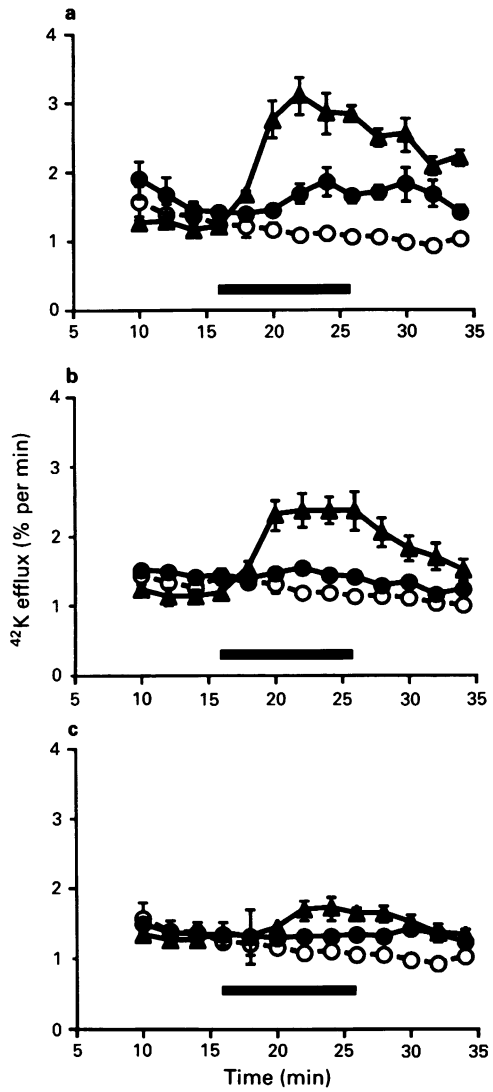

Figure 5 Effects of cromakalim, diazoxide and minoxidil sulphate on the efflux of ^{42}K from rat portal vein. (a) Effect of cromakalim 1 μM (●) and 10 μM (▲). (b) Effect of diazoxide 100 μM (●) and 1 mM (▲). (c) Effect of minoxidil sulphate 10 μM (●) and 100 μM (▲). In each case the basal ^{42}K efflux is represented by the broken line (○). The points signify mean values (n = 5) and the vertical lines represent s.e.mean. The horizontal bar indicates the period of drug or solvent exposure.

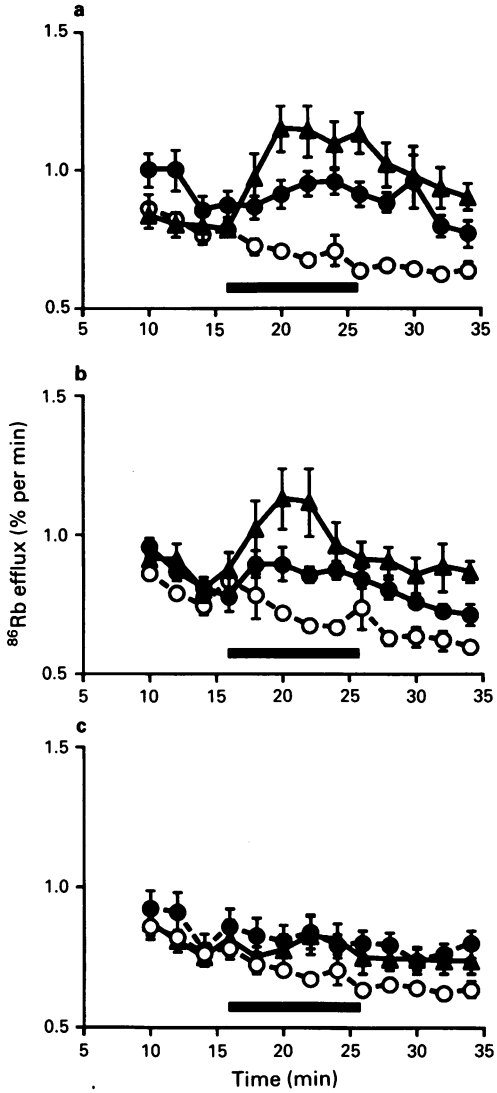

Figure 6 Effects of cromakalim, diazoxide and minoxidil sulphate on the efflux of ^{86}Rb from rat portal vein. (a) Effect of cromakalim 1 μM (●) and 10 μM (▲). (b) Effect of diazoxide 100 μM (●) and 1 mM (▲). (c) Effect of minoxidil sulphate 10 μM (●) and 100 μM (▲). In each case the basal ^{86}Rb efflux is represented by the broken line (○). The points signify mean values (n = 5) and the vertical lines represent s.e.mean. The horizontal bar indicates the period of drug or solvent exposure.

and ^{86}Rb efflux were investigated. Cromakalim (1 and 10 μM), diazoxide (0.1 and 1 mM) and minoxidil sulphate (3 and 30 μM) each increased ^{42}K efflux from rat aorta (Figure 7). The order of potency for this action was cromakalim > diazoxide > minoxidil sulphate. These effects were antagonised by glibenclamide (1 μM) which itself had no effect on basal ^{42}K efflux (Figure 8). Cromakalim (1 and 10 μM) and diazoxide (1 mM) each produced a significant increase in ^{86}Rb

Table 2 Effects of various relaxants on cyclic AMP and cyclic GMP concentrations in rat aorta

	Cyclic AMP (pmol mg^{-1} protein)	*Cyclic GMP* (pmol mg^{-1} protein)
Control	7.95 ± 1	0.058 ± 0.02
Cromakalim		
1 μM	5.3 ± 0.49	0.039 ± 0.01
10 μM	6.2 ± 0.46	0.049 ± 0.005
Diazoxide		
100 μM	8.8 ± 0.9	0.069 ± 0.018
1 mM	7.0 ± 1.4	0.13 ± 0.05
Minoxidil sulphate		
3 μM	10.5 ± 1.6	0.037 ± 0.016
30 μM	9.6 ± 2.6	0.039 ± 0.001
Sodium nitroprusside		
1 μM	Not determined	42.7 ± 6.1 $P < 0.01$
Forskolin		
1 μM	15.6 ± 2.2 $P < 0.05$	Not determined

Each value is the mean derived from 5 observations ± s.e.mean.

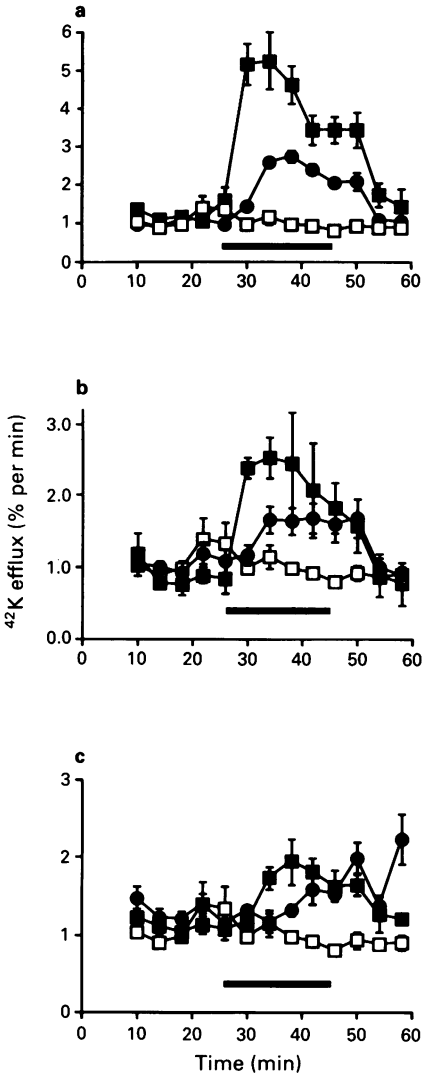

Figure 7 Effects of cromakalim, diazoxide and minoxidil sulphate on ^{42}K efflux from rat aorta. (a) Effect of cromakalim 1 μM (●) and 10 μM (■). (b) Effect of diazoxide 100 μM (●) and 1 mM (■). (c) Effect of minoxidil sulphate 3 μM (●) and 30 μM (■). In each case the basal ^{42}K efflux is represented by (□). The points signify mean values (n = 5) and the vertical lines represent the s.e.mean. The horizontal bar indicates the period of drug or solvent exposure.

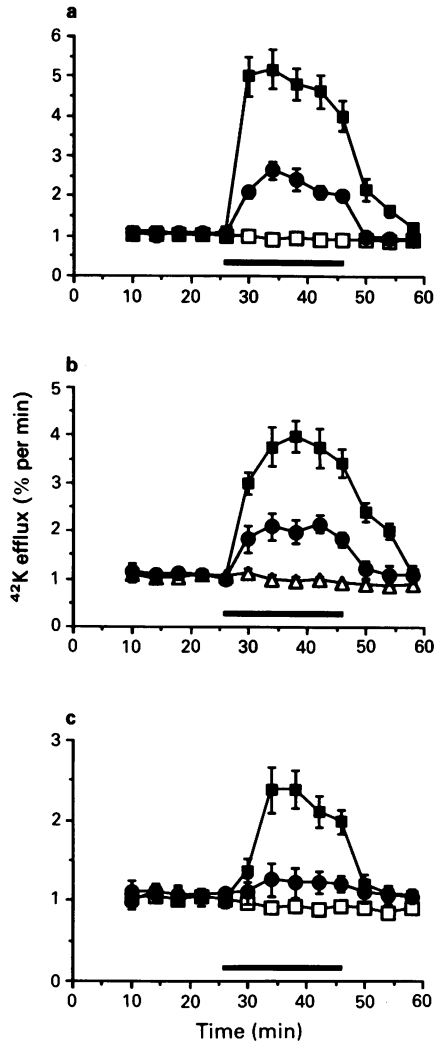

Figure 8 Effect of (a) cromakalim 10 μM, (b) diazoxide 1 mM and (c) minoxidil sulphate 30 μM on ^{42}K efflux from rat aorta in normal PSS (■) and in PSS containing glibenclamide 1 μM (●). In each case, basal ^{42}K efflux is represented by the open symbols. The points signify mean values (n = 5) and the vertical lines represent s.e.mean. The horizontal bar indicates the period of drug or solvent exposure.

efflux whereas minoxidil sulphate had no significant effect at either concentration tested (Figure 9). Glibenclamide 1 μM also antagonized the effects of cromakalim, 10 μM and diazoxide, 1 mM on ^{86}Rb exchange (data not shown). Cromakalim and diazoxide produced larger changes in ^{42}K efflux relative to ^{86}Rb efflux at both concentrations tested.

Cyclic nucleotide studies

The effects of IC_{100} and 10 fold IC_{100} concentrations of cromakalim, diazoxide and minoxidil sulphate on cyclic AMP and cyclic GMP levels in 20 mM KCl-contracted segments of rat aorta were examined. Cromakalim (1 and 10 μM), diazoxide (0.1 and 1 mM) and minoxidil sulphate (1 and 10 μM) had no significant effect on intracellular cyclic AMP concentration. Forskolin (1 μM) produced a significant increase in cyclic AMP concentration, indicating the viability of the assay technique. Cromakalim (1 and 10 μM) and minoxidil sulphate (3 and 10 μM) had no effect on intracellular cyclic GMP concentration. Diazoxide produced a small, but non-significant increase in cyclic GMP levels which was negligible compared with that obtained with a maximum relaxant concentration of sodium nitroprusside (1 μM). These effects are summarized in Table 2.

Discussion

Diazoxide and minoxidil sulphate each produced complete inhibition of spontaneous activity in the rat portal vein. The pattern of loss of activity was similar to that produced by cromakalim, used as a standard, and to that previously described for pinacidil (Southerton *et al.*, 1988). The order of potency for this action was cromakalim > minoxidil sulphate > diazoxide, with an approximate potency ratio of 1:10:100 when IC_{50} values were compared.

Diazoxide abolished spontaneous electrical activity and produced a concentration-dependent hyperpolarization of rat portal vein. Minoxidil sulphate exerted similar inhibitory effects on spontaneous electrical discharges, but the degree of hyperpolarization was less than that generated by diazoxide. No previous data on the effects of minoxidil sulphate on membrane potential in whole portal vein have been published, although hyperpolarization of isolated single cells from rabbit portal vein was detected by Leblanc *et al.* (1989). The results with diazoxide are consistent with those of a previous study by Rhodes & Sutter (1971) on rabbit anterior mesenteric vein. Using the sucrose gap method these workers showed that diazoxide had a hyperpolarizing action. Cromakalim also abolished spontaneous spike generation and produced a concentration-dependent hyperpolarization of the portal vein, confirming the results of Hamilton *et al.* (1986).

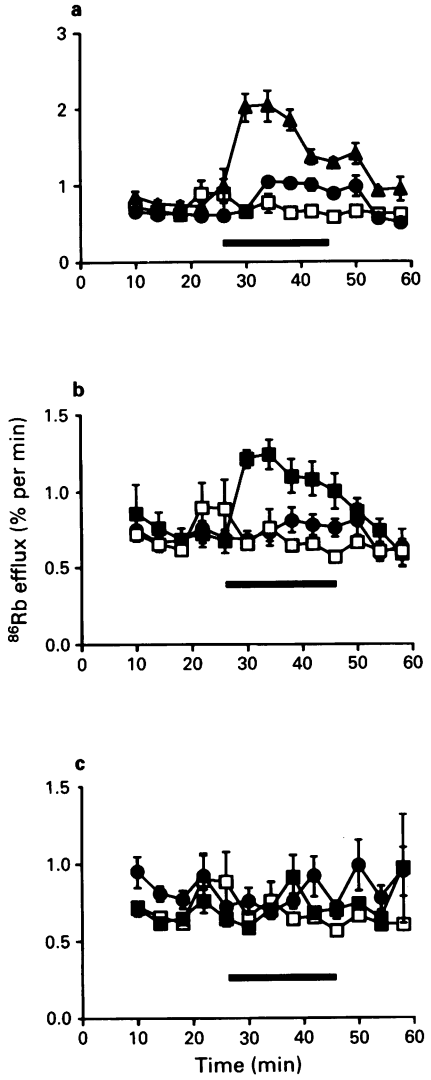

Figure 9 Effects of cromakalim, diazoxide and minoxidil sulphate on ^{86}Rb efflux from rat aorta. (a) Effect of cromakalim 1 μM (●) and 10 μM (▲). (b) Effect of diazoxide 100 μM (●) and 1 mM (■). (c) Effect of minoxidil sulphate 1 μM (●) and 10 μM (■). In each the basal ^{86}Rb efflux is represented by (□). The points show mean values (n = 5) and the vertical lines represent the s.e.mean. The horizontal bar indicates the period of drug or solvent exposure.

In rat portal vein, cromakalim produced the largest hyperpolarization followed by diazoxide, while minoxidil sulphate was the least effective when equivalent relaxant concentrations were compared. Furthermore, at concentrations which abolished spontaneous mechanical activity diazoxide produced only a 2 mV hyperpolarization and minoxidil sulphate had no effect on membrane potential. This observation suggests that diazoxide and minoxidil sulphate may have relaxant actions in rat portal vein in addition to K^+ channel opening. Alternatively, these agents may selectively inhibit pacemaker cells in the rat portal vein. Such a mechanism was originally suggested for cromakalim in this tissue by Hamilton *et al.* (1986) and has been proposed by Quast & Baumlin (1988) to explain discrepancies between the concentrations of cromakalim required to inhibit spontaneous activity and to stimulate ^{42}K efflux in guinea-pig portal vein. In the present study, diazoxide, minoxidil sulphate and cromakalim suppressed pacemaker potentials in rat portal vein before any marked hyperpolarization was observed. Thus these agents may open K^+ channels in pacemaker cells at concentrations lower than those required to hyperpolarize the other smooth muscle cells in the rat portal vein.

Diazoxide, minoxidil sulphate and cromakalim increased ^{42}K and ^{86}Rb efflux from rat portal vein. These results together with observed changes in membrane potential confirm that both diazoxide (Cook *et al.*, 1988) and minoxidil sulphate (Meisheri *et al.*, 1988) exert K^+ channel opening actions in vascular smooth muscle. In each case, the increase in ^{42}K efflux observed was greater than the increase in ^{86}Rb efflux. Quast & Baumlin (1988) showed that cromakalim stimulated quantitatively greater increases in ^{42}K efflux compared to ^{86}Rb efflux in rat aorta and guinea-pig portal vein. Such data support the general conclusion that ^{86}Rb can be used as a ^{42}K substitute, although absolute changes in K^+ efflux will be underestimated due to differences between the physical properties of the two ions (Smith *et al.*, 1986). Similar to the results obtained in rat portal vein, cromakalim and diazoxide produce larger changes in ^{42}K efflux relative to ^{86}Rb efflux from rat aorta. However, in the present study, minoxidil sulphate in contrast to diazoxide and cromakalim had no effect on ^{86}Rb efflux from rat aorta, even at a concentration ten times greater than that required for complete relaxation of a 20 mM KCl-induced contraction. Minoxidil sulphate did, however, increase ^{42}K efflux from rat aorta. Thus in rat aorta minoxidil sulphate may open a K^+ channel which is relatively impermeable to ^{86}Rb. Recently Foster *et al.* (1989) showed that cromakalim stimulated ^{43}K efflux, but not ^{86}Rb efflux, from guinea-pig urinary bladder indicating that a similar situation may exist in this tissue for cromakalim. These observations collectively suggest that ^{86}Rb may not be a totally adequate substitute for ^{42}K in evaluating putative K^+ channel openers.

In rat aortic strips, diazoxide, minoxidil sulphate and cromakalim were each able to relax fully a 20 mM KCl-induced contraction. The order of potency for this effect was the same as for inhibition of spontaneous activity in rat portal vein (cromakalim > minoxidil sulphate > diazoxide) with an approximate potency ratio of 1:3:160. Minoxidil sulphate, like cromakalim was without effect on aortic strips contracted with 80 mM KCl, while diazoxide was capable of producing maximal relaxation of these contractions. The ability of an agonist to relax contractions induced by low, but not high concentrations of extracellular K^+ is an indication of a K^+ channel opening action (Hamilton & Weston, 1989). Effects of other smooth muscle relaxants are inhibited in high K^+ solutions (isoprenaline: Allen *et al.*, 1985; sodium nitroprusside: Ito *et al.*, 1985). These observations are consistent with a relatively selective K^+ channel opening action of minoxidil sulphate and cromakalim, but suggest that diazoxide must exert additional effects. In rabbit pulmonary artery, Thorens & Haeusler (1979) showed that concentrations of diazoxide (300 μM–3 mM) which in the present study relaxed an 80 mM KCl-induced contraction of rat aorta, may exert a calcium entry-blocking action. Thus diazoxide may possess dual K^+ channel opening and calcium entry blocking activity.

The apparently selective K^+-channel opening action of both minoxidil sulphate and cromakalim contrasts with their effects in isolated single cells. In rabbit and rat portal veins under voltage clamp conditions, both minoxidil sulphate (Leblanc *et al.*, 1989) and cromakalim (Okabe *et al.*, 1990) inhibited calcium currents at concentrations which enhanced K^+-currents in these cells. The evidence for such an action on calcium currents in whole tissues is relatively weak and the contribution of this effect to the pharmacological profile of these agents is the subject of further study.

Glibenclamide, the hypoglycaemic sulphonylurea, which blocks ATP-regulated K^+ channels in pancreatic β-cells (Schmid-Antomarchi *et al.*, 1987; Sturgess *et al.*, 1988) inhibits the relaxant effects of cromakalim on rabbit aorta and mesenteric artery (Quast & Cook, 1988; Wilson *et al.*, 1988) and of diazoxide, minoxidil sulphate and cromakalim in rat portal vein (Winquist *et al.*, 1989). In the present study, the relaxation of 20 mM KCl-induced contractions produced by diazoxide, minoxidil sulphate and cromakalim in rat aortic strips was inhibited by glibenclamide with the order of potency; minoxidil sulphate > cromakalim > diazoxide. Schild analysis showed that the effect of glibenclamide on cromakalim and

diazoxide-induced relaxation was apparently competitive, whereas the relaxant action of minoxidil sulphate appeared to be non-competitively inhibited. Glibenclamide also antagonized the increase in ^{42}K efflux produced by cromakalim, diazoxide and minoxidil sulphate in rat aorta. In contrast, glibenclamide had no inhibitory effect on relaxations produced by sodium nitroprusside or by nifedipine, which were used as standards to produce relaxation by activation of guanylate cyclase or by blockade of L-type Ca^{2+}-channels respectively. It would appear therefore that glibenclamide is a relatively selective inhibitor of K^+ channel openers in vascular smooth muscle.

In pancreatic β-cells glibenclamide selectively inhibits the opening of ATP-regulated K^+ channels which can be opened by diazoxide (Trube *et al.*, 1986; Sturgess *et al.*, 1988; Zünkler *et al.*, 1988). The observation in the present study that glibenclamide inhibits the relaxant actions of diazoxide, minoxidil sulphate and cromakalim, coupled with the finding that all three agents open K^+ channels, suggests that the action of these K^+ channel openers may be exerted at a K^+ channel similar to the ATP-regulated K^+ channel in pancreatic β-cells. The *in vivo* hypotensive action of cromakalim in rats is also antagonized by glibenclamide (Buckingham *et al.*, 1989; Cavero *et al.*, 1989), while the hypotensive actions of diazoxide are known to be inhibited by the hypoglycaemic sulphonylurea, tolbutamide (Wales *et al.*, 1967), further supporting this view. High concentrations of cromakalim activate ATP-regulated K^+ channels in guinea-pig ventricular myocytes (Escande *et al.*, 1988; Osterrieder, 1988), an action which is also inhibited by glibenclamide (Escande *et al.*, 1988).

There are conflicting results about the ability of cromakalim to open the pancreatic ATP-regulated K^+ channel. Ashford *et al.* (1988) showed that cromakalim has no opening action against ATP-dependent K^+ channels in a rat pancreatic tumour cell line (CRI-GI). However, Dunne *et al.* (1989, 1990) have recently shown that high concentrations of cromakalim (100–400 μM) do open the ATP-dependent K channel in the RINm5F cell line, although to a much lesser extent than diazoxide. Cromakalim does not produce the marked increase in plasma glucose associated with antihypertensive doses of diazoxide *in vivo* (Cook *et al.*, 1988). Furthermore, the substance galanin which is located in nerves which innervate the pancreas (Dunning *et al.*, 1986) and which activates the pancreatic ATP-dependent K^+ channel (De Weille *et al.*, 1988) does not relax rat aorta or portal vein (Longmore *et al.*, 1989) but has contractile effects in these tissues.

These observations suggest that the ATP-regulated K^+ channel in pancreatic cells is not identical to that opened by the K^+ channel openers in vascular smooth muscle. However, the K^+ channel opened by these agents in smooth muscle may be similar to the ATP-regulated K^+ channel found in cardiac myocytes. Until recently, there was no direct evidence of an ATP-regulated K^+ channel in vascular smooth muscle, although many different voltage- and Ca^{2+}-gated channels have been described (for a review see Cook, 1988). However, evidence of an ATP-regulated K^+ channel in rat mesenteric artery and for an action of cromakalim at this site has recently been obtained (Standen *et al.*, 1989).

Several previous patch clamp studies have demonstrated that cromakalim interacts with a variety of smooth muscle K^+-channel types. Trieschmann *et al.* (1988) demonstrated that diazoxide and cromakalim increased the opening probability of the large conductance Ca^{2+}-dependent K^+ channel in isolated patches from human mesenteric arterial smooth muscle cells. Gelband *et al.* (1988) also showed that cromakalim increased the opening probability of the large conductance Ca^{2+}-dependent K^+ channel from rabbit aorta when isolated into planar lipid bilayers, by decreasing the mean closed time of the channel. In contrast, however, Beech & Bolton (1987) found no effect of cromakalim on the large conductance Ca^{2+}-dependent K^+ channel in isolated smooth muscle cells of rat portal vein by use of the whole cell voltage clamp method. Thus the exact nature of the K^+ channel opened by cromakalim and other K^+ channel openers remains to be resolved.

In the present study, no significant effect of diazoxide, minoxidil sulphate or cromakalim on intracellular cyclic AMP or cyclic GMP concentrations was detected. This suggests that the actions of these substances are independent of changes in these cyclic nucleotides. Previous studies have shown that the relaxant action of cromakalim is independent of changes in cyclic AMP and cyclic GMP in the bovine retractor penis (Gillespie & Sheng, 1988), rabbit mesenteric artery (Coldwell & Howlett, 1988) and rat aorta (Newgreen *et al.*, 1988a,b). The K^+ channel opening drug pinacidil also has no effect on either cyclic AMP or cyclic GMP levels in the rat aorta (Kauffman *et al.*, 1986; Southerton *et al.*, 1988). Conversely, the compound nicorandil has K^+ channel opening properties in rat portal vein (Weir & Weston, 1986) and it increases cyclic GMP concentration in bovine coronary artery (Holzmann, 1983) and rat aorta (Newgreen *et al.*, 1988b). However, these actions are believed to be independent via two separate pathways (Newgreen *et al.*, 1988a,b).

The results of the present study suggest that diazoxide and minoxidil sulphate have K^+ channel opening actions on vascular smooth muscle. However, diazoxide exerts an additional action which is not mediated by changes in cyclic nucleotide concentration. The ion flux experiments in rat aorta suggest that these agents may act on two different K^+ channels, one of which is permeable to Rb and the other relatively impermeable to this ion. The ability of minoxidil sulphate to exert a selective action on a Rb-impermeable channel suggests that the blood pressure lowering effect of minoxidil sulphate (and of cromakalim and diazoxide) may be causally related to this action. Such a conclusion is supported by the results obtained with the K^+ channel opener pinacidil in small rat blood vessels (Videbaek *et al.*, 1988) and is the subject of further investigation.

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Pharmacological evaluation of a guinea-pig tracheal epithelium-derived inhibitory factor (EpDIF)

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1 An epithelium-derived inhibitory factor (EpDIF) released by guinea-pig tracheal epithelium was evaluated in a co-axial bioassay system consisting of an epithelium-intact guinea-pig tracheal tube surrounding endothelium-denuded rat aortic strip.

2 Histamine and several muscarinic agonists induced concentration-dependent relaxation of phenylephrine-contracted rat aorta via the release of EpDIF. However, several other agonists did not induce the release of EpDIF from guinea-pig trachea. These included the nicotinic cholinoceptor agonists nicotine (25 μM), 1,1-dimethyl-4-phenylpiperazinium (DMPP) (25 μM), calcium ionophore A23187 (0.5 μM), bradykinin (0.05–0.5 μM), substance P (5 μM), platelet activating factor (PAF, 1–100 nM), the leukotrienes (LT) LTC_4, LTD_4 and LTE_4 (0.1–10 nM) as well as hyperosmotic stimuli.

3 Prostaglandin E_2 (PGE_2) induced concentration-dependent contraction of endothelium-denuded rat aortic preparations, indicating that this prostanoid could not be EpDIF. Furthermore, relaxation to histamine and methacholine, mediated via EpDIF, was not significantly altered in the presence of phenidone (50 μM) the cyclo-oxygenase/lipoxygenase inhibitor with radical scavenging properties or the cytochrome P-450 inhibitors metyrapone (1 mM) and SKF 525A (25 μM). This suggests that EpDIF is neither a prostanoid nor a cytochrome P-450 metabolite of arachidonic acid.

4 The soluble guanylate cyclase inhibitor, methylene blue (50 μM), caused small but significant increases in the potencies of both histamine and methacholine in co-axial assemblies, indicating that EpDIF did not activate this enzyme and therefore was not NO or a related substance. The β-adrenoceptor antagonist, (−)-propranolol (1 μM), and the PAF-receptor antagonist, WEB 2086 (50 μM), also failed to alter significantly EpDIF-modulated relaxations. These data suggest that EpDIF is neither a stimulant of β-adrenoceptors nor of PAF receptors.

5 The present study provides some evidence that this vascular smooth muscle-sensitive EpDIF may not be related to the putative EpDIF previously hypothesized to modulate directly spasmogen-induced airway smooth muscle tone.

Introduction

Asthma is often associated with non-specific bronchial hyperreactivity to spasmogens (Boushey *et al.*, 1980) as well as with damage to the airway epithelium (Laitinen *et al.*, 1985; Jeffery *et al.*, 1989). It is now well established *in vitro* that the epithelium has a significant role to play in modulating airway smooth muscle responsiveness to spasmogenic and relaxant agonists in several species including man (Barnes *et al.*, 1985; Flavahan *et al.*, 1985; Goldie *et al.*, 1986; Raeburn *et al.*, 1986; Aizawa *et al.*, 1988; Vanhoutte, 1988). It has been suggested that the airway epithelium may secrete an inhibitory factor which can modulate airway smooth muscle responsiveness to various agonists (Flavahan *et al.*, 1985). Thus substantial damage to or loss of airway epithelium, as commonly occurs in asthma (Laitinen *et al.*, 1985; Dunnill, 1987), with consequently reduced production of this inhibitory factor, may lead to increased airway sensitivity to spasmogens.

Direct evidence for the release of an inhibitory factor by airway epithelium has been demonstrated in co-axial bioassay systems consisting of guinea-pig trachea surrounding rabbit aorta (Ilhan & Sahin, 1986), rat anococcygeus muscle (Guc *et al.*, 1988) or rat aorta (Fernandes *et al.*, 1989). In the present study, we have used this co-axial bioassay technique to evaluate some of the pharmacological properties of epithelium-derived inhibitory factor (EpDIF) released by guinea-pig tracheal epithelium.

Methods

Tissue preparation for organ bath experiments

Male Wistar rats (220–250 g) were killed by stunning and exsanguination and the thoracic aorta removed and opened; cross-cut preparations (7–8 mm in length) were obtained as previously described (Fernandes *et al.*, 1989). The endothelium was removed from some vascular preparations with a cotton wool-coated probe. All preparations were suspended under 500 mg tension and equilibrated for 1 h in Krebs solution maintained at 37°C and aerated with 5% CO_2 in O_2. In all experiments, the Krebs solution also contained the cyclo-oxygenase inhibitor indomethacin (5 μM).

Endothelium-denuded rat aortic preparations were challenged with successively lower concentrations of phenylephrine (Phe) between 0.2 and 0.05 μM. The lowest concentration of Phe producing sustained stable, near maximal contractions was 0.05 μM and was used routinely to induce tone in vascular preparations. Preparations were then exposed to histamine (100 μM) or methacholine (25 μM). The presence of significant numbers of endothelial cells was indicated by relaxant responses to histamine and/or methacholine, while successful removal of vascular endothelium was confirmed by the absence of such relaxation. In some experiments, endothelium-intact and endothelium-denuded vascular preparations were precontracted with Phe (0.05 μM) and re-challenged with either histamine (100 μM) or methacholine (25 μM) in the presence or absence of the soluble guanylate cyclase inhibitor methylene blue (50 μM), the cyclo-oxygenase/lipoxygenase inhibitor BW 755C (150 μM), the cyclo-oxygenase/lipoxygenase inhibitor, radical scavenger and antioxidant phenidone (50 μM), the lipoxygenase inhibitor, radical scavenger and antioxidant nordihydroguaiaretic acid (NDGA) (10 μM), the lipoxygenase inhibitor and antioxidant gossypol (30 μM), the leukotriene receptor antagonist FPL 55712 (10 μM), the β-adrenoceptor antagonist (−)-propranolol (1 μM), the platelet activating factor (PAF) receptor antagonist WEB 2086 (50 μM), the cytochrome P-450 inhibitors metyrapone (1 mM) and SKF 525A (25 μM) or the free radical scavenger superoxide dismutase (50 u ml^{-1}). In some experiments, endothelium-denuded

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vessel strips alone, were challenged with prostaglandin E_2 (PGE_2) (0.05–200 μM).

Co-axial bioassay assemblies

Male guinea-pigs (SR/C Tricolour) weighing 450–500 g were killed by cervical dislocation and the trachea removed, dissected free of surrounding tissue and cut into tubular segments approximately 7 mm in length. The epithelium was deliberately removed from some tracheal segments with a cotton wool-coated probe as previously described for tracheal ring preparations (Goldie *et al.*, 1986). Complete removal of the airway epithelium from such preparations was confirmed by histological examination.

Following initial pharmacological verification of the presence or absence of endothelium, aortic preparations were set-up in co-axial assemblies within guinea-pig tracheal tube segments as previously described (Fernandes *et al.*, 1989). After equilibration for 45 min, the vascular preparation was precontracted with Phe (0.05 μM) and re-challenged with histamine (5–200 μM), methacholine (1–200 μM), acetylcholine (1–200 μM), carbachol (1–200 μM), bethanechol (1–200 μM), arecoline (5–500 μM), oxotremorine (5–500 μM), nicotine (25 μM), 1,1-dimethyl-4-phenylpiperazinium (DMPP) (25 μM), calcium ionophore A23187 (0.5 μM), bradykinin (0.05–0.5 μM), substance P (5 μM), PAF (1–100 nM), leukotriene C_4 (LTC_4), LTD_4, or LTE_4 (0.1–10 nM), urea (160 mM) or mannitol (160 mM).

The effects of single selective concentrations of methylene blue (50 μM; Martin *et al.*, 1985), BW 755C (150 μM), phenidone (50 μM), NDGA (10 μM) gossypol (30 μM), metyrapone (1 mM), SKF 525A (25 μM) (Forstermann *et al.*, 1988), FPL 55712 (10 μM; Jancar *et al.*, 1987), propranolol (1 μM), WEB 2086 (50 μM), or superoxide dismutase (50 u ml^{-1}) on responses to histamine (100 μM) and methacholine (25 μM) in co-axial bioassay systems were examined as previously described. In addition, the effects of methylene blue (50 μM) or phenidone (50 μM) on responses to histamine (5–200 μM) or methacholine (1–200 μM) were determined. The effects of the neutral endopeptidase inhibitor phosphoramidon (1 μM; Fine *et al.*, 1989) on responses to histamine (5–200 μM) were also examined.

Analysis of results

Unless otherwise stated, numerical data are presented as mean ± standard error of the mean (± s.e.mean). The potencies of agonists causing concentration-dependent responses of rat aortic preparations are expressed as pD_2 values or as molar EC_{50} values, i.e. concentrations producing 50% of the maximum response, derived from mean estimates of pD_2 where $pD_2 = -\log_{10}(EC_{50})$. Relaxation response data are expressed either as % reductions in the level of vascular tone induced by phenylephrine (0.05 μM), or as % E_{max} values, where E_{max} = maximal reduction in the level of tone induced by Phe (0.05 μM). The probability of differences between mean results was determined with Student's two-tailed t test (paired or non-paired) and was considered significantly different if $P < 0.05$. The effects of enzyme inhibitors on the potency of agonists were assessed as mean shifts (with 95% confidence limits) derived from paired data i.e. from pD_2 values for the agonists obtained in the presence and absence of the antagonist.

Drugs

Drugs used were: A23187, acetylcholine chloride, arecoline hydrobromide, bethanechol chloride, carbachol chloride, methacholine chloride, oxotremorine sesquifumarate, bradykinin acetate, gossypol-acetic acid, histamine diphosphate, indomethacin, metyrapone, nordihydroguaiaretic acid, PAF (L-α-phosphatidylcholine β-acetyl-γ-O-alkyl), phenidone, phenylephrine hydrochloride, phosphoramidon, prostaglandin E_2, substance P (Sigma); BW 755C (3-amino-1[*m*-(trifluoromethyl)-phenyl]-2 pyrazoline, Wellcome); DMPP (1,1-dimethyl-4-phenylpiperazinium, ICN-K&K Lab.); WEB 2086 (3-(4-(2-chlorophenyl)-9-methyl-6H-thieno-3,2-f),(1,2,4)-triazolo-(4,3-a),(1,4)-diazepine-2-yl)-1-(4-morpholinyl)-1-propanone, Boehringer Ingelheim); FPL 55712 (7-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid, Fisons); mannitol, urea (analytical grade, Ajax Chem.); methylene blue (Gurr); nicotine (B.D.H. Lab. Chem.); (−)-propranolol hydrochloride (I.C.I.); LTC_4, LTD_4, LTE_4, Proadifen (SKF 525A, Smith Kline, Beecham Pharmaceuticals); superoxide dismutase (Boehringer Mannheim).

All drugs tested in organ bath studies were prepared daily in 0.9% w/v NaCl solution (saline). BW 755C and FPL 55712 were prepared in distilled water. Prostaglandin E_2 was prepared in absolute ethanol. Gossypol was prepared in dimethylsulphoxide (DMSO). Indomethacin was prepared in 5 mg ml^{-1} sodium carbonate solution. PAF was obtained dissolved in chloroform. More dilute solutions of PAF were prepared in 0.25% bovine serum albumin (BSA; Sigma) in polypropylene containers. The Krebs solution used throughout the study had the following composition (mM): NaCl 117.6, KCl 5.4, $NaHCO_3$ 25, KH_2PO_4 1.03, $MgSO_4$ 0.57, D-glucose 11.1 and $CaCl_2$ 2.5.

Results

Neither endothelium-intact nor endothelium-denuded rat aorta preparations relaxed significantly in response to the β-adrenoceptor agonist (−)-isoprenaline (5 μM), indicating the absence of natural tone. Phe (0.05 μM) increased baseline tone (233 ± 8 mg; $n = 42$) which could be reduced in a concentration-dependent manner by (−)-isoprenaline (pD_2 = 7.77 ± 0.05; E_{max} = 113 ± 6%; $n = 8$ preparations). In endothelium-intact aortic strips, histamine (100 μM) and methacholine (25 μM) reduced Phe-induced tone by 81 ± 3% ($n = 19$ preparations from 11 animals) and 79 ± 4% ($n = 13$ preparations from 8 animals), respectively. These responses were abolished in the presence of the soluble guanylate cyclase inhibitor, methylene blue (50 μM), indicating that relaxation had been caused by the release of endothelium-derived relaxant factor (EDRF). Relaxation responses caused by this factor were enhanced by superoxide dismutase. In sharp contrast, endothelium-denuded rat aorta failed to respond to histamine or methacholine, thereby confirming the absence of endothelial cells. PGE_2 caused concentration-dependent contraction in endothelium-denuded rat aorta preparations and was therefore unlikely to be EpDIF. The mean pD_2 value obtained to this spasmogen was 6.01 ± 0.09 and the mean E_{max} value was 234 ± 23 mg ($n = 8$ preparations).

Effect of agonists in co-axial bioassay assemblies

When epithelium-intact guinea-pig tracheal tube segments were used as EpDIF donor preparations in a co-axial bioassay system, endothelium-denuded rat aorta, precontracted with Phe (0.05 μM) relaxed in a concentration-dependent manner in response to histamine and the muscarinic cholinoceptor agonists methacholine, acetylcholine, carbachol, bethanechol, oxotremorine and arecoline. While pD_2 values obtained for acetylcholine, carbachol, bethanechol and methacholine were similar, oxotremorine and arecoline were approximately 25 and 50 fold less potent respectively, than these other agonists. All of these agonists induced maximal or near maximal relaxation of Phe-induced tone (Table 1). Removal of the tracheal EpDIF donor preparations or the use of epithelium-denuded tracheal tubes as donor tissue, resulted in abolition of relaxation responses to histamine and methacholine.

In contrast, several agents failed to cause reductions in Phe-induced tone in rat aortic strips in co-axial assemblies. These

Table 1 Relaxant effects of histamine and various muscarinic agonists in co-axial assemblies

Agonist	pD_2	EC_{50}[a] (μM)	E_{max}[b] (%)	n
Histamine	5.04 ± 0.04	9.1	84 ± 5	15
Methacholine	5.69 ± 0.09	2.0	79 ± 6	14
Acetylcholine	5.52 ± 0.12	3.0	89 ± 5	10
Carbachol	5.76 ± 0.05	1.7	99 ± 7	10
Bethanechol	5.68 ± 0.08	2.1	90 ± 5	10
Oxotremorine	4.30 ± 0.33	50	88 ± 4	6
Arecoline	4.00 ± 0.09	100	105 ± 5	5

Data are expressed as mean ± s.e.mean. n = the number of preparations tested. [a] EC_{50} values are derived from mean pD_2 values. [b] E_{max} = maximal reduction in the level of tone induced by Phe (0.05 μM).

included nicotine (25 μM), DMPP (25 μM), calcium ionophore A23187 (0.5 μM), bradykinin (0.05–0.5 μM), substance P (5 μM), PAF (1–100 nM), LTC_4, LTD_4 and LTE_4 (0.1–10 nM), as well as urea (160 mM) and mannitol (160 mM). These agents were also found not to alter significantly Phe (0.05 μM)-induced tone in isolated endothelium-denuded aorta preparations in the absence of EpDIF donor tissue.

Effect of non-agonists in co-axial assemblies

Various enzyme inhibitors, receptor antagonists, radical scavengers and antioxidants were used in an attempt to modify the activity of EpDIF released in response to histamine or methacholine. The effect of these agents on the level of Phe-induced tone in endothelium-denuded rat aorta preparations alone, was also determined. BW 755C (150 μM), metyrapone (1 mM) and SKF 525A (25 μM) caused significant reductions in Phe-induced tone of between 25 and 45% (n = 12–14 preparations, $P < 0.05$, paired t test), while the remainder had no significant effect (n = 10–16 preparations, $P > 0.05$, paired t test).

The inhibitor of soluble guanylate cyclase, methylene blue (50 μM), abolished EDRF-induced relaxation, but failed to inhibit EpDIF activity induced by either histamine (100 μM) or methacholine (25 μM) (Table 2). Co-axial bioassay experiments were routinely performed in the presence of the cyclo-oxygenase inhibitor, indomethacin (5 μM). In addition, BW 755C (150 μM), phenidone (50 μM), NDGA (10 μM) and gossypol (30 μM) all failed to inhibit EpDIF activity (Table 2). The leukotriene receptor antagonist, FPL 55712 (10 μM), the β-adrenoceptor antagonist, propranolol (1 μM) and the PAF receptor antagonist, WEB 2086 (50 μM), all failed to inhibit EpDIF-induced vascular relaxation (Table 2). The cytochrome P-450 inhibitors, metyrapone (1 mM) and SKF 525A (25 μM), were also ineffective against EpDIF activity (Table 2).

In another series of experiments, concentration-effect curves to both histamine and methacholine were constructed in co-axial assemblies in the absence and presence of methylene blue (50 μM) or phenidone (50 μM) (Table 3). Methylene blue increased the potencies of histamine and methacholine by 3.1 fold (95% confidence limits: 1.9–4.9; n = 10 preparations) and 6.6 fold (95% confidence limits: 3.9–11.2; n = 9 preparations) respectively, while phenidone failed to alter the potencies of either agonist (Table 3). In addition, the maximal relaxant effects of histamine and methacholine (84 ± 7% and 76 ± 8%, respectively) were increased significantly in the presence of methylene blue (109 ± 14%, n = 10 and 115 ± 12%, n = 9, respectively; $P < 0.05$, paired t test). Phenidone however, was without significant effect in this regard.

Concentration-effect curves to histamine were also constructed in co-axial assemblies in the absence and presence of phosphoramidon (1 μM) to determine any effects of this neutral endopeptidase inhibitor on histamine potency and maximal relaxant effect. Potency values obtained to histamine in the absence and presence of phosphoramidon (5.02 ± 0.09 and 5.12 ± 0.07, respectively), were not significantly different ($P > 0.1$, paired t test; n = 9 preparations). Similarly, E_{max} values obtained to this agonist in the absence (84 ± 4) and presence (100 ± 10) of phosphoramidon were not significantly altered ($P > 0.1$).

In addition, superoxide dismutase did not significantly alter the relaxation response mediated by EpDIF. In co-axial assemblies, histamine (100 μM) caused mean relaxations of 66 ± 10% or 75 ± 15% in the absence or presence of superoxide dismutase (50 u ml^{-1}) respectively (n = 5 preparations, $P > 0.2$, paired t test).

Table 2 Effect of various agents on relaxant responses to histamine (100 μM) or methacholine (25 μM) in co-axial assemblies

	Histamine (100 μM)		*Methacholine (25 μM)*	
	Control	*Test (+ agent)*	*Control*	*Test (+ agent)*
Methylene blue (50 μM)	77 ± 7	71 ± 3† (8)	104 ± 5	96 ± 11† (10)
BW 755C (150 μM)	70 ± 11	84 ± 10† (7)	73 ± 7	73 ± 8† (6)
Phenidone (50 μM)	94 ± 20	108 ± 7† (6)	93 ± 8	91 ± 4† (6)
NDGA (10 μM)	90 ± 7	85 ± 7† (6)	103 ± 10	93 ± 6† (10)
Gossypol (30 μM)	74 ± 4	82 ± 5† (6)	90 ± 7	101 ± 9† (7)
FPL 55712 (10 μM)	80 ± 8	74 ± 6† (8)	85 ± 10	92 ± 9† (9)
Propranolol (1 μM)	97 ± 7	116 ± 8† (5)	97 ± 7	99 ± 5† (5)
WEB 2086 (50 μM)	74 ± 8	71 ± 7† (6)	94 ± 7	94 ± 9† (7)
Metyrapone (1 mM)	89 ± 7	83 ± 9† (7)	98 ± 7	81 ± 5† (5)
SKF 525A (25 μM)	86 ± 5	87 ± 6† (7)	75 ± 9	74 ± 11† (6)

Data are expressed as mean (± s.e.mean) % reductions in the level of Phe (0.05 μM)-induced tone. n = the number of paired observations.
† Relaxation response not significantly different from corresponding control response ($P > 0.05$, paired t test).

Table 3 Effect of methylene blue and phenidone on the relaxant potency (pD_2) of histamine and methacholine in co-axial assemblies

	Control	*pD_2*	*Test (+ agent)*	*Ratio¶*
Histamine				
Methylene blue (50 μM)	5.06 ± 0.06	(10)	5.55 ± 0.11*	3.1
Phenidone (50 μM)	4.99 ± 0.04	(5)	4.89 ± 0.08†	0.8
Methacholine				
Methylene blue (50 μM)	5.53 ± 0.07	(9)	6.35 ± 0.09*	6.7
Phenidone (50 μM)	5.45 ± 0.06	(6)	5.82 ± 0.18†	2.3

Data are expressed as mean ± s.e.mean. Numbers in parentheses indicate the number of paired observations.
¶ Ratio of EC_{50} values = EC_{50} Control: EC_{50} Test.
* pD_2 value significantly different from control value ($P < 0.05$, paired t test).
† pD_2 value not significantly different from control ($P > 0.05$, paired t test).

Discussion

In a co-axial bioassay system containing epithelium-intact guinea-pig trachea, histamine as well as the muscarinic agonists methacholine, acetylcholine, carbachol, bethanechol, oxotremorine and arecoline caused concentration-dependent relaxation of assay preparations via the release of EpDIF. Oxotremorine is a muscarinic agonist equal in potency to acetylcholine, but with no apparent nicotinic activity (Cho *et al.*, 1962). In contrast, arecoline has a higher intrinsic activity than acetylcholine, but is approximately 16 times less potent than acetylcholine in guinea-pig isolated ileum (Gloge *et al.*, 1966). In this study, however, oxotremorine and arecoline were found to be approximately 17 and 33 times less potent respectively than acetylcholine in inducing the release of EpDIF from guinea-pig trachea. Removal of the epithelium from EpDIF donor airway tissue resulted in abolition of this relaxation indicating that these responses were due to the release of EpDIF. Fernandes *et al.* (1989) showed that histamine- and methacholine-induced release of EpDIF was mediated via histamine H_1-receptors and muscarinic cholinoceptors respectively.

Prostaglandin E_2 is the major prostanoid produced by the epithelium in response to mediators such as major basic protein (Jacoby *et al.*, 1988). This prostanoid can reduce airway smooth muscle contraction (Barnett *et al.*, 1988; Manning *et al.*, 1989). In the present study however, PGE_2 caused concentration-dependent contraction of assay tissue and has previously been shown to induce further contraction of precontracted endothelium-denuded rat aorta preparations (Hay *et al.*, 1989), indicating that this prostanoid could not be the EpDIF detected in our co-axial bioassay.

The present studies were routinely performed in the presence of indomethacin (5 μM) in order to inhibit cyclo-oxygenase activity. In addition, BW 755C (150 μM), phenidone (50 μM), NDGA (10 μM) and gossypol (30 μM) did not inhibit EpDIF activity in this system, indicating that this inhibitory factor is neither a cyclo-oxygenase nor a lipoxygenase product and is apparently unaffected by radical scavengers and antioxidants.

The failure of methylene blue (50 μM) to inhibit relaxation responses to histamine (100 μM) and methacholine (25 μM) in co-axial assemblies, indicates that EpDIF does not act via stimulation of soluble guanylate cyclase. However, EpDIF-induced relaxation responses have been associated with increases in guanosine 3':5'-cyclic monophosphate levels, perhaps generated via membrane-bound guanylate cyclase (Hay *et al.*, 1989). The reasons for methylene blue-induced increases in the potencies and maximal relaxant effects of histamine and methacholine are unclear but presumably do not involve inhibition of soluble guanylate cyclase. The present study also showed that EpDIF was not a stimulant of leukotriene receptors, β-adrenoceptors or PAF receptors.

Superoxide dismutase (50 u ml^{-1}) caused enhancement of EDRF-induced relaxation responses of rat aorta but failed to alter those induced by EpDIF, indicating that the latter was apparently unaffected by superoxide radicals. Phosphoramidon, a neutral endopeptidase inhibitor was similarly ineffective in altering EpDIF activity in this system. If EpDIF was a peptide substrate for this enzyme, phosphoramidon might have been expected to alter its relaxant activity.

Removal of the epithelium has been shown to result in increased sensitivity of guinea-pig tracheal preparations to substance P (Tschirhart & Landry, 1986), LTC_4 and LTD_4 (Hay *et al.*, 1987; Hisayama *et al.*, 1988). While the increased sensitivity to substance P observed in epithelium-denuded guinea-pig tracheal preparations may be attributed to the loss of a metabolic site for this spasmogen (Fine *et al.*, 1989), it has been suggested that the leukotrienes may stimulate the production of an airway smooth muscle-sensitive inhibitory factor by the epithelium (Hay *et al.*, 1987; Hisayama *et al.*, 1988). However, stimulation of EpDIF release from guinea-pig trachea appears to be selective for some agonists including histamine and methacholine, whereas A23187 and hyperosmotic stimuli, stimulation of nicotinic cholinoceptors and receptors for bradykinin, substance P, PAF and the leukotrienes, all failed to induce EpDIF release that could be detected in this system. Furthermore, inhibition of cytochrome P-450 by metyrapone (1 mM) or SKF 525A (25 μM) failed to inhibit EpDIF-induced relaxation responses. Thus, while this enzyme system has been linked to epithelium-dependent modulation of smooth muscle contractility (Raeburn *et al.*, 1988), it was not involved in the production of EpDIF in the present system. These data suggest that the vascular smooth muscle-sensitive EpDIF detected with our co-axial bioassay was not the same factor as has been suggested to modulate airway smooth muscle tone.

It is important to note that several studies involving removal of the epithelium have shown that the potency of carbachol in isolated airway preparations was not significantly altered (Frossard & Muller, 1986; Goldie *et al.*, 1986; Tschirhart *et al.*, 1987; Hisayama *et al.*, 1988; Lundblad & Persson, 1988), suggesting that this agonist did not stimulate the production of an airway smooth muscle-sensitive EpDIF by the epithelium. However, the present study has demonstrated that carbachol induced EpDIF release that can be detected by a co-axial bioassay system. It may be that the co-axial bioassay is a more sensitive means of assessing the influence of cholinoceptor agonists on the epithelium than is the measurement of airway smooth muscle responsiveness before and after epithelium removal. Alternatively, the epithelium may secrete at least two inhibitory factors, one which selectively modulates airway smooth muscle tone (Vanhoutte, 1988) and one which modulates vascular smooth muscle tone (Vanhoutte, 1988; Fernandes *et al.*, 1989; the present study).

This study has evaluated an inhibitory factor released by guinea-pig tracheal epithelium in response to histamine and muscarinic agonists that is capable of relaxing vascular smooth muscle. The primary target for this, as yet unidentified, EpDIF may be the airway wall microcirculation. Increased bronchial blood flow might protect against severe bronchoconstriction by increasing the rate of removal of spas-

mogens from the airway wall. Reduction in EpDIF activity due to damage to the airway epithelium, as seen in asthma (Laitinen *et al.*, 1985; Dunnill, 1987) may therefore contribute towards bronchial hyperreactivity to spasmogens which is commonly associated with this disease (Boushey *et al.*, 1980; Jeffery *et al.*, 1989).

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The properties of the ATP-induced depolarization and current in single cells isolated from the guinea-pig urinary bladder

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1 The actions of exogenously applied ATP were investigated with the whole-cell patch clamp method in single cells isolated from guinea-pig urinary bladder with a modified concentration jump technique.

2 Rapid application of ATP (threshold ca. 100 nM) depolarized the cell membrane with superimposition of action potentials which was followed by transient hyperpolarization. In the presence of D600, the amplitude of the ATP-induced depolarization was a function of the ATP concentration (EC_{50}: 0.5–1 μM).

3 ATP activated a dose-dependent inward current with a short latency (18 ms with 10 μM ATP; measured as the time between the start of application and 10% of the peak). The relationship of the peak current versus ATP concentration was well fitted by a Michaelis-Menten equation with a Hill coefficient (n) of 1.7 and a dissociation constant (K_d) of 2.3 μM. The current desensitized rapidly and the time course of desensitization was a function of the ATP concentration and could be fitted by two exponentials.

4 The reversal potential of the ATP-activated current was near 0 mV. Replacement of extracellular Na by other monovalent or divalent cations indicated that the current flows through nonselective cation channels.

5 α,β-Methylene ATP also produced a dose-dependent inward current but was less potent than ATP (n: 1.6, K_d: 10.4 μM). α,β-Methylene ATP blocked the response to ATP by desensitization of the receptor.

6 α,β-Methylene ATP was 50–100 times more potent than ATP at eliciting a contractile response of strips of detrusor smooth muscle.

7 The relevance of the above results to the possible role of ATP as the fast excitatory transmitter is discussed.

Introduction

Since the first recognition of the atropine-resistant component of the parasympathetic nerve stimulated contraction in bladders of several species (Langley & Anderson, 1885), much work has been done to establish the nature of the non-adrenergic, non-cholinergic transmitter, and there is now considerable evidence that adenosine 5'-triphosphate (ATP) is an excitatory transmitter in the urinary bladder of small mammals. Firstly, the atropine-resistant contraction evoked by nerve stimulation can be mimicked by application of ATP (Burnstock, 1972) and both responses are blocked by quinidine. Secondly, the recently synthesized ATP analogue, α,β-methylene ATP, which is resistant to hydrolysis and has been shown to activate and desensitize P_{2x} purinoceptors, not only abolishes ATP-induced contraction but also the atropine-resistant response to excitatory nerve stimulation (Kasakov & Burnstock, 1983). Thirdly, excitatory junction potentials (e.j.ps) have been recorded with microelectrodes which show similar pharmacological properties to atropine-resistant nerve induced contractions (Creed *et al.*, 1983; Brading & Mostwin, 1989). Furthermore, use of the double-sucrose gap technique (Hoyle & Burnstock, 1985; Fujii, 1988) which allows simultaneous recording of contractions and e.j.ps, has confirmed that the e.j.ps are atropine resistant and abolished in parallel with the contractile response, on desensitization of the receptors with α,β-methylene ATP.

By use of the whole-cell patch clamp technique in isolated cells, the actions of ATP on smooth muscle membranes have been characterized in a number of preparations where non-cholinergic, non-adrenergic neurotransmission is known to occur (ear artery: Benham *et al.*, 1987; Benham & Tsien, 1987; vas deferens: Nakazawa & Matsuki, 1987; Friel, 1988; rat uterus: Honoré *et al.*, 1989). In these preparations, ATP depolarized the membrane by activation of non-selective cation channels (Benham & Tsien, 1987) with a relatively short latency (about 70 ms, Benham *et al.*, 1987).

The purpose of the present experiments was (1) to test whether ATP has the necessary properties to be the transmitter responsible for the fast excitatory neurotransmission in the guinea-pig bladder, with regard to the time course of ATP-induced depolarization and current studied with a concentration clamp technique (Krishtal & Pidoplichko, 1980), and (2) to compare the properties of the ATP-induced current with those described for other preparations. The effects of α,β-methylene ATP have also been examined. Preliminary results have appeared in abstract form (Inoue, 1990).

Methods

Cell preparation

Guinea-pigs of either sex, weighing between 450–750 g were killed either by stunning or by cervical dislocation and exsanguinated. Immediately after the abdominal cavity had been opened, the whole urinary bladder was excised and transferred into a dissecting dish filled with divalent cation-free Krebs solution. The mucosa was removed and small pieces of smooth muscle were removed from the detrusor and preincubated for 5 min in Ca-free solution. With constant spinning by a magnetic stirrer, pieces of the muscle were enzymatically digested in Ca-free Krebs solution containing 1–2 mg ml^{-1} collagenase and 0.5 mg ml^{-1} pronase at 36°C for 20–30 min. Sometimes the digested pieces were further agitated by a blunt tipped pipette or by spinning until sufficient cells were yielded. Isolated cells were stored in modified Krebs solution (Klöckner & Isenberg, 1985a) and were normally used within 3 h.

Whole-cell recording

The experimental set-up used was similar to that described previously (Hamill *et al.*, 1981; Inoue *et al.*, 1987). Briefly, patch electrodes (3–5 GΩ with KCl internal solution) were made from borosilicate glass capillary (GC150-15 225 pcs: Clark Electromedical Instruments, U.K.) with a Narishige

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puller (Tokyo, Japan). All input voltage signals were generated by a Neurolog stimulator (Digitimer Ltd, Welwyn Garden City, U.K.) and applied to the clamped cell through an EPC7 patch amplifier (List electronic, Darmstadt, West Germany). The corresponding current and membrane potentials were sampled through the amplifier with filtering (10 kHz, 3-pole Bessel), and then stored on video tape (Panasonic AG-6200, Osaka, Japan) after digitizing (16 bit resolution, Sony PCM-701, Tokyo, Japan) for later analysis, along with the voltage signals used to operate the electromagnetic valve in the concentration clamp apparatus (see below). The data were evaluated by replaying the video tape recorder into a storage oscilloscope (12 bit resolution, DSO1602, Gould Electronics Ltd., Instrument System, Hainault, Ilford, U.K.), and records were made by a brush pen recorder (Gould). Statistical values are all expressed as means ± s.e.mean.

Application of solutions

The small recording chamber (volume 0.8 ml) was constantly perfused with control solution (usually Krebs) at a rate of 0.03–0.04 ml s^{-1}. A diagram of the device used for the concentration clamp experiments is shown in Figure 1a. The device is essentially very similar to that described by Krishtal & Pidoplichiko (1980). A thin polyethylene tube (1 mm outer and 0.8 mm inner diameter) with a small hole of 200–500 μm in diameter was placed at one side of the chamber. One end of the polyethylene tube was connected to a reservoir containing test solution and an electromagnetic valve (normally closed, and opened by a 9 V signal within 3 ms, Lee Interface Fluidic, Gerrards Cross, U.K.) was used to stop the flow of the test solution. To avoid any leak of the test solution, the electromagnetic valve was placed at the same level as the hole. After the patch pipette seal with the cell had been made and the voltage-clamp condition established, the cell was lifted up from the bottom of the chamber and transferred to the side of the hole (within 100 μm). When the valve was opened, the test solution flowed rapidly onto the cell membrane. The rate of solution change depended on the hydrostatic pressure between the test solution and the hole. With a high hydrostatic pressure, the time constant of solution change could be shortened to about 10 ms (see Figure 1b). After closure of the valve, the test solution was quickly washed away by the constant flow of Krebs solution in the chamber, taking about 100 ms.

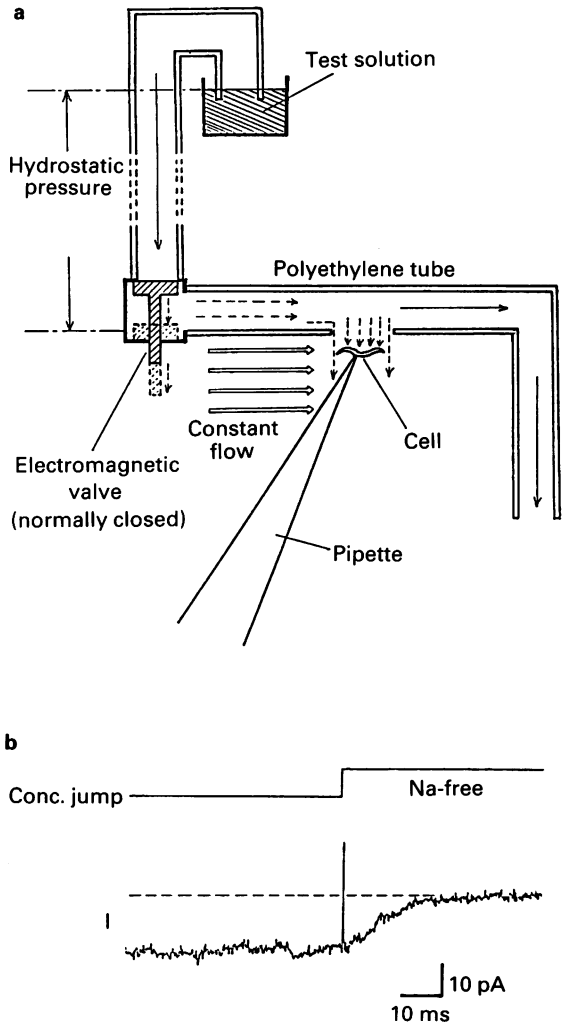

Figure 1 The modified concentration jump apparatus. (a) A diagram of apparatus. To reduce the dead space between the polyethylene tube and a clamped cell to minimum, the test solution was run out of the hole about 1 min before its application keeping the cell aside. (b) An example of a concentration jump experiment, where the effect of Na free (Tris replacement) solution was examined on the leak current (about −15 pA in this case at −100 mV) by setting the hydrostatic pressure to 65 cmH_2O in (a).

The composition of the Krebs solution was (mM): Na^+ 140, K^+ 6, Ca^{2+} 2, Mg^{2+} 1.2, Cl^- 152.4, glucose 10, HEPES/Tris 10 (pH 7.35–7.4). For Ca-free solution, Ca^{2+} and Mg^{2+} were simply omitted from the Krebs solution. In experiments to test the ionic selectivity of the ATP-activated channels to monovalent cations, extracellular Na^+ was replaced by equimolar monovalent cations (K^+, Li^+ and Cs^+) and Ca^{2+} was replaced by Mg^{2+} since the channels are less permeant to Mg^{2+} than Ca^{2+} (in the presence of 102 mM extracellular Mg^{2+} an inward current was induced by ATP, but it was too small to estimate its reversal potential). Divalent ion permeability was tested by replacing Na^+ with isosmotic divalent cations (Ca^{2+} or Ba^{2+}) and Mg^{2+} was removed. Then, reversal potentials were estimated as the intercepts of the voltage axis of the I-V relationship of the ATP-activated current, either by interpolation or by extrapolation. For the internal pipette solution, high KCl solution was used (mM): KCl 140, $MgCl_2$ 2, HEPES, 10, EGTA 10 μM, titrated to pH 7.2 with Tris base. In some experiments KCl was replaced by CsCl and 10 mM EGTA was used. Experiments were carried out at room temperature (20–25°C).

Contraction studies

Tension experiments were carried out on bladder smooth muscle strips measuring 1 × 1 × 7 mm, mounted in small organ baths (Brading & Sibley, 1983) and continuously superfused with Krebs solution at 36°C. Tissues were set up at an initial tension of 0.5 g weight, and tension was recorded isometrically with Pioden transducers and a Watanabe pen recorder. After an initial equilibration of at least 1 h, contractile responses to a 10 s application of 10^{-4} M carbachol were recorded, and the response to other agents is expressed as percentage of this control response.

Drugs and chemicals

The following chemicals were used: ATP, α,β-methylene ATP (Sigma, St. Louis, U.S.A.), D600 (methoxyverapamil, Knoll AG, West Germany), diltiazem (Tanabe, Osaka, Japan) Collagenase (Type I, Sigma), pronase (type E, Serva, Heidelberg, West Germany). Solutions containing these agents were freshly made just before use.

Results

The properties of single isolated cells from guinea-pig urinary bladder

Single cells isolated from guinea-pig detrusor had an input resistance of 2.7 ± 1.2 ($n = 20$) GΩ and a capacitance of

48.9 ± 14.1 pF ($n = 30$) when recorded with a pipette containing KCl internal solution. The membrane time constant evaluated by inward current injection was about 90 ms (88.3 ± 32.2, $n = 10$) at the resting membrane potential. These values are very similar to those described by Klöckner & Isenberg (1985a). The membrane potential of the cells ranged between −35 and −54 mV (−49.3 ± 6.7, $n = 14$) and the cells generated spontaneous action potentials with overshoot and after-hyperpolarization (Figure 2a). The size and duration of the action potential compares well with those obtained in multicellular preparations (Brading & Mostwin, 1989; Fujii, 1988), suggesting that the properties of single cells are not much changed by dispersion.

The effect of ATP on the membrane potential

When ATP (over 100 nM) was applied, the membrane depolarized rapidly, and action potentials were superimposed on the depolarization (Figure 2a). If only a single action potential was evoked, it was followed by an after-hyperpolarization (−65 to −70 mV, 1–2 s in duration) even in the continued presence of ATP (Figure 2a and b). The shape of this ATP-induced depolarization and hyperpolarization resembles the e.j.p. observed in multicellular preparations (Brading & Mostwin, 1989). When action potentials were abolished by addition of Ca antagonists to block Ca inward current (Klöckner & Isenberg, 1985b), the ATP-induced depolarization was isolated. Figure 3Aa shows recordings obtained in the presence of D600 and compares the amplitude and time course of ATP-induced depolarizations at various concentrations of ATP. The threshold concentration for depolarization occurred below 100 nM ATP, and the amplitude and time course were increased and accelerated by raising the concentration of ATP applied. Figure 3Ab summarizes the properties of the ATP-induced depolarization; half-maximal depolarization (about 18 mV) was observed between 0.5 and 1 μM ATP in spite of a large variation in the absolute values. For better comparison, examples from the same cells are marked with different symbols after normalization (○ and ×). These values nearly superimpose on the mean values (●). The time course of the ATP-induced depolarization was similar to that of ATP-activated current recorded near the resting membrane potential from the same cell (Figure 3B). At 10 μM ATP both ATP induced-depolarization and current were activated with a very short latency and rise time (18.4 ± 8.2 ms and 22.8 ± 7.4 ms measured with the current, respectively ($n = 8$)) and lasted about 200 ms. This similarity suggests that the depolarization is produced by an inward current. Although the time course of the ATP-induced depolarization may be complex due to the voltage-dependence of the membrane time constant, there was a consistent tendency for the lower concentrations of ATP to cause a slower repolarization from the

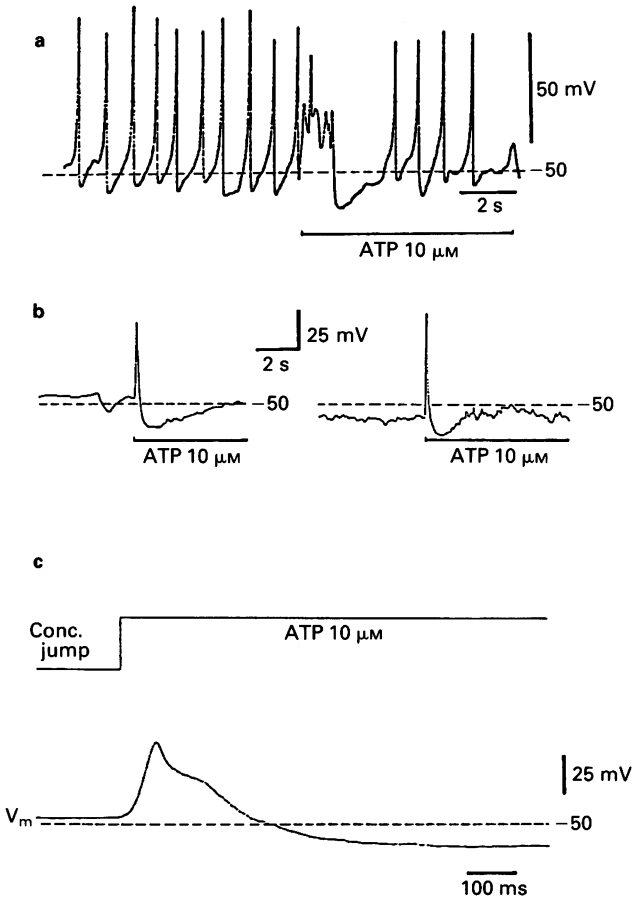

Figure 2 Current clamp recording from single isolated detrusor cells. KCl solution (10 μM EGTA) was used in the pipette. The bath contained Krebs solution. The effect of ATP on cells generating spontaneous action potentials frequently (a) and less frequently (b). (c) The ATP-induced depolarization on an expanded time scale. Note a notch on the depolarization which was not observed in the presence of Ca antagonist, thus indicating superimposition of an action potential.

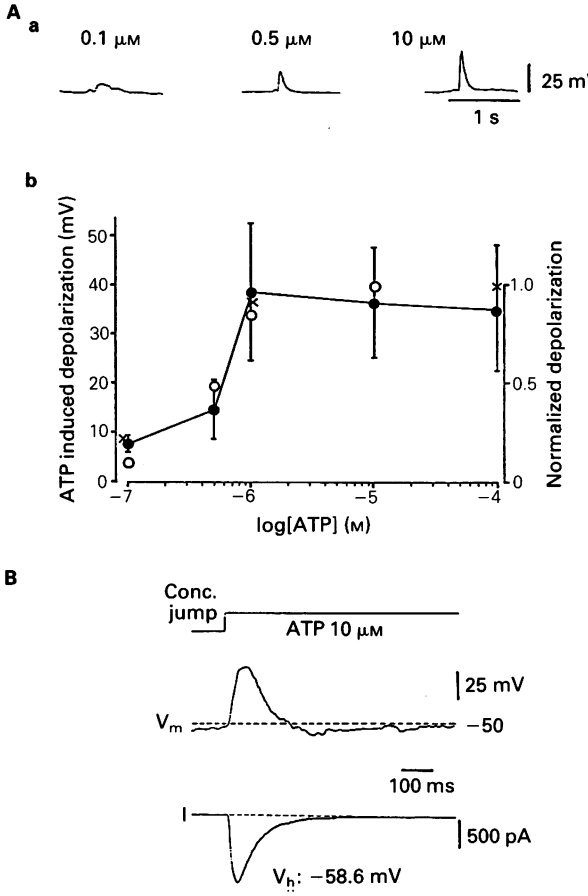

Figure 3 The ATP-induced depolarization recorded in the presence of Ca antagonist (D600, 10 μM). The recording conditions were the same as in Figure 2. (A,a) example of the ATP induced depolarization at three different concentrations of ATP (0.1, 0.5 and 10 μM) from the same cell. The duration of the depolarization was about 700, 350 and 250 ms for 0.1, 0.5 and 10 μM, respectively. (A,b) The concentration-depolarization relationship for ATP. (●) Mean obtained by averaging the absolute values of depolarization from more than 5 records and vertical lines represent s.e.mean. (○) and (×) indicate the depolarization normalized by the maximum for each cell. (B) The time course of ATP-induced depolarization and current recorded from the same cell in the presence of 10 μM D600. Latency and rise time were defined as the time between opening of the electromagnetic valve and the point at 10% of the peak of the ATP-activated current, and the time spent between 20 to 80% of the peak amplitude, respectively (see text). The pipette contained KCl internal solution.

peak, a feature which was also seen with the ATP-activated currents as will be described in the following section.

Concentration-dependence of the ATP-activated inward current

When various concentrations of ATP were applied, the evoked currents increased in amplitude and in rates of activation and desensitization in a concentration-dependent manner. The ATP-activated inward current was not essentially different when the pipette was filled with a solution in which K was replaced with Cs or the concentration of EGTA was raised to 10 mM in order to exclude any contaminating K current (Benham *et al.*, 1987; Friel, 1988). Figure 4a compares the time course of the ATP-activated currents using this pipette solution at four different ATP concentrations from the same cell. ATP 100 nM was the threshold concentration to produce an inward current, and the peak amplitude was produced with 10 μM ATP (1.5 nA at −60 mV on average; (●) in Figure 4b), but the rate of desensitization increased further by raising the concentration of ATP to 100 μM. The relationship of the normalized peak amplitude vs. ATP concentration is well fitted by a Michaelis Menten equation with a Hill coefficient of 1.7 and a dissociation constant of 2.3 μM ((○) in Figure 4b). A Hill coefficient of over 1.0 indicates some sort of cooperative action and more than one molecule of ATP needs to bind to the receptor to open the channel. This is consistent with observations obtained for atrial cells and rat vas deferens (Friel & Bean, 1988; Friel, 1988). The time course of desensitization of the ATP-activated current is also greatly affected by the concentration of ATP. This process was apparently well described by two exponentials (Figure 4c). The fast and the slow time constants were found to be 66.9 ± 12 ms and 480.0 ± 207 ms (n = 7), at −60 mV with 10 μM ATP. The contribution of the fast component became greater when the concentration of ATP was raised. With a saturating concentration of ATP (≥10 μM), the contribution of the fast component was more than 85% of the peak current (e.g., compare the 0 time interpolarions of fast and slow components in Figure 4c). This means that after desensitization of the fast component, the amplitude of the ATP-activated current would be greatly reduced. The fast time constant has not been clearly recognized in previous studies. This is probably due to the use of a less rapid solution change, because in early stages of our experiments, the time constant of the solution change was about 100 ms and the fast component was often absent. This would result in an underestimation of the peak amplitude of the ATP-activated current and would interfere with the accurate estimation of the dose-response curve for ATP. This problem might also influence the evaluation of the potency of other agonists for the purinoceptor as will be discussed below.

Properties of the ATP-activated current

The properties of the ATP-activated inward current were similar to those observed in other preparations (Benham *et al.*, 1987; Benham & Tsien, 1987; Nakazawa & Matsuki, 1987; Friel, 1988). As shown above, the current activated by ATP shows rapid desensitization. Intracellular perfusion with high EGTA (10 mM) or pretreatment with caffeine (10 mM) did not significantly change the amplitude of the ATP-activated current. Moreover, when every cation in the Krebs solution was substituted by the relatively impermeant cation Tris, ATP did not induce an inward current which indicates that Cl is not permeable. Figure 5a shows traces recorded at various membrane potentials under physiological conditions. The ATP-activated current reversed near 0 mV (see Figure 5c, +1.2 ± 1.9 mV, n = 5) and the outward current was very small (inward-going rectification). When extracellular Na^+ was substituted by equimolar Li^+, K^+ or Cs^+, the reversal potential was not significantly changed (−0.3 ± 3.7, −1.8 ± 5.2, 4.0 ± 3.1 mV, n = 3–6, respectively). Furthermore,

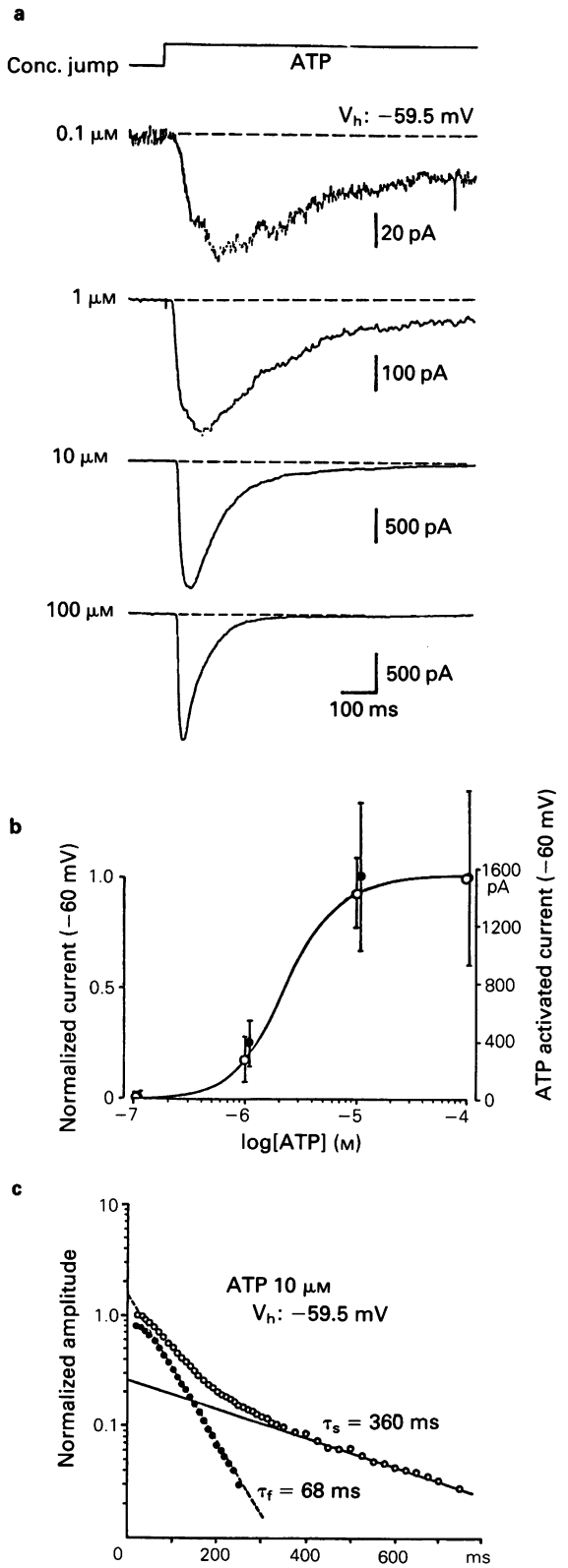

Figure 4 The influence of the concentration of ATP on the amplitude and time course of the ATP-activated current. The bath contained Krebs solution. For the pipette solution, CsCl internal solution (10 mM EGTA) was used to avoid contamination with outward K current. (a) Example of ATP activated currents at 0.1, 1, 10 and 100 μM from the same cell. (b) Concentration-current curve for ATP with absolute values (●) ($n \geq 8$) and after normalization by the maximum value (100 μM, ○) (n = 6). Points represent the mean and vertical lines show s.e.mean. The curve was obtained by the equation: normalized current = $1/(1 + (K_d/[ATP])^n)$, with K_d of 2.3 μM and n, 1.7. (c) The amplitude of the ATP-activated inward current (normalized by its peak) plotted on a semilogarithmic scale (○). (●) Indicate the amplitude of the current after subtracting the slowly desensitizing component (continuous line).

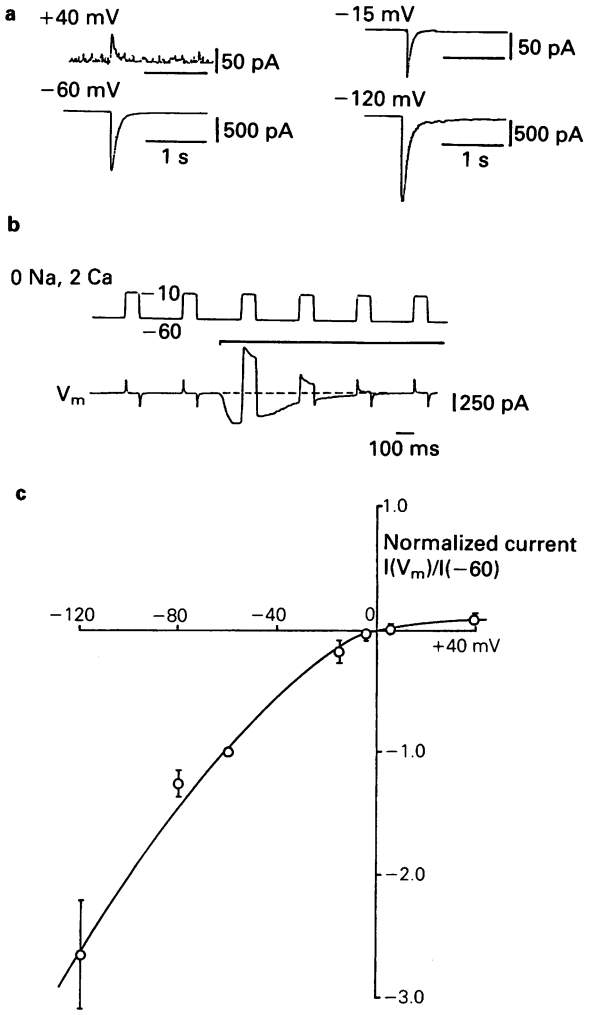

Figure 5 The reversal potential and I-V relationship of the ATP-activated current. (a) The ATP-activated current recorded with KCl internal solution at various membrane potentials. The bath contained Krebs. (b) ATP evoked a considerable inward current at −60 mV in Na free and 2 mM Ca-containing solution. Voltage steps to −10 mV of 100 ms were applied at intervals of 400 ms from a holding potential of −60 mV. The pipette contained CsCl internal solution with 10 mM EGTA. (c) I-V relationship of the ATP-activated currents evaluated from 5 cells. After normalization to the value at −60 mV, the peak amplitude of the ATP-activated current was plotted against the membrane potential.

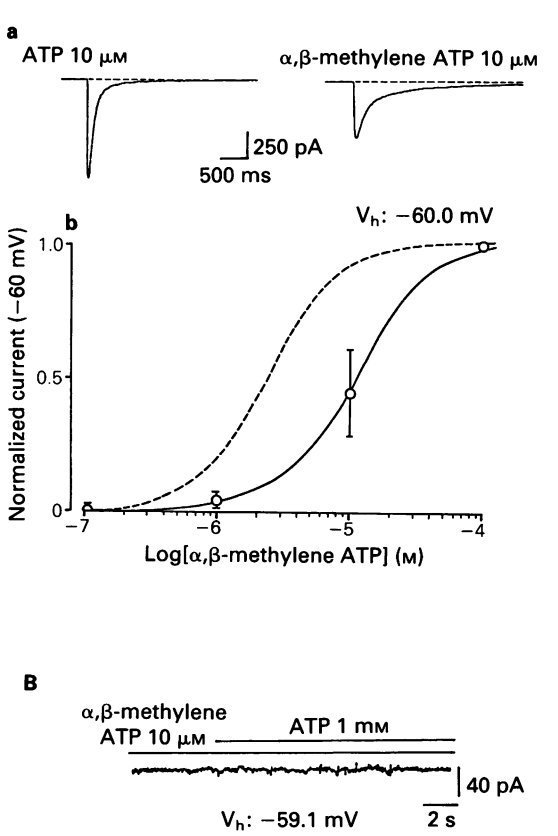

Figure 6 Effect of α,β-methylene ATP on a single detrusor cell. The current was recorded with CsCl internal solution (10 mM EGTA) in Krebs bathing solution. (A,a) α,β-Methylene ATP activates a smaller inward current than was obtained with the same concentration of ATP. (A,b) Concentration-current curve for α,β-methylene ATP (continuous curve). The current was normalized to the maximum value (at 100 μM). The curve was drawn using the equation in Figure 4b, by setting K_d to 10.4 μM and n to 1.6. The dashed curve indicates the concentration-current curve obtained for ATP (Figure 4b). (B) The response to ATP (1 mM) was blocked by pretreatment with 10 μM α,β-methylene ATP, which was applied 1.5 min before the recording shown.

isosmotic substitution of Na^+ by Ba^{2+} or Ca^{2+} did not shift the reversal potential much (−3.1 ± 4.9, 0.2 ± 1.9 mV, $n = 5$, respectively). When Na^+ was absent and only 2 mM Ca^{2+} was present in the bath solution, ATP still produced an inward current (−84.8 ± 49.5 pA, $n = 4$, at −60 mV), but its reversal potential moved to more negative membrane potentials (−36.6 ± 4.0 mV, $n = 4$). These results are very similar to those obtained elsewhere (Benham *et al.*, 1987; Benham & Tsien, 1987). Therefore, we conclude that ATP opens similar channels in guinea-pig bladder to those described in other preparations.

α,β-Methylene ATP is a potent agonist for the purinoceptor and blocks the response to ATP

α,β-Methylene ATP is a methylated analogue of ATP which is resistant to hydrolysis. It is an agonist for the P_{2x} receptor and has been used to desensitize this purinoceptor. After pretreatment with this agent, both nerve-stimulated contraction and the e.j.p. disappeared in guinea-pig urinary bladder (Fujii, 1988; Brading & Mostwin, 1989), which suggests that ATP may be involved in neurotransmission. Figure 6A shows that α,β-methylene ATP can also activate an inward current, but is less potent than ATP. With the same concentration (in this case, 10 μM), the current activated by α,β-methylene ATP has a smaller amplitude and slower time course of desensitization than that of ATP (Figure 6Aa). As summarized in Figure 6Ab, the concentration-response curve for α,β-methylene ATP (continuous curve) is to the right of that for ATP (dashed curve). The curve is fitted by the Michaelis Menten equation with a Hill coefficient of 1.6 and a dissociation constant of 10.4 μM. The time constant desensitization could also be fitted by two exponentials but these were longer than those of ATP (the fast and slow time constants were 276.8 ± 79.0 and 1659.3 ± 364.4 ms, $n = 6$, respectively, at −60 mV with 10 μM α,β-methylene ATP). The maximal inward current evoked by 100 μM α,β-methylene ATP (1016.9 ± 444.5 pA, at −60 mV, $n = 8$) was smaller than that for ATP (10 μM, 1554.2 ± 537 pA, $n = 11$). These results suggests that α,β-methylene ATP is less potent in activating the receptor than ATP.

Figure 6B shows the blocking action of α,β-methylene ATP on the ATP-activated current. In the continued presence of α,β-methylene ATP (control currents for ATP and α,β-methylene ATP not shown), even a high concentration of ATP could not induce inward current. The same observation was obtained with ATP itself. After the application of ATP, 5 to 10 min was needed for recovery from desensitization of the receptor, and in some cases with long exposure to ATP, the recovery was not complete.

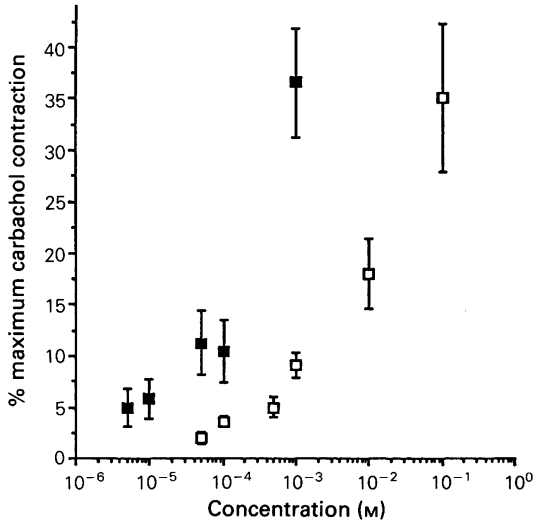

Figure 7 The contractile response of strips of guinea-pig detrusor, (■) response to α,β-methylene ATP; (□) response to ATP. Note that the tissue is 50–100 times more sensitive to α,β-methylene ATP than to ATP. Means of 6–12 results. Vertical lines show s.e.mean.

The effects of α,β-methylene ATP and ATP on the contractile response

Figure 7 shows dose-response curves of the contractile response of strips of guinea-pig bladder to α,β-methylene ATP and to ATP. The drugs were applied in the superfusion solution at 36°C. As can be seen, with this method of application, α,β-methylene ATP is about 50–100 times more potent than ATP. This was also true at room temperature (not shown).

Discussion

The present work supports the hypothesis that ATP is the neurotransmitter responsible for fast neurotransmission in the guinea-pig urinary bladder. In previous experiments using microelectrodes and bath application (Fujii, 1988), ATP depolarized the membrane with a slow time course (tens of seconds) only at very high concentrations (1 mM) and the response was very different from the e.j.p. Furthermore, the possibility could not be totally excluded that ATP caused release of a transmitter from nerve terminals (e.g. from cholinergic nerves: Northway & Burks, 1980). However, in the present experiments, with single isolated cells and a concentration jump technique (time constant about 10 ms), we have demonstrated that ATP can mimic the e.j.p. (Fujii, 1988; Brading & Mostwin, 1989) to a considerable extent: (1) a fairly low concentration of ATP (100–500 nM: see Figure 3Ab) depolarized the membrane enough to evoke an action potential, (2) the ATP-induced response is a complex of depolarization, action potential and after hyperpolarization like the nerve-evoked response, and its duration is of the same order as the e.j.p. (200–700 ms), (3) α,β-methylene ATP completely blocked the inward current underlying the ATP-induced depolarization. The real time course of the ATP concentration profile is unknown. It is affected not only by the process of release and diffusion of the transmitter in the synaptic cleft, but also by the degrading enzymes, ATPase, 5'-nucleotidase and adenosine deaminase. Furthermore, propagation of the e.j.p. in three dimensions might result in attenuation or summation of the response and thereby result in a more complicated time course. However, since in guinea-pig urinary bladder, smooth muscles may only be rather sparsely innervated (Brading & Mostwin, 1989) and the cells of the smooth muscles less well coupled electrically than in most other tissues (impedance measurement, Brading *et al.*, 1989), the time course of the depolarization observed in a single cell might correspond well to that in the multicellular preparation.

The non-selectivity of the ATP-activated current has been seen in other preparations. In rabbit ear artery, Benham & Tsien (1987) demonstrated that the permeability ratio of Ca to Na is about 3.0 and therefore concluded that sufficient calcium could flow into the cell to raise the intracellular Ca concentration to about 500 nM (Indo-1 measurement, Benham, 1989). Our results in the present experiments showed very similar values of reversal potential, although very precise measurement was impossible because the reversal potentials were estimated by interpolation or extrapolation of the I-V relationship of the ATP-activated current instead of with a ramp potential. In experiments with only 10 μM EGTA in the pipette, application of ATP to a cell occasionally caused contraction even when the membrane potential was clamped at the resting potential, and an outward current, probably a Ca-activated K current, followed the fast desensitizing inward current (it was not observed using 10 mM EGTA in the pipette: unpublished observation). Therefore, a significant increase in the intracellular Ca concentration may occur at potentials close to the resting membrane potential in response to agonist application. However, in physiological conditions, since ATP only causes very short-lasting depolarization and activates an action potential, the role of this receptor-operated Ca-influx might be very small in guinea-pig urinary bladder.

The latency of the ATP-activated inward current (18 ms at 10 μM) was considerably shorter than previously estimated (ionophoretic application: rabbit ear artery single cells, 70 ms, Benham *et al.*, 1987; rat anococcygeus muscle, 30 ms, Byrne & Large, 1984). In some smooth muscles, the latency estimated for non-cholinergic, non-adrenergic neurotransmission is, however, rather short. For example, in guinea-pig vas deferens, α,β-methylene ATP sensitive excitatory junction current can be evoked with a delay of less than 3 ms using extracellular recording and nerve stimulation (Brock & Cunnane, 1988). The exact background for this discrepancy is still unclear in the present experiments. A possible explanation is the efficiency of the concentration clamp. Thus, in our experiments, the measured latency tended to be shortened by raising the hydrostatic pressure for the concentration jump apparatus (see Figure 1), even when the cell was placed close to the side of the hole in the polyethylene tube. Therefore, with an even more rapid system for solution change, it might be possible to obtain latencies of less than 20 ms. However, it is quite clear that activation of the ATP receptor is very different from receptors requiring intracellular second messengers like inositol trisphosphate or cyclic AMP, where the latency exceeds 500 ms (Tsien, 1983; Horn & Marty, 1988; Miledi & Parker, 1989). It is likely that simple association of ATP to the receptor activates the channel proteins as for the nicotinic-receptor, the glutamate and the γ-amino-butyric acid receptor-activated currents (Hille, 1984; Franke *et al.*, 1987; Akaike *et al.*, 1987; Strange, 1988). With these receptors the latency of the current is less than 10 ms, two molecules of agonist bind to the receptor and the currents show two exponential desensitization processes, all features shared with the ATP-activated current. More careful experiments are, however, in progress to clarify the underlying mechanism.

The effect of α,β-methylene ATP is thought to be mediated by activation of the same receptor as ATP. The responses caused by ATP and α,β-methylene ATP are not additive. Their concentration-current curves have similar Hill coefficients and the time course of desensitization shows two exponentials in both cases. These findings suggest that a common molecular mechanism is involved in both responses. Therefore, it is clear from the present experiments that the blocking action of α,β-methylene ATP on the ATP-induced depolarization is not an indirect effect through an increase in membrane conductance as suggested in other preparations (Ishikawa, 1985), but is by desensitization of the receptor. The order of potency of ATP and α,β-methylene ATP to produce a membrane current is the reverse of that obtained for the con-

tractile response. This has also been seen by Burnstock & Kennedy (1985), who showed that α,β-methylene ATP causes more potent vasoconstriction than ATP but less potent vasorelaxation, which led them to suggest a subdivision of the P_2 purinoceptors into P_{2x} and P_{2y}. In rat vas deferens, a similar reversed order has been obtained with single isolated cells and the patch clamp technique (Friel, 1988). We suppose that ATP is rapidly broken down when it is applied to multicellular preparations, and that this, combined with the relatively slow solution change which occurs in tension measurement might result in the rapidly desensitizing component of ATP response (in order of 50 ms) not to be observed, therefore reducing the response. The greater resistance of α,β-methylene ATP to enzymic breakdown would result in a higher concentration reaching the receptors, and the more sustained response (slow time constant 1700 ms) might cause the larger and more prolonged depolarization observed with microelectrodes (Fujii, 1988), which could in turn cause a larger Ca influx than ATP. However, since tension is the result of the integration of many cellular processes, more information is necessary to explain the discrepancy, including possible effects of ATP on other nerve terminals or on the sarcoplasmic reticulum.

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The role of adenosine in the hyperaemic response of the hepatic artery to portal vein occlusion (the 'buffer response')

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1 Adenosine has been shown to be responsible for the hyperaemic response of the hepatic artery to portal vein occlusion (the hepatic arterial 'buffer response').

2 The effect of adenosine receptor blockade and of adenosine uptake inhibition on the hepatic arterial response to portal vein occlusion was investigated in three groups of anaesthetized dogs.

3 Venous return and arterial blood pressure were maintained during periods of portal occlusion by establishing a side-to-side portacaval shunt. Hepatic artery and portal vein blood flows were measured with electromagnetic flowmeters.

4 Hepatic arterial infusions of 8-phenyltheophylline ($500\,\mu g\,kg^{-1}\,min^{-1}$) and 3-isobutyl-1-methylxanthine ($75\,\mu g\,kg^{-1}\,min^{-1}$), doses sufficient to block the vasodilator response of the hepatic artery to exogenously applied adenosine, reduced the magnitude of the 'buffer response' by 50% and 75%, respectively.

5 Intravenous infusion of dipyridamole ($100\,\mu g\,kg^{-1}\,min^{-1}$), a dose sufficient to potentiate the vasodilator response of the hepatic artery to exogenously applied adenosine, had little effect on the 'buffer response'.

6 It is concluded that adenosine is an important, but not the sole, agent responsible for the hepatic arterial 'buffer response'.

Introduction

The compensatory hyperaemic response of the hepatic artery (HA) to portal vein (PV) occlusion (the hepatic arterial 'buffer response': Lautt, 1981) has been the subject of many investigations during the past 20 years, such as those by Kock *et al.* (1972), Lautt (1983) and Mathie & Blumgart (1983).

A putative mediator of the response has recently been identified in studies in the cat by Lautt and his colleagues (Lautt *et al.*, 1985), who have concluded that the 'buffer response is mediated entirely by local adenosine concentration' (Lautt & Legare, 1985). They demonstrated that the adenosine receptor antagonist 8-phenyltheophylline totally blocked, and that the adenosine uptake inhibitor dipyridamole potentiated, both the 'buffer response' and the HA vasodilatation induced by injected adenosine. Our own studies have previously shown that, in the dog, neither an intrinsic neurogenic phenomenon (Mathie *et al.*, 1980) nor an hepatic β-adrenoceptor mechanism is involved in the 'buffer response' (Alexander *et al.*, 1989).

The current investigation was carried out to determine the role of adenosine in the HA 'buffer response' in the dog, by use of the experimental model previously established in our laboratory (Mathie *et al.*, 1980; Mathie & Blumgart, 1983). A pharmacological approach has been adopted, employing two adenosine receptor antagonists and an adenosine uptake inhibitor, in three groups of animals. According to the widely accepted classification of Burnstock (1978), purine receptors comprise two distinct populations: P_1-purinoceptors (mediating responses to adenosine and adenosine 5'-monophosphate (AMP)) located on vascular smooth muscle, and P_2-purinoceptors (mediating responses to adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP)) located on the endothelial cell membrane (Burnstock & Kennedy, 1986). We have used both 3-isobutyl-1-methylxanthine (IBMX) and 8-phenyltheophylline (8-PT) as P_1-purinoceptor antagonists, and the adenosine uptake inhibitor, dipyridamole (DPD), to potentiate the effects of adenosine. The magnitude of the 'buffer response' has been examined before and after administration of each of these agents.

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Methods

Experiments were carried out in a total of 14 greyhounds of either sex, weighing 22.2–32.3 kg (mean 27.8 kg). The animals were deprived of food but not water for 24 h before the operation. Anaesthesia was induced with thiopentone ($25\,mg\,kg^{-1}$) i.v. and maintained with pentobarbitone ($30\,mg\,kg^{-1}$) i.v. After endotracheal intubation, the dogs were ventilated with a 3:1 mixture of nitrous oxide and oxygen by a Starling pump. The minute volume and the inspired oxygen concentration were adjusted to maintain the Po_2 and Pco_2 at normal levels (approximately 100 mmHg (13.3 kPa) and 40 mmHg (5.3 kPa), respectively). The base deficit was maintained at $4\,mmol\,l^{-1}$ with sodium bicarbonate i.v. as required. Fluid balance was achieved by infusion of 150 mM sodium chloride i.v.; haematocrit remained above 40% throughout each experiment. Body temperature remained at 36–38°C, assisted when necessary by means of radiant heat lamps.

Operative procedures

The experimental model has been described in detail elsewhere (Mathie *et al.*, 1980; Mathie & Blumgart, 1983). After right femoral artery cannulation (for blood pressure measurement), a mid-line laparotomy was performed. A precalibrated electromagnetic flow probe (Statham) was applied to both the HA and the PV (3 mm and 6 mm diameter respectively). The HA probe was positioned about 2 cm from the coeliac axis while the PV probe was placed mid-way between the gastroduodenal and splenic veins (see illustration in Alexander *et al.*, 1989).

The gastroduodenal artery and vein were then ligated and cannulated, the former to allow injections into the HA and the latter for PV pressure measurement. Hepatic vein (HV) cannulation was achieved via the right external jugular vein, the location of the catheter tip being confirmed by direct palpation; the catheter was withdrawn about 5 mm from a 'wedged' position for 'free' HV pressure measurement.

A side-to-side mesocaval shunt was performed, with 5/0 silk suture, by the construction of an anastomosis between the inferior vena cava and the superior mesenteric vein, just below the entry of the splenic vein. After haemostasis had been achieved, the shunt was closed by means of a small bulldog clip placed along the suture line, thus restoring normal PV flow to the liver until the start of the experimental measurements.

Experimental protocol

The effect of PV flow interruption was investigated in all dogs approximately 1 h after the completion of the operative procedures. Measurements were made of the basal HA and PV blood flows and of the pressures in the femoral artery, PV and HV, before any alterations in blood flow. The PV was then cross-clamped just proximal to the flow probe, and the shunt immediately opened to divert PV blood into the vena cava. The PV occlusion was maintained for 5–10 min, at which time blood flow and pressure measurements were repeated. PV flow was then restored by re-closing the shunt with the bulldog clip after release of the cross-clamp on the PV. These manoeuvres were repeated in order to obtain duplicate information on the magnitude of the normal 'buffer response' for every animal; the value ascribed to each dog was the arithmetic mean of the two measurements, the second measurement showing, on average, a 1.7% greater response than the first (range: −25% to +37%).

Drug administration

The effect of intra-portally injected adenosine (Sigma, A-9251) was then investigated to establish the normal response of the HA circulation. Five doses in the range (50 ng–0.5 mg kg^{-1}) were employed. The peak of the HA hyperaemic response to adenosine injection was plotted against the dose given in each case.

One of 3 agents was then administered as follows: 3-isobutyl-1-methylxanthine (IBMX, Sigma I-5879; $n = 5$) was infused into the HA at a rate of 50–100 (mean 75) $\mu g\,kg^{-1}\,min^{-1}$; 8-phenyltheophylline (8-PT, Sigma P-2278; $n = 4$) was infused into the HA at 250–750 (mean 500) $\mu g\,kg^{-1}\,min^{-1}$; dipyridamole (DPD, Sigma D-9766; $n = 5$) was infused i.v. at 50–150 (mean 100) $\mu g\,kg^{-1}\,min^{-1}$, following a bolus i.v. injection of 400 $\mu g\,kg^{-1}$. In general, the rate of infusion of a drug was reduced from the higher to the lower value of the stated range during the course of an experiment.

After 20–30 min, the potency of the blockade or potentiation was established by the construction of a further dose-response curve to exogenously applied adenosine. Good repetition of responses to adenosine over a similar duration of experiment was confirmed in a pilot series of animals.

When maximal blockade or potentiation had been achieved, two further 'buffer responses' were then elicited over the subsequent 15–20 min, and the mean response compared with that of the control (normal) response. Repetition for 'buffer responses' after drug administration was similar to that in the normal situation (mean difference between first and second measurement: −3.6%; range: −17% to +14%), except for two dogs in the 8-PT series and for one dog in the IBMX series in which the second 'buffer response' was less than half the magnitude of the first; the 'buffer response' was completely eliminated in both measurements in another of the dogs given IBMX.

Calculations

Blood flows were recorded on the flowmeters in ml min^{-1} and subsequently recalculated in ml min^{-1} 100 g^{-1} by relating the readings to the wet weight of the liver, determined at the end of each experiment. Total liver blood flow was calculated by addition of the individual HA and PV flows.

The 'buffering capacity' of the HA was calculated as the increase in HA flow/decrease in PV flow, expressed in %.

Statistics and presentation of data

Student's paired t test was used to test the significance of differences in: (a) measured indices before and after PV occlusion; (b) measured indices due to drug administration alone; (c) the magnitude of responses either to adenosine injection or to PV occlusion before and after drug administration. All results are quoted as mean ± s.e.mean.

Results

Dose-response to adenosine

The changes in HA blood flow produced by intraportal injections of adenosine before and after IBMX, 8-PT or DPD are shown in Figure 1(a–c). Increasing doses of adenosine caused increasing dilatation of the HA up to the maximum dose

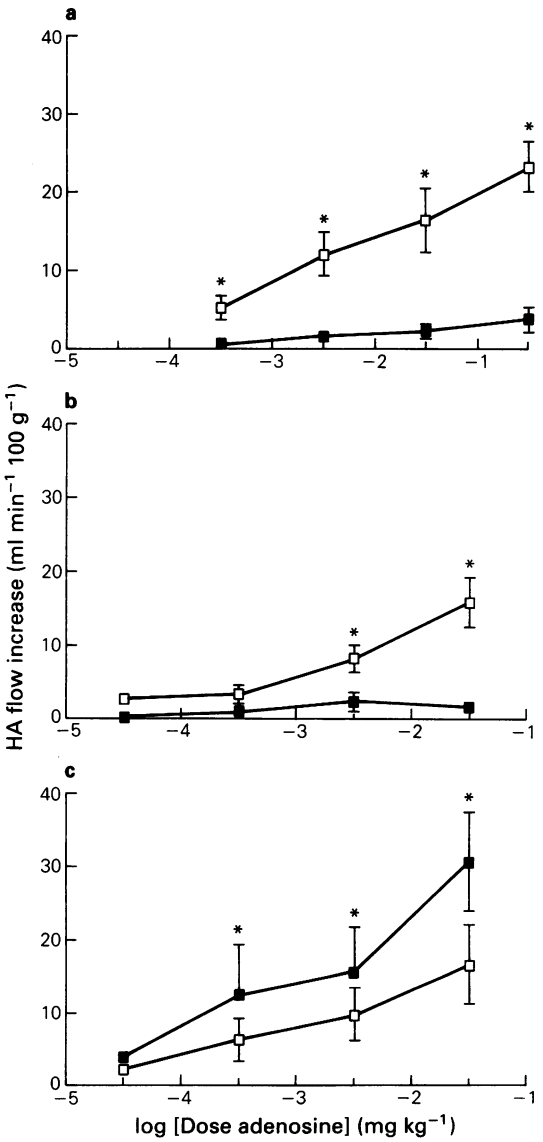

Figure 1 Hepatic arterial (HA) blood flow response to increasing doses of adenosine injected intraportally, before (□) and during (■) drug administration in 3 groups of dogs: (a) IBMX (3-isobutyl-1-methylxanthine); (b) 8-PT (8-phenyltheophylline); (c) DPD (dipyridamole). All values are mean and vertical lines represent s.e.mean. * Significant difference between responses before and during drug adminstration ($P < 0.05$).

injected; an ED_{50} for adenosine could therefore not be calculated. IBMX and 8-PT produced a similar degree of attenuation of the response ($P < 0.05$), while DPD produced a potentiation which was statistically significant at adenosine doses from 0.5–50 $\mu g\,kg^{-1}$ ($P < 0.05$).

Selection of the appropriate infusion rate for each of the three drugs proved somewhat difficult. In each case, the infusion rate often had to be altered by a factor of about 2 around the mean level quoted above, in order to achieve the desired effect on the response to injected adenosine, but with minimal detrimental effect on other haemodynamic indices: IBMX caused a decrease in arterial blood pressure; 8-PT tended to diminish PV blood flow; DPD reduced basal HA blood flow (see below).

Hepatic arterial 'buffer response'

The changes in the 'buffer response' induced by IBMX, 8-PT and DPD are illustrated in Figure 2. In the normal situation, PV occlusion caused HA blood flow to increase, on average over the three groups, by 19.6 ml min^{-1} 100 g^{-1}. Both IBMX and 8-PT reduced the magnitude of the 'buffer response' significantly, though 8-PT was noted to cause a less pronounced mean inhibition of the response (49%) than IBMX (71%). However, the inhibition was much more reproducible with 8-PT than with IBMX (coefficients of variation: 59% and 104% respectively). DPD produced little or no change in the magnitude of the response.

There was a statistically significant increase in HA flow during PV occlusion in all cases, both with and without drug administration, except during infusion of IBMX (see Table 1). Basal HA flow was not altered by either IBMX or 8-PT, but was decreased by DPD. PV flow was increased by IBMX, unaltered by DPD, and decreased (though not in a statistically significant manner) by 8-PT.

'Buffering capacity' was reduced by IBMX from 24.2 ± 4.2% to 6.4 ± 3.2% ($P < 0.05$). 8-PT also reduced 'buffering capacity', from 26.4 ± 5.8% to 18.4 ± 3.7%, but the difference was not statistically significant. DPD caused a non-significant reduction in 'buffering capacity', from 24.5 ± 6.2% to 20.9 ± 4.0%.

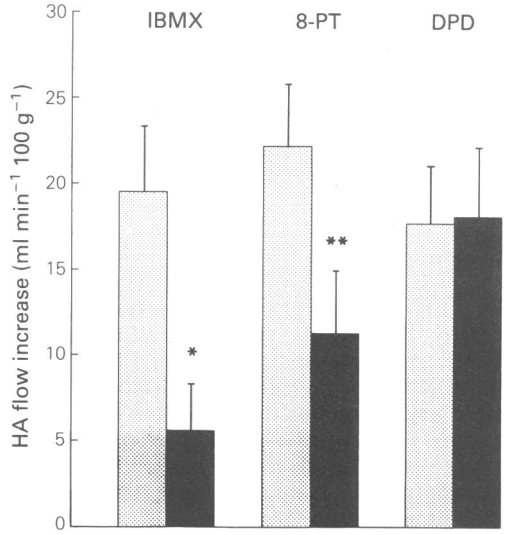

Figure 2 Hepatic arterial (HA) blood flow increase ('buffer response') before (stippled columns), and during (solid columns) drug administration in 3 groups of dogs: IBMX (3-isobutyl-1-methylxanthine); 8-PT (8-phenyltheophylline); DPD (dipyridamole). All values are mean and vertical lines represent s.e.mean. Significant differences between response before and during drug administration: * $P < 0.05$, ** $P < 0.01$.

Basal mean arterial blood pressure was significantly decreased by IBMX, but unchanged by either 8-PT or DPD (see Table 1). PV occlusion did not affect arterial pressure on any occasion. HV pressure was not changed by any procedure. Basal PV pressure was increased by approximately 1.0 mmHg by all 3 drugs, significantly in the cases of IBMX and DPD. The effects of PV occlusion on true pre-hepatic PV

Table 1 Haemodynamic indices before and during hepatic artery (HA) 'buffer response' to portal vein occlusion (PVO) in 3 groups of dogs

	Normal		*Drug*	
	Before PVO	*During PVO*	*Before PVO*	*During PVO*
(a) IBMX ($n = 5$)				
HA flow (ml min^{-1} 100 g^{-1})	35.5 ± 9.3	55.0* ± 11.4	36.2 ± 4.8	41.8 ± 4.4
PV flow (ml min^{-1} 100 g^{-1})	83.3 ± 12.4	0 ± 0	107.4‡ ± 12.4	0 ± 0
Total LBF (ml min^{-1} 100 g^{-1})	118.8 ± 17.0	55.0* ± 11.4	143.6‡ ± 15.1	41.8* ± 4.4
Blood pressure (mmHg)	122.6 ± 7.0	119.0 ± 9.6	68.0‡ ± 6.5	68.4 ± 6.0
PV pressure (mmHg)§	7.2 ± 1.2	(5.5)* ± (1.1)	8.1‡ ± 1.3	(5.0)* ± (1.0)
HV pressure (mmHg)	3.0 ± 1.1	3.1 ± 1.0	2.7 ± 1.0	2.8 ± 0.8
(b) 8-PT ($n = 4$)				
HA flow (ml min^{-1} 100 g^{-1})	31.7 ± 5.7	53.9* ± 5.8	27.6 ± 14.3	38.9* ± 17.1
PV flow (ml min^{-1} 100 g^{-1})	90.2 ± 13.4	0 ± 0	58.8 ± 7.1	0 ± 0
Total LBF (ml min^{-1} 100 g^{-1})	121.9 ± 13.4	53.9* ± 5.8	86.4 ± 20.5	38.9* ± 17.1
Blood pressure (mmHg)	152.0 ± 7.6	152.3 ± 8.6	159.3 ± 6.2	162.8 ± 6.2
PV pressure (mmHg)§	7.3 ± 0.5	(4.5) ± (0.3)	8.0 ± 0.3	(4.9)* ± (0.4)
HV pressure (mmHg)	2.7 ± 0.9	3.1 ± 0.9	1.9 ± 1.1	2.1 ± 1.1
(c) DPD ($n = 5$)				
HA flow (ml min^{-1} 100 g^{-1})	61.9 ± 13.1	79.6* ± 12.5	41.3‡ ± 12.6	59.4* ± 13.3
PV flow (ml min^{-1} 100 g^{-1})	77.7 ± 7.4	0 ± 0	85.4 ± 9.5	0 ± 0
Total LBF (ml min^{-1} 100 g^{-1})	139.6 ± 19.4	79.6* ± 12.5	126.7 ± 19.1	59.4* ± 13.3
Blood pressure (mmHg)	147.8 ± 12.5	146.8 ± 12.6	136.0 ± 9.2	133.5 ± 8.9
PV pressure (mmHg)§	7.6 ± 0.8	(5.8)* ± (0.8)	8.7‡ ± 0.9	(6.0)* ± (0.5)
HV pressure (mmHg)	3.1 ± 0.4	3.2 ± 0.4	3.0 ± 0.4	3.1 ± 0.3

PV: portal vein; LBF: liver blood flow; HV: hepatic vein. IBMX: 3-isobutyl-1-methylxanthine; 8-PT: 8-phenyltheophylline; DPD: dipyridamole.

* Significant difference from baseline value ('Normal, before PVO' or 'Drug, before PVO', as appropriate), $P < 0.05$.

‡ Significant difference from baseline value before drug administration ('Normal, before PVO'), $P < 0.05$.

§ PV pressure measurements during PV occlusion do not represent prehepatic pressure readings, due to the position of the recording catheter (see text).

All values are mean ± s.e.mean.

pressure could not be assessed, due to the absence of a recording catheter below the cross-clamp.

Discussion

The experimental model employed in this study has been used in a number of earlier investigations by the authors: it achieves complete PV occlusion in a repeatable manner without loss of systemic venous return or blood pressure (Mathie *et al.*, 1980; Mathie & Blumgart, 1983; 1987; Alexander *et al.*, 1989). The resultant HA 'buffer response' is highly reproducible both within and between individual animals, as demonstrated by observations in the current and in the previous experiments described by the authors.

The present experiments have demonstrated that blockade of adenosine receptors in the hepatic arterial circulation of the dog reduced, but did not eliminate, the HA 'buffer response' following PV occlusion. In addition, the adenosine uptake inhibitor dipyridamole (DPD) did not potentiate the response, despite potentiating the HA hyperaemia induced by exogenously applied adenosine. We therefore conclude that, while adenosine does exert a significant degree of control over HA blood flow, the 'buffer response' is unlikely to be 'mediated entirely by local adenosine concentration', as proposed by Lautt & Legare (1985).

Effectiveness of adenosine receptor blockade was confirmed in our experiments by the careful measurement of dose-responses to injected adenosine immediately before the time of PV occlusion. 8-Phenyltheophylline (8-PT) and 3-isobutyl-1-methylxanthine (IBMX) had very similar inhibitory effects on the adenosine dose-response curve, though 8-PT caused a less marked (but more reproducible) attenuation of the 'buffer response'.

We found both of these antagonists quite difficult drugs to use: IBMX infusion resulted in progressive systemic hypotension, which necessitated prompt measurement of haemodynamic indices before blood pressure fell below 50% of control. Indeed, it is possible that the profound hypotensive effect of IBMX may have limited the HA 'buffer response'. However, previous studies have shown that the 'buffer response' is unaffected by a 25% reduction in mean arterial blood pressure (Alexander *et al.*, 1989), and it is possible that the 50% reduction in pressure observed with IBMX in the present study may also not have influenced the response, particularly since neither HA nor PV blood flow was reduced by the drug. 8-PT had no effect on blood pressure, even at the dose required to produce virtually complete adenosine blockade, and was therefore considered a preferable drug to use than IBMX. In addition, unlike IBMX, 8-PT is only a very weak phosphodiesterase inhibitor (Smellie *et al.*, 1979), and is a potent and selective P_1-purinoceptor antagonist (Bruns *et al.*, 1988; Collis, 1988). However, 8-PT tended to cause a decrease in PV blood flow.

Lautt & Legare (1985) found the antagonist effect of 8-PT varied considerably between different cats, and they had to administer the drug in the range 1–16 mg min^{-1} kg^{-1} in order to obtain complete blockade. They also found that the 'buffer response' required a higher dose of 8-PT to achieve the same degree of block as seen with injected adenosine, and that an overdose of 8-PT could produce serious HA vasoconstriction. We found a smaller and much less variable dose of 8-PT produced nearly complete blockade of the response to injected adenosine in our dogs; HA vasoconstriction was never observed. It remains possible that we might have achieved more complete blockade of the 'buffer response' with higher doses of 8-PT, but it seems very unlikely that PV occlusion could stimulate the release of adenosine in amounts exceeding the highest level we administered in our dose-response curves.

DPD significantly potentiated the vasodilator response of the HA to exogenously applied adenosine, but did not alter the magnitude of the 'buffer response'. This finding contrasts with that obtained by Lautt *et al.* (1985), who showed a marked increase in both the response to injected adenosine and the 'buffer response'. We found that potentiation of the hyperaemic response to injected adenosine could only be achieved within a narrow range of DPD infusion rates, and that the measurements of dose-response curves and the 'buffer response' had to be very carefully timed. An additional problem with DPD was its tendency to decrease basal HA blood flow, though to a mean level similar to that in the other two groups. We share Lautt's concern that DPD itself did not dilate the HA, as might be expected if adenosine plays a major role in the control of HA flow. DPD, however, does not have very specific actions: it may block efflux as well as influx of purines (Stone, 1985), and it is a potent inhibitor of some phosphodiesterases (Van Belle, 1988). Results obtained with DPD must therefore be viewed with considerable caution.

Throughout this investigation, we employed the technique whereby adenosine was injected into the PV in order to elicit changes in flow in the HA (Lautt *et al.*, 1984). We have found that this approach provides a highly reproducible vasoactive response in the HA on first pass through the liver. Such a 'transhepatic' mode of drug action is indicative of some form of communication between PV blood and the pre-sinusoidal HA resistance sites. While the mechanism of action is unknown, its existence was used to advantage in the current study, as it enabled the local infusion of blocking agents into the HA to proceed uninterrupted; furthermore, HA rather than PV injection of adenosine was found in pilot experiments to result in a significantly longer duration of response, which would have been incompatible with the precise timing required in our experimental protocol.

The 'buffering capacity' of the HA was significantly reduced by IBMX, but only slightly decreased by 8-PT. The 'buffering capacity' is a useful index of the responsiveness of the HA specifically to abrupt interruption of PV flow, because it accommodates variations in basal PV flow. The absence of a statistically significant decrease in this index with 8-PT may have been due to the decrease in basal PV flow caused by the drug, but this implies an even lesser blocking effect of 8-PT on the 'buffer response' than discussed above. By contrast, the large decrease in 'buffering capacity' with IBMX took place despite an increase in basal PV flow, thus implying a rather greater inhibitory effect of IBMX than previously concluded; the hypotensive effect of IBMX is probably not responsible (see discussion above). DPD did not enhance 'buffering capacity'.

Adenosine has been shown to be an important regulator of blood flow in a number of organs (Berne *et al.*, 1983), including the brain (Phillis *et al.*, 1984; Winn *et al.*, 1985), the coronary circulation (Berne, 1963; Leung *et al.*, 1985; Randall & Jones, 1985), skeletal muscle (Berne *et al.*, 1983), the kidney (Osswald, 1988) and the intestine (Granger & Norris, 1980; Lautt, 1986). It has also been shown to control post-occlusion reactive hyperaemia of the HA (Ezzat & Lautt, 1987). Several mechanisms have been proposed for the release of adenosine from tissue. Its release may be continuous, so that during PV occlusion, for example, it may accumulate and result in HA dilatation (Lautt, 1985; 1988). It may, however, be regulated by tissue hypoxia (Berne *et al.*, 1983), or by an imbalance between oxygen supply and demand (Bardenheuer & Schrader, 1986). The present results do not allow these mechanisms to be separated. Nevertheless, it is important to recognise the fact that the PV normally provides the major supply of oxygen to the liver (Mathie *et al.*, 1988), and that its loss could lead to a degree of hepatic tissue hypoxia.

P_1-purinoceptors in the cardiovascular system comprise two subtypes: A_1 receptors (which mediate the prejunctional inhibition by adenosine of neurotransmitter release) and A_2 receptors (which mediate vasodilatation due to adenosine). They are believed to be present in a number of peripheral vascular beds (Collis, 1985; Burnstock & Kennedy, 1986; Williams, 1987). Evidence also exists to support the existence of P_1-purinoceptors in the HA (A.L. Brizzolara & G. Burnstock, personal communication), and recent work in the rabbit by

the present authors has shown these to include an A_2 receptor subtype (R.T. Mathie, B. Alexander, V. Ralevic & G. Burnstock, unpublished observations).

We therefore suggest that following PV occlusion, adenosine, released from the liver (either from HA endothelial cells or produced from the breakdown of ATP) in response to tissue hypoxia or to diminished oxygen delivery, may act upon P_1 receptors in the HA to result in dilatation of the HA vascular bed. Our results indicate that adenosine is unlikely to be the sole regulator of HA blood flow, and further studies are needed to elucidate the nature of the other mediator(s) of the 'buffer response'.

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Co-operation between interleukin-1 and the fibrinolytic system in the degradation of collagen by articular chondrocytes

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1 The interaction between interleukin 1 (IL-1) and the fibrinolytic system in the control of collagen degradation by rabbit chondrocytes has been investigated in a tissue-culture system where cells are grown on a ^{14}C-labelled collagen matrix.

2 Culture of rabbit chondrocytes in the presence of human recombinant IL-1β at a concentration of 57 pM for 48 h led to the presence of procollagenase but not active collagenase in the medium. The latent collagenase could be activated by incubation with an organomercurial, aminophenylmercuric acetate (APMA).

3 Addition of IL-1β to chondrocytes grown on a ^{14}C-labelled collagen matrix did not increase the degradation of the matrix compared to control over a 48 h period. However, in the presence of plasmin (200 μg ml^{-1}) or plasminogen (100 μg ml^{-1}), IL-1β (57 pM) caused almost complete degradation of the collagen matrix. Plasmin or plasminogen alone caused only slight degradation of the collagen matrix.

4 Tissue inhibitor of metalloproteinases (TIMP) or the selective metalloproteinase inhibitor, SC44463, inhibited the degradation induced by IL-1β and plasminogen in a concentration-related manner and at concentrations that were correlated with inhibition of collagenase.

5 When concentrations of IL-1β which caused only minimal degradation of the matrix in the presence of plasminogen were combined with fibrin (1 μg ml^{-1}), there was almost total degradation of the matrix by 48 h.

6 These results indicate there is a synergistic interaction between IL-1 and the fibrinolytic system in the degradation of collagen by rabbit chondrocytes in culture.

Introduction

Cartilage cells or chondrocytes are now recognised as playing an active role in the degradation of articular cartilage that occurs in chronic arthritis. Destruction of extracellular matrix occurs in areas surrounding chondrocytes in early rheumatoid arthritis (RA) (Hamerman, 1969) and culture of cartilage in the presence of the cytokine, interleukin 1 (IL-1), results in the degradation of both its proteoglycan (Saklatvala *et al.*, 1985; Ratcliffe *et al.*, 1986) and its collagen (Kraukauer *et al.*, 1985; Tyler, 1988) components. These effects may be due to the induction and release from chondrocytes of the metalloproteinases, stromelysin and collagenase, which can degrade proteoglycan and collagen at neutral pH (Murphy & Reynolds, 1985). However, these enzymes are tightly regulated: metalloproteinases are released in a latent form and, if activated, can be avidly bound by an endogenous inhibitor, termed tissue inhibitor of metalloproteinases or TIMP (Murphy & Reynolds, 1985).

Latent collagenase can be activated in cell-free systems by incubation with trypsin or organomercurials (Werb & Burleigh, 1974), although the mechanism of activation *in vivo* is unclear. Some studies have suggested that plasmin may be a physiological activator of metalloproteinases (Eeckhout & Vaes, 1977) and thereby be important in chondrocyte-mediated degradation of cartilage proteoglycan (Meats *et al.*, 1985; Collier & Ghosh, 1988). However, other studies have indicated that the degradation of proteoglycan induced by IL-1 in cartilage explants occurs maximally in the absence of plasminogen (Saklatvala & Sarsfield, 1988). Similarly, the degradation of collagen by a range of connective tissue cells in culture is reported to be plasminogen dependent (Werb *et al.*, 1977; Reynolds *et al.*, 1988) while others find that the degradation of both proteoglycan and collagen by synovial cells occurs in the absence of plasminogen (Peeters-Joris *et al.*, 1981; Peeters-Joris & Vaes, 1984).

In order to clarify further the importance of the fibrinolytic system in collagenolysis, we have examined the effects of IL-1 on the degradation of collagen by rabbit articular chondrocytes in the presence and absence of plasminogen or plasmin. We have also determined whether the degradation of collagen is collagenase-dependent by using the selective metalloproteinase inhibitors TIMP and SC 44463 (Docherty *et al.*, 1985; Reich *et al.*, 1988). The role of fibrin in the degradation of collagen was also investigated since this substance is found at sites of degradation in rheumatoid joints and may interact with cells to modulate the erosive process.

Methods

Isolation of rabbit chondrocytes

New Zealand white rabbits (2–2.5 kg) were killed by overdose of phenobarbitone. Shavings of articular cartilage from the knees and shoulders of these rabbits were sequentially digested with hyaluronidase type II (Sigma; 1 mg ml^{-1}) and pronase (Sigma; 1 mg ml^{-1}) for 1 h, followed by collagenase type II (Sigma; 1 mg ml^{-1}) for 2 h. All incubations were carried out at 37°C in Dulbecco's modification of Eagle's medium (DMEM) containing 1% acid-treated foetal calf serum. The cells were filtered through a Nitex filter (R. Cadisch and Sons, London) and washed 3–4 times in calcium-free phosphate-buffered saline. The cells were then plated at high density in culture flasks, which prevents the cells from dedifferentiating (Watt, 1988) and passaged on to collagen films after 4–7 days. Some cells (5×10^5 cells per well) were seeded on to 24-well plates (Costar) without collagen films for assay of collagenase in the medium.

Preparation of ^{14}C-labelled collagen films

Type 1 collagen was prepared from rat skin and acetylated with [^{14}C]-acetic anhydride (Amersham; 100 mCi mmol^{-1}) as

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described by Cawston & Barrett (1979). Collagen films were prepared by the method of Gavrilovic et al. (1985). Aliquots (100 μl) of a [^{14}C]-collagen solution (2 mg ml^{-1}) were placed in each well of 24-well culture plates (Costar). The plates were incubated at 37°C for 1 h to polymerise the collagen to form a fibrillar gel and then allowed to dry overnight at room temperature. The films were then washed with phosphate buffered saline and incubated with DMEM containing antibiotics (100 u ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, Gibco) and fungizone (Gibco, 0.5 μg ml^{-1}).

Stimulation of rabbit chondrocytes

Once the cells were confluent, combinations of IL-1β (human recombinant, Wellcome) with plasminogen (human, Biopool) or plasmin (human, Sigma) were added to the chondrocyte monolayers on the radiolabelled collagen films. In some experiments human fibrin (1 μg ml^{-1}) was added to the cells. After a 48 h period, an aliquot of the medium was removed and the radioactivity measured in a liquid scintillation counter. The undegraded collagen remaining on the plate was digested with bacterial collagenase (0.5 mg ml^{-1}, Sigma) and the radioactivity estimated thus allowing % degradation to be calculated. Parallel cultures of rabbit chondrocytes were stimulated with IL-1β (57 pM) for the assay of collagenase in the medium. In some experiments human recombinant tumour necrosis factor α (TNFα; Genentech) was added to the chondrocytes.

Effect of metalloproteinase inhibitors

Once optimal conditions for collagen film degradation were established the collagenase inhibitors, TIMP (human recombinant, Celltech) and SC 44463, N-[3-(N'-hydroxycarboxamido)2 - (2 - methylpropyl)propanoyl] - O - methyl - L - tyrosine-N-methylamide (synthesized by Wellcome Laboratories) were included in the incubation mixture and the degradation over 48 h measured. The reversibility of the inhibitory effect on collagen degradation was investigated by incubating the cells for a further 48 h after removal of the inhibitors and then measuring the degradation.

Assay of collagenase

Collagenase was assayed by a fibril-based assay. Type 1 ^{14}C-labelled collagen was used as a substrate as described by Cawston & Barrett (1979). ^{14}C-labelled collagen (100 μg) was diluted in 100 mM Tris buffer (250 μl) and allowed to form fibrils at 37°C for 2 h. After fibril formation, a 50 μl aliquot of standard enzyme (purified human gingival collagenase from Strangeways Research Laboratories, Cambridge) was added to give a final concentration of 0.125 μg ml^{-1}–1.0 μg ml^{-1}. Aliquots of chondrocyte-conditioned medium were assayed before and after incubation with APMA (0.7 mM) for 20 h. In some experiments concentrations of TIMP and SC 44463 were included in the incubation mixture.

Statistical analysis

Data were expressed as means ± s.e.mean and statistically analysed by Student's t test and $P < 0.01$ was considered significant.

Results

Induction of latent collagenase by interleukin 1

The medium supporting chondrocytes for a 48 h period contained less than 0.075 μg ml^{-1} active and latent collagenase. Addition of IL-1β (57 pM) did not increase the concentration of active collagenase in the medium compared to control. However, treatment of the medium with APMA (0.7 mM) led to an increase in activity equivalent to 0.35 ± 0.04 μg ml^{-1} ($n = 4$) purified human collagenase. This indicates that IL-1β induces the synthesis and release of latent, but not active, collagenase.

In the presence of IL-1 (57 pM) and plasminogen (100 μg ml^{-1}) 0.67 ± 0.05 μg ml^{-1} ($n = 3$) active collagenase was detected.

Effect of cytokines on rabbit chondrocytes in the presence or absence of plasminogen or plasmin

Culture of rabbit chondrocytes on ^{14}C-labelled collagen films resulted in less than 5% degradation over a 48 h period which was not enhanced in the presence of IL-1β at concentrations up to 570 pM (10 ng ml^{-1}). However, when plasminogen (100 μg ml^{-1}) was added to the chondrocytes on the film, degradation was increased to 18.1 ± 1.1% ($n = 6$) and was significantly enhanced when IL-1β was added in combination with plasminogen (Figure 1). This effect of plasminogen was maximal at 100 μg ml^{-1} with less effect at 50 μg ml^{-1} (data not shown). Synergy was also noted when IL-1β and plasmin (200 μg ml^{-1}) were added to the chondrocytes together (Figure 1). The effect of IL-1β on collagen degradation in the presence of plasminogen was dose-related and was maximal at 20 pM (Figure 2). Interestingly, TNFα which shares many of the activities of IL-1 did not cause degradation at concentrations up to 10 ng ml^{-1} in the absence or presence of plasminogen.

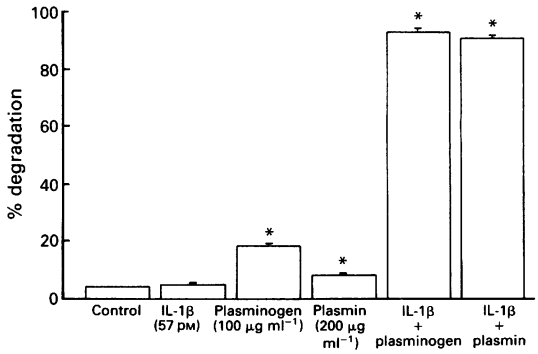

Figure 1 Effect of human recombinant IL-1β (57 pM) on the degradation of collagen matrix by rabbit chondrocytes. Interleukin 1 was added in the presence or absence of plasminogen or plasmin. Results are expressed as % degradation of the collagen matrix. Each column represents the mean of data from 6 experiments with s.e.mean shown as vertical bars. * Indicates a significant difference ($P < 0.01$) between control and treated cultures.

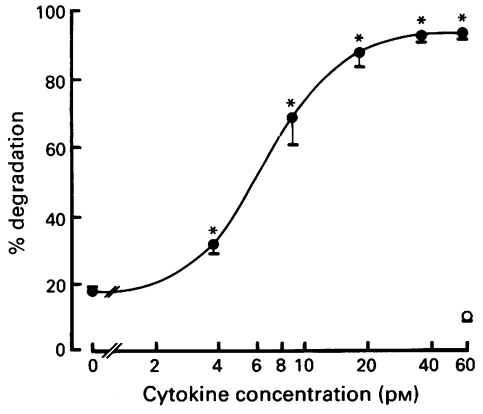

Figure 2 Degradation of collagen by rabbit chondrocytes induced by increasing concentrations of human recombinant interleukin-1β (IL-1β, ●) in the presence of plasminogen (100 μg ml^{-1}). The effect of human recombinant tumour necrosis factor α (TNFα, ○), in the presence of plasminogen, is also shown. Each point is the mean of data from 6 cultures with s.e.mean shown as vertical bars; * $P < 0.01$ compared to control.

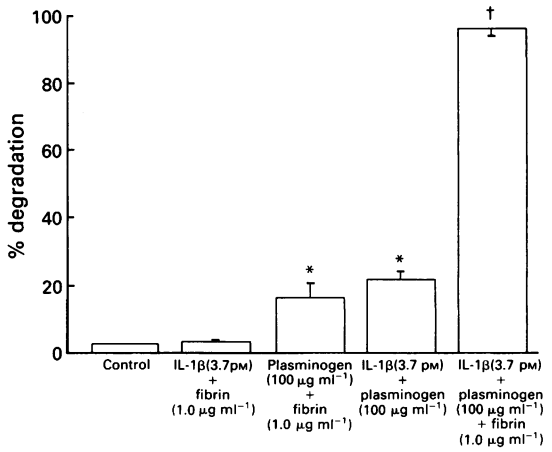

Figure 3 Effect of fibrin on the degradation of collagen by chondrocytes induced by a low dose of human recombinant interleukin-1β (IL-1β). Each point represents the mean of data from 6 cultures with s.e.mean shown as vertical bars. * Indicates a significant difference from control and † represents a significant difference compared to IL-1β and plasminogen alone.

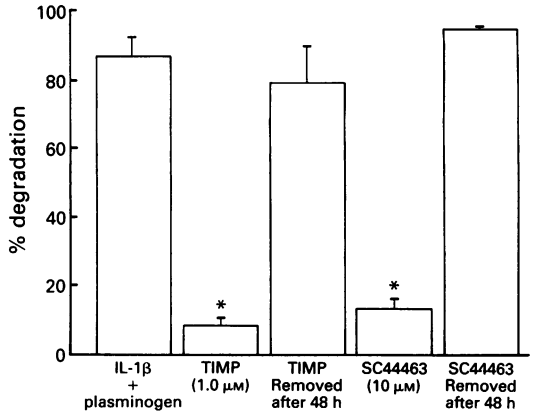

Figure 5 The effect of removing the inhibitors on the ability of the chondrocytes to degrade the collagen matrix when supplied with fresh interleukin-1β (IL-1β) and plasminogen. Each column is the mean of data from 6 cultures with s.e.mean shown by vertical bars (* $P < 0.01$ compared to IL-1β and plasminogen).

Effect of fibrin on the degradation of collagen by rabbit chondrocytes

When fibrin (1 μg ml^{-1}) was combined with low doses of IL-1β (3.7 pM) there was a further synergistic interaction in the presence of plasminogen such that the collagen matrix was almost totally degraded over a 48 h period (Figure 3). It was necessary to combine fibrin with both IL-1β and plasminogen to exert this effect while no effect was observed with fibrin alone.

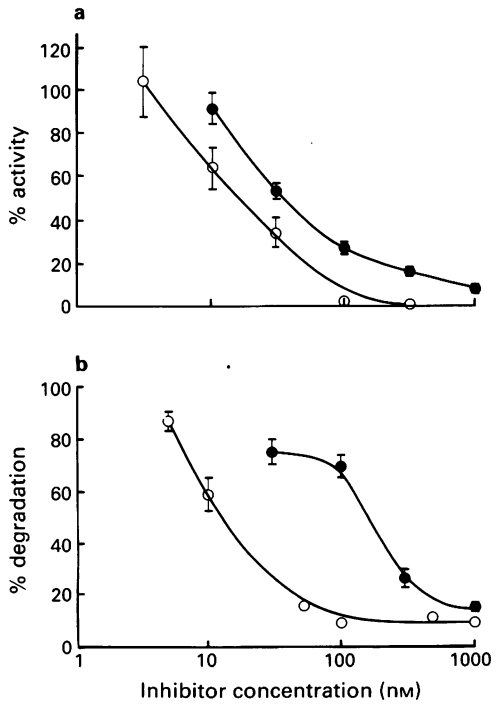

Figure 4 In (a) the concentration-dependent inhibition of the activity of human gingival collagenase by TIMP (○) and SC 44463 (●) in a fibril-based assay is shown. Each point is the mean from 3 assays with s.e.mean shown by vertical bars. (b) Shows the concentration-dependent inhibition of collagen degradation by rabbit chondrocytes by TIMP (○) and SC 44463 (●). Each point is the mean from 6 cultures with s.e.mean shown by vertical bars.

Effect of TIMP on collagenase activity and on collagen degradation by rabbit articular chondrocytes

Recombinant TIMP (3–1000 nM) inhibited collagenase in a cell-free system with an IC_{50} of 16 nM (Figure 4a). Inhibition of collagenase correlated closely with the ability of TIMP to block the degradation of collagen by chondrocytes in response to plasminogen and IL-1β (Figure 4b; IC_{50}, 12 nM). After removal of the TIMP and replacement with fresh IL-1β and plasminogen the chondrocytes regained their ability to degrade the collagen film (Figure 5).

Effect of SC 44463 on collagenase activity and on collagen degradation by rabbit articular chondrocytes

SC 44463 (10–1000 nM) inhibited collagenase in a cell-free system in a dose-related manner with an IC_{50} of 45 nM (Figure 4a). However, the concentrations required to inhibit collagen degradation by rabbit chondrocytes in culture were higher (Figure 4b; IC_{50}, 190 nM). When the media supporting the chondrocytes was assayed for SC 44463 on collagenase after the 48 h incubation period, there was an apparent reduction in potency (IC_{50}, 780 nM) which suggests that the compound is unstable to some extent under the incubation conditions or is metabolised by the cells. This may explain the higher concentrations required to inhibit collagen degradation by cells in culture.

Upon removal of the SC 44463 after a 48 h period, the chondrocytes regained their ability to degrade the collagen film in the presence of IL-1β and plasminogen (Figure 5).

Discussion

In osteoarthritis and RA the degradation of cartilage collagen is believed to result in irreversible joint destruction and this process is resistant to current drug therapy (Wright & Amos, 1980). The proteoglycan depletion from cartilage, which precedes the collagen degradation, is also resistant to drug therapy but is reversible provided the chronic synovitis wanes, at least in experimental arthritis (Pettipher & Henderson, 1988). Therefore, efforts have been made to design therapies to prevent the degradation of proteoglycan and collagen in chronically inflamed joints. This is unlikely to be achieved by drugs designed to inhibit neutrophil migration or by inhibitors of neutrophil-derived enzymes since cartilage degradation is not reduced in arthritic rabbits which have been rendered neutropenic (Pettipher *et al.*, 1988) or in mice which are

genetically deficient in neutrophil elastase or cathepsin G (Schalkwijk *et al.*, 1988).

Since there is increasing evidence that articular chondrocytes, under the influence of cytokines, are actively engaged in destroying their own matrix in arthritic joints (for review, see Henderson *et al.*, 1987), it is more likely that enzymes from these cells, such as the metalloproteinases are responsible for the degradation of proteoglycan and collagen. The metalloproteinases include collagenase, stromelysin and gelatinase, the combined action of which can lead to the degradation of all components of the extracellular matrix at neutral pH. We have examined the processes involved in collagen degradation by rabbit chondrocytes in a cell culture model.

When chondrocytes are grown on a collagen matrix, they cause little degradation of the collagen even in the presence of doses of IL-1 which cause proteoglycan degradation in cartilage explants *in vitro* (Saklatvala *et al.*, 1985; Ratcliffe *et al.*, 1986) or in rabbit joints *in vivo* (Pettipher *et al.*, 1986). However, the present study demonstrates that in the presence of plasminogen or plasmin, IL-1 stimulated the cells to cause almost complete degradation of the collagen matrix over a 48 h period, while plasminogen or plasmin alone had little effect. As plasmin did not degrade the collagen matrix directly, it is probable that it is acting by converting latent collagenase to active collagenase as has been demonstrated in cell-free systems (Eeckhout & Vaes, 1977).

Indeed, IL-1 stimulated the chondrocytes to produce collagenase only in a latent form and activity was only apparent after incubation with an organomercurial. In the chondrocyte/collagen matrix system this activation was presumably brought about by plasmin as has been demonstrated previously (Gavrilovic *et al.*, 1985). We have now confirmed these findings and also demonstrated that there is a further synergistic interaction, in that incubation of fibrin with concentrations of IL-1β which cause only minimal degradation in the presence of plasminogen, leads to almost complete degradation of the collagen matrix.

Fibrin may be acting to increase the activity of tissue-type plasminogen activator as occurs when this enzyme binds to fibrin *in vitro* (Hoylaerts *et al.*, 1982). Whatever the mechanism of the synergistic interaction between IL-1β and fibrin it may be particularly relevant to the rheumatoid joint where fibrin is deposited at sites of cartilage damage (Kulka *et al.*, 1955; Scott *et al.*, 1981).

The degradation of the collagen by chondrocytes in this cell culture system is due to collagenase since the process is blocked by TIMP at concentrations that correlate with inhibition of collagenase. This confirms the observations of Gavrilovic *et al.* (1985) and we also demonstrate that the low molecular weight collagenase inhibitor, SC 44463, is effective in inhibiting collagen degradation.

Interestingly, TNFα which shares many properties with IL-1, did not induce the chondrocytes to degrade the collagen matrix either in the presence or absence of plasminogen. This is in keeping with previous studies which indicate that rabbit chondrocytes are unresponsive to TNFα *in vitro* (Schnyder *et al.*, 1987; Gilman, 1987) or *in vivo* (Henderson & Pettipher, 1989). However, human chondrocytes do respond to TNFα by producing neutral protease activity (Bunning & Russell, 1989).

In summary, this study supports the view that collagenase plays a key role in the degradation of collagen by chondrocytes (Reynolds *et al.*, 1988). We have also observed a synergistic interaction between IL-1 and the fibrinolytic system in the degradative process since the presence of plasminogen and fibrin facilitates collagen degradation by rabbit chondrocytes in response to IL-1.

We would like to thank Dr Gillian Murphy of Strangeways Research Laboratory for supplying the human collagenase and Celltech for supplying the recombinant TIMP. We are also grateful to Genentech for the gift of recombinant TNFα.

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Temperature-dependence of desensitization induced by acetylcholine and histamine in guinea-pig ileal longitudinal muscle

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1 The effects of temperature on the time course of desensitization induced by acetylcholine and histamine, and on the recovery from desensitization were studied in the longitudinal muscle of the guinea-pig ileum.

2 Self- and cross-desensitization produced by acetylcholine (10^{-5} M) occurred rapidly in the first 10 min of exposure to the agonist, with the same time course and the same degree of desensitization over the temperature range of 11°C to 31°C.

3 Self-desensitization produced by histamine (10^{-5} M) also occurred rapidly in the first 10 min of exposure to the agonist, and showed great temperature-dependence, especially at 11°C and 21°C, but scarcely occurred at 6°C.

4 Cross-desensitization produced by histamine developed gradually with time and showed a moderate temperature-dependence between 11°C and 31°C, but scarcely occurred at 6°C.

5 The recovery processes from desensitization showed marked temperature-dependence. Recovery was halted completely at 11°C.

6 These studies suggest that acetylcholine-induced desensitization may be attributed to a single non-specific mechanism. Histamine-induced desensitization may be due to at least two mechanisms: it occurs in both a specific and non-specific manner. Each of these desensitizations can be characterized by its unique temperature-dependence.

Introduction

Prolonged treatment of smooth muscles with high concentrations of agonists leads to reduced responsiveness to the agonists. This phenomenon, called desensitization, is well studied in the guinea-pig ileal longitudinal muscle and may be either specific or non-specific, the former affecting only the responses to a desensitizing agonist and the latter affecting the responses to multiple agonists after desensitizing treatment with one agonist. Treatment with either acetylcholine or histamine induces non-specific desensitization in guinea-pig ileum (Cantoni & Eastman, 1946; Dale, 1958; Paton, 1961; Gosselin & Gosselin, 1973). It has been assumed that desensitization is due to a change in some common process beyond the receptor/ligand level, such as a gain of Na^+ and a loss of K^+ by the smooth muscle cells (Paton & Rothschild, 1965; Joiner, 1973; James-Kracke & Roufoglis, 1981; Aboulafia *et al.*, 1987) or depletion of intracellular stores of Ca^{2+} (Hishinuma & Uchida, 1988). However, a specific component of desensitization may be masked by a larger non-specific component, and efforts have been made to uncover the specific component of desensitization (Gosselin & Gosselin, 1973; Morgenstern & Bluth, 1976; Siegel *et al.*, 1984). In the present study, we undertook to examine further the specific and non-specific components of desensitization produced by acetylcholine and histamine. In an attempt to discriminate between these components of desensitization, we have studied the effect of temperature on the development of and recovery from desensitization. Assuming multiple mechanisms are concerned with the desensitization process in guinea-pig ileum, these respective components of desensitization may show distinctive temperature-dependence.

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Methods

Isolated preparations

Guinea-pigs of either sex, weighing 250–450 g, were killed by a blow on the head followed by severance of the major blood vessels in the throat. The ileum was removed, placed in aerated Tyrode solution and cut into segments. The longitudinal muscle layer was obtained according to the method of Rang (1964). The strips were suspended in Tyrode solution at 31°C and bubbled with air under a resting tension of 500 mg. The Tyrode solution had the following composition (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 5.6. Isotonic contractions were recorded with a lever on a smoked drum.

The drugs used were acetylcholine chloride (Daiichi Pharmaceutical Co.) and histamine dihydrochloride (Wako Chemical Industry).

Measurement of desensitization

The time course of desensitization was studied as follows. First, a dose-response curve to acetylcholine or histamine was measured on a longitudinal muscle strip at 31°C. Then, the muscle strip was treated with a desensitizing agent (10^{-5} M of acetylcholine or 10^{-5} M of histamine) for a period of time varying from 3 to 30 min. After the muscle was washed out with Tyrode solution for 2 min, the dose-response curve for either acetylcholine or histamine was re-examined. The dose-response curve shifted almost in parallel to the right in the desensitized state. The dose-ratio for shifted dose-response curves was determined at 50% response to assess the degree of desensitization (Paton, 1961).

The temperature-dependence of desensitization was studied as follows. After a control dose-response curve was obtained

at 31°C, the muscle was treated with the desensitizing agent at various temperatures (6°C, 11°C, 21°C, and 31°C). The tissue was washed with Tyrode solution at 31°C for 2 min and the dose-response curve was re-examined at 31°C. For the control experiments, the muscle was held at various temperatures (6°C–31°C) for 30 min in normal Tyrode solution, then at 31°C for 2 min, and the dose-response curve re-examined. This treatment had no significant effect on the dose-response curve. Incubation of the tissue at low temperatures in Tyrode solution had practically no effect on the dose-response curve.

Measurement of recovery from desensitization

After the control dose-response curve was examined, desensitization was induced by treating the tissue with a large dose of desensitizing agent (10^{-4} M acetylcholine or 10^{-4} M histamine) at 31°C for 30 min. Then the tissue was washed with Tyrode solution for 5 min and the responses to a test agonist were examined at 31°C. After washing, the tissue was incubated with Tyrode solution at 11°C, 21°C and 31°C. Then, the recovery of response was examined at 31°C at varying times. Before each examination the tissue was transferred to solution at 31°C and left for 2 min. Dose-ratios were determined from the dose-response curves, as explained in 'Measurement of desensitization'.

In some experiments in which the effects of low temperature (11°C) on the time course of recovery from desensitization were examined, procedures were almost the same as above except that contractile responses at a test dose of acetylcholine (10^{-6} M) or histamine (10^{-6} M) were examined. In these experiments the desensitized responses were expressed as percentage responses of test control.

Calculations of kinetic parameters

Half-times of development of desensitization ($t_{1/2}$) were determined as the times required for desensitization to reach 50% of the maximally desensitized state which was attained after 30 min-desensitizing treatment. Half-times of recovery from desensitization ($t_{1/2}$) were determined as the times required for desensitization to recover 50% from the maximally desensitized state. Rates of desensitization at various temperatures (6°C–31°C) were calculated from 'dose-ratios' obtained by 10 min-desensitizing treatment according to the equation: rate of desensitization = log(dose-ratio)/10(min^{-1}). Q_{10} (temperature coefficient for temperature change of 10°C) was then calculated as the ratio of rate of desensitization between two temperatures.

Results

Time course of desensitization

To study the desensitization phenomenon quantitatively, we used the following method. As shown in Figure 1, the desensitizing treatment (pretreatment of the tissue with 10^{-5} M histamine) shifted the dose-response curves to the right according to the length of exposure to the agent. Thus dose-ratios for the shifted dose-response curves were used to assess the degree of desensitization. Essentially, the same method was used in the study of acetylcholine-induced desensitization (pretreatment with 10^{-5} M acetylcholine).

This method enabled us to compare precisely self- and cross-desensitization produced by acetylcholine and histamine. As shown in Figure 2a, a similar time course was observed in acetylcholine-induced self- and cross-desensitization. Both desensitizations developed rapidly in the first 10 min and proceeded more slowly in the next 20 min. The half-times of development of desensitization ($t_{1/2}$) were 3.8 ± 0.3 min and 3.4 ± 0.3 min for self- and cross-desensitization, respectively. On the other hand, different time courses were observed in histamine-induced self- and cross-desensitization (see Figure 2b). Self-desensitization developed rapidly in the first 10 min and then more slowly in the next 20 min ($t_{1/2}$ = 3.8 ± 0.4 min), whereas cross-desensitization developed at a more uniform and slower rate ($t_{1/2}$ = 11.1 ± 1.0 min).

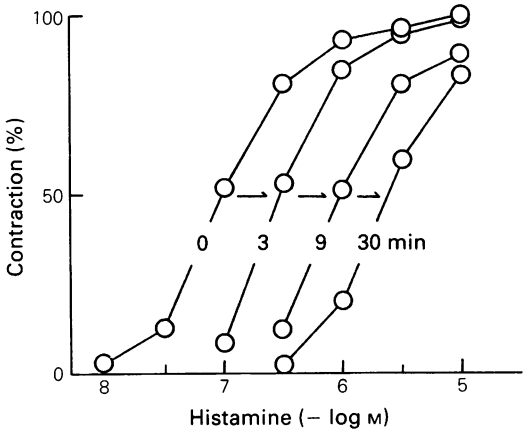

Figure 1 Effect of desensitization induced by histamine on the dose-response curves to histamine in guinea-pig ileal longitudinal muscle. The control dose-response curve is shown as the left hand curve (0 min). Dose-response curves after desensitizing treatment (10^{-5} M histamine) for 3, 9 and 30 min are shown as marked in the figure. The results are typical examples from those obtained in 4 experiments. Temperature 31°C.

Temperature-dependence of desensitization

To examine further the differences between self- and cross-desensitization produced by acetylcholine and histamine, we studied the temperature-dependence of each desensitization. In this case, desensitizing treatments were carried out at 6°, 11°, 21°, and 31°C, for periods of 10–30 min, during which significant contractile responses were observed even at the lowest temperature of 6°C, indicating that receptor-effector systems remained in operation.

As shown in Figure 3a,b, similar temperature-dependence was observed in acetylcholine-induced self- and cross-desensitization (Figure 3a,b). In both cases, desensitization occurred to the same extent at temperatures between 11°–31°C, but failed at 6°C. The values of Q_{10} for the rate of desensitization at 11°–21°C and 21°–31°C were 1.16 and 1.03 for self-desensitization, and 1.16 and 0.97 for cross-desensitization, indicating that desensitization had virtually

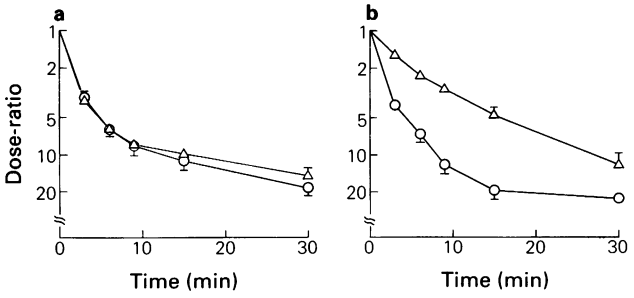

Figure 2 Time course for self- and cross-desensitization induced by acetylcholine and histamine. (a) Desensitization induced by acetylcholine (10^{-5} M) against acetylcholine response (○) and histamine response (△). (b) Desensitization induced by histamine (10^{-5} M) against histamine response (○) and acetylcholine response (△). Tissues were desensitized for the periods of time indicated on the abscissa scales of the graph. Dose-ratios on the ordinate scale were determined as described under Methods to express the degree of desensitization. Each point is the mean of 4 measurements and s.e.means are indicated by vertical bars. Temperature 31°C.

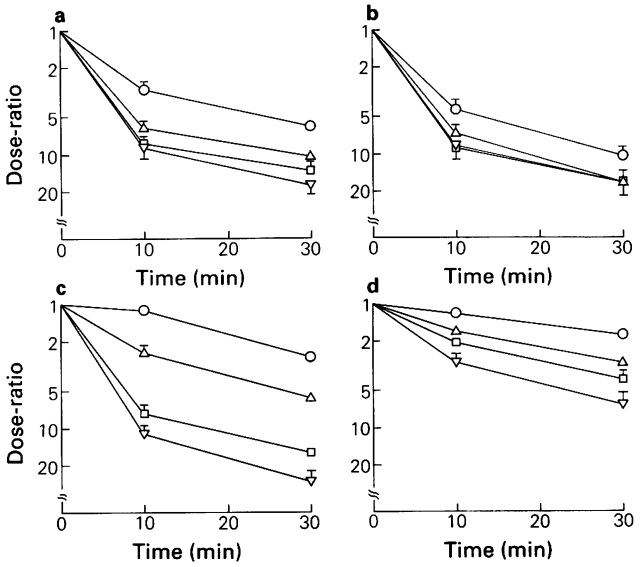

Figure 3 Effect of temperature on the time course of (a) desensitization induced by acetylcholine (10^{-5} M) against acetylcholine response; (b) desensitization induced by acetylcholine (10^{-5} M) against histamine response; (c) desensitization induced by histamine (10^{-5} M) against histamine response; (d) desensitization induced by histamine (10^{-5} M) against acetylcholine response. Tissues were desensitized at 6°C (▽), 11°C (□), 21°C (△), or 31°C (○) for the periods of time indicated on the abscissa scales. Dose-ratios on the ordinate scale were determined as described under Methods to express the degree of desensitization. Each point is the mean of 4–5 measurements and s.e.means are indicated by vertical bars.

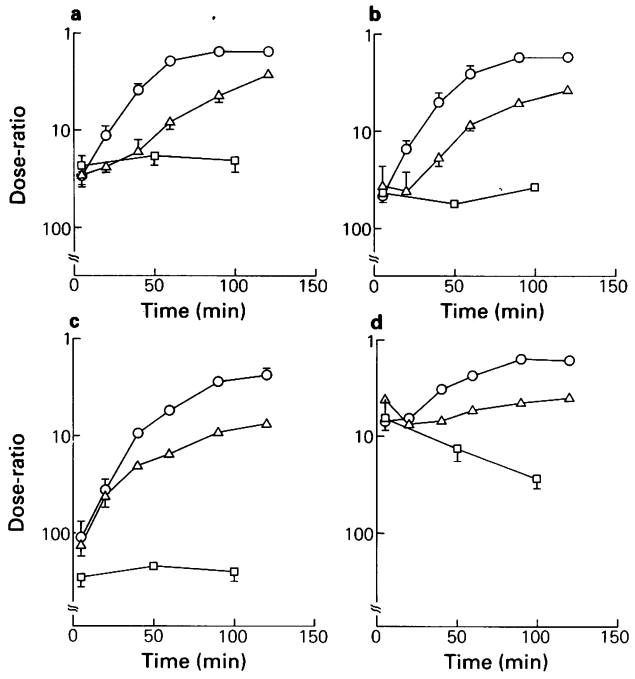

Figure 4 Effect of temperature on the time course of (a) recovery of acetylcholine response from desensitization induced by acetylcholine (10^{-4} M, 30 min); (b) recovery of histamine response from desensitization induced by acetylcholine (10^{-4} M, 30 min); (c) recovery of histamine response from desensitization induced by histamine (10^{-4} M, 30 min); (d) recovery of acetylcholine response from desensitization induced by histamine (10^{-4} M, 30 min). Recovery processes were examined at 11°C (□), 21°C (△) and 31°C (○). At the times indicated on the abscissa scale, recovery of responses were measured and dose-ratios were obtained as described under Methods. Each point is the mean of 4–10 experiments and s.e.means are indicated by vertical bars.

no temperature-dependence between 11°–31°C. On the contrary, a significant decrease in the rate of desensitization occurred at 6°C, where the values of Q_{10} at 6°–11°C were 2.69 and 1.81 for self- and cross-desensitization, respectively.

Thoroughly different temperature-dependence was observed in histamine-induced desensitization (Figure 3c,d). In this case, self- and cross-desensitization were also different from each other in their temperature-dependence. Self-desensitization showed large temperature-dependence over the temperature range of 6°–31°C. In particular, a marked change in the rate of desensitization was observed between 11°–21°C, where the value of Q_{10} was 2.24. Cross-desensitization showed moderate temperature-dependence in the range of 11°–31°C, where the values of Q_{10} at 11°–21°C and at 21°–31°C were 1.43 and 1.54, respectively. Both self- and cross-desensitization produced by histamine were extremely influenced by lowering the temperature to 6°C, where the rate of desensitization became virtually zero.

The recovery from the desensitized states to the initial level may occur in different ways, depending on the types of desensitization. Therefore we compared the recovery processes from desensitization of various types in terms of their time courses and the temperature-dependence. To study the recovery processes quantitatively, we employed the method used in the study of desensitization. In this case, however, to desensitize tissues fully, the desensitizing treatment was done using a higher concentration of acetylcholine (10^{-4} M) and histamine (10^{-4} M).

Both the recovery from self- and cross-desensitization produced by acetylcholine occurred in the same manner (see Figure 4a,b). At 31°C, the recovery was complete within 80–90 min and followed first order kinetics with half times of recovery ($t_{1/2}$) of 33.9 ± 2.1 min and 34.5 ± 2.8 min for self- and cross-desensitization, respectively. At 21°C, the recovery did not follow first order kinetics showing a lag period of about 20 min. At 11°C, the recovery did not occur at all. Essentially the same results were obtained in self-desensitization produced by histamine (see Figure 4c). That is, the recovery was complete within 100 min and followed first order kinetics with $t_{1/2}$ of 38.4 ± 2.8 min at 31°C. The recovery did not follow first order kinetics at 21°C, and did not occur at all at 11°C. Thus the recovery from self- and cross-desensitization produced by acetylcholine and the recovery from self-desensitization produced by histamine can be grouped together.

On the other hand, the recovery from cross-desensitization produced by histamine occurred in a different manner from the other cases studied (see Figure 4d). At 31°C, the recovery was complete within about 90 min, but did not follow first order kinetics. At 21°C, only slight recovery was observed, and at 11°C, the development of further desensitization was observed. Thus, from our present study the recovery from desensitization was divided into two classes.

As described above, recovery from desensitization was profoundly inhibited at 11°C. This phenomenon was not observed with the contractile response or the desensitization process, each of which significantly occurred at 11°C. Further experiments were, therefore, performed to examine the recovery processes at 11°C. First, we examined the effect of incubating the desensitized tissues at 11°C for 60 min before the recovery process proceeded at 31°C. As shown in Figure 5a, the recovery started immediately after the tissue was re-incubated at 31°C following the same time course as the control. In the next experiment, 11°C-incubation for 60 min was put in the course of recovery process at 31°C. As shown in Figure 5b, no recovery was observed during the 11°C-incubation for 60 min, but once re-incubated at 31°C, recovery proceeded with the same time course as control. Thus, 11°C-incubation had no effect on the ability of the desensitized tissues to recover, whether the treatment was carried out before the beginning of recovery process or in the midst of the recovery process. The above results were obtained on recovery from self-desensitization produced by histamine, and similar

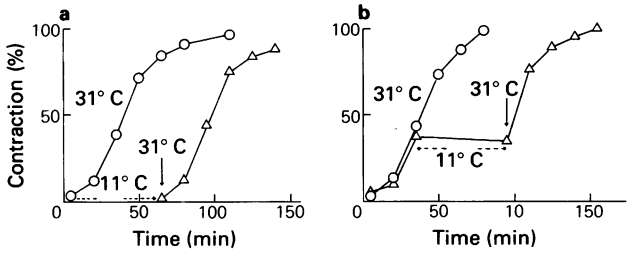

Figure 5 Effect of low temperature on the time course of recovery from self-desensitization induced by histamine. The desensitized responses were examined at a test dose of histamine (10^{-6} M) and were expressed as percentage of test control responses. (a) Tissues were desensitized by 10^{-4} M histamine for 30 min at 31°C. Then recovery of response was measured at 31°C at the times indicated on the abscissa scale (○), or the tissues were first incubated at 11°C for 60 min and then incubated at 31°C and recovery of response was measured at the times indicated (△). (b) Tissues were desensitized by 10^{-4} M histamine for 30 min at 31°C. Recovery of response was measured at 31°C (○), or the tissues were first allowed to recover at 31°C for 35 min, then incubated at 11°C for 60 min, and thereafter incubated at 31°C and recovery of response was measured at the times indicated (△). The results are typical examples from those obtained in 3 experiments.

results were obtained with self- and cross-desensitization produced by acetylcholine (data not shown). In the case of cross-desensitization produced by histamine, the above experiments were not performed because of the further development of desensitization during incubation at 11°C.

Discussion

Desensitization is a temperature-dependent process. Several reports have revealed various types of desensitization having their own temperature-dependencies: for example, desensitization usually ceases to develop at temperatures as low as 4°C (El-Fakahany & Richelson, 1980; Higuchi *et al.*, 1981), while in a few cases it develops significantly at this temperature (Magazanik & Vyskocil, 1975; Homburger *et al.*, 1980). Assuming that a desensitization phenomenon is a complex response involving several processes and that these processes have their own temperature-dependence, it may be possible to dissociate them according to their different temperature-dependence. Thus for the β-adrenoceptor, the dissociation of desensitization steps, an alteration in the receptor-effector coupling and a loss of β-adrenoceptors, was uncovered by lowering the temperature to 4°C (Homburger *et al.*, 1980).

The aim of the present study was to reveal various types of desensitization processes involved in guinea-pig ileal muscle by use of essentially the method mentioned above. The temperature range used was 6°–31°C, where receptor-effector coupling appeared to operate well since significant contractile responses to both acetylcholine and histamine were observed. However, desensitization was dramatically influenced over this temperature range. It is possible that cooling of the muscle strips had an appreciable influence on sensitivity to the agonists, thus showing apparently reduced desensitization at low temperatures. However, this possibility was excluded by control experiments which revealed an unchanged sensitivity to the agonists even after the muscle strips were pretreated at low temperatures (6°–21°C).

It was evident from our study that desensitization in guinea-pig ileum could be separated into three types, i.e., desensitization produced by acetylcholine, self-desensitization by histamine, and cross-desensitization by histamine, according to their temperature-dependence (Figure 3). However, one striking feature common to all the types is that desensitization was greatly inhibited at 6°C. This suggests that a certain degree of membrane fluidity is required for the desensitization processes (El-Fakahany & Richelson, 1980; Higuchi *et al.*, 1981). In the present study acetylcholine-induced desensitization was inhibited only partially at 6°C, while histamine-induced desensitization was complete. Possibly, different mechanisms are operating in each desensitization process which may be influenced differently by membrane fluidity. For example, histamine-induced desensitization may include the process of receptor internalization or sequestration which is reported to be completely inhibited at low temperatures (Homburger *et al.*, 1980; Higuchi *et al.*, 1981; Harden *et al.*, 1985). At above 11°C, the differences between each type of desensitization were uncovered. Firstly, acetylcholine-induced desensitization was distinguished by its relative absence of temperature-dependence. It should be noted that the Q_{10} for desensitization calculated in the present study was about 1.0–1.16, a value quite different from the Q_{10} values (approximately 2) reported for the nicotinic receptor system of frog muscle (Magazanik & Vyskocil, 1975) and muscarinic receptor system of neuroblastoma cells (El-Fakahany & Richelson, 1980). The cause of the absence of temperature dependence between 11°–31°C is not clear. Secondly, histamine-induced self-desensitization was characterized by its large temperature-dependence between 11°–21°C. At above 21°C, however, this desensitization process showed little temperature-dependence. This situation can be explained by assuming that two steps are concerned in the process, one having considerable and the other little temperature-dependence. Thirdly, histamine-induced cross-desensitization was characterized by moderate temperature-dependence between 11°–31°C.

Acetylcholine-induced desensitization in guinea-pig ileum has been considered non-specific, being attributed to a gain of Na^+ and a loss of K^+ in the smooth muscle cells (Paton & Rothschild, 1965; Joiner, 1973; James-Kracke & Roufogalis, 1981; Aboulafia *et al.*, 1987). However, several mechanisms have been reported to contribute to a specific desensitization of the muscarinic receptor; receptor phosphorylation (Kwatra *et al.*, 1987), receptor sequestration (Harden *et al.*, 1985), and changes in receptor distribution (Siegel & Triggle, 1983). If these changes are the cause of desensitization, they could be distinguished from non-specific desensitization in terms of differences in their temperature-dependence. However, there were no differences between self- and cross-desensitization produced by acetylcholine (Figure 3). Acetylcholine-induced desensitization in the ileum may, therefore, be non-specific.

Histamine-induced desensitization may occur at the receptor level (Kenakin & Cook, 1980); Bielkiewicz & Cook, 1984), or at a non-specific site (Gosselin & Gosselin, 1973; Morgenstern & Bluth, 1976; Aboulafia *et al.*, 1987). The present finding that histamine-induced self- and cross-desensitization had different temperature-dependencies suggests that two types of mechanism, specific and non-specific, are involved. This idea is further supported by our findings of differences in the time course ($t_{1/2}$ = 3.8 min vs. 11.1 min at 31°C) and in the degree (dose-ratio = 22.4 vs. 11.9 at 31°C, 30 min) of histamine-induced self- and cross-desensitization (Figure 2).

We have classified the recovery processes from desensitization according to their temperature-dependence (Figure 4). Most of the recovery processes, recovery from acetylcholine-induced self- and cross-desensitization and histamine-induced self-desensitization, occurred in a similar way. They occurred according to first order kinetics at 31°C, non-first order kinetics at 21°C, and were completely halted at 11°C. This kind of temperature-dependence is similar to that observed in the pattern of mobility of proteins in the cell membrane (Frye & Edidin, 1970; Yahara & Edelman, 1972), where the mobility of proteins is completely inhibited at 10°C, partially inhibited at 20°C, and scarcely inhibited at 30°C. This similarity suggests that free movement of certain protein(s) in the membrane lipid is prerequisite for the recovery process to proceed. This idea can explain the observation that lowering the temperature to 11°C in the midst of the recovery process, completely halted recovery. Recovery from histamine-induced cross-desensitization was distinguished from others by its dif-

ferent temperature dependence. In this case further development of desensitization was observed at 11°C. At present, the reason for this phenomenon cannot be explained, but it was not due to some irreversible effect of incubating the tissue at 11°C, since 11°C-pretreatment for 2 h had no lasting effect on the sensitivity to the agonists.

Previous observations (Magazanik & Vyskocil, 1975; El-Fakahany & Richelson, 1980) show that both the development of, and the recovery from desensitization have essentially the same temperature-dependence. These results differ from our results in that our data show that the recovery process is more sensitive to temperature change than the desensitization process. In guinea-pig ileum, the recovery process may, therefore, be operated by a mechanism which is influenced profoundly by lowering the temperature, which is distinct from that operated in the desensitization process.

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Enhanced α-adrenoceptor responsiveness and receptor number during global ischaemia in the Langendorff perfused rat heart

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1 Idioventricular rate responses and adrenoceptor number have been examined in normal Langendorff hearts perfused at 70 cmH_2O and in 'ischaemic' hearts perfused at 10 cmH_2O.

2 In hearts perfused at normal pressure, ventricular rate responses to phenylephrine in the presence of propranolol were biphasic with low concentrations of phenylephrine reducing, and high concentrations increasing, ventricular rate. Both responses were abolished in the presence of prazosin (100 nM). In hearts perfused at low pressure, the negative chronotropic responses to phenylephrine were no longer present and positive chronotropic responses were enhanced compared with those of control tissues. The number of ventricular [^{3}H]-prazosin binding sites was also significantly increased during ischaemia.

3 Idioventricular rate responses to isoprenaline were depressed in ischaemic tissues compared with controls, but [^{3}H]-dihydroalprenolol binding was not altered in these hearts.

4 The incidence and duration of arrhythmias which occurred during 30 min of global ischaemia and reperfusion were not significantly altered in the presence of 100 nM prazosin.

5 These results demonstrate that reducing the perfusion pressure of rat isolated hearts enhances α-adrenoceptor-mediated responses and increases α-adrenoceptor density. Whether the increase in α-adrenoceptor responsiveness during ischaemia is involved in arrhythmogenesis requires further investigation.

Introduction

Ventricular arrhythmias form an important complication of acute myocardial ischaemia and reperfusion, but the factors involved in arrhythmogenesis have not yet been fully established. Enhanced sympathetic activity and locally induced noradrenaline release, have been identified as factors involved in the genesis of ventricular arrhythmias (Dart & Riemersma, 1985; Schomig, 1988) and activation of myocardial β-adrenoceptors with the resulting increase in cyclic AMP has been proposed as a critical event in the genesis of ventricular arrhythmias and fibrillation in the ischaemic heart (Podzuweit, 1982; Podzuweit *et al.*, 1989).

α-Adrenoceptors are also present in the myocardium and mediate positive inotropic responses (Bruckner *et al.*, 1985). Activation of these receptors in the cat heart increases ventricular automaticity and has been implicated in the genesis of the arrhythmias which occur during myocardial ischaemia and reperfusion (Sheridan *et al.*, 1980). In this species, ventricular α-adrenoceptor number (Corr *et al.*, 1981) and responsiveness (Sheridan *et al.*, 1980) are increased during periods of myocardial ischaemia and the α-adrenoceptor antagonists, prazosin and phentolamine, abolish ventricular fibrillation induced by coronary artery occlusion or reperfusion. The evidence supporting an increased role for α-adrenoceptors during myocardial ischaemia in other species is less clear. Ischaemia-induced increases in α-adrenoceptor density have been recorded in both the dog (Mukherjee *et al.*, 1980) and rat heart (Allely & Brown, 1988), but Dillon *et al.* (1988) were unable to confirm this finding in the rat. The mechanism for the anti-arrhythmic activity of α-adrenoceptor antagonists, demonstrated in many species (Benfey *et al.*, 1984; Sheridan & Culling, 1985; Williams *et al.*, 1987), has also been questioned and it has been suggested that these effects may be explained by membrane stabilizing properties (Dukes & Vaughan Williams, 1984; Daugherty *et al.*, 1986).

In the present study, we have examined α- and β-adrenoceptor responsiveness and receptor density in ischaemic and control perfused hearts of the rat. We have also examined the effects of prazosin on the arrhythmias induced by global ischaemia and reperfusion in these isolated hearts. A preliminary account of this work has been presented to the British Pharmacological Society (Butterfield & Chess-Williams, 1989).

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Methods

Isolated perfused hearts

Male Wistar rats were killed by a blow to the head and exsanguinated. Hearts were rapidly removed, flushed with heparinized saline (20 units ml^{-1}), and perfused at 70 cmH_2O by the Langendorff method with Krebs-bicarbonate solution (composition in mM: NaCl 118.4, KCl 4.7, $CaCl_2$ 1.9, $NaHCO_3$ 25.0, $MgSO_4$ 1.2, KH_2PO_4 1.2) at 37°C. The perfused hearts were mounted horizontally in an organ bath and superfused with Krebs solution to maintain the temperature at 37°C during periods of reduced coronary flow. The hearts were connected, via a thread and clip attached to the apex of the heart, to an isometric force transducer (Dynamometer UF1, 280g sensitivity). Developed tension was recorded on a Devices MX2 recorder. ECG and heart rate were displayed on a Hewlett Packard ECG monitor and recorded on a Devices MX2 recorder. Hearts were paced at 4 Hz (5 ms pulse-width, twice threshold voltage), with a Grass S48 stimulator and a bipolar silver electrode in contact with the right ventricle.

Preliminary experiments demonstrated that the tension developed by hearts was greatest when the resting tension on the tissue was 2 g and the perfusion pressure was 70 cmH_2O. These values were therefore used in all subsequent experiments.

Radioligand binding studies

Hearts were equilibrated for 20 min until developed tension was stable. The prefusion pressure was then reduced to 10 cmH_2O for 30 min ('ischaemic' perfusion) or maintained at 70 cmH_2O (control perfusion). At the end of this period, the ventricles were quickly immersed in ice-cold Tris buffer

(50 mM) and a crude membrane fraction was prepared from the ventricular tissue by homogenizing the tissue in ice-cold Tris buffer (50 mM) for 15 s with an Ultra-Turrax homogenizer, followed by 4 times 4 strokes of a Teflon homogenizer. The homogenate was centrifuged at 1000*g* for 10 min in an M.S.E. Chillspin centrifuge. The supernatant was then recentrifuged at 30,000*g* for 20 min in an M.S.E.18 centrifuge. The pellet was finally resuspended in 3 ml of Tris buffer and used in a receptor binding assay. These procedures were carried out at 4°C.

The crude membrane fraction was assayed for α-adrenoceptor binding sites with [^{3}H]-prazosin and β-adrenoceptor binding sites with [^{3}H]-dihydroalprenolol (DHA). Samples, 100 μl, of the membrane suspension were incubated at 37°C for 30 min with a range of radioligand concentrations (0.125–4.0 nM for [^{3}H]-prazosin and 0.5–16.0 nM for [^{3}H]-DHA) in a final volume of 250 μl. Non-specific binding was determined by displacement with 10 μM phentolamine in [^{3}H]-prazosin experiments or 200 μM isoprenaline in [^{3}H]-DHA experiments. Incubations were terminated by the addition of 1 ml ice-cold buffer, followed by rapid filtration through Whatman GF/B glass fibres filters. The filters were rapidly washed with 3 times 5 ml ice-cold buffer. Radioactivity remaining on the filters was measured by standard scintillation counting techniques. The specific binding as a percentage of the total binding was approximately 60% for [^{3}H]-DHA and 80% for [^{3}H]-prazosin. Protein content of the membrane fraction was determined by the method of Lowry *et al.* (1951).

Idioventricular rate responses

To examine changes in ventricular automaticity induced by adrenoceptor stimulation, rat isolated hearts were set up as described previously. Before the hearts were paced, the atria were removed and the atrioventricular node was crushed. When the ventricular rate was stable, the tissues were paced at 4 Hz for an equilibration period of 20 min, followed by 30 min control (70 cmH_2O) or ischaemic (10 cmH_2O) perfusion. Without changing the perfusion pressure, the pacing stimulus was then removed, metanephrine (10 μM) and cocaine (10 μM) were added to the perfusate and the tissues were allowed to equilibrate until the ventricular rate was stable. Cumulative concentration-response curves were then constructed to isoprenaline or phenylephrine, by perfusing the tissue with a given concentration of drug until the ventricular rate had stabilized at the new level. The concentration of drug in the perfusate was increased in 3 fold increments until a maximum rate was obtained. When phenylephrine was the agonist, propranolol (0.1 μM) was also added to the perfusate at the same time as the metanephrine and was then present throughout the rest of the experiment. At the end of the experiment, when the maximum response had been obtained to phenylephrine, a maximum rate response to isoprenaline was also obtained.

Effect of prazosin on ventricular arrhythmias

To investigate the effect of prazosin on ventricular arrhythmias in this model, Langendorff perfused hearts were set up as previously described and paced at 4 Hz. Following 20 min equilibration, the perfusion pressure was reduced to 10 cmH_2O for 30 min, before the tissue was reperfused by returning the perfusion pressure to 70 cmH_2O. The arrhythmias occurring during the 30 min perfusion at reduced pressure and during the first 10 min after reperfusion were recorded and classified according to the Lambeth Conventions (Walker *et al.*, 1988). The incidence, time of onset and duration of ventricular tachycardia (VT) and ventricular fibrillation (VF) were recorded and also the incidence and time of onset of premature ventricular complexes (PVCs). Experiments were performed in the absence or in the presence of prazosin (100 nM) in the perfusate.

Statistical analysis

The dissociation constants (K_d) and the maximum number of sites (B_{max}) for [^{3}H]-prazosin and [^{3}H]-DHA binding were determined by Scatchard analysis of saturation curves. The number of binding sites was expressed as the number of sites per mg protein and the mean values with s.e.mean were calculated. Students unpaired *t* test was employed to test for statistically significant differences between control and ischaemic tissue.

In experiments in which idioventricular rate responses were examined, increases in rate were plotted as a percentage of individual maximum responses and EC_{50} values (concentration for a half-maximal response) were determined. Geometric mean EC_{50} values with 95% confidence limits were then calculated and differences between values for control and ischaemic tissues examined by performing Student's *t* test on individual logarithmic EC_{50} values. For phenylephrine, responses were also plotted as a percentage of the maximum isoprenaline response.

In arrhythmia experiments, the incidences of VT, VF and PVCs were compared between prazosin and control groups by use of Chi square test. The duration and time of onset of VT and VF in control and test animals were compared with a Mann Whitney U-test.

Drugs used

Prazosin was kindly supplied as a gift by Pfizer Central Research, Sandwich, Kent. (±)-Isoprenaline, (−)-phenylephrine, (±)-metanephrine and (±)-propranolol were obtained commercially as the hydrochloride salts from Sigma. [^{3}H]-prazosin (23 Ci mmol^{-1}) and [^{3}H]-DHA (110 Ci mmol^{-1}) were obtained from the Radiochemical Centre, Amersham.

Results

Idioventricular rate responses

Removing the atria from perfused hearts resulted in a significant fall in the spontaneous rate of beating (Figure 1) which was reduced even further when the atrioventricular node was crushed, the final resting rate stabilising at 75.9 ± 6.7 beats min^{-1}. Perfusing the ventricles for 30 min at 70 cmH_2O,

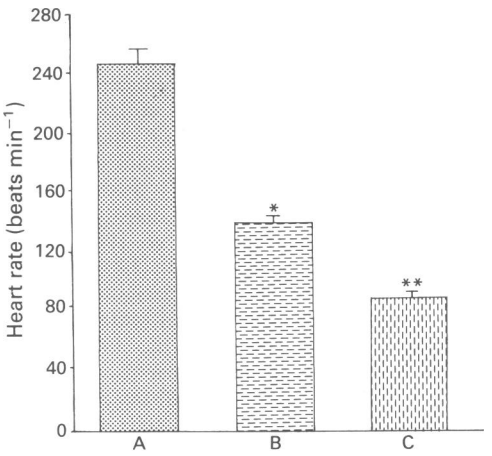

Figure 1 Mean spontaneous rates (beats min^{-1}) of tissues perfused at 70 cmH_2O (A) intact hearts, (B) hearts with the atria removed and (C) hearts with the atria removed and the atrioventricular node crushed. *Significantly different from intact hearts ($P < 0.001$), **Significantly different from hearts with atria removed ($P < 0.001$). Bars show s.e.mean.

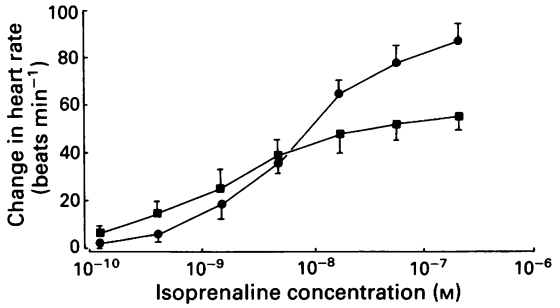

Figure 2 Mean concentration-response curves to isoprenaline of rat isolated perfused ventricles during normal (70 cmH_2O) perfusion (●) and during 'ischaemic' (10 cmH_2O) perfusion (■). Responses are plotted as the mean increase in spontaneous rate. Vertical lines show s.e.mean.

while pacing at 4 Hz did not affect the spontaneous rate, the resting spontaneous rate being 72.8 ± 6.0 beats min^{-1} when the pacing stimulus was removed. However, when the perfusion pressure was reduced to 10 cmH_2O, the spontaneous rate on removing the pacing stimulus after 30 min pacing was significantly reduced to 39.1 ± 2.0 beats min^{-1} ($P < 0.05$). When isoprenaline (1–300 nM) was perfused through the hearts the idioventricular rate was increased in both the control and the ischaemic tissues (Figure 2). The EC_{50} values for isoprenaline were similar in the two groups of tissues (3.5(1.5–8.3) nM and 1.8(0.5–6.3) nM in control and ischaemic tissues respectively), but the maximum increase in rate was greater ($P < 0.05$) in the tissues perfused at 70 cmH_2O (86.3 ± 7.5 beats min^{-1}) than in the tissues perfused at 10 cmH_2O (53.5 ± 8.2 beats min^{-1}).

In contrast, idioventricular rate responses to phenylephrine in control perfused hearts were biphasic: low concentrations of phenylephrine (< 10 μM) reducing spontaneous rate; higher concentrations (> 10 μM) increasing spontaneous ventricular rate (Figure 3). Both the positive and the negative chronotropic responses to phenylephrine were abolished in the presence of prazosin (100 nM). In hearts perfused at 10 cmH_2O, phenylephrine elicited positive chronotropic responses only. The EC_{50} values for these responses in ischaemic tissues (3.0(0.8–18.0) μM) were significantly lower ($P < 0.05$) than those obtained in control tissues (28.8(12.4–67.0) μM), indicating an increase in tissue sensitivity in tissues perfused at only 10 cmH_2O. The maximum increase in spontaneous rate to phenylephrine was also greater in ischaemic tissues (54.8 ± 8.2 beats min^{-1}) than in controls (44.8 ± 11.9 beats min^{-1}), although this was not statistically significant. However, when expressed as a percentage of the isoprenaline maximum response in each tissue, the change was significant ($P < 0.05$), the maximum increasing from 49.6 ± 9.6% to 73.0 ± 5.6%.

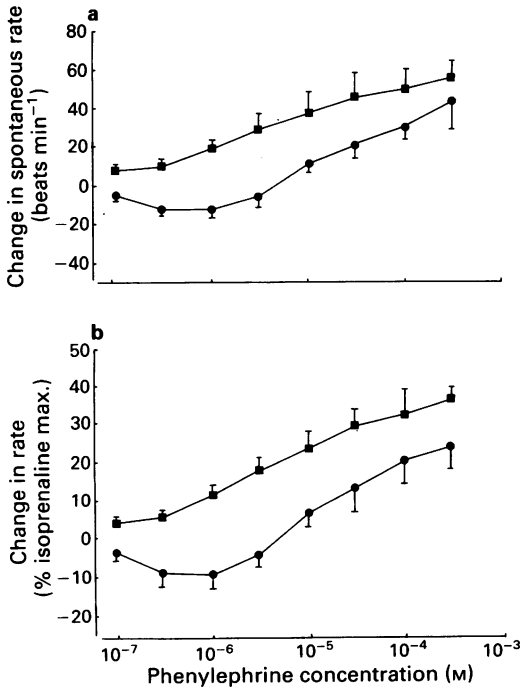

Figure 3 Mean concentration-response curves to phenylephrine of rat isolated perfused ventricles during normal (70 cmH_2O) perfusion (●) and during 'ischaemic' (10 cmH_2O) perfusion (■). Responses are plotted as the change in spontaneous rate in beats min^{-1} (a) and also as a percentage of the isoprenaline maximum response (b). Vertical lines show s.e.mean.

Table 1 The mean number of binding sites (B_{max}) and dissociation constants (K_d) for [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) and [^{3}H]-prazosin binding to crude membrane fractions prepared from control and ischaemic perfused ventricles of the rat

Tissue	B_{max} (fmol mg^{-1} protein)	K_d (nM)	n
[^{3}H]-DHA			
Control	52.1 ± 4.6	1.4 ± 0.1	5
Ischaemic	48.0 ± 4.5	1.9 ± 0.8	5
[^{3}H]-prazosin			
Control	123.6 ± 9.9	0.09 ± 0.04	9
Ischaemic	177.9 ± 16.2*	0.10 ± 0.03	6

Data shown are means ± s.e.mean. *n* is the number of saturation curves studied. Individual hearts were used in each assay procedure. *$P < 0.01$.

Radioligand binding experiments

In membranes prepared from the ventricular tissue of control hearts perfused at 70 cmH_2O for 30 min, the number of [^{3}H]-prazosin binding sites was significantly greater ($P < 0.001$) than the number of [^{3}H]-DHA binding sites (Table 1). Perfusing hearts at a reduced perfusion pressure of 10 cmH_2O for 30 min resulted in a 30% increase in the number of [^{3}H]-prazosin binding sites, without a change in the dissociation constant for [^{3}H]-prazosin binding. Reducing coronary flow had no effect on either the number or the affinity of [^{3}H]-DHA binding sites (Table 1).

Effect of prazosin on arrhythmias

An analysis of the arrhythmias occurring during 30 min of low pressure perfusion and reperfusion, failed to demonstrate an antiarrhythmic effect of prazosin in this model. In control experiments ($n = 10$), PVCs occurred in 70% of the hearts, but the incidence was identical in hearts perfused with 100 nM prazosin ($n = 10$). The time of onset of PVCs and the actual number of PVCs occurring during the 30 min of perfusion at 10 cmH_2O were also similar in control (714 ± 56 s; 32 ± 8 PVCs) and prazosin-perfused (587 ± 147 s; 35 ± 11 PVCs) hearts. No PVCs were recorded during reperfusion in either group of hearts.

Prazosin also failed to alter the incidence or duration of VT and VF induced during low pressure perfusion and reperfusion of hearts (Table 2). The times of onset of VT and VF during ischaemia and reperfusion in the two groups were also not significantly different.

Discussion

In the present study, we have established a model for the examination of ventricular spontaneous rate in the rat. Ventricular automaticity during ischaemia has previously been examined in the anaesthetized cat, where idioventricular rate was recorded during stimulation of the vagus nerve (Sheridan

Table 2 Incidence (%), time of onset (s) and duration (s) of ventricular tachycardia (VT) and ventricular fibrillation (VF) during 30 min ischaemia and 10 min reperfusion of Langendorff rat hearts perfused with Krebs solution alone ($n = 10$) or Krebs solution containing 100 nM prazosin ($n = 10$)

	Control		*Prazosin*	
	VT	*VF*	*VT*	*VF*
Ischaemia				
Incidence (%)	100	30	90	40
Onset (s)	725 ± 100	1473 ± 99	699 ± 109	1008 ± 140
Duration (s)	859 ± 121	88 ± 32	743 ± 147	81 ± 36
Reperfusion				
Incidence (%)	80	60	100	60
Onset (s)	40 ± 35	162 ± 73	18 ± 8	124 ± 47
Duration (s)	337 ± 78	397 ± 89	322 ± 65	327 ± 63

Data shown are means ± s.e.mean.

et al., 1980). However, this method is not ideal for studying adrenergic responses, since muscarinic stimulation itself may alter adrenergic responsiveness (Simpson *et al.*, 1981). We have recorded ventricular rate following removal of the sinoatrial and atrioventricular pacemakers. In preliminary experiments ($n = 4$), the tension developed by hearts before (2.7 ± 0.1 g) and after (2.6 ± 0.1 g) this procedure was similar, indicating that the ventricular tissue was not damaged. Furthermore, the idioventricular rate and developed tension remained stable over a period of several hours of control perfusion.

Idioventricular rate responses to phenylephrine were found to be biphasic in rat isolated perfused ventricles, low concentrations reducing spontaneous ventricular rate, while concentrations greater than 10 μM caused an increase in rate. Negative chronotropic effects of phenylephrine have previously been demonstrated on isolated Purkinje fibres from the dog, the response being mediated via α-adrenoceptors (Rosen *et al.*, 1984). The rate response of canine Purkinje fibres to adrenaline is also biphasic, low concentrations reducing spontaneous rate via α-adrenoceptor activation, while higher concentrations increase ventricular rate via β-adrenoceptor stimulation (Rosen *et al.*, 1977). In the present study, phenylephrine was employed as a selective α-adrenoceptor agonist. However, this drug also has weak β-adrenoceptor agonist activity (Chess-Williams *et al.*, 1989) and responses to phenylephrine were therefore performed in the presence of propranolol. The antagonism of responses by low concentrations of prazosin, which abolished both the negative and positive chronotropic ventricular responses, confirmed that both components of the response to phenylephrine were mediated via α-adrenoceptor activation.

In experiments where perfusion pressure was reduced, positive chronotropic responses to phenylephrine were enhanced. This supports previous *in vivo* studies in the cat (Sheridan *et al.*, 1980; Corr & Crafford, 1981) and dog (Juhasz-Nagy & Aviado, 1976), where ventricular chronotropic responses to α-adrenoceptor stimulation were potentiated during periods of ischaemia. The mechanism for the enhanced α-adrenoceptor responsiveness, may be explained at least partially, by an increase in the density of [^{3}H]-prazosin binding sites in the ischaemic ventricle, indicating an increase in the number of ventricular α-adrenoceptors. Such an increase in the density of α-adrenoceptor binding sites induced by ischaemia has been demonstrated in the cat (Corr *et al.*, 1981; Corr & Crafford, 1981, Dillon *et al.*, 1988) and the dog heart (Mukherjee *et al.*, 1980). No such change can be demonstrated in the guinea-pig heart (Broadley *et al.*, 1985), although the number of ventricular α-adrenoceptors in this species is low, making the demonstration of small changes in receptor number difficult, Dillon *et al.* (1988) were similarly unable to find a change in [^{3}H]-prazosin binding during myocardial ischaemia in the rat. However, the present results disagree, and support the findings of Allely *et al.* (1988) who obtained an increase in α-adrenoceptor number after 30 min of ischaemia in the perfused heart of the rat. This ischaemia-induced increase in α-adrenoceptor number may combine with the lack of a negative chronotropic response to enhance ventricular automaticity during periods of ischaemia.

Ventricular rate responses to isoprenaline were also altered during ischaemic perfusion. Although EC_{50} values were similar in control and ischaemic tissues, maximum responses to isoprenaline were reduced during low pressure perfusion. This may be due to the general depression of spontaneous rate during ischaemia, or to a depressed adenosine 3′:5′-cyclic monophosphate (cyclic AMP) response to β-adrenoceptor stimulation. Short periods of ischaemia have been shown to result in a depressed cyclic AMP response to β-adrenoceptor stimulation (Krausse & England, 1982).

Thus, during ischaemia, β-adrenoceptor responses were depressed while α-adrenoceptor responses were enhanced and the maximum phenylephrine response relative to isoprenaline was increased from 50% to 73%. A similar situation also occurs in hypothyriodism and results in the inotropic response to noradrenaline being mediated via α-adrenoceptors, whereas in the normal hearts of euthyroid animals responses to noradrenaline are mediated solely via β-adrenoceptors (Chess-Williams & Critchley, 1987). The observations made in the present study may therefore explain the change in the idioventricular rate response to sympathetic nerve stimulation during ischaemia in the cat heart, where responses were β-mediated in the normal heart but α-mediated during ischaemia (Sheridan *et al.*, 1980).

Whether the enhanced ventricular α-adrenoceptor responsiveness contributes to the genesis of arrhythmias is unclear at the present time. Manning *et al.* (1984) have found that the increase in cardiac α-adrenoceptor density during ischaemia does not coincide with the onset of arrhythmias, suggesting a dissociation of the two events. On the other hand, α-adrenoceptor activation has been shown to play an important role during reperfusion, where α-adrenoceptor antagonists significantly attenuate calcium uptake (Sharma *et al.*, 1983). α-Adrenoceptor antagonists have also been shown to prevent reperfusion-induced arrhythmias in the cat (Sheridan *et al.*, 1980), dog (Benfey *et al.*, 1984), guinea-pig (Sheridan & Culling, 1985) and rat (Williams *et al.*, 1987), although it has been suggested that these effects may be due to membrane stabilizing properties (Dukes & Vaughan-Williams, 1984; Daugherty *et al.*, 1986). In the present study, 100 nM prazosin failed to reduce the incidence of premature ventricular complexes, ventricular tachycardia or ventricular fibrillation induced during periods of low pressure perfusion and reperfusion. This concentration of prazosin completely abolished chronotropic responses to phenylephrine in perfused ventricles, thereby confirming that this concentration of prazosin produced a substantial blockade of α-adrenoceptors. The increase in α-adrenoceptor responsiveness therefore, does not appear to contribute to arrhythmogenesis in this model of global ischaemia.

It has been suggested that where myocardial ischaemia is localised to small regions, local α-adrenoceptor mediated prolongation of action potential duration may combine with β-adrenoceptor-mediated shortening of action potential duration in areas of normal coronary flow, to produce the electrical inhomogeneity suitable for the emergence of ventricular arrhythmias (Uprichard *et al.*, 1988). If this is correct, the ischaemia-induced increase in α-adrenoceptor responsiveness may not be an important factor in the genesis of arrhythmias in a model of global ischaemia, where α-mediated prolongation of action potential duration may be more uniform. Whether an α-adrenoceptor antagonist would have been more effective against ischaemia- and reperfusion-induced arrhythmias following coronary artery occlusion is unknown. Manning *et al.* (1984) could not demonstrate a protective effect for prazosin in coronary artery occluded isolated hearts of the rat, but Bernauer & Ernenputsch (1988) have found the α_1-selective antagonist corynanthine to be effective in this model.

α_1-selective antagonist corynanthine to be effective in this model.

The present study has therefore demonstrated both positive and negative idioventricular rate responses to α-adrenoceptor stimulation, the positive chronotropic response being potentiated during ischaemia. The increase in α-adrenoceptor sensitivity during ischaemia is associated with an increase in the number of [^{3}H]-prazosin binding sites, but further work is required to establish the relationship between these receptor changes and the genesis of arrhythmias during myocardial ischaemia and reperfusion.

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Modulation of fluoroaluminate-induced inositol phosphate formation by increases in tissue cyclic AMP content in bovine tracheal smooth muscle

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1 The effect of fluoroaluminate complexes ($AlCl_3$ plus NaF) upon smooth muscle tone, [^{3}H]-inositol phosphate accumulation and [^{3}H]-cyclic AMP accumulation has been investigated in slices of bovine tracheal smooth muscle.

2 Fluoroaluminate (10 μM $AlCl_3$ + various concentrations of NaF) elicited concentration-dependent contractions of bovine tracheal smooth muscle strips at concentrations of NaF in the range 1–10 mM. The resultant contractile response was reversed by isoprenaline (50 nM) and was preserved in calcium-free medium.

3 Fluoroaluminate stimulated [^{3}H]-inositol phosphate formation at concentrations of NaF over 1 mM. The response to 20 mM NaF + 10 μM $AlCl_3$ was 164 ± 29% of the response to 1 mM histamine. Fluoroaluminate also increased the incorporation of [^{3}H]-*myo*-inositol into membrane phospholipids.

4 Fluoroaluminate produced a small rise in [^{3}H]-cyclic AMP levels (2.1 fold increase over basal with 20 mM NaF). The response to forskolin (1 μM, 8.6 fold over basal) was reduced by fluoroaluminate in a concentration-dependent manner, but still remained significantly ($P < 0.05$) elevated over the response to fluoroaluminate alone.

5 The [^{3}H]-inositol phosphate response to fluoroaluminate was inhibited by salbutamol (maximum inhibition 60%, IC_{50} = 0.08 μM), forskolin (1 μM, 46% inhibition) and isobutylmethylxanthine (1 mM, 73% inhibition).

6 These data suggest that inhibition of agonist-induced inositol phospholipid turnover by cyclic AMP in this tissue can occur at the post-receptor level.

Introduction

Histamine H_1-receptor stimulation in bovine tracheal smooth muscle is associated with inositol phospholipid hydrolysis and a contractile response (Barnes, 1986; Hall & Hill, 1988; Hall *et al.*, 1989). The second messenger products of inositol phospholipid hydrolysis, inositol 1,4,5-trisphosphate and diacylglycerol, have both been implicated as mediators of the contractile response to histamine in this tissue. Inositol 1,4,5-trisphosphate is thought to be involved in the initiation of the contractile response by mobilising calcium from intracellular stores (Hashimoto *et al.*, 1985; Takuwa *et al.*, 1987; Kotlikoff *et al.*, 1987; Berridge, 1987), whilst diacylglycerol has been proposed to have a role in the maintenance of the tonic contractile phase by activating protein kinase C (Park & Rasmussen, 1985; Takuwa *et al.*, 1986).

We have previously demonstrated that the inositol phosphate response induced by histamine in bovine tracheal smooth muscle can be inhibited by a range of smooth muscle relaxants which are capable of elevating tissue adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels through a variety of different mechanisms, including β_2-adrenoceptor stimulation, direct stimulation of adenylate cyclase and phosphodiesterase inhibition (Hall & Hill, 1988; Hall *et al.*, 1989). One possible explanation of this effect is that cyclic AMP is phosphorylating some component(s) of the inositol phospholipid receptor-effector system, via the activation of protein kinase A, leading to inhibition of receptor-mediated inositol phospholipid hydrolysis. This could occur either at the H_1-receptor itself, or at the post receptor (G protein/phosphoinositidase C) level.

There is increasing evidence that a guanosine 5'-triphosphate (GTP)-binding protein (termed G_p) is responsible for transducing information between calcium mobilising receptors, such as the H_1-receptor, and phosphoinositidase C (Cockcroft & Gomperts, 1985; Berridge 1987; Cockcroft & Stutchfield, 1988; Claro *et al.*, 1989). In intact tissues and membrane preparations from a number of tissues, phosphoinositidase C activity can be stimulated by the fluoroaluminate complex AlF_4^- formed when Al^{3+} and F^- are present in solution together (Sternweis & Gilman, 1982). AlF_4^- is believed to stimulate phosphoinositidase C activity by activating G_p directly via the GTP binding site (Blackmore *et al.*, 1985; Harden, 1989). In addition, fluoroaluminates have been shown to induce both contraction and inositol phosphate formation in uterine smooth muscle (Marc *et al.*, 1988).

In this study we have utilized the ability of fluoroaluminates to stimulate phosphoinositidase C activity via activation of G_p, in order to examine the potential of the inositol phospholipid system in airway smooth msucle for modulation by raised intracellular cyclic AMP levels at the post-receptor level. A preliminary account of this work has been presented to the British Pharmacological Society (Hall & Hill, 1989a).

Methods

Contractile responses

The contractile response of strips of bovine tracheal smooth muscle (1 cm by 0.3 cm), denuded of epithelium and mucosa, was measured by isometric recording in a 20 ml organ bath by use of Grass FT03 isometric transducers. The tissue was allowed to equilibrate under 1 g of tension in Krebs-Henseleit buffer, continuously gassed with 95% O_2/5% CO_2 for 1 h, before the addition of 10 μM $AlCl_3$ and various concentrations of NaF. In some experiments, the medium was changed to a nominally calcium-free Krebs-Henseleit buffer, and contractile responses monitored again after a 30 min equilibration period.

Formation of [^{3}H]-inositol phosphates

Accumulation of total [^{3}H]-inositol phosphates in response to stimulation with histamine was measured in slices of bovine tracheal smooth muscle as describe previously (Hall & Hill, 1988). In brief, the trachealis muscle was dissected free from

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surrounding tissue in trachea obtained from freshly slaughtered bullocks, and smooth muscle slices (300 μm) prepared with a McIlwain tissue chopper. Washed slices were incubated for 30 min in 25 ml of Krebs-Henseleit buffer at 37°C under an atomosphere of 95% O_2/5% CO_2, and then labelled in a minimal volume of Krebs-Henseleit buffer containing 30 μCi [^{3}H]-myo-inositol (final concentration 0.4 μM, total volume 8 ml) for a further 75 min under the same conditions. The slices were then washed in Krebs-Henseleit buffer containing 5 mM lithium chloride and resuspended in 8 ml of medium. One hundred μl aliquots of the slice suspension were transferred to flat-bottomed insert vials containing Krebs-Henseleit buffer and 5 mM LiCl (final volume 300 μl). The vials were gassed with 95% O_2/5% CO_2, capped and incubated for 20 min at 37°C in the presence or absence of phosphodiesterase inhibitors. Agonists or cyclic AMP analogues were finally added in 10 μl of medium and the incubation terminated after 45 min by the addition of 100 μl of ice-cold perchloric acid (10% w/v). Samples were neutralised with 0.75 ml KOH (0.15 M), centrifuged (3000 g, 10 min, 4°C) and 0.75 ml aliquots of the supernatant diluted to 3 ml with 50 mM Tris buffer (pH 7.0). Total [^{3}H]-inositol phosphates were finally separated from free [^{3}H]-*myo*-inositol by anion-exchange chromatography (Hill & Kendall, 1987).

In order to measure incorporation of [^{3}H]-inositol into inositol-containing phospholipids, 1.2 ml chloroform/methanol/10 M HCl (100:200;1 v/v/v) was added to each sample after removal of the supernatant as described above. The tubes were vortexed and left to stand for 30 min. Chloroform (0.4 ml) and water (0.4 ml) were then added and the tubes centrifuged at *circa* 1500 g for 5 min to separate the phases. Two hundred μl of the chloroform phase was then removed into a scintillation vial and dried overnight. Tritium was determined by liquid scintillation counting.

Accumulation of [^{3}H]-cyclic AMP

Formation of [^{3}H]-cyclic AMP by slices of bovine tracheal smooth muscle was measured as previously described (Hall *et al.*, 1989). Essentially, washed slices prepared as described above were incubated for 40 min at 37°C in 20 ml of Krebs-Henseleit medium containing 0.08 μM (40 μCi) [^{3}H]-adenine under an atmosphere of 95% O_2/5% CO_2. These slices were washed with Krebs-Henseleit, resuspended in 8 ml of medium, and 100 μl aliquots of slice suspension were added to flat bottomed insert vials containing Krebs-Henseleit medium. Agonists were then added in 10 μl of medium and the incubations terminated after 10 min by the addition of 200 μl of 1 M HCl. After the samples had been left on ice for 5 min, 0.75 ml of distilled water was added to dilute the samples, and slices precipitated by centrifugation at 1500 g for 10 min. One ml aliquots of the supernatant were analysed for [^{3}H]-cyclic AMP by column chromatography, as described previously (Donaldson *et al.*, 1988). Recovery of [^{3}H]-cyclic AMP from the columns was corrected for by spiking the samples with [^{14}C]-cyclic AMP (Donaldson *et al.*, 1988). Additional 100 μl samples were taken from the supernatant and used to determine the total radioactivity present in each sample; these 'totals' were then used to correct for variations in the amount of tissue present in each sample.

Data analysis

The effect of the various agents on inositol phosphate formation was performed by paired t tests, and in addition with the Wilcoxon signed rank test where applicable. Concentration-response curves were drawn by inspection. All values given in the text or figure legends represent mean ± s.e.mean of n separate experiments.

Chemicals

Dowex 50W, H^+-form (200–400 mesh), Dowex-1 (X8, 100–200 mesh, chloride form), neutral alumina (type WN-3), imidazole, isobutylmethylxanthine (IBMX), salbutamol, forskolin, $AlCl_3$ and NaF were obtained from Sigma. Histamine dihydrochloride was purchased from BDH. [^{3}H]-*myo*-inositol (16.5 Ci mmol^{-1}) and 8-[^{14}C]-cyclic AMP (specific activity 42.4 mCi mmol^{-1}) were purchased from N.E.N., and 8-[^{3}H]-adenine (specific activity 26 Ci mmol^{-1}) was obtained from Amersham.

Results

Effect of fluoroaluminate on tracheal smooth muscle strips

A combination of $AlCl_3$ (10 μM) and NaF elicited a marked contraction of bovine tracheal smooth muscle strips at concentrations of NaF above 1 mM NaF (Figure 1). The maximum contractile response seen (10 mM NaF) accounted for 59 ± 7% of the response to a maximal dose of carbachol (10 μM; $n = 4$). The contractile response to fluoroaluminate was reversed by isoprenaline (50 nM) (Figure 1a and c). Switching to a nominally calcium-free buffer before the addition of fluoroaluminate had little effect upon the ensuing contractile response in the majority of experiments (Figure 1d), although in one experiment some increase in the contractile response was noted (Figure 1b). The contractile response to fluoroaluminate was usually delayed by a period of 2–4 min after the addition of fluoroaluminate in calcium-containing medium (but see Figure 1c), and this delay was increased in the absence of added calcium (Figure 1). A similar delay has been noted in the contractile response of uterine smooth muscle (Marc *et al.*, 1988).

Effect of fluoroaluminate on [^{3}H]-inositol phosphate formation

The effect of varying the concentration of the fluoroaluminate ion upon total [^{3}H]-inositol phosphate formation in slices of bovine tracheal smooth muscle is shown in Figure 2. It can be seen that at concentrations of fluoroaluminate ≥ 5 mM there is marked stimulation of [^{3}H]-inositol phosphate formation,

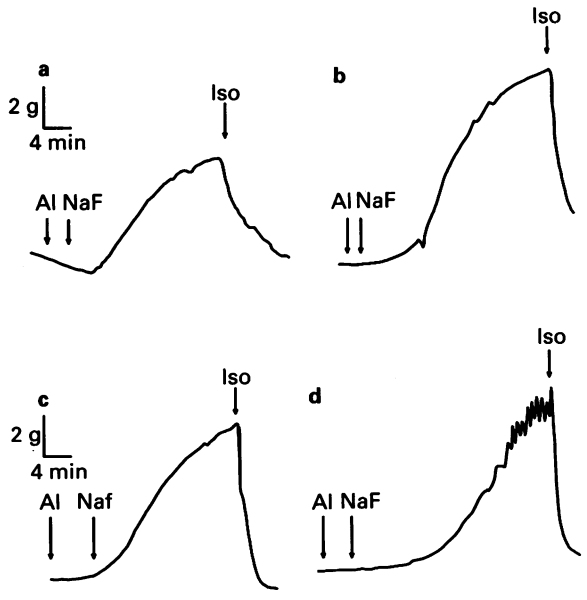

Figure 1 The effect of 4 mM NaF, in the presence of 10 μM $AlCl_3$ (Al), on the smooth muscle tone of bovine tracheal smooth muscle strips, and the inhibition of this response by 50 nM isoprenaline (Iso). Traces show the response to NaF obtained in calcium-containing (a and c) or calcium-free (b and d) Krebs-Henseleit medium. Traces (a and b) or (c and d) were obtained on the same tracheal smooth muscle strip.

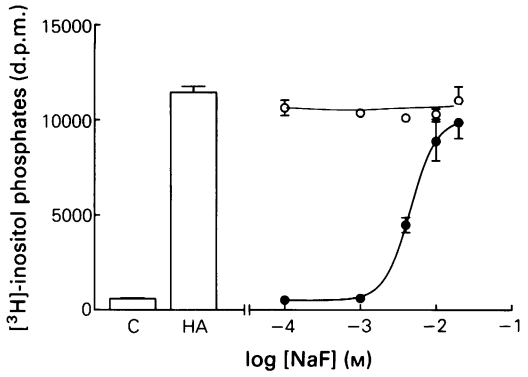

Figure 2 The effect of increasing concentrations of NaF in the presence of 10 μM $AlCl_3$ (●) on the accumulation of total [^{3}H]-inositol phosphates in slices of bovine tracheal smooth muscle. The control (unstimulated) response is shown by the column marked C, and the response to 1 mM histamine by the column marked HA. (○) Simultaneous addition of NaF, 10 μM $AlCl_3$ and 1 mM histamine. Each data point represents the mean of triplicate determinations and vertical lines show s.e.mean. Where no error bars are shown they are within the size of the symbol. Data are from a single experiment repeated twice more with similar results.

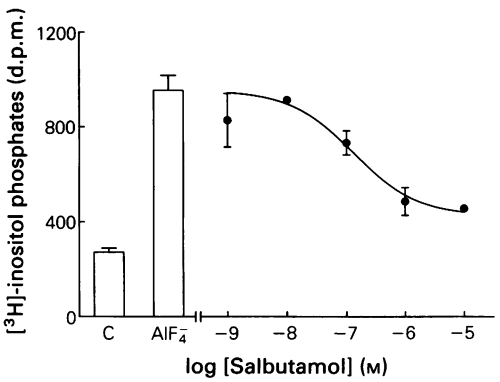

Figure 3 Inhibition of the inositol phosphate response to 20 mM NaF and 10 μM $AlCl_3$ by salbutamol. The basal (unstimulated response) is shown by the column marked C, and the response to fluoraluminate by the column marked AlF_4^-. The effect of adding various concentrations of salbutamol with fluoroaluminate is shown in the graph on the right of the figure. Each data point shows the mean of triplicate determinations in a representative experiment and vertical lines show s.e.mean. The experiment was repeated twice with similar results.

compatible with fluoroaluminate stimulating hydrolysis of phosphatidylinositol 4,5-bisphosphate via its action on the G protein coupled to phosphoinositidase C. The relative sizes of the responses obtained with 1 mM histamine and a standard dose of fluoroaluminate (10 μM $AlCl_3$ + 20 mM NaF) varied quite widely between preparations. The relative size of the fluoroaluminate response (10 μM $AlCl_3$ + 20 mM NaF) ranged from 60 to 345% of the response to 1 mM histamine with a mean value of 164 ± 29% (n = 11). Figure 2 also indicates that the inositol phosphate responses to histamine and fluoroaluminate ions were not additive. As has been demonstrated for other agonists in this tissue (Hall & Hill, 1988; 1989b; Chilvers *et al.*, 1989), 20 mM fluoroaluminate also induced an increase in the incorporation of [^{3}H]-*myo*-inositol into tissue slices to 171 ± 32% (n = 4) compared with control (unstimulated, = 100%) levels.

Modulation of inositol phosphate response to fluoroaluminate by cyclic AMP

To investigate the ability of changes in tissue cyclic AMP content to modulate the [^{3}H]-inositol phosphate response to fluoroaluminate, we examined the effect of varying the concentration of the β_2-adrenoceptor agonist salbutamol upon the [^{3}H]-inositol phosphate response to 20 mM fluoroaluminate. A typical example of this series of experiments is shown in Figure 3, demonstrating that concentration-dependent inhibition of the response is seen with salbutamol. The calculated IC_{50} value for salbutamol was 0.08 ± 0.02 μM (n = 3), which is in excellent agreement with the previously obtained IC_{50} for salbutamol-induced inhibition of the inositol phosphate response to histamine (0.03 μM) in this tissue (Hall & Hill, 1988). The mean inhibition seen with 1 μM salbutamol was 60 ± 6% (n = 8, $P < 0.05$), again agreeing well with the inhibitory effect of 1 μM salbutamol on histamine-stimulated inositol phosphate formation.

It is notable that the absolute magnitude (in terms of d.p.m.) of the inositol phosphate response to fluoroaluminate and histamine varied extensively between experiments (cf Figures 2 and 3 which show respectively the largest and smallest fluoroaluminate responses observed in this study). The reason for this is unclear, but it probably reflects inter-animal differences in both the size of the response and the extent to which [^{3}H]-inositol is taken up by different bovine tracheal slice preparations. However, within individual experiments reproducibility was good (Figures 2 and 3) and the extent of the inhibition produced by cyclic AMP-elevating agents (in percentage terms) was consistent between experiments (Table 1).

The effect of a range of agents capable of elevating tissue cyclic AMP levels by (a) phosphodiesterase inhibition (IBMX, a non-selective phosphodiesterase inhibitor, Reeves *et al.*, 1987), (b) direct stimulation of adenylate cyclase (forskolin) or (c) receptor mediated stimulation of adenylate cyclase (salbutamol) is shown in Table 1, together with results previously obtained for the inhibitory effect of these agents on histamine-stimulated inositol phosphate formation (Hall *et al.*, 1989). These results show that the inositol phosphate response to fluoroaluminate is subject to modulation in a similar manner to the histamine-induced inositol phosphate response.

Effect of fluoroaluminate on G_s and G_i

In addition to stimulating the G protein linked to phosphoinositidase C, fluoroaluminate might be expected to stimulate other G proteins present in the tissue including G_s and G_i linked to adenylate cyclase. This might in turn be expected to modulate the effect of agents working through adenylate cyclase on the inositol phosphate response to fluoroaluminate. In order to examine this possibility the effect of various concentrations of fluoroaluminate on [^{3}H]-cyclic AMP formation was quantified. Alone, fluoroaluminate produced a small, but significant increase in tissue cyclic AMP levels at the highest concentration examined (20 mM, 2.1 ± 0.4 fold stimulation over basal, n = 8, $P < 0.05$) (Figure 4). Forskolin (1 μM) produced a marked stimulation (8.6 ± 1.7 fold, n = 7) of [^{3}H]-

Table 1 Inhibition of fluoroaluminate- and histamine-stimulated [^{3}H]-inositol phosphate formation in bovine tracheal smooth muscle

	% inhibition of response to:	
Agent	*AlF_4^- 20 mM*	*Histamine 0.1 mM†*
Salbutamol (1 μM)	60 ± 6* (8)	66 ± 3* (15)
IBMX (1 mM)	73 ± 2* (6)	81 ± 6* (8)
Forskolin (1 μM)	46 ± 5* (6)	48 ± 6* (8)

Data shown are means ± s.e.mean; n = number in parentheses.
* $P < 0.05$ (paired t and Wilcoxon signed rank tests). IBMX = 3-isobutyl-1-methylxanthine. AlF_4^- 20 mM represents a mixture of 10 μM $AlCl_3$ and 20 mM NaF.
† Data from Hall *et al.* (1989).

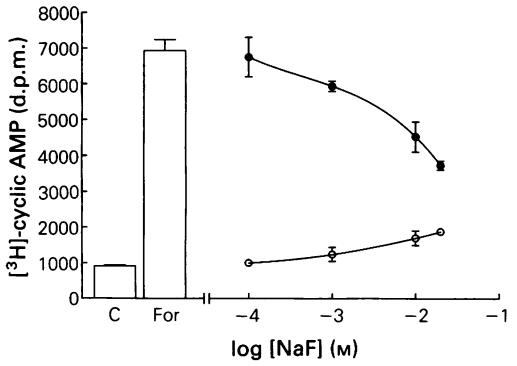

Figure 4 The effect of varying concentrations of NaF in the presence of 10 μM $AlCl_3$ on the accumulation of [^{3}H]-cyclic AMP in slices of bovine tracheal smooth muscle in the presence and absence of 1 μM forskolin. The basal (unstimulated) response is shown by the column marked C, and the response to forskolin alone by the column marked For. The responses to various concentrations of NaF in the presence of 10 μM $AlCl_3$ in the absence (○) and presence (●) of forskolin are shown. Each data point represents the mean of triplicate determinations in a representative experiment repeated at least twice with similar results. Vertical lines represent s.e.mean and where no error bars are shown they are within the size of the symbol.

cyclic AMP formation as previously demonstrated (Hall *et al.*, 1989). When 20 mM fluoroaluminate and 1 μM forskolin were added together, the net effect of fluoroaluminate seen was a significant ($P < 0.05$) inhibition of the [^{3}H]-cyclic AMP response to forskolin, the fold stimulation being reduced to 3.7 ± 0.8 ($n = 7$) (see Figure 4). However, the net effect of forskolin and fluoroaluminate was still a significant ($P < 0.05$) accumulation of cyclic AMP over basal levels. At lower concentrations of fluoroaluminate, a smaller stimulation of [^{3}H]-cyclic AMP accumulation was observed in the absence of forskolin, and the inhibitory effect on the response to 1 μM forskolin was less marked (Figure 4).

Discussion

We have previously shown that elevation of tissue cyclic AMP levels through a variety of different mechanisms, with a range of agents capable of relaxing airway smooth muscle, can inhibit the inositol phosphate responses to histamine and 5-hydroxytryptamine in bovine tracheal smooth muscle (Hall *et al.*, 1989; Hall & Hill, 1989b). The studies described here provide further insight into the mechanisms underlying the modulatory effect of elevation of tissue cyclic AMP on inositol phosphate responses.

Fluoroaluminate (AlF_4^-) is thought to induce inositol phosphate responses in smooth muscle and other tissues by a receptor-independent mechanism involving stimulation of G_p, the G protein which acts as a link in the signal transduction pathway between agonist receptors and phosphoinositidase C (Sternweis *et al.*, 1982; Cockcroft & Gomperts, 1985; Blackmore *et al.*, 1985; Gilman, 1987; Cockcroft & Stutchfield, 1988). This is probably a result of the fluoroaluminate complex substituting for GTP and activating the G protein, causing dissociation of α from $\beta\gamma$ subunits in a manner similar to the proposed mechanism of action of the stable analogue of GTP, GTPγS (Harden, 1989). Fluoride is able to activate membrane phosphoinositidase activity at similar concentrations (>1 mM) to those activating G proteins (Cockcroft & Stutchfield, 1988), and has been shown to induce inositol phosphate formation in uterine smooth muscle (Marc *et al.*, 1988). As shown above, at the concentrations at which it is effective in stimulating phosphoinositidase C activity in other tissues, fluoroaluminate causes bovine tracheal smooth muscle contraction and stimulates [^{3}H]-inositol phosphate formation, implicating the involvement of a G protein in this response, as in many other tissues which have been studied (Harden, 1989). These results therefore provide support for the involvement of the phosphoinositide system in pharmaco-mechanical coupling in this tissue. It was notable, particularly in calcium-free conditions, that a delay of 2–4 min was seen before the contractile response commenced: the explanation for this remains at present uncertain. A similar observation has been made in uterine smooth muscle (Marc *et al.*, 1988).

A relationship between intracellular cyclic AMP and inhibition of agonist-induced inositol phosphate formation has been observed in a number of tissues including bovine and canine tracheal smooth muscle (Hall & Hill, 1988; Madison & Brown, 1988; Hall *et al.*, 1989), neutrophils (Della Bianca *et al.*, 1986), platelets (Takai *et al.*, 1982; Watson *et al.*, 1984), gastric mucosal cells (Puurunen *et al.*, 1987) and rat kidney (Neylon & Summers, 1988), but little is known of the mechanism underlying the interaction between these two intracellular second messenger pathways. The present study suggests that, in bovine tracheal smooth muscle, cyclic AMP exerts a modulatory effect on inositol phosphate responses at the post-receptor level. Thus, the inositol phosphate response, induced by direct stimulation by fluoroaluminate of the G protein linked to phosphoinositidase C, is inhibited by agents which elevate cyclic AMP levels in this tissue. The most likely target for cyclic AMP would therefore seem to be either the G protein itself or phosphoinositidase C.

In addition to stimulating the G protein coupled to phosphoinositidase C, fluoroaluminate would be expected to stimulate other G proteins present in the tissue, including G_s and/or G_i linked to adenylate cyclase. When added on its own in bovine tracheal smooth muscle, fluoroaluminate produced a small stimulation of [^{3}H]-cyclic AMP formation at the higher concentration used. However, when added with the adenylate cyclase activator forskolin, the net effect of fluoroaluminate was an inhibition of the cyclic AMP response to forskolin at concentrations of fluoroaluminate above 10 mM, suggesting that fluoroaluminate inhibits adenylate cyclase activity when this is stimulated with other agents in intact tissues, as has been previously demonstrated (Marc *et al.*, 1988). This effect is presumably due to stimulation of G_i by fluoroaluminate. The combination of forskolin and fluoroaluminate, however, still produced a significant stimulation of cyclic AMP accumulation over basal levels (i.e. in the presence of fluoroaluminate alone) of a magnitude that we have previously shown to be associated with inhibition of histamine-stimulated inositol phosphate (Hall *et al.*, 1989). Hence the net effect of adding forskolin with fluoroaluminate on [^{3}H]-inositol phosphate formation would be expected to be the cyclic AMP-mediated inhibition that was observed.

The increase in cyclic AMP accumulation observed in the presence of fluoroaluminate alone (Figure 4) is within the range that one would expect to observe some inhibition of inositol phospholipid hydrolysis (Hall *et al.*, 1989). This suggests that the fluoroaluminate inositol phosphate response is blunted to some extent by the effect of this ion on the cyclic AMP messenger system. No marked inhibition of the inositol phosphate response to histamine was obvious when increasing concentrations of fluoroaluminate were added simultaneously (Figure 1). However, the possible presence of an inhibitory cyclic AMP-dependent component in the combined fluoroaluminate/histamine response makes it difficult to draw any conclusions regarding the additivity of the two responses and *vice versa*. The fact that further decrements in fluoroaluminate-induced inositol phosphate responses can be achieved by salbutamol, IBMX and forskolin does, however, indicate that the raised cyclic AMP levels produced by fluoroaluminate alone are not sufficient to produce a maximal inhibition of the inositol phosphate response.

In summary, this study suggests that a G protein is likely to be involved in the signal transduction pathway thought to be responsible for pharmaco-mechanical coupling in bovine airway smooth muscle, and that stimulation of the G protein

with fluoroaluminate produces an inositol phosphate response in this tissue, which is subject to regulation by changes in tissue cyclic AMP content. The target of action of cyclic AMP in this response is likely to be either phosphoinositidase C or the G protein itself. These observations lend support to the proposal that part of the relaxant properties of agents, which elevate tissue cyclic AMP levels against histamine- or 5-hydroxytryptamine-contracted airway smooth muscle, is due to the inhibitory effect of raised cyclic AMP levels on inositol phosphate production at the post-receptor level.

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Characterization of cardiac angiotensin converting enzyme (ACE) and *in vivo* inhibition following oral quinapril to rats

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1 Angiotensin converting enzyme (ACE) from the rat heart and lung was studied by use of the radioligand [^{125}I]-351A.

2 Displacement of the bound radioinhibitor [^{125}I]-351A was used to assess the relative potency of six ACE inhibitors in rat heart and lung homogenates and estimate the binding association constant (K_A).

3 The K_A for atrial preparations was significantly higher than that of the lung ($P < 0.025$) and also the ventricles ($P < 0.005$). Ventricular preparations and preparations from the lung also differed significantly ($P < 0.05$). These differences in K_A were noted for all six ACE inhibitors used to displace the radioligand.

4 The rank order of potency of the ACE inhibitors was quinaprilat = benazeprilat > perindoprilat > 351A > lisinopril > fosinoprilat.

5 Cardiac ACE inhibition was studied *ex vivo* following oral administration of quinapril to rats. Following 0.3 mg kg^{-1} quinapril, the time course and degree of inhibition of ventricular and atrial ACE were similar.

6 These results suggest that the detected differences in K_A noted have only a limited potential biological significance. The difference in K_A may reflect variations in the structure or conformation of ACE in different tissues.

Introduction

The renin angiotensin system (RAS) has traditionally been regarded as a circulating endocrine system. The concept that an endogenous RAS exists in tissues has recently been supported by the demonstration of all components of the system in several tissues, including the heart, where locally generated angiotensin may exert an autocrine or paracrine influence on myocardial function (Ganten *et al.*, 1984; Campbell, 1987; Dzau, 1987).

The possible physiological role of the RAS in the heart is suggested by clinical and experimental data on the cardiac effects of angiotensin converting enzyme (ACE) inhibitors. ACE inhibitors have been shown to (1) reduce ventricular arrhythmias associated with coronary artery occlusion and reperfusion (van Gilst *et al.*, 1984; 1986), (2) reduce ischaemic injury following coronary artery ligation (Ertl *et al.*, 1982; Daniell *et al.*, 1984; Hock *et al.*, 1985; de Graeff *et al.*, 1987), (3) increase coronary flow *in vivo* and in the isolated perfused heart (Ertl *et al.*, 1983; Linz *et al.*, 1986), (4) prevent ventricular dilatation and remodelling after myocardial infarction (Pfeffer & Pfeffer, 1988) and (5) prevent or reduce cardiac hypertrophy in experimental hypertension (Sen *et al.*, 1980; Pfeffer *et al.*, 1982; Sharma *et al.*, 1983; Dussaule *et al.*, 1986).

The results of animal studies support the clinical observations of a decreased incidence of arrhythmias (Cleland *et al.*, 1984; 1985; Webster *et al.*, 1985), improvement in haemodynamic indices, clinical symptoms (Dzau *et al.*, 1980; Fouad *et al.*, 1982; Sharpe *et al.*, 1988) and improved mortality (The Consensus Trial Study Group, 1987; Newman *et al.*, 1988) in patients with congestive heart failure, and decrease in left ventricular mass (Fouad *et al.*, 1983) in patients with essential hypertension after treatment with ACE inhibitors.

The rapid increase in the clinical use of ACE inhibitors and the further development of a new generation of compounds with the potential for differential binding characteristics to tissue ACE suggested the present study. The effect of a panel of ACE inhibitors on myocardial ACE has been compared to pulmonary ACE in the rat. We have also examined the ability of the ACE inhibitor quinapril to inhibit myocardial ACE when administered *in vivo*.

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Methods

Radioinhibitor binding assay of ACE

Radioligand preparation 351A, (N-(1carboxy-3-phenylpropyl) -L-lysyl-[εN-4hydroxy benzimido]-L-proline, a potent competitive inhibitor of ACE was used as the ligand for ACE. 351A was radioiodinated by the chloramine T method of Hunter & Greenwood (1962). Two hundred and fifty ng of 351A was dissolved in 25 μl of 0.5 M potassium phosphate buffer (pH 7.5) and, 1 mCi Na ^{125}I and 10 μl chloramine T (1 mg ml^{-1}) were added sequentially and mixed for 45 s. The reaction was stopped by addition of 10 μl sodium metabisulphite (5 mg ml^{-1}) followed by 50 μl sodium iodide and bovine serum albumin each at a concentration of 10 mg ml^{-1}. The mixture was applied to a 1 × 15 cm column of SP Sephadex C25 equilibrated with 0.01 M ammonium acetate buffer (pH 3). The column was washed with the same buffer, then [^{125}I]-351A was eluted with 0.1 M ammonium acetate buffer (pH 3.5). The purity of the radioligand was examined by high performance liquid chromatography and a single peak of activity was obtained. The specific activity of the iodinated 351A was 1600 μCi μg^{-1}.

Tissue ACE preparation Lungs and heart were collected from male Sprague-Dawley rats (250–300 g). Immediately after decapitation, the heart was removed and divided into right atrium (RA), left atrium (LA), right ventricle (RV), left ventricle (LV) and stored at −20°C. Tissues were thawed, homogenized in assay buffer (0.05 M Tris buffer, pH 7.0, containing 0.3% bovine serum albumin, 75 mM NaCl, 50 μM $ZnSO_4$ and 0.02% NaN_3) for 10 s with an Ultra Turrax T-25 (13,500 r.p.m.), centrifuged for 15 min (4°C at 1800 *g*) in the same buffer and the pellet resuspended and recentrifuged. The pellet

from the second centrifugation was filtered through gauze and used in subsequent assays. Protein in the homogenate was measured by the method of Lowry et al. (1951).

In vitro *estimation of ACE and its inhibition by ACE inhibitors* Binding experiments were initially conducted on heart (RA, LA, RV, LV) and lung membrane preparations to establish, for each tissue, the protein concentration necessary for approximately 30% binding of [^{125}I]-351A. Two hundred and fifty μl of tissue resuspended membrane homogenate and 50 μl of [^{125}I]-351A (40,000 c.p.m.; 10 pg per assay tube) were added to duplicate tubes, and after overnight equilibration [^{125}I]-351A bound to ACE was separated from unbound radioinhibitor by alcohol precipitation. The optimal conditions for [^{125}I]-351A binding have previously been established (Jackson et al., 1986). Protein concentration ranged from 0.012 mg ml^{-1} (in lung) to 2.9 mg ml^{-1} (in LV) to give 30% binding. Non-specific binding was estimated in the presence of an excess of unlabelled ligand (1 μM), or 1 mM EDTA.

Subsequent ligand binding displacement experiments were performed on a pooled homogenate preparation for each tissue. Diluted pooled tissue preparation (250 μl) was mixed with 50 μl [^{125}I]-351A and 10 μl of serial dilutions of the ACE inhibitor to achieve a final concentration in the range 10^{-11} to 10^{-6} mol l^{-1}. After 12 h equilibration at 20°C, 1 ml of absolute ethanol was added to separate free radioinhibitor from bound. The tubes were vortexed and then centrifuged for 5 min (4°C at 1800 g). The supernatant was discarded and the pellet containing ACE-bound radioligand was counted in a gamma counter (LKB 1260 Multigamma II). All samples were estimated in duplicate. Total counts were corrected for non-specific binding which was always less than 6% of total. Radioinhibitor binding was expressed as a percentage of counts bound measured in tubes without ACE inhibitors.

Ex vivo *study with quinapril*

Quinapril was administered to adult male Sprague-Dawley rats (250–300 g) by gavage at a dose of 0.3 mg kg^{-1}. Rats were killed by decapitation at 0, 1, 2, 4, 8 and 24 h after administration of the drug (n = 3 at each time). Trunk blood was collected for determination of drug concentration. The heart was removed and immediately stored at −20°C for ACE evaluation.

Plasma drug level Quinaprilat was extracted from 100 μl plasma by the addition of 10 μl of 1 M HCl solution. After an equilibrium period of 10 min at room temperature, ethanol (890 μl) was added and the mixture vortexed and centrifuged for 10 min (4°C at 1800 g). The ethanol supernatant containing extracted quinaprilat was decanted, a 10 μl aliquot evaporated to dryness by air and reconstituted in 10 μl of buffer. Ten μl of the reconstituted extract was then mixed with 250 μl pooled normal rat sera containing ACE diluted 1:40 in binding buffer and with 50 μl [^{125}I]-351A (40,000 c.p.m.).

The plasma concentration of quinaprilat, the active diacid of quinapril, was then measured by radioinhibitor binding displacement assay as previously described in rat plasma (Jackson et al., 1987). Results were expressed as ng quinaprilat ml^{-1}.

Radioinhibitor binding assay of cardiac ACE

Specific binding of radioligand in pooled homogenates of right and left atrium and ventricle was determined over a range of protein concentrations as described above. Specific binding of [^{125}I]-351A to heart ACE in rats treated with quinapril was compared with that of untreated animals at the protein concentration that gave 50% of maximal [^{125}I]-351A specific binding in homogenates from hearts of untreated animals. The degree of ACE inhibition in treated rats was then expressed as a percentage of the binding in tissues from untreated rats (ACE ratio).

Drugs used

ACE inhibitors examined were: 351A, lisinopril; quinaprilat, quinapril; benazeprilat; perindoprilat; fosinoprilat.

Data analysis and statistics

Estimation of K_A was performed by the interative curve fitting programme 'LIGAND' (Munson & Rodbard, 1980). Results from multiple experiments were first analysed simultaneously. Comparison of the mean K_A value between tissues or between ACE inhibitors was made by two-way analysis of variance (Armitage & Berry, 1987).

Results

Characterization of ACE by 351A radioligand binding and displacement by ACE inhibitors

[^{125}I]-351A bound to ACE in a protein concentration-dependent manner. Binding of [^{125}I]-351A to ACE was displaced by all six ACE inhibitors in a concentration-related manner in the heart, as well as the lung (Figures 1, 2 and 3). Computerized analysis of binding displacement data gave best statistical fit with a single class of binding site model.

Calculated K_A values for each ACE inhibitor with ACE from heart and lung are shown in Table 1. K_A values were higher in the right and left atrium than in the lung ($P < 0.025$), or the right and left ventricle ($P < 0.005$). Ventricular preparations and preparations from the lung also differed significantly ($P < 0.05$). No differences were observed between the calculated K_A values of the right and left atrium, or of the right and left ventricle. In competitive binding displacement experiments the rank order of potency of the ACE inhibitors tested was quinaprilat = benazeprilat > perindoprilat > 351A > lisinopril > fosinoprilat. The same rank order of potency was found in each of the tissue homogenates studied (Figure 4).

Ex vivo *studies with quinapril*

The time course of ACE inhibition in preparations from the atria and ventricles of rats following gavage with quinapril

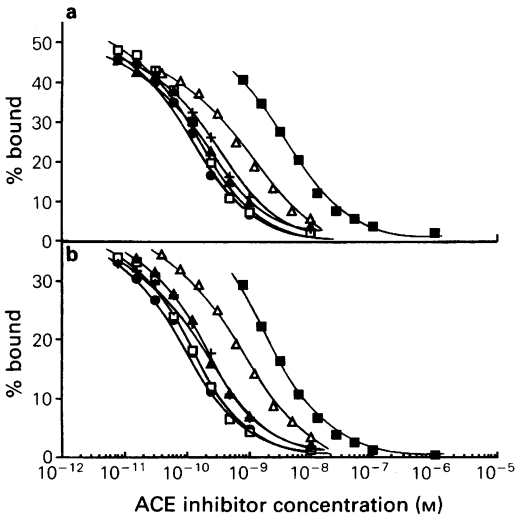

Figure 1 Binding displacement of the radioligand [^{125}I]-351A (expressed as % bound) to angiotensin converting enzyme (ACE) in the right atrium (a) and left atrium (b) of Sprague-Dawley rats by (●) quinaprilat, (□) benazeprilat, (▲) perindoprilat, (+) 351A, (△) lisinopril and (■) fosinoprilat.

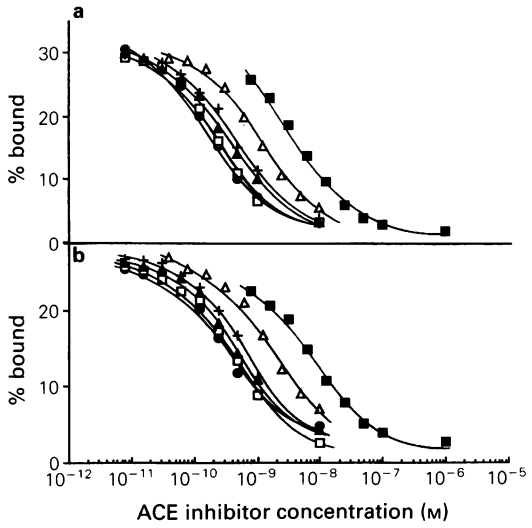

Figure 2 Binding displacement curves of [^{125}I]-351A binding (expressed as % bound) to angiotensin converting enzyme (ACE) derived from right ventricle (a) and left ventricle (b) of Sprague-Dawley rats by (●) quinaprilat, (□) benazeprilat, (▲) perindoprilat, (+) 351A, (△) lisinopril and (■) fosinoprilat.

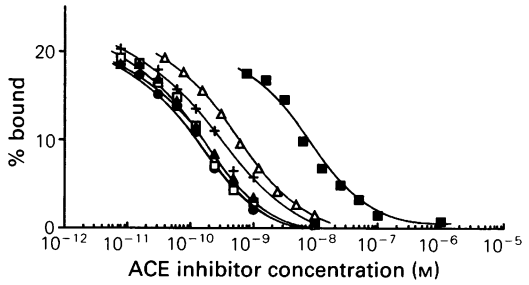

Figure 3 Binding displacement of [^{125}I]-351A (expressed as % bound) to angiotensin converting enzyme (ACE) from rat lung by (●) quinaprilat, (□) benazeprilat, (▲) perindoprilat, (+) 351A, (△) lisinopril and (■) fosinoprilat.

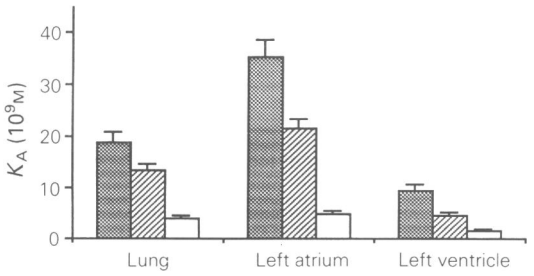

Figure 4 Binding association constant (K_A) for quinaprilat (shaded columns), perindoprilat (hatched columns) and lisinopril (open columns) from heart and lung.

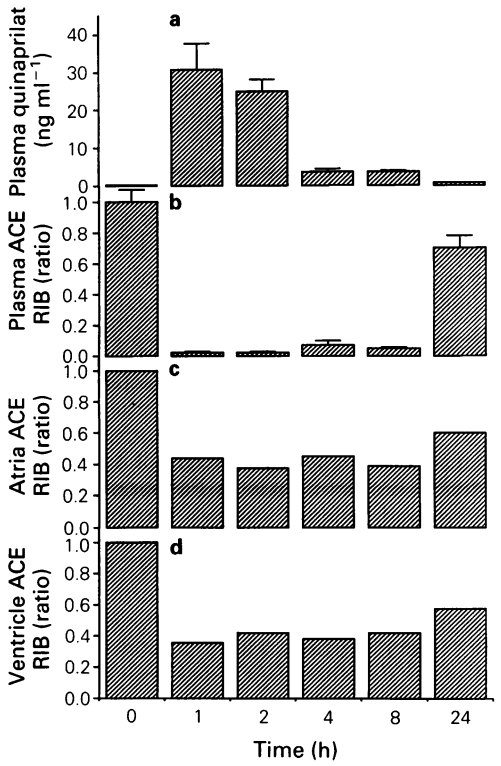

Figure 5 Plasma quinaprilat concentration (ng ml^{-1}) (a), plasma angiotensin converting enzyme (ACE, b), time course (h) of binding [^{125}I]-351A to ACE in membrane homogenates from atria (c) and ventricles (d), of rats following quinapril (0.3 mg kg^{-1}). Tissue ACE inhibition radioinhibitor binding (RIB) is expressed as a ratio of ACE in untreated rats/treated rats.

(0.3 mg kg^{-1}) is shown in Figure 5. Maximal ACE inhibition in plasma atria and ventricles occurred within the first 2 h following drug administration, coincident with the highest plasma levels of quinaprilat and inhibition of plasma ACE by more than 90% (Figure 5). Inhibition of cardiac ACE persisted throughout the 24 h study, and was poorly correlated with plasma drug concentration over this time. Following quinapril the time course and degree of inhibition of ACE derived either from the ventricles or from the atria were very similar.

Discussion

In this study the active site of ACE from different regions of the heart, and from the lung was examined by use of the radioligand [^{125}I]-351A, an iodinated ACE inhibitor. This ligand is specific for ACE and binds with high affinity (Jackson *et al.*, 1986). Binding was protein concentration-related and, like ACE enzyme activity, has been shown to be dependent on both chloride and zinc concentration. Previous

Table 1 Binding association constant (K_A) for angiotensin converting enzyme (ACE) inhibitor to ACE in homogenate of lung and the heart of the rat

Tissue	*Quinaprilat (CI 906)*	*Benazeprilat (CGS14831)*	*Perindoprilat (S 9780)*	*351A*	*Lisinopril (MK 521)*	*Fosinoprilat (SQ 27519)*	
Lung	18.92 ± 2.08	19.96 ± 2.39	13.28 ± 1.33	9.93 ± 1.70	4.08 ± 0.49	0.42 ± 0.05	
Heart							
RA	36.31 ± 2.18	31.99 ± 2.88	20.85 ± 1.67	15.72 ± 1.62	4.36 ± 0.44	0.94 ± 0.07	*
LA	35.18 ± 3.52	38.70 ± 2.71	21.64 ± 1.73	16.23 ± 1.95	4.93 ± 0.44	1.13 ± 0.16	*
RV	11.70 ± 0.70	11.58 ± 0.46	6.40 ± 0.38	4.99 ± 0.59	1.94 ± 0.12	0.46 ± 0.03	‡§
LV	9.51 ± 1.24	5.70 ± 1.08	4.54 ± 0.73	3.27 ± 0.93	1.59 ± 0.24	0.27 ± 0.05	‡§

RA = right atrium; LA = left atrium; RV = right ventricle; LV = left ventricle; K_A values ($\times 10^9$ M) are mean ± s.e. * $P < 0.025$ ‡ $P < 0.05$ for K_A values relative to lung; § $P < 0.005$ for K_A values relative to right and left atrium, by two-way analysis of variance.

studies have also demonstrated that the ligand is stable under binding conditions in tissue homogenates of the rat (Jackson *et al.*, 1986).

Small, but statistically significant differences in the calculated K_A were demonstrated between the rat atria, ventricles and the lung. This is in keeping with a growing body of evidence that suggests ACE may be different in various tissues. For instance Sakharov *et al.* (1987) have recently found that some of the physico-chemical properties of ACE isolated from human heart differed markedly, on the basis of differential protein gel electrophoretic mobility and of chromatofocusing isoelectric point, from ACE from other organs. Also the effect of sodium chloride on the heart ACE activity showed an organ specificity. At higher sodium chloride concentrations the activity of the heart enzyme sharply decreased, in contrast to that of serum and lung ACE which was non-specifically activated at high ionic strength. Also, other workers have noted differences in the molecular weight of ACE derived from different organs (Polsky-Cynkin & Fanburg, 1979; El-Dorry *et al.*, 1982a), and different carboxy terminal amino acids (Iwata *et al.*, 1982). Immunological cross-reactivity (Polsky-Cynkin & Fanburg, 1979; El-Dorry *et al.*, 1982a), and heat lability studies (Velletri *et al.*, 1985) have further supported the notion that ACE derived from different tissues may have differences in tertiary structure. However, convincing proof for differences in the structure of ACE has been obtained only in the case of testis (El Dorry *et al.*, 1982b).

The role of ACE in the myocardium has yet to be delineated in detail, but the conversion of angiotensin I to angiotensin II has been already demonstrated in human heart (Sakharov *et al.*, 1987) and in isolated heart preparations from animals (Nakashima *et al.*, 1982; Lindpaintner *et al.*, 1987). Experimental studies support a role for angiotensin II in modulating cardiac myosin expression, particularly in remodelling during hypotensive therapy (Dussaule *et al.*, 1986; Re, 1987). Laboratory studies during myocardial ischaemia have also suggested that ACE inhibition leads to a reduction in ischaemic injury, protection against ventricular arrhythmias and reduced catecholamine release (Ertl *et al.*, 1982; Daniell *et al.*, 1984; Ziogas *et al.*, 1984; van Gilst *et al.*, 1984; 1986; Hock *et al.*, 1985; de Graeff *et al.*, 1987; Sumners *et al.*, 1987). The therapeutic potential thus exists for ACE inhibitors to be of benefit in cardiac hypertrophy, ischaemic heart disease and cardiac arrhythmias (Dzau *et al.*, 1980; Fouad *et al.*, 1982; 1983; Cleland *et al.*, 1984; 1985; Webster *et al.*, 1985; The Consensus Trail Study Group, 1987; Newman *et al.*, 1988; Sharpe *et al.*, 1988). The demonstration of different classes of tissue ACE with differences in K_A and variable lipophilicity raises the potential of tissue-specific ACE inhibitor therapy. In this study, we have used a variety of ACE inhibitors covering a wide range of molecular structures. Displacement of the index radioligand 351A occurred with each compound, with the same rank order of displacement potency being observed in each tissue studied. Thus amongst the compounds screened there was no apparent variation in their relative affinities for interaction with ACE derived from atria, ventricles or the lung.

To examine further any potential biological significance of the differences noted in the values of K_A, we measured the degree of ACE inhibition in atria and ventricles following oral gavage of rats with the new ACE inhibitor quinapril. As can be seen from *in vitro* binding displacement studies, quinaprilat, the active diacid derivative of quinapril, is a very potent ACE inhibitor, and showed some three fold difference in K_A between atria and ventricles. Following oral gavage with quinapril ($0.3\,mg\,kg^{-1}$), the time course and degree of ACE inhibition in the atria and the ventricles of rat hearts was found to be similar. This would suggest that the observed differences in K_A have only a limited potential biological significance. The dose of quinapril used produces near maximum inhibition of plasma and renal ACE. It is possible that smaller doses of quinapril may show differential inhibition between atrium and ventricles.

However, differences in K_A could be related to a different amino acid sequence of ACE in different tissues or more probably differences in tertiary structure of ACE. The potential development of novel ACE inhibitors, related not to the natural substrate but to the architecture of the active site, could offer the possibility of differential ACE inhibition in different tissues.

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Bradykinin-induced depolarization of primary afferent nerve terminals in the neonatal rat spinal cord *in vitro*

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1 Application of bradykinin (BK) to the spinal cord of the neonatal rat evoked depolarizations which could be recorded via either the dorsal or ventral roots. However, responses recorded via the ventral root were abolished by removal of extracellular Ca^{2+} or the addition of Cd^{2+}, while responses recorded via the dorsal root were unaffected.

2 The response recorded via the ventral root was inhibited by the substance P antagonist spantide, while responses recorded via the dorsal root were unaffected.

3 Depolarizations recorded via the dorsal root were concentration-dependent with an EC_{50} of 30 nM. These responses were not antagonized by the BK_1 selective antagonist Leu^8des-Arg^9BK, but were antagonized by D-$Arg^0Hyp^3Thi^{5,8}$D-Phe^7BK with a pA_2 of 6.8 ± 0.6, which is similar to the values determined for other BK_2-mediated responses.

4 Application of phorbol dibutyrate (PDBu) to the spinal cord also evoked a depolarization with respect to the dorsal root. This response to PDBu was enhanced by removal of extracellular Ca^{2+}, while the response to BK was unaffected.

5 The potent protein kinase inhibitor staurosporine reduced the response to PDBu, but did not affect the response to BK.

6 These results suggest that BK by acting on BK_2 receptors can depolarize the central terminals of primary afferent nerve fibres. This response to BK does not appear to be mediated via the activation of protein kinase C. The depolarization to BK recorded via the ventral root of the spinal cord is indirect and may be secondary to the action of BK on the primary afferent terminals.

Introduction

In the periphery, the nonapeptide bradykinin (BK) is formed from kininogen by the enzyme kallikrein in response to tissue injury. However, there are several pieces of evidence which suggest a role for BK in the central nervous system. Bradykinin and BK-like immunoreactivity have been detected in the brain (Correa *et al.*, 1979; Perry & Snyder, 1984) and BK binding sites have been demonstrated in the spinal cord (Steranka *et al.*, 1988). Intracerebroventricular and intrathecal injection of BK produces selective autonomic and behavioural effects and antinociception (Ribeiro & Rocha E Silva, 1973; Laneuville & Couture, 1987). However, central administration of BK receptor antagonists alone appear to have no effect on nociception (Laneuville & Couture, 1987; Dray *et al.*, 1988a), indicating that BK does not play an important role in the central transmission of nociceptive information. The precise mechanism of the antinociceptive action of intrathecal BK is unknown, but the effect is blocked by α_2-adrenoceptor antagonists, and it has been suggested that BK is acting on the terminals of bulbo-spinal noradrenergic neurones (Laneuville *et al.*, 1989).

Application of BK to the spinal cord of the neonatal rat evokes a depolarization of the ventral root (Dray *et al.*, 1988a). However, since BK binding sites are located mainly on primary afferent nerve fibres and in the dorsal horn of the spinal cord (Steranka *et al.*, 1988), the response recorded via the ventral root is probably secondary to an action in the dorsal horn, possibly on the terminals of the primary afferent nerve fibres. In this study, we have examined further the actions of BK on the neonatal rat spinal cord *in vitro*. By recording responses via both the ventral and dorsal roots, we have attempted to determine the site of action of BK.

In many cells, BK activates the enzyme phospholipase C (PLC) (Yano *et al.*, 1985; Derian & Moskowitz, 1986; Thayer *et al.*, 1988), giving rise to the formation of inositol trisphosphate (IP_3), and diacylglycerol (DAG) which is the endogenous activator of protein kinase C (PKC) (see Nishizuka, 1988). In the periphery, BK stimulates the peripheral terminals of nociceptive sensory neurones (Erdos, 1979; Martin *et al.*, 1987; Dray *et al.*, 1988b). This activation of sensory neurones probably results from a depolarization of the nerve terminal due to an increase in sodium permeability of the membrane, which may be mediated via the activation of PKC by DAG (Burgess *et al.*, 1989). We have therefore compared the action of BK in the spinal cord with its action on sensory neurones in the periphery, by looking at the ionic basis of the BK response and the possible involvement of PKC in this response. Some of these data have already been presented to the British Pharmacological Society (Dunn & Rang, 1989).

Methods

Preparation

One day old Sprague-Dawley rat pups were killed by decapitation and the entire vertebral column removed rapidly, and placed in a dish containing Locke solution. A dorsal laminectomy was performed and the dura overlying the cord was removed. The cord with attached dorsal and ventral roots was then removed. The cord was hemisected sagitally and cut transversely, to produce a section of lumbar cord extending to one root either side of the root selected for recording (usually L3).

Recording

The section of spinal cord was placed in a 2 chamber bath, with the spinal cord in one compartment and a single dorsal root projecting into the second compartment. The d.c. potential between the two compartments was recorded via silver-silver chloride electrodes connected to a differential amplifier (WPI; DAM50) and displayed on a chart recorder (Gould BS272). The compartment containing the cord (volume $\approx$ 1 ml) was perfused by gravity with Locke solution, at room temperature (22 to 25°C), at a constant rate of

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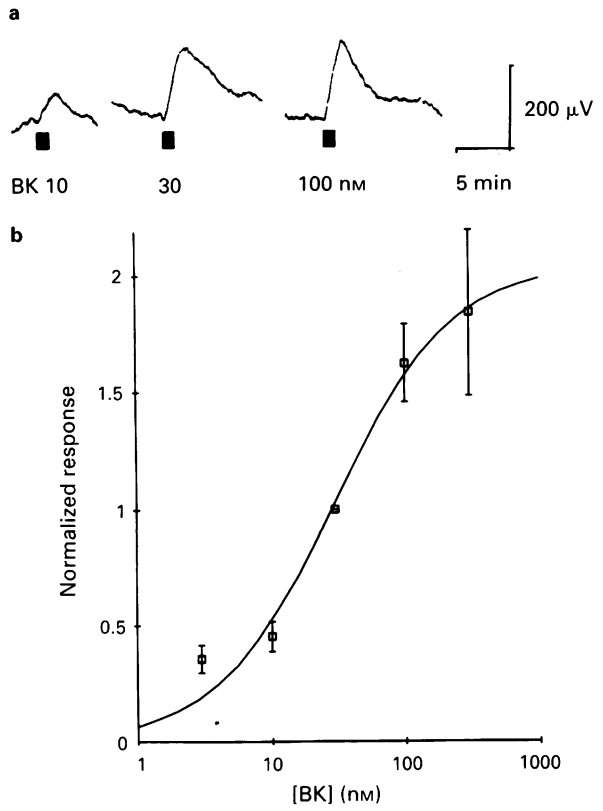

Figure 1 Responses to bradykinin (BK) recorded via the dorsal root of the neonatal rat spinal cord. (a) Depolarizations evoked by 1 min application of 10, 30 and 100 nM BK. (b) Log concentration-response curve for the depolarization to BK; combined data from 11 experiments with 3–5 concentrations applied in each experiment. Points represent the mean and vertical lines s.e.mean of 4–11 observations normalized with respect to the response evoked by 30 nM BK. The EC_{50} computed by an iterative curve fitting routine was 30 nM.

2 ml per min. Drugs were dissolved in the perfusate at the required final concentration.

In some experiments, the same technique was used to record responses from a single ventral root instead of a dorsal root.

The BK concentration-response curve was determined by recording responses to 3–5 concentrations applied in a pseudo-random order, in each experiment. The responses were

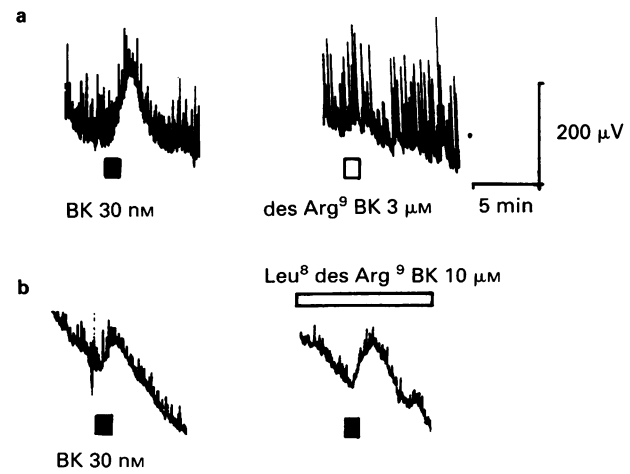

Figure 2 Action of selective BK_1 receptor agonists and antagonists on the neonatal rat spinal cord. (a) Comparison of the effect of bradykinin (BK, 30 nM) with the selective BK_1 agonist des-Arg^{9}BK (3 μM). (b) Responses to 30 nM BK recorded in the absence and presence of the selective BK_1 antagonist Leu^{8}des-Arg^{9}BK. The antagonist at concentrations up to 10 μM failed to alter responses to BK.

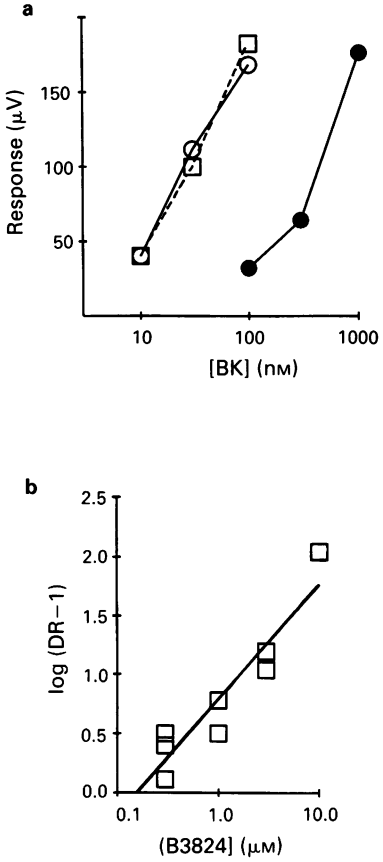

Figure 3 Effect of the antagonist D-Arg^{0}Hyp^{3}Thi^{5,8}D-Phe^{7}BK (B3824) on the response to bradykinin (BK). (a) Log concentration-response curves to BK recorded in a single experiment, before (○), during (●) and after (□) perfusion with solution containing 3 μM B3824. (b) A Schild plot for the antagonism produced by B3824. The data were fitted well by a straight line ($r = 0.93$, $n = 8$) with a slope of 0.97 which was not significantly different from unity ($P > 0.05$ by one sample Student's t test). The pA_2 value determined from this line was 6.8 ± 0.6.

then normalized with respect to the response produced by 30 nM BK (a concentration used in every experiment). The normalized data were fitted with an unconstrained mass action curve by an iterative curve fitting routine (RS/1, BBN Software Products Corp.)

Analysis of results

Means were compared by Student's t test (paired or unpaired as stated).

Drugs and solutions

The composition of the normal Locke solution was (mM): NaCl 154, KCl 5.6, $CaCl_2$ 2, HEPES 10 and D-glucose 5; buffered to pH 7.4 with NaOH. Sodium-free solution contained (mM): N-methyl glucamine 154, KCl 0.7, $CaCl_2$ 2, HEPES 10 and D-glucose 5; buffered to pH 7.4 with 4.9 mM KOH.

Drugs used were bradykinin (Sandoz, Basle), D-Arg^{0}Hyp^{3}Thi^{5,8}D-Phe^{7}BK (B3824), des-Arg^{9}BK, Leu^{8}des-Arg^{9}BK (Bachem, AG), staurosporine (Fluka), tetrodotoxin (TTX), spantide ([D-Arg^{1}D-Trp^{7,9}Leu^{11}]-substance P), 4β-phorbol-12,13-dibutyrate (PDBu), capsaicin (Sigma). Stock solutions (10 mM) of staurosporine, PDBu and capsaicin were made up in dimethylsulphoxide. All other drugs were dissolved in Locke solution.

Results

Application of BK (3 to 1000 nM) to the spinal cord for 1 min evoked depolarizations which could be recorded via the dorsal root (Figure 1a). These responses reached a peak in

approximately 2 min, and then declined during the following 5 to 10 min. Longer applications of BK did not produce significantly larger responses and to prevent problems of desensitization, BK applications were limited to 1 min and responses were recorded at 40 min intervals. This effect of BK was concentration-dependent, with an EC_{50} of 30 nM (Figure 1b).

Receptor type

We used some of the selective BK receptor agonists and antagonists to investigate the type of receptor mediating the depolarization recorded via the dorsal root. The selective BK_1 agonist des Arg^9 BK at concentrations up to 3 μM failed to evoke any depolarization (Figure 2a), and the BK_1 antagonist Leu^8des Arg^9BK at a concentration of 10 μM failed to antagonize the response to BK (Figure 2b). However, the response to BK was antagonized by the antagonist D-$Arg^0Hyp^3Thi^{5,8}$D-Phe^7BK (B3824). This compound produced a parallel displacement of the BK log concentration-response curve (Figure 3a). The Schild plot for the antagonism by B3824 was linear with a slope of 0.97, which was not significantly different from unity (Figure 3b) and yielded a pA_2 value of 6.8 $\pm$ 0.6.

Ionic mechanism

We examined the dependence of the dorsal root response to BK on extracellular sodium. When extracellular sodium was replaced by N-methylglucamine, there was a reversible reduction in the amplitude of the response to BK (Figure 4a). Application of TTX to the preparation produced a small hyperpolarization and abolished any spontaneous activity, but the response to BK was unaffected (Figure 4b). Removal of extracellular Ca^{2+} produced no change in the response to BK (see below and Figure 5a).

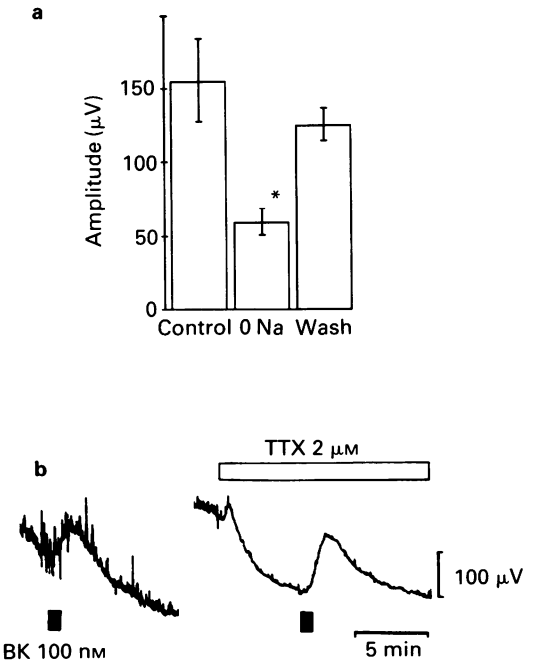

Figure 4 Dependence of the response to bradykinin (BK) on Na^+ ions. (a) The response amplitude before (control), during (0Na) and after (Wash) perfusion with solution in which Na^+ was replaced by an equimolar amount of N-methylglucamine. Columns represent mean and bars s.e.mean from 4 experiments. (b) Responses to BK recorded before and during perfusion with a solution containing 2 μM tetrodotoxin (TTX). TTX produced a small hyperpolarization and abolished the spontaneous activity, but did not affect the depolarization evoked by BK. *Significantly different from control, $P < 0.05$ by paired Student's t test.

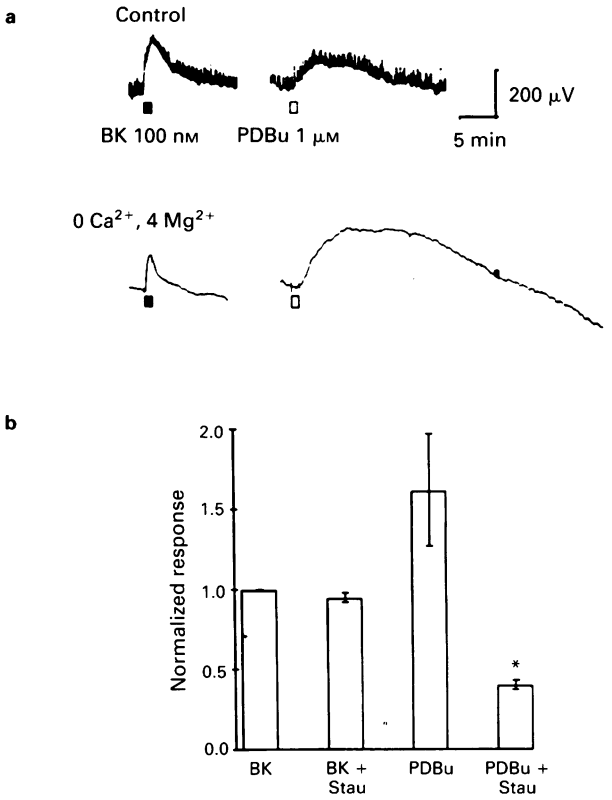

Figure 5 Role of second messengers in the response to bradykinin (BK). (a) Responses to 100 nM BK and 1 μM phorbol dibutyrate (PDBu) recorded from the same preparation, in control (2 mM Ca^{2+}) solution and in 0 mM Ca^{2+}, 4 mM Mg^{2+} solution. While the response to BK was essentially unaltered in Ca^{2+}-free solution, the response to PDBu was greatly enhanced. (b) Comparison of the effect of the protein kinase C inhibitor staurosporine (Stau, 200 nM) on responses to BK (30 nM) and PDBu (1 μM). Responses were normalized with respect to an initial response to 30 nM BK. Each column represents the mean and bars s.e.mean from 4 experiments. *Significantly different from control, $P < 0.05$ by unpaired Student's t test.

Second messengers

When 1 μM PDBu was applied to the spinal cord, a small slow depolarization was recorded via the dorsal root (Figure 5a). As found by Rang & Ritchie (1988), this response was greatly enhanced by removal of Ca^{2+} from the bathing solution (Figure 5a), with the response in Ca^{2+}-free solution being 210 $\pm$ 50% (mean $\pm$ s.e.mean, $n = 5$) of the response recorded in normal (2 mM Ca^{2+}) solution. In contrast, the response to BK was unaffected in Ca^{2+}-free solution (Figure 5a).

We examined the effect of the PKC inhibitor staurosporine on responses to BK and PDBu. In these experiments an initial normalizing response to 30 nM BK was recorded, followed by a response to either PDBu alone, BK in the presence of staurosporine, or PDBu in the presence of staurosporine. Equilibration of the preparation with staurosporine (200 nM) for 20 min produced a large reduction in the response to PDBu, but had no effect on the response to BK (Figure 5b).

Site of action

When the same recording technique was applied to the isolated vagus nerve ($n = 2$), dorsal roots ($n = 2$), or dorsal root ganglia ($n = 2$), we were unable to detect any response to BK, although depolarizations to γ-aminobutyric acid (GABA) and capsaicin were observed. In agreement with the observations of Dray *et al.* (1988a), when BK was applied to the spinal cord

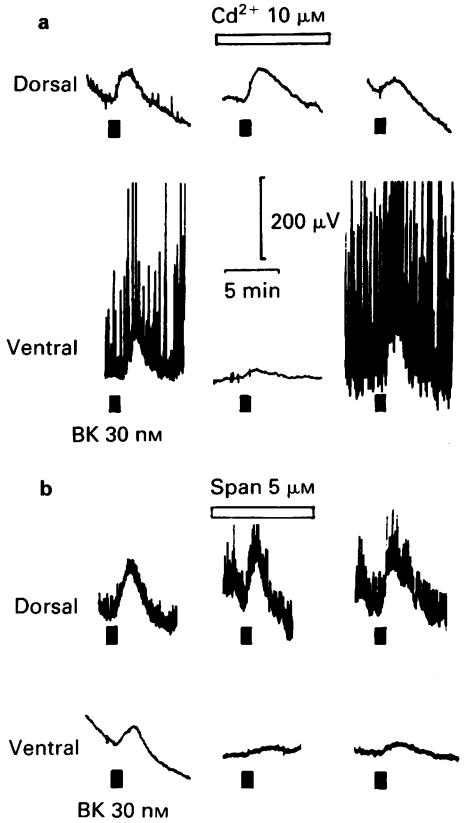

Figure 6 Comparison of the effects of Cd^{2+} (a) and the substance P antagonist spantide (Span) (b) on responses to bradykinin (BK) recorded via the dorsal and ventral roots from 4 preparations. Cd^{2+} reversibly inhibited both the spontaneous activity and the response to BK recorded via the ventral root, while the response to BK recorded via the dorsal root was unaffected. Similarly, the response to BK recorded via the ventral root was abolished by spantide, while the response recorded via the dorsal root was unaffected.

depolarizations could also be recorded via the ventral root (Figure 6). However, when synaptic transmission was blocked by the removal of calcium from the bathing solution, or by addition of cadmium (10–100 μM), responses recorded via the ventral root were abolished, while responses recorded via the dorsal root were unaltered (Figure 6a), suggesting that the response recorded via the dorsal root was direct, while the response recorded via the ventral root was secondary to the release of neurotransmitter within the spinal cord.

To see whether substance P might have a transmitter role in the ventral root response to BK, we examined the effect of the substance P antagonist, spantide, on the responses recorded via the dorsal and ventral roots. The response to 30 nM BK recorded via the ventral root was reversibly reduced by 5 μM spantide, while responses recorded via the dorsal root were unaffected (Figure 6b).

To see whether the depolarization recorded via the dorsal root was due to an action of BK on the central projections of nociceptive (capsaicin-sensitive) neurones, we examined the effect of capsaicin on the response to BK. Capsaicin 10 μM produced a large depolarization and when BK was applied during the capsaicin response the effect of BK was greatly reduced (Figure 7), indicating that there was at least a considerable overlap in the populations of afferent fibres responding to BK and capsaicin.

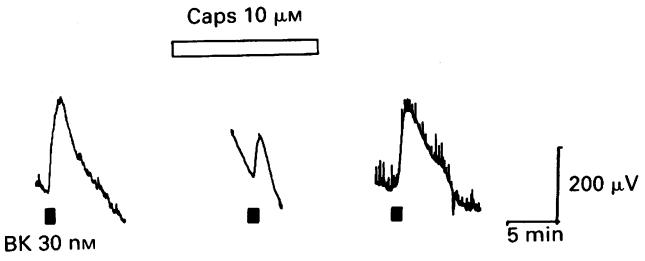

Figure 7 Effect of capsaicin on the response to bradykinin (BK) recorded via the dorsal root. Responses to bradykinin (BK) were recorded before, during and after perfusion with a solution containing 10 μM capsaicin (Caps). Capsaicin evoked a depolarization of approximately 4000 μV (not shown). Application of BK during the slowly declining capsaicin depolarization evoked a considerably smaller response.

Discussion

Bradykinin binding sites have been demonstrated in the dorsal horn of the spinal cord (Steranka *et al.*, 1988), although the precise location of these sites is unknown. Bradykinin is known to have several effects within the spinal cord; it can activate dorsal horn neurones (Henry, 1976), cause depolarization of motor neurones (Dray *et al.*, 1988a) and produce antinociception (Laneuville & Couture, 1987). We have shown that BK can also depolarize primary afferent fibres within the spinal cord, although we have been unable to record similar responses from isolated vagus nerve, dorsal roots or dorsal root ganglia. Since these preparations did respond to GABA and capsaicin, it suggests that, although BK binding sites are present on dorsal roots and dorsal root ganglia (Steranka *et al.*, 1988), the electrophysiological response to BK is in some way localized to the terminal region of the primary afferent nerve fibres. Because the response recorded via the ventral root can be inhibited by the susbtance P antagonist spantide, the response recorded via the ventral root may be secondary to the release of substance P, presumably from the primary afferents. It is possible that the BK binding sites in the dorsal horn are exclusively on the primary afferent terminals and that other effects of BK within the spinal cord are all secondary to the depolarization of the primary afferent terminals. Although it has been suggested that BK can act on the terminals of bulbo-spinal noradrenergic neurones (Laneuville *et al.*, 1989), the depolarization recorded via the dorsal root was unaffected by TTX and Ca^{2+} free solution, indicating that it was the result of a direct action of BK on the afferent terminals and not via the release of noradrenaline within the spinal cord.

Capsaicin can selectively depolarize unmyelinated sensory neurones (Heyman & Rang, 1985; Marsh *et al.*, 1987; Bevan & Forbes, 1988) and activates the same polymodal nociceptive neurones as BK (Martin *et al.*, 1987). The response to BK recorded via the dorsal root was greatly reduced in the presence of capsaicin, confirming that BK was acting on capsaicin-sensitive fibres.

Whether BK receptors within the spinal cord have any physiological role is at present unclear. Small amounts of BK have been detected in the spinal cord (Perry & Snyder, 1984) and neurones containing BK-like immunoreactivity have been observed elsewhere in the central nervous system (Correa *et al.*, 1979). However, BK receptor antagonists failed to modify nociception *in vivo* (Laneuville & Couture, 1987), or the ventral root response to peripheral noxious stimuli *in vitro* (Dray *et al.*, 1988a). Thus if BK does have a role in the spinal cord, it is not directly involved in the transmission of nociceptive information.

Bradykinin receptors have been divided into BK_1, BK_2 and BK_3 subtypes, based on the activity of a number of BK analogues (Regoli & Barabe, 1980; Farmer *et al.*, 1989). We have shown that the BK receptors present on the primary afferent terminals are of the BK_2 subtype since the BK_1 agonist des-Arg^{9}BK and the BK_1 antagonist Leu^{8}desArg^{9}BK were inac-

tive, while the response to BK was antagonized by D-Arg^{0}Hyp^{3}Thi^{5,8}D-Phe^{7}BK, which is inactive at BK_3 sites. The pA_2 value of 6.8 for this antagonist is similar to values of 6.0 and 6.9 found for BK_2 receptors in isolated smooth muscle (Schacter *et al.*, 1987; Steranka *et al.*, 1988). These observations are consistent with experiments on cultured sensory neurones (Burgess *et al.*, 1989) and with other effects of BK on the spinal cord (Laneuville & Couture, 1987; Dray *et al.*, 1988a), which are also mediated via BK_2 receptors.

The depolarization of cultured sensory neurones by BK is due mainly to an increase in sodium conductance as a result of the activation of PKC by DAG, although other mechanisms may also be involved in some cells (Burgess *et al.*, 1989). Activation of PKC by phorbol esters also causes a depolarization of vagal C fibres, due to an increase in sodium conductance (Rang & Ritchie, 1988). The depolarization of afferent terminals by BK is also due, in part, to an increase in sodium conductance, since the response was attenuated by the removal of extracellular sodium, although it was unaffected by TTX. However, other ions may also be involved in this response since it was not completely abolished in sodium-free solution.

There are two results which indicate that this response to BK is not mediated via activation of PKC. Although PDBu, an activator of PKC (Castagna *et al.*, 1982), evoked a similar depolarization to BK, the response to PDBu was greatly enhanced by the removal of extracellular Ca^{2+}, while the response to BK was unaffected. This effect of Ca^{2+} on the response to PDBu is similar to that observed by Rang & Ritchie (1988) and, although the underlying mechanism is unclear, the differential effect of Ca^{2+} on the response to PDBu and BK suggests that these responses do not share a common mechanism. More importantly, while the response to PDBu was greatly reduced by staurosporine, a potent though not very selective inhibitor of PKC (Ruegg & Burgess, 1989), the response to BK was unaffected, providing further evidence that this response to BK is not mediated via PKC.

In conclusion, we have shown that BK by acting on BK_2 receptors can depolarize the terminals of primary afferent fibres within the spinal cord. This depolarization is due mainly to an increase in sodium conductance, but does not appear to be mediated via the activation of PKC. It is possible that other effects of BK on the spinal cord occur as a consequence of the depolarization of the afferent terminals and the subsequent release of neurotransmitters.

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VOLUME 100 (3) JULY 1990

SPECIAL REPORT

R. Micheletti, L. Giudici, M. Turconi & A. Donetti. 4-DAMP analogues reveal heterogeneity of M_1 muscarinic receptors 395

SHORT COMMUNICATION

S. Sanjar, D. Smith, M.A. Kings & J. Morley. Pretreatment with rh-GMCSF, but not rh-IL3, enhances PAF-induced eosinophil accumulation in guinea-pig airways 399

PAPERS

S. Ryang, S. Takei, T. Kawai, Y. Imaizumi & M. Watanabe. Atropine-resistant relaxation induced by high K^+ in iris dilator muscle of the rat and pig 401

Y.W. Kwan, R.M. Wadsworth & K.A. Kane. Modification of the ischaemic-induced contraction in the sheep circumflex coronary artery by various pharmacological antagonists 407

H. Yamada, F. Yoneyama, K. Satoh & N. Taira. Specific but differential antagonism by glibenclamide of the vasodepressor effects of cromakalim and nicorandil in spinally-anaesthetized dogs 413

S. Brunelleschi, L. Vanni, F. Ledda, A. Giotti, C.A. Maggi & R. Fantozzi. Tachykinins activate guinea-pig alveolar macrophages: involvement of NK_2 and NK_1 receptors 417

R.G. Evans & J. Ludbrook. Effects of μ-opioid receptor agonists on circulatory responses to simulated haemorrhage in conscious rabbits 421

A.S.O. Adeagbo & K.U. Malik. Endothelium-dependent and BRL 34915-induced vasodilatation in rat isolated perfused mesenteric arteries: role of G-proteins, K^+ and calcium channels 427

G.B. Kaplan, N.T. Tai, D.J. Greenblatt & R.I. Shader. Caffeine-induced behavioural stimulation is dose- and concentration-dependent 435

M.L. Fann, C. Souccar & A.J. Lapa. Phenthonium, a quaternary derivative of (−)-hyoscyamine, enhances the spontaneous release of acetylcholine at rat motor nerve terminals 441

D.A. Yeats & Y.S. Bakhle. Cationic factors affecting phospholipase activities from human lung 447

T. Knudsen, H.C. Berthelsen & T. Johansen. Inhibition of Na^+-K^+ pump activity by divalent cations in intact peritoneal mast cells of the rat 453

J.L. Ellis & G. Burnstock. Neuropeptide Y neuromodulation of sympathetic co-transmission in the guinea-pig vas deferens 457

M.G. Persson, L.E. Gustafsson, N.P. Wiklund, P. Hedqvist & S. Moncada. Endogenous nitric oxide as a modulator of rabbit skeletal muscle microcirculation *in vivo* 463

P.W. Gage, J.J. McArdle & D.A. Saint. Effects of butanedione monoxime on neuromuscular transmission 467

O. Rascol, J.-L. Montastruc, G. Gauquelin, M.-A. Tran, G. Geelen, C. Gharib & P. Montastruc. Cardiovascular effects of central injection of acetylcholine in anaesthetized dogs: a role for vasopressin release 471

B.J. Northover. Continuous fluorimetric assessment of the changes in cytoplasmic calcium concentration during exposure of rat isolated myocardium to conditions of simulated ischaemia 477

A.D. King, M. Milavec-Krizman & E. Müller-Schweinitzer. Characterization of the adenosine receptor in porcine coronary arteries 483

S. Thesleff, L.C. Sellin & S. Tågerud. Tetrahydroaminoacridine (tacrine) stimulates neurosecretion at mammalian motor endplates 487

J.V. Esplugues, E.G. Ramos, L. Gil & J. Esplugues. Influence of capsaicin-sensitive afferent neurones on the acid secretory responses of the rat stomach *in vivo* 491

H.E. Moss & G.J. Sanger. The effects of granisetron, ICS 205-930 and ondansetron on the visceral pain reflex induced by duodenal distension 497

A. Subissi, M. Guelfi & M. Criscuoli. Angiotensin converting enzyme inhibitors potentiate the bronchoconstriction induced by substance P in the guinea-pig 502

K. Muraki, Y. Imaizumi, T. Kojima, T. Kawai & M. Watanabe. Effects of tetraethylammonium and 4-aminopyridine on outward currents and excitability in canine tracheal smooth muscle cells 507

R.C. Small, D.M. Good, J.S. Dixon & I. Kennedy. The effects of epithelium removal on the actions of cholinomimetic drugs in opened segments and perfused tubular preparations of guinea-pig trachea 516

X.-F. Zhou & B.G. Livett. Effect of capsaicin-sensitive sensory nerves on plasma glucose and catecholamine levels during 2-deoxyglucose-induced stress in conscious rats 523

E.J.F. Carlisle, J.D. Allen, W.G. Kernohan, W. Leahey & A.A.J. Adgey. Pharmacological analysis of established ventricular fibrillation 530

R. Matran, K. Alving & J.M. Lundberg. Cigarette smoke, nicotine and capsaicin aerosol-induced vasodilatation in pig respiratory mucosa 535

J. Davidson, A.S. Milton & D. Rotondo. A study of the pyrogenic actions of interleukin-1α and interleukin-1β: interactions with a steroidal and a non-steroidal anti-inflammatory agent 542

V. Mollace, Y. Masuda, T. Pelaggi & G. Nisticò. The effects of phosphatidylserine on the decreased cardiovascular response to clonidine microinfused into the nucleus tractus solitarii of old rats 547

N.C.B. Nyborg. Action of noradrenaline on isolated proximal and distal coronary arteries of rat: selective release of endothelium-derived relaxing factor in proximal arteries 552

C. Richer, P. Mulder, M.-P. Doussau, P. Gautier & J.-F. Giudicelli. Systemic and regional haemodynamic interactions between K^+ channel openers and the sympathetic nervous system in the pithed SHR 557

S. Etoh, M. Ohashi, A. Baba & H. Iwata. Inhibition by ibudilast of leukotriene D_4-induced formation of inositol phosphates in guinea-pig lung 564

H. Moritoki, T. Matsugi, H. Takase, H. Ueda & A. Tanioka. Evidence for the involvement of cyclic GMP in adenosine-induced, age-dependent, vasodilatation 569

R.B. Rose'Meyer & W. Hope. Evidence that A_2 purinoceptors are involved in endothelium-dependent relaxation of the rat thoracic aorta 576

R. Pilsudski, O. Rougier & Y. Tourneur. Action of cromakalim on potassium membrane conductance in isolated heart myocytes of frog 581

C.A. Maggi, R. Patacchini, S. Giuliani, P. Rovero, S. Dion, D. Regoli, A. Giachetti & A. Meli. Competitive antagonists discriminate between NK_2 tachykinin receptor subtypes 588

T. Fukumitsu, H. Hayashi, H. Tokuno & T. Tomita. Increase in calcium channel current by β-adrenoceptor agonists in single smooth muscle cells isolated from porcine coronary artery 593

J. Feldman, E. Tibiriça, G. Bricca, M. Dontenwill, A. Belcourt & P. Bousquet. Evidence for the involvement of imidazoline receptors in the central hypotensive effect of rilmenidine in the rabbit 600

D.T. Newgreen, K.M. Bray, A.D. McHarg, A.H. Weston, S. Duty, B.S. Brown, P.B. Kay, G. Edwards, J. Longmore & J.S. Southerton. The action of diazoxide and minoxidil sulphate on rat blood vessels: a comparison with cromakalim 605

L.B. Fernandes & R.G. Goldie. Pharmacological evaluation of a guinea-pig tracheal epithelium-derived inhibitory factor (EpDIF) 614

R. Inoue & A.F. Brading. The properties of the ATP-induced depolarization and current in single cells isolated from the guinea-pig urinary bladder **619**

R.T. Mathie & B. Alexander. The role of adenosine in the hyperaemic response of the hepatic artery to portal vein occlusion (the 'buffer response') **626**

S.C. Cruwys, D.E. Davies & E.R. Pettipher. Co-operation between interleukin-1 and the fibrinolytic system in the degradation of collagen by articular chondrocytes **631**

S. Horio, M. Shima, H. Ueda & Y. Ishida. Temperature-dependence of desensitization induced by acetylcholine and histamine in guinea-pig ileal longitudinal muscle **636**

M.C. Butterfield & R. Chess-Williams. Enhanced α-adrenoceptor responsiveness and receptor number during global ischaemia in the Langendorff perfused rat heart **641**

I.P. Hall, J. Donaldson & S.J. Hill. Modulation of fluoroaluminate-induced inositol phosphate formation by increases in tissue cyclic AMP content in bovine tracheal smooth muscle **646**

B. Fabris, H. Yamada, R. Cubela, B. Jackson, F.A.O. Mendelsohn & C.I. Johnston. Characterization of cardiac angiotensin converting enzyme (ACE) and *in vivo* inhibition following oral quinapril to rats **651**

P.M. Dunn & H.P. Rang. Bradykinin-induced depolarization of primary afferent nerve terminals in the neonatal rat spinal cord *in vitro* **656**

NOTICE CONCERNING SPECIAL REPORTS

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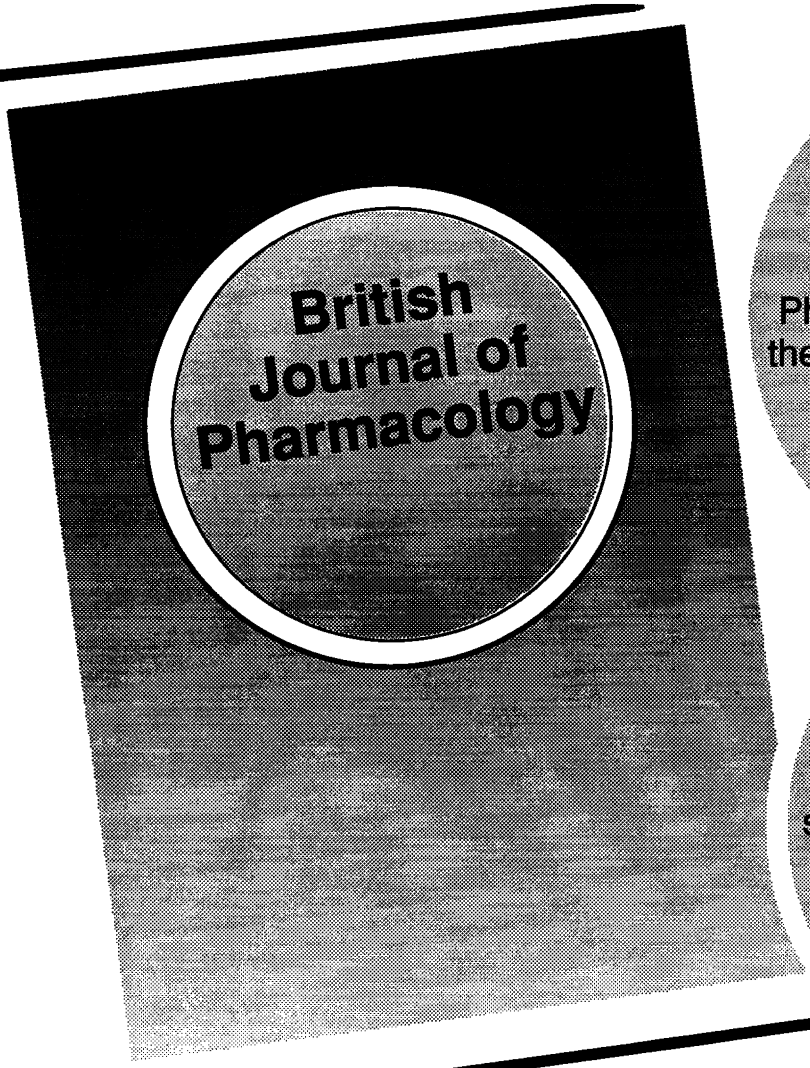

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BRITISH JOURNAL OF PHARMACOLOGY VOLUME 100 (3) 1990

SPECIAL REPORT

R. Micheletti, L. Giudici, M. Turconi & A. Donetti. 4-DAMP analogues reveal heterogeneity of M_1 muscarinic receptors 395

SHORT COMMUNICATION

S. Sanjar, D. Smith, M.A. Kings & J. Morley. Pretreatment with rh-GMCSF, but not rh-IL3, enhances PAF-induced eosinophil accumulation in guinea-pig airways 399

PAPERS

S. Ryang, S. Takei, T. Kawai, Y. Imaizumi & M. Watanabe. Atropine-resistant relaxation induced by high K^+ in iris dilator muscle of the rat and pig 401

Y.W. Kwan, R.M. Wadsworth & K.A. Kane. Modification of the ischaemic-induced contraction in the sheep circumflex coronary artery by various pharmacological antagonists 407

H. Yamada, F. Yoneyama, K. Satoh & N. Taira. Specific but differential antagonism by glibenclamide of the vasodepressor effects of cromakalim and nicorandil in spinally-anaesthetized dogs 413

S. Brunelleschi, L. Vanni, F. Ledda, A. Giotti, C.A. Maggi & R. Fantozzi. Tachykinins activate guinea-pig alveolar macrophages: involvement of NK_2 and NK_1 receptors 417

R.G. Evans & J. Ludbrook. Effects of μ-opioid receptor agonists on circulatory responses to simulated haemorrhage in conscious rabbits 421

A.S.O. Adeagbo & K.U. Malik. Endothelium-dependent and BRL 34915-induced vasodilatation in rat isolated perfused mesenteric arteries: role of G-proteins, K^+ and calcium channels 427

G.B. Kaplan, N.T. Tai, D.J. Greenblatt & R.I. Shader. Caffeine-induced behavioural stimulation is dose- and concentration-dependent 435

M.L. Fann, C. Souccar & A.J. Lapa. Phenthonium, a quaternary derivative of (−)-hyoscyamine, enhances the spontaneous release of acetylcholine at rat motor nerve terminals 441

D.A. Yeats & Y.S. Bakhle. Cationic factors affecting phospholipase activities from human lung 447

T. Knudsen, H.C. Berthelsen & T. Johansen. Inhibition of Na^+-K^+ pump activity by divalent cations in intact peritoneal mast cells of the rat 453

J.L. Ellis & G. Burnstock. Neuropeptide Y neuromodulation of sympathetic co-transmission in the guinea-pig vas deferens 457

M.G. Persson, L.E. Gustafsson, N.P. Wiklund, P. Hedqvist & S. Moncada. Endogenous nitric oxide as a modulator of rabbit skeletal muscle microcirculation *in vivo* 463

P.W. Gage, J.J. McArdle & D.A. Saint. Effects of butanedione monoxime on neuromuscular transmission 467

O. Rascol, J.-L. Montastruc, G. Gauquelin, M.-A. Tran, G. Geelen, C. Gharib & P. Montastruc. Cardiovascular effects of central injection of acetylcholine in anaesthetized dogs: a role for vasopressin release 471

B.J. Northover. Continuous fluorimetric assessment of the changes in cytoplasmic calcium concentration during exposure of rat isolated myocardium to conditions of simulated ischaemia 477

A.D. King, M. Milavec-Krizman & E. Müller-Schweinitzer. Characterization of the adenosine receptor in porcine coronary arteries 483

S. Thesleff, L.C. Sellin & S. Tågerud. Tetrahydroaminoacridine (tacrine) stimulates neurosecretion at mammalian motor endplates 487

J.V. Esplugues, E.G. Ramos, L. Gil & J. Esplugues. Influence of capsaicin-sensitive afferent neurones on the acid secretory responses of the rat stomach *in vivo* 491

H.E. Moss & G.J. Sanger. The effects of granisetron, ICS 205-930 and ondansetron on the visceral pain reflex induced by duodenal distension 497

A. Subissi, M. Guelfi & M. Criscuoli. Angiotensin converting enzyme inhibitors potentiate the bronchoconstriction induced by substance P in the guinea-pig 502

K. Muraki, Y. Imaizumi, T. Kojima, T. Kawai & M. Watanabe. Effects of tetraethylammonium and 4-aminopyridine on outward currents and excitability in canine tracheal smooth muscle cells 507

R.C. Small, D.M. Good, J.S. Dixon & I. Kennedy. The effects of epithelium removal on the actions of cholinomimetic drugs in opened segments and perfused tubular preparations of guinea-pig trachea 516

X.-F. Zhou & B.G. Livett. Effect of capsaicin-sensitive sensory nerves on plasma glucose and catecholamine levels during 2-deoxyglucose-induced stress in conscious rats 523

E.J.F. Carlisle, J.D. Allen, W.G. Kernohan, W. Leahey & A.A.J. Adgey. Pharmacological analysis of established ventricular fibrillation 530

R. Matran, K. Alving & J.M. Lundberg. Cigarette smoke, nicotine and capsaicin aerosol-induced vasodilatation in pig respiratory mucosa 535

J. Davidson, A.S. Milton & D. Rotondo. A study of the pyrogenic actions of interleukin-1α and interleukin-1β: interactions with a steroidal and a non-steroidal anti-inflammatory agent 542

V. Mollace, Y. Masuda, T. Pelaggi & G. Nisticò. The effects of phosphatidylserine on the decreased cardiovascular response to clonidine microinfused into the nucleus tractus solitarii of old rats 547

N.C.B. Nyborg. Action of noradrenaline on isolated proximal and distal coronary arteries of rat: selective release of endothelium-derived relaxing factor in proximal arteries 552

C. Richer, P. Mulder, M.-P. Doussau, P. Gautier & J.-F. Giudicelli. Systemic and regional haemodynamic interactions between K^+ channel openers and the sympathetic nervous system in the pithed SHR 557

S. Etoh, M. Ohashi, A. Baba & H. Iwata. Inhibition by ibudilast of leukotriene D_4-induced formation of inositol phosphates in guinea-pig lung 564

H. Moritoki, T. Matsugi, H. Takase, H. Ueda & A. Tanioka. Evidence for the involvement of cyclic GMP in adenosine-induced, age-dependent, vasodilatation 569

R.B. Rose'Meyer & W. Hope. Evidence that A_2 purinoceptors are involved in endothelium-dependent relaxation of the rat thoracic aorta 576

R. Pilsudski, O. Rougier & Y. Tourneur. Action of cromakalim on potassium membrane conductance in isolated heart myocytes of frog 581

C.A. Maggi, R. Patacchini, S. Giuliani, P. Rovero, S. Dion, D. Regoli, A. Giachetti & A. Meli. Competitive antagonists discriminate between NK_2 tachykinin receptor subtypes 588

T. Fukumitsu, H. Hayashi, H. Tokuno & T. Tomita. Increase in calcium channel current by β-adrenoceptor agonists in single smooth muscle cells isolated from porcine coronary artery 593

J. Feldman, E. Tibiriça, G. Bricca, M. Dontenwill, A. Belcourt & P. Bousquet. Evidence for the involvement of imidazoline receptors in the central hypotensive effect of rilmenidine in the rabbit 600

D.T. Newgreen, K.M. Bray, A.D. McHarg, A.H. Weston, S. Duty, B.S. Brown, P.B. Kay, G. Edwards, J. Longmore & J.S. Southerton. The action of diazoxide and minoxidil sulphate on rat blood vessels: a comparison with cromakalim 605

L.B. Fernandes & R.G. Goldie. Pharmacological evaluation of a guinea-pig tracheal epithelium-derived inhibitory factor (EpDIF) 614

R. Inoue & A.F. Brading. The properties of the ATP-induced depolarization and current in single cells isolated from the guinea-pig urinary bladder 619

R.T. Mathie & B. Alexander. The role of adenosine in the hyperaemic response of the hepatic artery to portal vein occlusion (the 'buffer response') 626

S.C. Cruwys, D.E. Davies & E.R. Pettipher. Co-operation between interleukin-1 and the fibrinolytic system in the degradation of collagen by articular chondrocytes 631

S. Horio, M. Shima, H. Ueda & Y. Ishida. Temperature-dependence of desensitization induced by acetylcholine and histamine in guinea-pig ileal longitudinal muscle 636

M.C. Butterfield & R. Chess-Williams. Enhanced α-adrenoceptor responsiveness and receptor number during global ischaemia in the Langendorff perfused rat heart 641

I.P. Hall, J. Donaldson & S.J. Hill. Modulation of fluoroaluminate-induced inositol phosphate formation by increases in tissue cyclic AMP content in bovine tracheal smooth muscle 646

B. Fabris, H. Yamada, R. Cubela, B. Jackson, F.A.O. Mendelsohn & C.I. Johnston. Characterization of cardiac angiotensin converting enzyme (ACE) and *in vivo* inhibition following oral quinapril to rats 651

P.M. Dunn & H.P. Rang. Bradykinin-induced depolarization of primary afferent nerve terminals in the neonatal rat spinal cord *in vitro* 656

NOTICE CONCERNING SPECIAL REPORTS

ERRATA

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Errata

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'Protective effect of beraprost sodium, a new chemically stable prostacyclin analogue, against the deterioration of baroreceptor reflex following transient global cerebral ischaemia in dogs' by Junichi Kurihara, Tatsuhiro Sahara & Hitoshi Kato.

In the Summary of this paper, the last two words of the last sentence of point 3 are illegible: they should read 'higher dose'.

On page 93, there is an error in Table 2. The first entry in the *During infusion* column should read 26.1 ± 3.7 instead of 6.1 ± 3.7.