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Low dihydropyridine receptor density in vasa deferentia of castrated rats

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Radioligand binding studies in crude membrane preparations of vasa deferentia of normal rats, with the 1,4-dihydropyridine (+)-[^{3}H]-PN200-110 (isradipine) showed typical saturation isotherms. The binding exhibited a K_D of 259 ± 60 pM and B_{max} of 144 ± 20 fmol mg^{-1} protein. The low K_D and the stereoselective displacement of (+)-[^{3}H]-PN200-110 binding by (+)- and (−)-PN200-110 and by nifedipine suggests that these tissues contain dihydropyridine receptors probably coupled to voltage-sensitive, L-type calcium channels. In membrane preparations from vasa deferentia from rats castrated 30 days previously the maximum specific binding was 25 ± 10 fmol mg^{-1} protein, representing only 11% of total binding; thus, the calculation of reliable K_D values was not feasible. These findings suggest that a testicular hormone, possibly testosterone, plays an important role in the regulation of dihydropyridine-sensitive, voltage-dependent calcium channels in the rat vas deferens.

Keywords: Dihydropyridine receptors; vas deferens; calcium channels; castration

Introduction Functional studies of isolated tissues have indicated that castration influences calcium translocation in smooth muscle cells of the rat vas deferens (Jurkiewicz *et al.*, 1977). In this organ, the presence of voltage-dependent calcium channels of the L-type (dihydropyrine-sensitive), was demonstrated by means of electrophysiological techniques (Nakazawa *et al.*, 1988) and by binding of radiolabelled nitrendipine (Triggle *et al.*, 1989). The present investigation was undertaken to determine if the dihydropyridine-sensitive calcium channels of this organ can be influenced by castration, as suggested by functional analysis. Therefore, the binding of the dihydropyridine (+)-[^{3}H]-PN200-110 was examined in membrane preparations of vasa deferentia of normal and castrated animals.

Methods Orchiectomy was performed in 3-month-old Wistar rats, under ether anaesthesia. Animals were killed 30 days after castration and vasa deferentia excised to obtain membrane preparations. Vasa deferentia of castrates or normal controls were cut into small segments. Homogenization was carried out in 50 volumes (w/v) ice-cold 50 mM Tris/HCl buffer, pH 7.5 (measured at 25°C), with an Ultraturrax homogenizer at 20,500 r.p.m., for 3 × 1 min. This was followed by 10 strokes of a glass potter homogenizer. After filtration under vacuum through 4 layers of gauze, the homogenate was centrifuged at 45,000 *g* for 20 min at 0–4°C. The resulting pellet was washed in buffer, followed by rehomogenization (3 × 1 min) and centrifugation at 45,000 *g* for 20 min. The final pellet was then resuspended in 10 volumes (w/v) buffer for subsequent use in the binding assay.

Saturation binding studies were made as described by Castillo *et al.* (1989). Aliquots of the membrane suspension (final protein concentration of 200 μg ml^{-1}) were incubated at 37°C with 10 to 1500 pM of (+)-[^{3}H]-PN200-110 (isradipine) in a final volume of 1 ml for 90 min, under sodium light. Incubations were terminated by dilution of the samples with 5 ml ice-cold buffer, followed by rapid filtration under reduced pressure Whatman GF/C glass fibre filters and washing of the filters with 3 × 5 ml buffer. After drying at 80°C for 15–20 min, filters were added to a scintillation cocktail, and the radioactivity measured in a Beckman liquid scintillation counter with 50% efficiency. Specific binding of (+)-[^{3}H]-PN200-110 was defined as the difference between the total binding and that obtained in the presence of 1 μM (±)-PN200-110.

For displacement studies, (+)-[^{3}H]-PN200-110 (150 pM) was incubated with various concentrations of (+)-PN200-110, (−)-PN200-110, and nifedipine, under the same conditions as for saturation experiments. Termination of incubation and processing of the filters were as described above.

Binding parameters were determined through Scatchard analysis, by plotting the concentration of bound ligand (fmol mg^{-1} protein) in relation to bound/free ligand (fmol mg^{-1} protein pM^{-1}).

The dihydropyridine, (+)-[^{3}H]-PN200-110 (84.1 Ci mmol^{-1}) was obtained from New England Nuclear; (+)-, (±)-, and (−)-PN200-110 was from Dr P. Hof (Sandoz, Ltd, Basel, Switzerland) and nifedipine from Prof. F. Hoffmeister (Bayer A.G. Wuppertal, Germany).

Results Specific binding of (+)-[^{3}H]-PN200-110 to membrane preparations of normal rats was linear up to 300 μg of protein ml^{-1} and typically represented more than 60% of total binding. The radiolabelled ligand was displaced (Figure 1) by (+)-PN200-110 (K_i = 0.18 nM), nifedipine (K_i = 22 nM) and (−)-PN200-110 (K_i = 12 nM). The clear stereospecificity, the range of K_i (nM or sub-nM), and the order of potencies found suggest that we are dealing with a true dihydropyridine receptor, likely to be associated with a L-type calcium channel.

In vas deferens membrane preparations of normal rats, the values of K_D for (+)-[^{3}H]-PN200-110 was 259 ± 60 pM and the B_{max} 144 ± 20 fmol mg^{-1} protein. The mean saturation curve for (+)-[^{3}H]-PN200-110 binding, and the respective Scatchard plot are shown in Figure 2.

In membrane preparations of castrated animals, the maximum specific binding of (+)-[^{3}H]-PN200-110 was only about 10% of the total binding, and the corresponding concentration was reduced to 25 ± 10 fmol mg^{-1} protein (Figure 2). In these conditions, the values of K_D and B_{max} could not be determined.

Discussion The specific binding of (+)-[^{3}H]-PN200-110 in membrane preparations of castrated rats was strikingly smaller than in normal preparations. These results suggest

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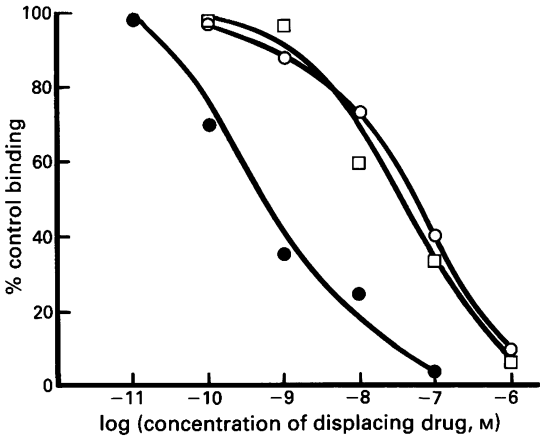

Figure 1 Displacement by (+)-PN200-110 (●), nifedipine (□) and (−)-PN200-110 (○) of (+)-[^{3}H]-PN200-110 binding to vas deferens membrane preparations from normal rats. Data represent typical experiment in duplicate.

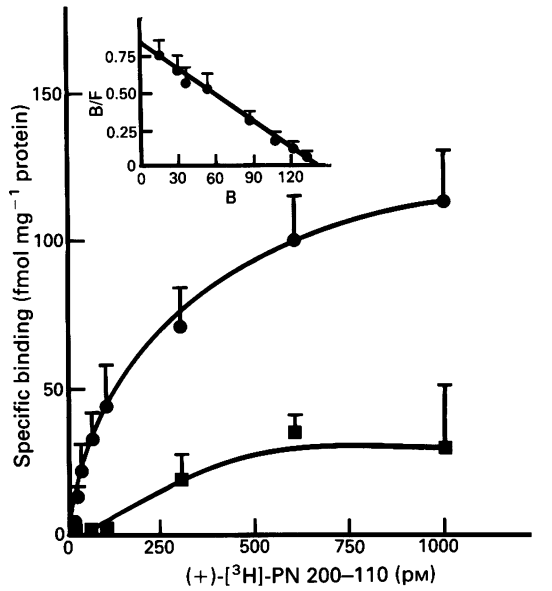

Figure 2 Specific binding of (+)-[^{3}H]-PN200-110 membrane preparations of vas deferens of normal (●) and 30-day castrated rats (■). Inset shows the respective Scatchard plot, from which the values of K_D and B_{max} were obtained. Vertical lines show one s.e.mean.

that the (+)-[^{3}H]-PN200-110 binding sites can be influenced by the lack of testosterone or other testicular products, induced by castration.

This decrease of receptor density is not a consequence of protein loss due to organ atrophy, since our results are expressed in relation to the protein content of the tissue. Nor is it due to a variation in the balance between smooth muscle and non-muscular tissue, since it is known through histometry of cross-sections of the vas that a reduction of about 45%–55% is observed on the thickness of both the epithelium (from $34.8 \pm 2.3\,\mu m$ to $15.4 \pm 0.5\,\mu m$) and muscle (from $371.5 \pm 26.7\,\mu m$ to $207.1 \pm 6.6\,\mu m$) after 1-month castration (C.S. Porto, personal communication).

A survey of several drugs which might regulate the number and functioning of calcium channels has recently been presented (Ferrante & Triggle, 1990). The only references to the influence of sex hormones has been from the work of Ishii *et al.* (1986), Batra (1987) and Ruzycky *et al.* (1987) who reported that circulating oestrogens and progesterone can alter the binding characteristics of dihydropyridines in smooth muscle of female rats. However, to our knowledge, the possibility derived from the present results that male sex hormones also influence calcium channel regulation, has not been demonstrated previously.

It has been shown previously that the contractile effects of several agonists in isolated vas deferens of normal rats are drastically reduced if calcium is removed from the nutrient solution (Jurkiewicz *et al.*, 1975a). These results were explained through a theoretical model in which calcium is considered as one of the constituents of the chain of events leading from drug-receptor interaction to the effect (Jurkiewicz *et al.*, 1975b). In vasa deferentia of castrates, it was shown that several agonists exhibit reduced contractile effects, which are restored to control values if the concentration of calcium in the nutrient solution is increased above normal values (Jurkiewicz *et al.*, 1977). This calcium-dependent change in mechanical response after castration is compatible with our present findings, since one would expect a decrease of calcium availability for contraction, in a situation in which the density of L-type calcium channels is substantially reduced.

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SPECIAL REPORT

Direct action of BRL 38227 and glibenclamide on intracellular calcium stores in cultured airway smooth muscle of rabbit

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The effects of BRL 38227 and glibenclamide on intracellular calcium stores were investigated in permeabilized cultured airway smooth muscle cells of the rabbit using ^{45}Ca effluxes. BRL 38227 (10 μM) reduced loading of the inositol 1,4,5-trisphosphate ($InsP_3$)-sensitive intracellular store by 26.5% ± 1.0; this effect was antagonized by 1 μM glibenclamide. BRL 38227 itself did not release calcium and had no effect on guanosine 5'-0-(3-thiotriphosphate)-induced calcium release. Glibenclamide ($\geqslant$5 μM) also reduced calcium loading of the intracellular store, and enhanced calcium release. These results suggest that BRL 38227 has a direct effect on intracellular calcium handling.

Keywords: BRL 38227; Ca^{2+} uptake; Ca^{2+} release; glibenclamide; GTP; inositol 1,4,5-trisphosphate; sarcoplasmic reticulum; smooth muscle

Introduction Cromakalim has been shown to promote ^{86}Rb efflux, hyperpolarization and subsequent relaxation of airway smooth muscle (Allen *et al.*, 1986). There has been much interest recently in effects of potassium channel openers that are additional to their hyperpolarizing action and Bray *et al.* (1991) have suggested that cromakalim may exert a direct inhibitory action on intracellular Ca^{2+} stores in intact rabbit aorta.

We have investigated whether the active enantiomer of cromakalim, BRL 38227, has a direct effect on Ca^{2+} uptake and release from intracellular stores in airway smooth muscle and whether such an effect can be antagonized by glibenclamide.

Methods *Cell culture* Smooth muscle cells (passages 5–8) isolated from rabbit trachea were cultured as described in detail for human airway smooth muscle by Twort & van Breemen (1989). Cells were grown to confluency on 35 mm dishes (0.2 mg cell protein/dish) in Dulbecco's Modified Eagle's medium supplemented by 10% foetal calf serum.

Ca^{2+} uptake and efflux from permeabilized cells Experiments were performed at room temperature (22°C). Ethylene glycol-*bis*(β-aminoethylether)N,N,N,N-tetraaectic acid (EGTA) solutions also contained KCl 130 mM, $MgCl_2$ 5 mM, Tris-maleate buffer 20 mM, pH 6.8. The procedure was similar to that previously described (Twort & van Breemen, 1989).

The cells were permeabilized with 40 μM digitonin and preincubated for 15 min in a 0.1 mM EGTA solution containing BRL 38227 and/or glibenclamide. This was replaced with a loading solution containing ^{45}Ca (10 μCi ml^{-1}) with 1 μM free [Ca^{2+}], Na_2ATP (3.15 mM), and BRL 38227 and/or glibenclamide where appropriate. Control dishes were incubated with identical solutions including the appropriate solvents, but containing no BRL 38227 or glibenclamide. After 30 min the loading solution was replaced with an efflux solution containing 0.1 mM EGTA. This was changed every minute and collected in scintillation vials for estimation of ^{45}Ca efflux.

The effect of BRL 38227 on inositol 1,4,5-trisphosphate ($InsP_3$) and guanosine 5'-0-(3-thio trisphosphate) (GTPγS)-induced Ca^{2+} release was examined by adding 10 μM BRL 38227 during the ^{45}Ca loading period. The efflux procedure was carried out as described but either $InsP_3$ was added at 13 min for 1 min or, as GTPγS had a longer response time, GTPγS was added at 25 min for 5 min.

The effects of BRL 38227 or glibenclamide on Ca^{2+} release were investigated by loading cells in normal solution and adding either BRL 38227 or glibenclamide during the efflux procedure.

Identical dishes of cells from the same cell line and subcultured at the same time were grouped into pairs with one member of the pair acting as the control and one as the test dish. The difference between each pair of control and test dishes used at the same time were compared by Student's *t* test for paired values. Results are expressed as the means ± s.e.mean.

Results BRL 38227 decreased both the rate and amount of Ca^{2+} uptake (Figure 1a). At 30 min, Ca^{2+} uptake in the presence of 10 μM BRL 38227 was significantly reduced (control: 2.473 ± 0.161; +BRL 38227: 1.888 ± 0.107 nmol Ca^{2+} mg^{-1} protein; $n = 4$, $P < 0.01$). This effect was concentration-dependent, (Figure 1b). Addition of BRL 38227 (10 μM) during the efflux procedure did not release intracellular calcium ($n = 5$). Addition of 1 μM glibenclamide during the uptake period did not itself affect Ca^{2+} uptake ($n = 8$, $P > 0.5$), but abolished the inhibitory effect of BRL 38227 (Figure 1b).

Preincubation with 10 μM BRL 38227 during loading reduced $InsP_3$ (10 μM)-induced Ca^{2+} release from 1.91 ± 0.37 to 1.41 ± 0.26 nmol Ca^{2+} mg^{-1} protein ($n = 6$, $P < 0.05$), equivalent to a reduction of 23.3% ± 5.3. Addition of BRL 38227 during the efflux phase alone had no effect on $InsP_3$-induced Ca^{2+} release. Preincubation with 10 μM BRL 38227 had no effect on Ca^{2+} released by GTPγS (100 μM); (control: 0.439 ± 0.01; +BRL 38227: 0.441 ± 0.05 nmol Ca^{2+} mg^{-1} protein; $n = 5$).

Glibenclamide (5–50 μM) could itself reduce loading of Ca^{2+} into the intracellular store (Figure 2), and caused a small slow release of Ca^{2+} when added during the efflux procedure; (1 μM glibenclamide: 0.005 ± 0.003; 10 μM: 0.044 ± 0.014; 50 μM: 0.213 ± 0.010 nmol Ca mg^{-1} protein; $n = 4$, Figure 2).

Discussion We have demonstrated that BRL 38227 has a direct effect on Ca^{2+} uptake into intracellular stores of permeabilized airway smooth muscle. This is consistent with the results obtained by Bray *et al.* (1991) for intact rabbit aorta,

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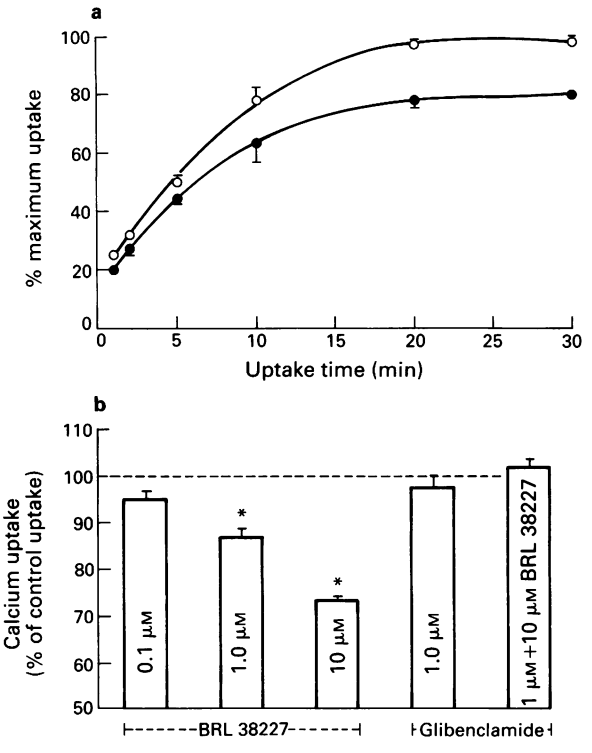

Figure 1 Action of BRL 38227 on intracellular Ca^{2+} uptake: (a) effect of BRL 38227 on the time course of ^{45}Ca uptake by permeabilized cells. Control: (○); BRL 38227 (10 μM) (●); $n = 4$. (b) reduction of Ca^{2+} uptake by BRL 38227 and inhibition of this effect by 1 μM glibenclamide. Results are expressed as % control Ca^{2+} uptake; $n = 4$–8, * $P < 0.01$.

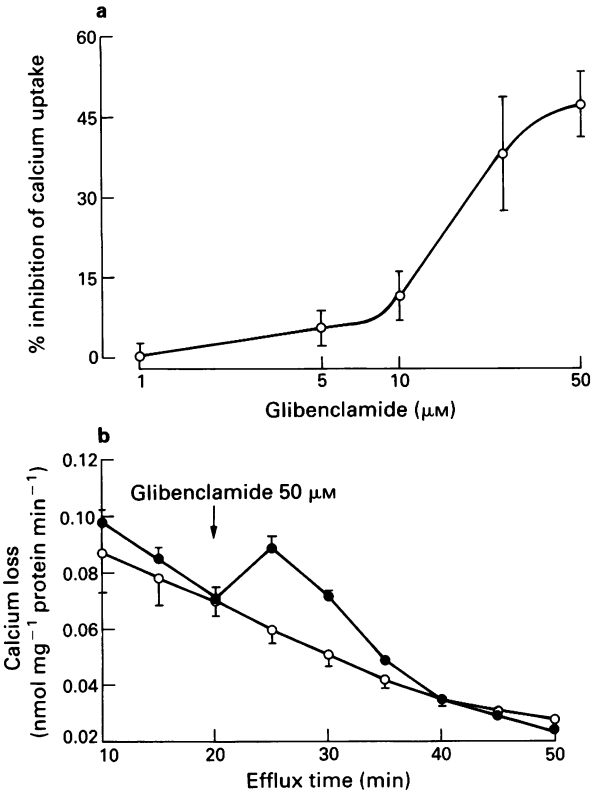

Figure 2 Action of glibenclamide on intracellular Ca^{2+} uptake and release. (a) reduction of Ca^{2+} uptake by glibenclamide; $n = 4$; (b) effect of glibenclamide on ^{45}Ca efflux from permeabilized cells. Control: (○); 50 μM glibenclamide added at 20 min for 15 min (●); $n = 4$.

who found that in the presence of cromakalim both the rate and extent of store refilling, as estimated by the magnitude of noradrenaline-induced contraction, were reduced. Xiong *et al.* (1991) have also demonstrated that pinacidil can inhibit release, or deplete Ca^{2+} from intracellular stores in intact portal vein cells of rabbit. As in our experiments this effect was antagonized by 1 μM glibenclamide.

BRL 38227 had no direct effect on $InsP_3$-induced Ca^{2+} release, but inhibited loading of the $InsP_3$-, but not GTPγS-, sensitive Ca^{2+} store. This suggests that $InsP_3$ and GTPγS release Ca^{2+} from different sites, and that the mechanism by which the GTPγS-sensitive Ca^{2+} store is refilled is different from that of the $InsP_3$-sensitive store.

At 1 μM, glibenclamide antagonized the effects of BRL 38227. At higher concentrations it could reduce uptake substantially and caused a slow release of Ca^{2+} when added to full stores. This was unlike BRL 38227 which did not cause Ca^{2+} release. Enhanced release of Ca^{2+} will reduce uptake so these two effects of glibenclamide may be related. Xiong *et al.* (1991) have also shown that 30 μM glibenclamide has a similar action to pinacidil.

The sarcoplasmic reticulum has K^+ selective channels which are thought to allow charge balancing against movement of Ca^{2+} ions (Somlyo *et al.*, 1981). In skeletal muscle (Fink & Stephenson, 1987) the amount of releasable Ca^{2+} is markedly increased by reducing the K^+-conductance of the sarcoplasmic reticulum. An increase in K^+ conductance should therefore decrease the ability of the sarcoplasmic reticulum to load Ca^{2+}. Whether BRL 38227 and low concentrations of glibenclamide (1 μM) act at such K^+ channels, and whether they are similar to the ATP-sensitive K^+ channel of the cell membrane remains to be seen.

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Inhibition of neurogenic plasma exudation in guinea-pig airways by CP-96,345, a new non-peptide NK_1 receptor antagonist

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A new non-peptide tachykinin antagonist, CP-96,345, inhibited airway plasma exudation induced in guinea-pigs by i.v. substance P in a dose-dependent manner with dose-ratios in the main bronchi of 5 at 1 nmol kg^{-1} and 19 at 100 nmol kg^{-1}. At 100 nmol kg^{-1}, CP-96,345 completely inhibited plasma exudation induced by either electrical stimulation of the cervical vagus nerves or i.v. capsaicin, indicating inhibition of the effects of endogenous tachykinins, but did not inhibit the bronchoconstrictor response to neurokinin A, suggesting selectivity for NK_1 receptors. CP-96,345 may be useful in examining the role of endogenous tachykinins *in vivo*.

Keywords: CP-96,345; tachykinin receptor; NK_1 receptor; NK_1 receptor antagonist; substance P; plasma exudation; sensory nerves; vagus nerve; capsaicin; asthma

Introduction Tachykinins have several effects on airway function including bronchoconstriction, mucus secretion, plasma exudation and vasodilatation (Barnes *et al.*, 1990). Tachykinins may be released from capsaicin-sensitive sensory nerves in airways by capsaicin itself and by antidromic electrical stimulation of the vagus nerves (Lundberg & Saria, 1982).

Three classes of tachykinin receptor are currently recognised, denoted as NK_1, NK_2 and NK_3, which exhibit preferential affinity for substance P (SP), neurokinin A (NKA) and NKB respectively (Guard & Watson, 1991). SP is more potent than NKA or NKB in inducing plasma exudation in guinea-pig airways (Rogers *et al.*, 1988), which indicates that neurogenic plasma exudation is mediated via NK_1 receptors. In contrast, bronchoconstriction is presumed to be mediated by NK_2 receptors (Advenier *et al.*, 1987). The role of tachykinins released from sensory nerves has proved difficult to determine in the absence of specific and potent antagonists, since sensory nerves may release several other peptides including calcitonin gene-related peptide.

CP-96,345 has been demonstrated recently to be a potent NK_1 receptor antagonist at central and peripheral SP binding sites and in *in vitro* and *in vivo* functional assays (Mclean *et al.*, 1991; Snider *et al.*, 1991). In the present study, we assessed the pharmacological profile of CP-96,345 on airway plasma exudation induced by exogenous SP, capsaicin, or vagus nerve stimulation, and on bronchoconstriction induced by capsaicin, vagus nerve stimulation or NKA.

Methods Male Dunkin-Hartley guinea-pigs (320–380 g) were anaesthetized with an initial dose of 2 g kg^{-1} urethane (diluted to 25% w/v in 0.9% saline) intraperitoneally and laid supine. Additional urethane was given as required to maintain anaesthesia. The animals were mechanically ventilated at a tidal volume of 10 ml kg^{-1} and a rate of 60 strokes per min via a cannula inserted into the lumen of the upper cervical trachea. A side-arm connected to a pressure transducer recorded pulmonary insufflation pressure (PIP). A catheter was inserted into the left carotid artery to monitor blood pressure. Both external jugular veins were exposed for administration of drugs. In one group of animals, both cervical vagus nerves were exposed and sectioned for electrical stimulation. Atropine (1 mg kg^{-1}, i.v.) was given 15 min before stimulation. Evans blue dye (25 mg kg^{-1}, dissolved in 0.9% saline) was used as a marker of plasma exudation and injected 5 min after CP-96,345 ((2*S*,3*S*-*cis*-2-(diyhenylmethyl)-*N*-[(2-methoxyphenyl)-methyl]-1-azabi-cyclo[2.2.2]octan-3-amine) or saline control. One min later control saline, SP (Sigma, 1 pmol–100 nmol kg^{-1}), capsaicin (Sigma; 0.3 mg kg^{-1}: 10 mg ml^{-1} in ethanol diluted in saline to 0.3 mg ml^{-1}) or NKA (Aldrich; 6 nmol kg^{-1}) were administered, or the vagus nerves were electrically stimulated (5 V, 10 Hz, 5 ms for 3 min). Ten min after saline, SP, NKA or capsaicin, or 5 min after vagus nerve stimulation, the systemic circulation was perfused with saline to remove intravascular dye. The trachea and main bronchi were removed and tissue Evans blue was extracted in 2 ml formamide (40°C for 16 h). Evans blue dye concentrations were measured with a spectrophotometer (Phillips, U8630, at 620 nm wavelength), interpolated on a standard curve and expressed as ng dye mg^{-1} wet weight tissue.

Results SP induced a dose-dependent increase in the Evans blue content of the trachea and main bronchi (Figure 1a). The maximal increase above controls was 429% in the lower trachea at a dose of 1 nmol kg^{-1} and was 360% in the main bronchi at a dose of 10 nmol kg^{-1}. CP-96,345, given 5 min previously, caused a rightward shift in the SP dose-response curve: at 1 nmol kg^{-1} the dose-ratios for the trachea and main bronchi were 8.9 and 4.6; and at 100 nmol kg^{-1} were 20.4 and 18.6 respectively. CP-96,345 also inhibited the increase in the tissue dye content induced by SP 1 nmol kg^{-1} with ID_{50} values of 0.7 nmol kg^{-1} and 1.4 nmol kg^{-1} in trachea and main bronchi respectively and with complete inhibition at a dose of 5 μmol kg^{-1} for both airways (Figure 1b). Dye leakage was inhibited by 86% and 110% in the trachea and main bronchi when 100 nmol kg^{-1} CP-96,345 was given 30 min before 0.1 nmol kg^{-1} SP.

CP-96,345 100 nmol kg^{-1} inhibited dye leakage induced by capsaicin or vagus nerve stimulation in both lower trachea and main bronchi (Figure 2a).

Increases above baseline in PIP induced by NKA or by capsaicin were not inhibited by 100 nmol kg^{-1} CP-96,345. Vagus nerve stimulation increased PIP and although CP-96,345 partially inhibited this response, the reduction was not significant ($P = 0.1$).

CP-96,345 up to 1 μmol kg^{-1} alone had no effect on blood pressure although higher doses (2–10 μmol kg^{-1}, i.v.) caused decreases in blood pressure of up to 45% at the highest dose used.

Discussion In the present *in vivo* study we found that the non-peptide SP receptor antagonist, CP-96,345, caused a rightward shift of the SP dose-response curve for plasma leakage. The shift was approximately parallel for two doses of CP-96,345 which indicated probable competitive antagonism and is consistent with *in vitro* binding and functional data (Snider *et al.*, 1991; McLean *et al.*, 1991). Our present finding that 5 μmol kg^{-1} CP-96,345 blocked leakage induced by 1 nmol kg^{-1} SP is similar to previous *in vivo* data in the rat where 8.2 μmol kg^{-1} CP-96,345 blocked salivation induced by 1 nmol kg^{-1} SP (Snider *et al.*, 1991).

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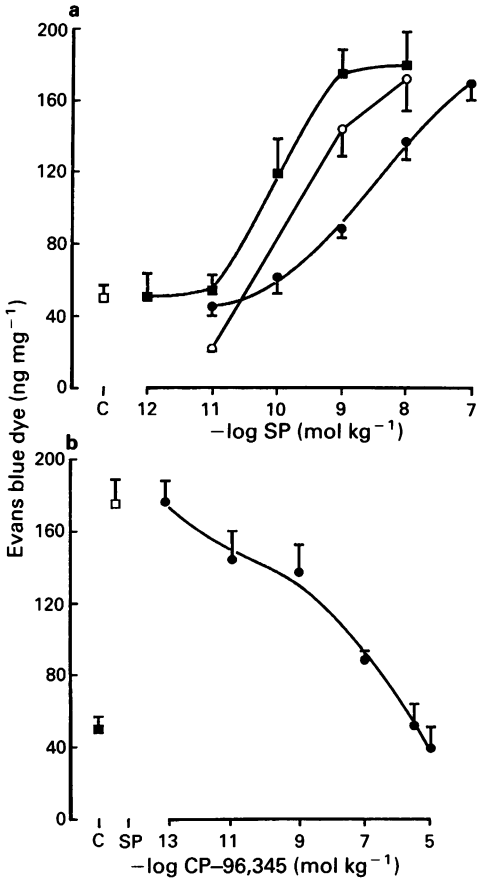

Figure 1 Effects of CP-96,345 on substance P (SP)-induced plasma exudation in guinea-pig main bronchi. (a) Inhibition of SP-induced leakage (■) by CP-96,345 1 nmol kg^{-1} (○) or 100 nmol kg^{-1} (●). (b) Inhibition of SP (1 μmol kg^{-1})-induced leakage by CP-96,345; (□) control values. Mean tissue content of Evans blue dye for 4–6 animals; s.e.mean shown by vertical bars.

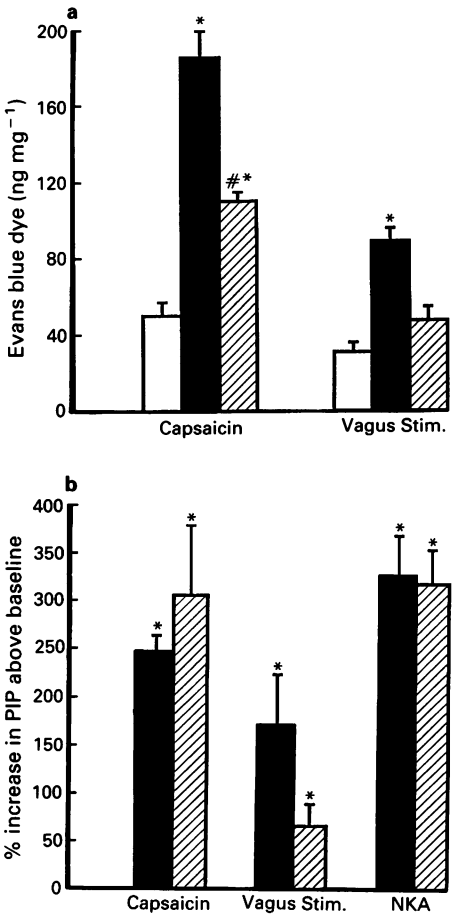

Figure 2 Effects of CP-96,345 in guinea-pig airways: (a) plasma exudation in main bronchi or (b) change in pulmonary insufflation pressure (PIP) induced by capsaicin (0.3 mg kg^{-1}, i.v.), electrical stimulation of the vagus nerves (Vagus Stim) or neurokinin A (NKA, 6 nmol kg^{-1}). Open column, control; solid column, response to stimulation; hatched column, effect of CP-96,345 100 nmol kg^{-1} on response to stimulation. Data are expressed as mean for 4–6 animals; s.e.mean shown by vertical bars. * $P < 0.05$ vs control; # $P < 0.05$ vs stimulation.

Neurogenic plasma exudation induced by capsaicin or vagus nerve stimulation (Lundberg & Saria, 1982) is mediated via tachykinins released from capsaicin-sensitive sensory nerves. Agonist potency studies indicate that an NK_1 receptor mediates this response (Rogers *et al.*, 1988). Our present data using CP-96,345 indicate that this antagonist is active at NK_1 receptors. In contrast, NKA, capsaicin and vagus nerve stimulation-induced bronchoconstriction, which is mediated predominately via NK_2 receptors (Advenier *et al.*, 1987), was not significantly inhibited by the dose of CP-96,345 which completely blocked the plasma exudation.

Increased microvascular permeability with plasma exudation leads to tissue oedema and is a feature of bronchial diseases including asthma (Chung *et al.*, 1990). Release of neuropeptides via an axon reflex has been suggested as a mechanism which may contribute to the inflammation of asthma (Barnes, 1986). CP-96,345, a potent antagonist *in vivo*, may be a useful tool in elucidating the mechanisms of airway neurogenic inflammation and may have therapeutic potential in the treatment of bronchial diseases including asthma.

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Evidence for the absence of a functional role for muscarinic M_2 inhibitory receptors in cat trachea *in vivo*: contrast with *in vitro* results

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1 The effect of the selective muscarinic M_2 receptor antagonist, gallamine and the selective M_2 receptor agonist, pilocarpine, on airway constriction induced by vagal stimulation was studied in anaesthetized cats. In addition, the effect of gallamine on contraction of cat isolated tracheal and bronchi preparations induced by electrical field stimulation was also investigated.

2 In *in vivo* experiments, extrathoracic airway constriction was measured with an electromechanical caliper that was attached to the outer surface of tracheal ring 4. Intrathoracic airway constriction was determined by measuring the changes in total lung resistance and dynamic compliance during vagal stimulation.

3 Intravenous gallamine (0.1, 1, and 10 mg kg^{-1}) augmented the rise in total lung resistance induced by vagal stimulation in a dose- and frequency-dependent manner. At stimulation frequencies of 8 and 12 Hz the fall in dynamic compliance provoked by vagal stimulation was also significantly increased by gallamine (10 mg kg^{-1}). Gallamine was without effect on airway constriction induced by acetylcholine.

4 Vagal stimulation at 4 Hz produced significant tracheal constriction, but the amount of constriction did not change following injection of increasing doses of gallamine. Similarly, there was no difference in tracheal constriction at any frequency of stimulation (0.5–16 Hz) when frequency-response curves before and after gallamine injection (10 mg kg^{-1}) were compared.

5 Pilocarpine (0.01–10 μg kg^{-1}, i.v.) diminished changes in total lung resistance and dynamic compliance induced by vagal stimulation, an effect that was reversed by gallamine (10 mg kg^{-1}, i.v.). Vagally-induced tracheal constriction was not significantly affected by any dose of pilocarpine, nor was it modified by gallamine (10 mg kg^{-1}) given subsequently.

6 Atropine (0.5 mg kg^{-1}) completely blocked tracheal constriction induced by vagal stimulation, indicating that the changes in tracheal ring diameter provoked by stimulation were mediated by muscarinic receptors and that intravenous drugs could reach the cervical trachealis muscle.

7 *In vitro* tissue bath studies demonstrated a significant leftward shift of the frequency-response curve to electrical field stimulation in both tracheal strips and bronchial rings following gallamine (10^{-4} M) administration.

8 Although the functional presence of muscarinic M_2 autoreceptors was demonstrated in feline isolated tracheal and bronchial preparations, a corresponding functional role was not detected in cat trachea *in vivo*. This was despite repeated demonstration of muscarinic M_2 receptor-mediated limitation of airway constriction of intrathoracic airways *in vivo*.

Keywords: Muscarinic receptors; neuromodulator receptors; parasympathetic nervous system; airway resistance.

Introduction

Inhibitory muscarinic (M_2) autoreceptors in airways were first demonstrated with *in vivo* experiments using the selective M_2 muscarinic antagonist, gallamine (Fryer & Maclagan, 1984). When the autoreceptors were blocked with gallamine, the effect of vagally-induced bronchoconstriction was potentiated in guinea-pig airways. Gallamine had little effect on the bronchoconstrictor response to injected acetylcholine (ACh), indicating that gallamine was not a potent antagonist for the postjunctional M_3 muscarinic receptors in airway smooth muscle. A connection between an increased magnitude of vagally-induced bronchonconstriction during viral respiratory infections and alteration of M_2 autoreceptors in the lungs of guinea-pigs has been reported (Fryer *et al.*, 1990; Fryer & Jacoby, 1991). The investigators hypothesized that damage to M_2 receptors could result in the loss of negative feedback inhibition of ACh release at the airway smooth muscle neuromuscular junction.

Cats experimentally infected with herpesvirus-I demonstrate tracheal hyperreactivity to vagal stimulation, which is completely blocked by intravenous atropine (Killingsworth *et al.*, 1990). An increased tracheal response to ACh injection is not detected. Intrathoracic airway hyperreactivity is not present because herpesvirus-I infects the trachea and proximal air passages but seldom infects the lungs (Gaskell & Povey, 1979; Killingsworth *et al.*, 1990). These findings suggest that herpesvirus-induced tracheal hyperreactivity is mediated by a presynaptic mechanism, which could be inhibitory M_2 receptor dysfunction.

Other investigators have reported that following gallamine, vagal stimulation results in a significant increase of total lung resistance and a significant decrease of dynamic compliance. They concluded that inhibitory muscarinic receptors were present in cats in parasympathetic nerves of the trachea, bronchi, and respiratory bronchioles (Blaber *et al.*, 1985). However, previous studies were not designed to investigate specifically the role of M_2 inhibitory receptors in limiting airway narrowing in the extrathoracic trachea. The studies described in the present paper were designed to examine whether M_2 receptors can inhibit both extra- and intrathoracic airway constriction. Changes in airway calibre induced by exogenous ACh and bilateral vagal stimulation at 4 Hz were measured in healthy cats that were not infected with herpesvirus. Cervical tracheal constriction and intrathoracic airway responses were quantitated before and after graded intravenous doses of gallamine. A separate group of *in vivo* experiments was performed to determine the effect of the muscarinic

receptor agonist, pilocarpine, on airway responses measured during vagal stimulation. Cervical tracheal constriction and the changes in total lung resistance and dynamic compliance were measured before and after increasing doses of intravenous pilocarpine. The M_2 receptor antagonist, gallamine, was injected at the completion of the pilocarpine dose-response study. A further group of *in vivo* experiments was designed to explore the effect of the M_2 receptor antagonist, gallamine, on frequency-dependent activation of M_2 receptors. Airway responses were measured during vagal stimulation at increasing frequencies (0.5–16 Hz) before and after gallamine injection. Atropine was injected intravenously (i.v.) at the end of the experiments to demonstrate the role of muscarinic receptors in the measured tracheal contraction and to show that i.v. drugs reach the cervical trachealis muscle. Finally, a series of muscle bath experiments was conducted to determine the effect of increasing concentrations of gallamine on electrical field stimulation-induced and ACh-induced contraction of tracheal strips and bronchial rings *in vitro*.

Methods

Cats were managed in accordance with the National Institutes of Health standards defined by the United States Department of Agriculture Animal Welfare Acts. Experiments were approved by the All-University Committee on Animal Use and Care at Michigan State University, East Lansing, MI, U.S.A.

Surgical preparation for in vivo *experiments*

Adult, mixed breed cats of either sex were anaesthetized with thiamylal (15–20 mg kg^{-1}, i.v.) administered to maintain a corneal reflex but to prevent withdrawal response following paw pinch. A midline ventral cervical incision exposed the trachea and an endotracheal tube was placed by tracheostomy at the thoracic inlet so that the cranial cervical tracheal segment was undisturbed. The endotracheal tube was connected to a fixed volume ventilator (Harvard Model 665 Small Animal Ventilator) and respiratory frequency was adjusted to maintain $Paco_2$ between 28–32 mmHg and Pao_2 between 85 and 100 mmHg. Sodium bicarbonate was infused i.v. to maintain arterial pH between 7.360 and 7.440. Cats were placed on a thermostatically controlled heating pad to maintain body temperature at 38°C. Lactated Ringer solution was administered by continuous infusion (10 ml kg^{-1} h^{-1}, i.v.). A catheter in the left femoral artery allowed for continuous measurement of blood pressure and for collection of arterial blood samples.

Following intubation, anaesthesia was maintained with urethane (500 mg kg^{-1}, i.v.) and α-chloralose (100 mg kg^{-1}, i.v.). These doses produce surgical anaesthesia lasting 8 to 10 h (Green, 1982). The experiments described lasted from 3 to 4 h and depth of anaesthesia was monitored by observing for fluctuations in blood pressure. In the initial series of *in vivo* experiments in which the single (4 Hz) stimulation frequency was used, the cats were paralyzed with pancuronium bromide (0.6 mg kg^{-1}, i.v.). In later experiments (effect of pilocarpine, frequency-response curves) pancuronium was not administered so as to exclude any possible antagonistic effects of this agent on muscarinic receptors. In comparative studies (data not shown), conducted with and without pancuronium, no statistically significant differences were found in the effect of vagal stimulation on tracheal ring 4 diameter, total lung resistance, or dynamic compliance.

The vagus nerves were isolated from the sympathetic trunk in the mid to distal cervical region. The sympathetic trunk was identified by demonstration of mydriasis during nerve stimulation and transected bilaterally. The vagus nerves were transected at the angle of the mandible, immersed in a pool of mineral oil warmed to 38°C, and placed over platinum electrodes connected to a dual output square pulse stimulator (Grass Instruments, Model S88 Multifunction Solid-State Square Wave Stimulator). The right common carotid artery was occluded proximal to the cranial thyroid artery and distal to tracheal ring 4. The tip of an indwelling catheter was positioned at the origin of the cranial thyroid artery for local intra-arterial infusion of ACh. Preliminary experiments in which Evan's blue dye was injected through the carotid catheter demonstrated even staining of the entire cervical trachea. The left common carotid artery was undisturbed and arterial blood could be aspirated through the catheter in the right carotid artery, indicating an intact blood supply to the segment studied.

Changes in extrathoracic airway calibre were determined by measuring the decrease in the external diameter of cervical tracheal ring 4. Tracheal responses were recorded with a polygraph (Model 7E, Grass Instruments). The outer diameter of tracheal ring 4 was measured initially with a micrometer. A customized electromechanical tracheal caliper was attached to tracheal ring 4 at its widest diameter with single 4–0 silk sutures. A description of the construction and calibration of this measurement device has been published previously (Killingsworth *et al.*, 1990).

Changes in intrathoracic airway calibre were determined by measurement of total lung resistance and dynamic compliance. Air flow was measured with a Fleisch 00 pneumotachograph attached to the endotracheal tube that had been placed through a tracheostomy at the thoracic inlet to bypass the extrathoracic cervical trachea. The Fleisch pneumotachograph was connected to a differential pressure transducer (Model DP45-22, Validyne Engineering Corp.). Tidal volume was obtained by electronic integration of the flow signal. A 6 cm incision was made in the right lateral thoracic wall so that pleural pressure was equal to ambient pressure. Transpulmonary pressure was equal to the pressure at the distal end of the endotracheal tube and was measured with a differential pressure transducer (Model MP 45-34, Validyne Engineering Corp.). Respiratory variables were displayed on a VR-12 Simultrace Polygraph (Electronics for Medicine, Honeywell Inc.). Dynamic compliance (C_{dyn}) was calculated as the ratio of the changes in tidal volume and transpulmonary pressure between points of zero flow. Total lung resistance (R_L) was calculated by the isovolume technique (Amdur & Mead, 1958).

In vivo *gallamine and pilocarpine dose-response experiments*

Airway smooth muscle contraction was produced in ten cats by bilateral stimulation of the distal ends of the cut vagus nerves and by intra-arterial (i.a.) injection of 10^{-4} M ACh through the carotid catheter. This was the maximal bolus of ACh that could be injected consistently without causing severe hypotension and death in some cats (Killingsworth *et al.*, 1990). Each cat received local i.a. infusions of the vehicle (lactated Ringer solution) as a control injection prior to receiving ACh. The vagus nerves were stimulated with supramaximal voltage (24 V, 0.5 ms duration) at 4 Hz for 30 s continuous stimulation.

Following vagotomy, baseline measurements were made of tracheal ring 4 diameter, R_L, and C_{dyn}. Secondly, with a 10 min stabilization period between each stimulation, airway responses to vagal stimulation, and vehicle and ACh injections were recorded before beginning the gallamine dose-response study. Ten minutes after each dose of gallamine (0.1, 1.0, 10.0 mg kg^{-1}, i.v.), bilateral vagal nerve stimulation was repeated and the changes in R_L, C_{dyn}, and the diameter of tracheal ring 4 were recorded. To determine the effect of gallamine on ACh-induced airway constriction, the second vehicle and ACh injections were given following the last dose of gallamine and nerve stimulation. A separate group of cats underwent the same treatments but received vehicle injections (0.9% sodium chloride) without gallamine in order to determine the effect of repeated vagal stimulation, and vehicle and ACh injections (time controls, $n = 5$).

Pilocarpine studies ($n = 5$) were conducted in a similar fashion to the gallamine dose-response studies. These experiments were designed to examine the effect of the selective M_2 receptor agonist pilocarpine on airway responses measured during vagal stimulation. In addition to bilateral transection of the sympathetic trunk, cats were pretreated 30 min before beginning the experiments with guanethidine (5 mg kg^{-1}, i.v.) to deplete neuronal catecholamines and prevent any effects of sympathetic nerve stimulation. At the end of these experiments, depletion of noradrenaline was determined by absence of a pressor response following injection of tyramine (10 mg kg^{-1}, i.v.). The vagus nerves were stimulated as described above, and variables were measured during 30 s continuous stimulation. Changes in tracheal ring 4, R_L and C_{dyn} were recorded before and after administering increasing doses of pilocarpine (0.01, 0.1, 0.3, 1.0, and 10.0 μg kg^{-1}, i.v.). Gallamine (10 mg kg^{-1}, i.v.) was injected at the end of the experiment and airway responses to vagal stimulation were recorded.

In vivo *frequency-response experiments with gallamine*

The second group of experiments examined the role of M_2 receptors in limiting airway constriction during nerve stimulation at stimulation frequencies greater than 4 Hz by use of the M_2 muscarinic antagonist, gallamine. Cats were surgically sympathectomized and pretreated with guanethidine 30 min before starting the protocol.

Following vagotomy, baseline measurements were made of tracheal ring 4 diameter, R_L, and C_{dyn}. Prior to gallamine administration, the changes in these three variables were measured during vagal stimulation at increasing frequencies (24 V, 0.5 ms, 0.5–16 Hz) and following i.a. injections of vehicle and 10^{-4} M ACh. A 10 min stabilization period was allowed between each stimulation. Gallamine (10.0 mg kg^{-1}, i.v.) was injected and 10 min later, the frequency-response curve and ACh injection were repeated in random order ($n = 10$). Five additional cats did not receive gallamine and served as time controls. Atropine (0.5 mg kg^{-1}, i.v.) was injected at the end of the experiment and airway responses to vagal stimulation and ACh were measured a third time. All cats in the *in vivo* experiments were killed with an overdose of i.v. sodium pentobarbitone.

In vitro *experiments*

Adult mixed breed cats were killed with an overdose of sodium pentobarbitone (50 mg kg^{-1}, i.v.). The trachea and lungs were removed immediately from the thoracic activity and placed into Krebs-Henseleit solution (composition in mM: KCl 4.7, KH_2PO_4 1.2, $MgSO_4 7\ H_2O$ 1.19, NaCl 118.4, dextrose 11.7, $NaHCO_3$ 25.0, $CaCl_2 2\ H_2O$ 2.6). Eight rectangular strips from the cervical and intrathoracic trachea (approximately 5.0 mm long and 3.0 mm wide) or eight bronchial rings (3.0–3.5 mm long and 1.0–1.5 mm outside diameter) were prepared from each cat (Stephens & Kroeger, 1970). Wet tissue weights were obtained at the end of each experiment after the strips were blotted dry on gauze pads.

Tracheal strips or bronchial rings were placed between platinum ring electrodes in 15 ml jacketed glass muscle baths filled with Krebs-Henseleit buffer that was maintained at 38°C. The bathing solution was aerated with a gas mixture of 95% O_2-5% CO_2. Isometric force generation was recorded continuously by tying each tracheal strip or bronchial ring to a Grass FT.03 transducer that was connected to a polygraph (Model 7E, Grass Instruments).

Electrical field stimulation (EFS) was provided by a stimulator (Grass Instruments, Model S88 Multifunction Solid-State Square Wave Stimulator) and a stimulus power booster (Med Lab Stimu-splitter II). The optimal preloads for strips of trachealis and bronchial rings were determined in preliminary experiments. The tension at optimal length was 0.5 g in the tracheal strips and 0.3 g in bronchial rings. In the experiments described in this paper, tissues were equilibrated for 80–90 min at the optimal length and were maintained at this length throughout the protocol. During equilibration, the tissues were stimulated submaximally (15 V, 0.5 ms duration, 16 Hz, 15 s continuous stimulation) at regular intervals to stabilize tension development to EFS. Krebs-Henseleit solution (38°C) was changed every 15 min.

Before addition of gallamine, the contractile response to 10^{-3} M ACh was determined. This response was defined as a maximal response, and all subsequent forces developed to EFS or ACh were expressed as a percentage of this response. The tissues were rinsed until the tension returned to baseline. After pretreating the tissues with 10^{-5} M guanethidine for 20 min, control frequency-response curves were obtained at frequencies of stimulation from 1 to 64 Hz in trachea and from 0.5 to 32 Hz in bronchi. A 5 min interval elapsed between each stimulation frequency. A second frequency-response curve to EFS was performed after a 20 min incubation period with gallamine (10^{-6} to 10^{-4} M in half-log increments). Each preparation was exposed to only one concentration of gallamine. One tracheal strip or bronchial ring from every cat was not exposed to gallamine and served as a time control.

The effect of gallamine on ACh-induced smooth muscle contraction was studied in separate studies ($n = 3$ cats). Cumulative ACh dose-response curves (10^{-8} to 10^{-3} M in log increments) were completed. The tissues were washed until tension returned to baseline. A second concentration-response curve to ACh was performed following incubation for 20 min with 10^{-4} M gallamine. Two ACh dose-response curves were completed in an additional three tracheal strips and three bronchial rings that were not exposed to gallamine and served as time controls.

In separate experiments, the effects of atropine (10^{-7} M, $n = 3$ cats), tetrodotoxin (10^{-6} M, $n = 3$ cats), or hexamethonium (10^{-6} M, $n = 1$ cat) on EFS-induced tension development were determined in tissues incubated with gallamine (10^{-5} M). Tension development during EFS was measured before and after gallamine administration. A third frequency-response curve was obtained following incubation of the tissues with one of the three drugs listed above.

Drugs

Drugs used in these studies included urethane, α-chloralose, gallamine triethiodide, pilocarpine nitrate, hexamethonium bromide, tyramine hydrochloride, tetrodotoxin, atropine sulphate for *in vitro* studies, and acetylcholine chloride (Sigma Chemical Company, St. Louis, MO, U.S.A.), atropine sulphate (*in vivo* studies; Anpro Pharmaceutical, Arcadia, CA, U.S.A.), sodium bicarbonate (Abbott Laboratories, North Chicago, IL, U.S.A.), thiamylal sodium (Boehringer Ingelheim, St. Joseph, MO, U.S.A.), sodium pentobarbitone (The Butler Company, Columbus, OH, U.S.A.), and pancuronium bromide (Organon Inc., West Orange, NJ, U.S.A.). Guanethidine monosulphate was supplied by CIBA Pharmaceutical Company, Summit, NJ, U.S.A. All drugs for the *in vitro* studies were prepared fresh daily and dissolved in Krebs-Henseleit solution. Drugs used in the *in vivo* studies were also prepared fresh daily and were dissolved in 0.9% saline or lactated Ringer solution (ACh).

Statistics

All values are given as mean ± s.e.mean. Single factor repeated measures analysis of variance with Tukey's test of multiple comparisons was used to determine the effect of drug doses or increasing frequency of stimulation within a group of cats. Split-plot analysis of variance (ANOVA) compared the frequency-response curves before and after gallamine injection. Student's t test for independent samples was used to test the change in tracheal ring 4 diameter, R_L, or C_{dyn} at a single frequency of stimulation before and after gallamine, or to

compare changes in response between the first and second ACh injections (Steel & Torrie, 1980).

In vitro, the responses of time-control preparations to EFS tended to decrease between the first and second stimulus-response curves. Therefore, *in vitro* data were corrected for the loss of tissue sensitivity that occurred with time (Kenakin, 1987). Tension development before and after gallamine administration at a single frequency of stimulation was compared by a paired Student's t test. In all experiments, n represents the number of cats studied. Differences were considered significant when $P < 0.05$.

Results

The baseline outer diameter of tracheal ring 4 after vagotomy was 1.0 ± 0.04 cm. Baseline R_L was $17.4 \pm 1.94\,cmH_2O\,l^{-1}\,s$ and C_{dyn} was $10.0 \pm 0.93\,ml\,cmH_2O^{-1}$. Because R_L and C_{dyn} measurements tended to vary between stimulations, the increase in R_L and the decrease in C_{dyn} during stimulation are given as the change from prestimulation measurements (ΔR_L; ΔC_{dyn}, respectively). Injection of vehicle prior to ACh produced no change in any of the airway calibre measurements. Vagal stimulation increased R_L and decreased C_{dyn} and the diameter of tracheal ring 4 in every cat. The magnitude of the change in R_L, C_{dyn}, and tracheal ring 4 constriction in both series of *in vivo* experiments was unaltered by repeated vagal stimulations or ACh injections in time-control cats that received vehicle rather than gallamine.

In vivo *gallamine and pilocarpine dose-response experiments*

Vagal stimulation at 4 Hz produced significant tracheal constriction, but the amount of constriction did not change following injection of increasing doses of gallamine. In contrast, total lung resistance during vagal stimulation increased with each dose of gallamine. The fall in C_{dyn} produced by vagal stimulation was not modified by gallamine (Figure 1).

Gallamine had no significant effect on airway constriction produced by ACh. The decrease in tracheal calibre following ACh injection was $350.7 \pm 54.5\,\mu m$ before and $266.5 \pm 56.9\,\mu m$ after the highest dose of gallamine. The ΔR_L was unchanged before and after gallamine ($0.41 \pm 0.18\,cmH_2O\,l^{-1}\,s$ and $0.39 \pm 0.20\,cmH_2O\,l^{-1}\,s$, respectively). Similarly, the ΔC_{dyn} after ACh injection was unchanged by gallamine ($0.25 \pm 0.06\,ml\,cmH_2O^{-1}$ and $0.21 \pm 0.08\,ml\,cmH_2O^{-1}$).

The muscarinic agonist, pilocarpine, tended to have the opposite effect to gallamine in the intrathoracic airways (Figure 2). The ΔR_L was diminished during vagal nerve stimulation by pilocarpine doses ranging from 0.01 to $10.0\,\mu g\,kg^{-1}$ compared to ΔR_L prior to pilocarpine administration. Gallamine ($10\,mg\,kg^{-1}$, i.v.) reversed this effect of pilocarpine. The ΔC_{dyn} produced by vagal nerve stimulation was decreased by pilocarpine doses of 0.1, 0.3, and $1.0\,\mu g\,kg^{-1}$. Gallamine significantly increased the ΔC_{dyn} recorded during vagal stimulation (Figure 2). Because ΔC_{dyn} is the value for C_{dyn} during stimulation minus the pre-stimulation C_{dyn} measurement, a decrease in ΔC_{dyn} represents inhibition of vagally-induced airway constriction. A rise in ΔC_{dyn} signifies an increase in airway constriction. Pilocarpine lacked a bronchoconstrictor effect at the drug-range administered because there was no significant change in prestimulation R_L or C_{dyn} measurements following each dose of pilocarpine. Vagally-induced tracheal constriction was not significantly affected by any dose of pilocarpine, nor was tracheal constriction enhanced by gallamine ($10\,mg\,kg^{-1}$, i.v.) given after pilocarpine (Figure 2).

In vivo *frequency-response experiments with gallamine*

Vagal stimulation caused a progressive reduction in the diameter of tracheal ring 4 as the frequency of stimulation was increased. However, there was no significant difference in tracheal constriction at any frequency of stimulation when frequency-response curves before and after gallamine injection were compared (Figure 3). Similarly, gallamine ($10\,mg\,kg^{-1}$, i.v.) did not significantly alter tracheal constriction produced by ACh injection ($333.9 \pm 70.3\,\mu m$ before and $191.6 \pm 40.6\,\mu m$ after gallamine treatment). Intravenous atropine ($0.5\,mg\,kg^{-1}$) completely eliminated tracheal constriction induced by ACh and vagal stimulation (results not shown).

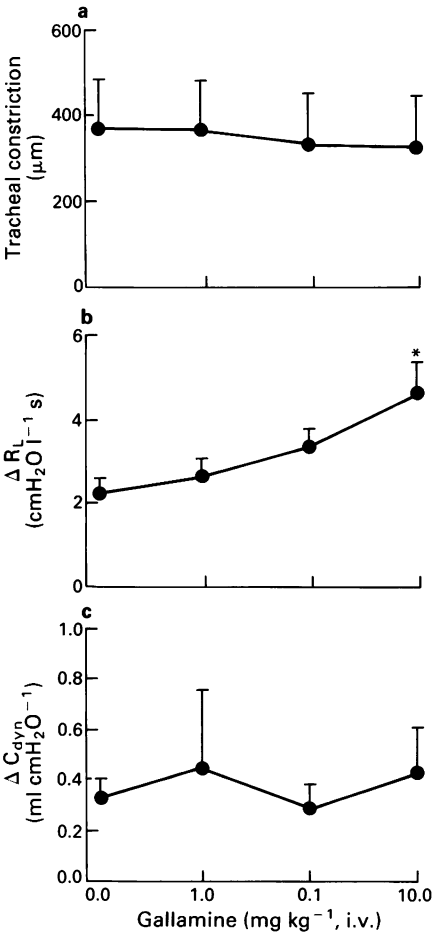

Figure 1 Effect of increasing doses of gallamine on tracheal ring 4 constriction, total lung resistance (R_L), and dynamic compliance (C_{dyn}) in 10 anaesthetized cats during bilateral vagal nerve stimulation (24 V, 0.5 ms, 4 Hz, 30 s continuous stimulus). Tracheal constriction is represented by an upward deflection in (a). The external diameter of tracheal ring 4 prior to stimulation was 1.0 ± 0.04 cm. Vagal stimulation constricted tracheal ring 4, but constriction was not augmented at any dose of gallamine compared to the pre-gallamine measurement ($0.0\,mg\,kg^{-1}$). Because R_L and C_{dyn} measurements tended to vary between stimulations, the increases in R_L and decreases in C_{dyn} are given as the change from pre-stimulation measurements (ΔR_L and ΔC_{dyn}, respectively; prior to stimulation, $R_L = 17.4 \pm 1.94\,cmH_2O\,l^{-1}\,s$ and $C_{dyn} = 10.0 \pm 0.93\,ml\,cmH_2O^{-1}$; respectively). At a gallamine dose of $10\,mg\,kg^{-1}$, ΔR_L was significantly greater than before gallamine injection (b; * $P < 0.005$, ANOVA with Tukey's test of multiple comparisons). At 4 Hz, the change in C_{dyn} was not augmented by gallamine at any dose (c).

Vagal stimulation significantly increased R_L at 12 and 16 Hz before, and at 8, 12, and 16 Hz after gallamine injection (Figure 3). Comparison of the two frequency-response curves indicated significant augmentation of the ΔR_L responses at 4, 8, and 16 Hz following gallamine injection. Gallamine administration did not significantly increase the ΔR_L measurement following ACh injection ($1.26 \pm 0.63\,cmH_2O\,l^{-1}\,s$ before and $1.71 \pm 1.37\,cmH_2O\,l^{-1}\,s$ after gallamine injection).

Before gallamine, vagal stimulation caused a significant change in ΔC_{dyn} at 8, 12, and 16 Hz (Figure 3). Following gallamine, the change in C_{dyn} was enhanced and significantly dif-

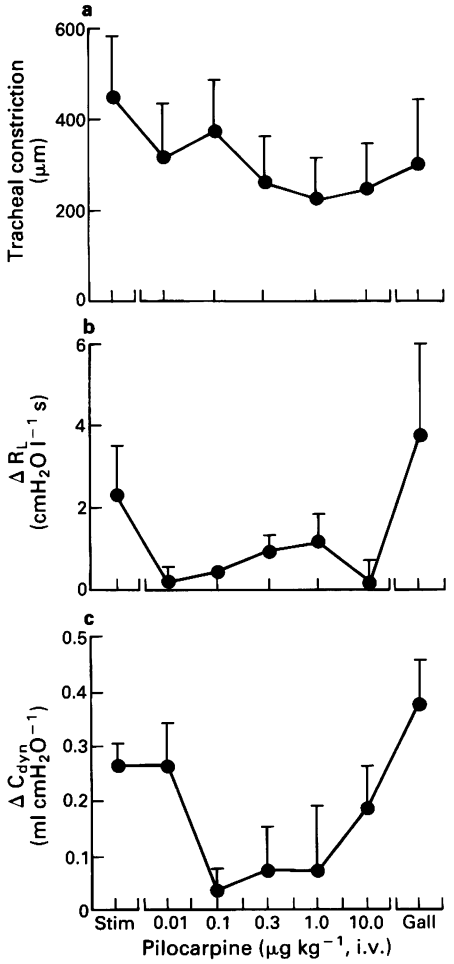

Figure 2 Effect of increasing doses of pilocarpine on tracheal ring 4 constriction, total lung resistance (R_L), and dynamic compliance (C_{dyn}) in 5 anaesthetized cats during bilateral vagal nerve stimulation. Vagally-induced tracheal constriction was not significantly affected by any dose of pilocarpine ($P = 0.09$), nor was it enhanced by 10 mg kg^{-1}, i.v. gallamine. The ΔR_L during vagal nerve stimulation was diminished by pilocarpine doses ranging from 0.01 to 10.0 μg kg^{-1} compared to ΔR_L prior to pilocarpine administration. Gallamine (10 mg kg^{-1}, i.v.) reversed the pilocarpine effect. The ΔC_{dyn} was decreased by pilocarpine doses of 0.1, 0.3, and 1.0 μg kg^{-1}, resulting in a significant treatment effect ($P = 0.01$). Gallamine significantly increased the ΔC_{dyn} recorded during vagal stimulation compared to ΔC_{dyn} measured following injections ranging from 0.1 to 1.0 μg kg^{-1} pilocarpine ($P < 0.05$). Because ΔC_{dyn} and ΔR_L represent the change in C_{dyn} and R_L during stimulation minus the pre-stimulation measurement, decreases in ΔC_{dyn} and ΔR_L represent inhibition of vagally-induced airway constriction. Nerve stimulation was performed and data calculated as described in Figure 1. Data were analyzed statistically by ANOVA with Tukey's test of multiple comparisons. Each point is the mean of 5 animals with s.e.mean shown by vertical bars.

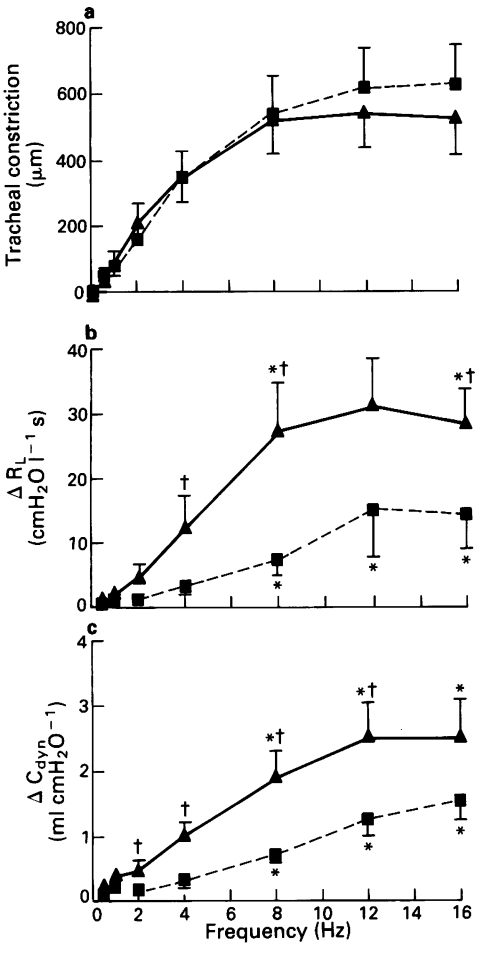

Figure 3 Tracheal ring 4 constriction, total lung resistance (R_L), and dynamic compliance (C_{dyn}) during bilateral vagal nerve stimulation (24 V, 0.5 ms, 0.5–16 Hz) before (■) and after (▲) gallamine administration in 10 anaesthetized cats. The amount of tracheal constriction increased with increasing frequencies of stimulation, but gallamine (10 mg kg^{-1}, i.v.) did not augment the constriction (a). Vagal stimulation increased resistance at 12 and 16 Hz before gallamine injection and at 8, 12, and 16 Hz after injection of 10 mg kg^{-1} gallamine (b; * $P < 0.05$). Comparison of the two frequency-response curves indicated augmentation of ΔR_L at 4, 8, and 16 Hz († $P < 0.05$). Vagal stimulation changed ΔC_{dyn} at 8, 12, and 16 Hz prior to gallamine and also at 4, 8, 12, and 16 Hz following gallamine (c; * $P < 0.05$). The ΔC_{dyn} was enhanced by gallamine at 4, 8, and 12 Hz († $P < 0.05$). Data were analyzed statistically by ANOVA with Tukey's test of multiple comparisons or Student's t test to determine differences between two curves at a single frequency of stimulation before and after gallamine. Each point is the mean of 10 animals with s.e.mean shown by vertical bars. See Figure 1 for description of measurements.

ferent from pre-gallamine measurements at 4, 8, and 12 Hz. The ΔC_{dyn} measurements after ACh injection were unchanged before (0.25 ± 0.04 ml cmH_2O^{-1}) and after (0.29 ± 0.21 ml cmH_2O^{-1}) gallamine injection.

In vitro *experiments*

The mean wet weights of 40 tracheal strips and 40 bronchial rings were 11.8 ± 0.55 mg and 10.7 ± 0.33 mg, respectively. In response to 10^{-3} M ACh, active tension development of tracheal strips and bronchial rings was 1.28 ± 0.17 g and 1.0 ± 0.05 g, respectively. Gallamine shifted the frequency-response curves to EFS leftward in a dose-dependent manner in both trachea and bronchi (Figures 4 and 5).

Gallamine (10^{-4} M) did not significantly enhance tension development at any ACh concentration in either trachea or in bronchi (Figures 6a and b). Atropine (10^{-7} M) and tetrodotoxin (10^{-6} M) inhibited tracheal tension development during EFS completely (data not shown). Hexamethonium (10^{-6} M) had no effect on tracheal response to EFS after gallamine treatment (data not shown). The effects of atropine, tetrodotoxin, and hexamethonium on bronchial ring responses were not determined.

Discussion

Most nerve endings contain autoreceptors that inhibit further release of neurotransmitter and act as a negative presynaptic feedback mechanism to reduce neurotransmission (Starke *et al.*, 1989). The primary role of inhibitory receptors may be in self-regulation of neuronal activity by limiting the neurone's

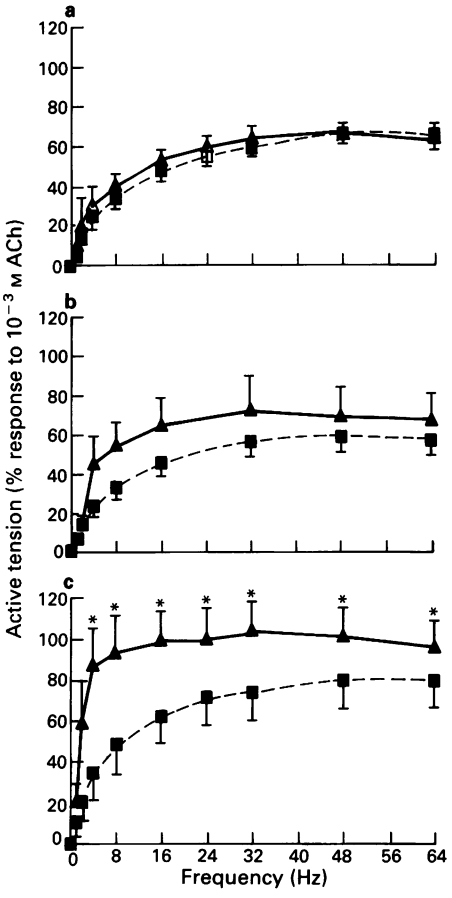

Figure 4 Frequency-response curves to electrical field stimulation in tracheal strips in the absence (■) and presence (▲) of increasing concentrations of gallamine. Each preparation was exposed to only one concentration of gallamine. Active tension data are expressed as a percentage of the contractile response of each strip to 10^{-3} M acetylcholine (ACh). Gallamine shifted the frequency-response curves leftward in a dose-dependent manner (a: 10^{-6} M; b: 10^{-5} M; c: 10^{-4} M). Tension development in the trachea was augmented significantly with 10^{-4} M gallamine at stimulation frequencies of 4 Hz and greater (c; * $P < 0.05$, Student's t test). Each point is the mean of 5 animals with s.e.mean shown by vertical bars.

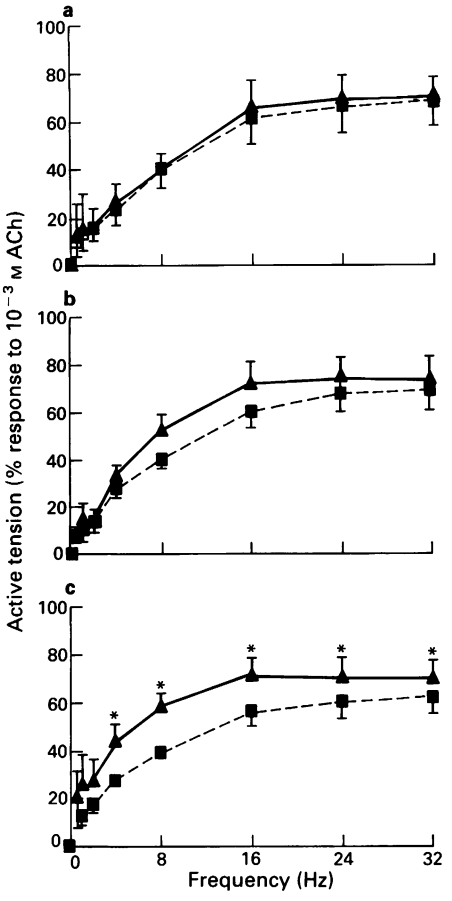

Figure 5 Frequency-response curves to electrical field stimulation in bronchial rings in the absence (■) and presence (▲) of increasing concentrations of gallamine. Each preparation was exposed to only one concentration of gallamine. Active tension data are expressed as percentage of contractile response of each ring to 10^{-3} M acetylcholine (ACh). Gallamine shifted the frequency-response curves leftward in a dose-dependent manner (a: 10^{-6} M; b: 10^{-5} M; c: 10^{-4} M). Tension development in the bronchi was augmented significantly with 10^{-4} M gallamine at stimulation frequencies of 4 Hz and greater (c; * $P < 0.05$, Student's t test). Each point is the mean of 5 animals with s.e.mean shown by vertical bars.

chemical signal. Autoreceptors associated with cholinergic nerves supplying airway smooth muscle have been reported in a number of mammalian species (Fryer & Maclagan, 1984; Blaber *et al.*, 1985, Ito & Yohitomi, 1988; Minette & Barnes, 1988). Previous *in vivo* studies in the cat have used the M_2 receptor agonist, pilocarpine and M_2 receptor antagonist, gallamine, to demonstrate the existence of neuronal muscarinic receptors that inhibit parasympathetic transmission (Blaber *et al.*, 1985). These authors reported changes in R_L and C_{dyn} as indicators of central and peripheral airway constriction, respectively. However, studies to examine the role of M_2 receptors in limiting cervical tracheal constriction have not been described.

The gallamine dose-response studies confirmed previous investigations in which M_2 receptors have been demonstrated to modulate intrathoracic airway constriction (Fryer & Maclagan, 1984; Blaber *et al.*, 1985; Fryer & Jacoby, 1990). In the current studies, the ΔR_L induced by vagal stimulation was augmented by gallamine in a dose-dependent manner. The muscarinic agonist, pilocarpine, tended to have the opposite effect to gallamine in the intrathoracic airways. The M_2 receptor agonist, pilocarpine, inhibited bronchoconstriction as measured by ΔR_L and ΔC_{dyn}, whereas the M_2 receptor antagonist, gallamine, reversed the pilocarpine effect. A similar role of M_2 receptors in limiting constriction in the cranial cervical trachea was not demonstrated, because the constriction was neither inhibited by pilocarpine nor augmented by gallamine.

Activation of M_2 receptors is frequency-dependent (Blaber *et al.*, 1985; Wessler, 1989). The effect of M_2 agonists is more obvious during nerve stimulation at lower frequencies, while the influence of antagonists at these receptors is more apparent when the nerves are stimulated at higher frequencies (Fryer & Maclagan, 1984; Fryer & Jacoby, 1991). A frequency of 4 Hz may have been too low to demonstrate best an effect of gallamine on airway responses. Although this is unlikely, because a gallamine effect was demonstrated in intrathoracic airways at a stimulus frequency of 4 Hz, the second set of *in vivo* experiments was designed to search further for M_2 receptors in cat cervical trachea at stimulation frequencies greater than 4 Hz.

Vagal stimulation (24 V, 0.5 ms duration) caused extra- and intrathoracic airway constriction that increased with greater frequencies of stimulation (0.5–16 Hz). Following gallamine administration, the ΔR_L and ΔC_{dyn} were enhanced significantly. A plateau in intrathoracic airway constriction occurred at the highest frequencies. Although vagal stimulation significantly decreased tracheal diameter with increasing stimulation frequencies, gallamine injection had no effect on the amount of tracheal constriction. Intravenous atropine blocked tracheal constriction completely, indicating that the constriction was mediated through muscarinic receptors and that drugs

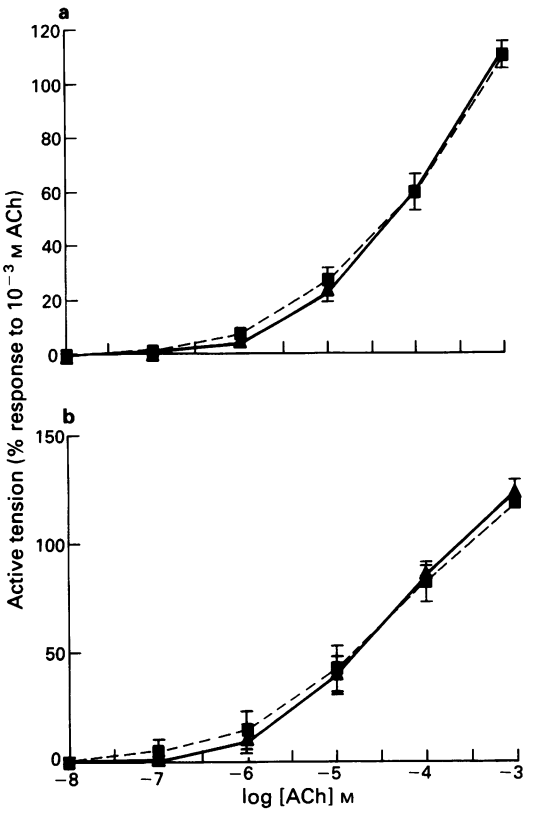

Figure 6 Concentration response-curves to ACh in trachea and bronchi. Active tension data are expressed as a percent contractile response of each tissue to 10^{-3} M ACh. There was no difference in the two curves when active tension before (■) and after (▲) gallamine administration (10^{-4} M) was compared in either tracheal strips or bronchial rings. Each point is the mean of 3 animals with s.e.mean shown by vertical bars.

injected i.v. into the systemic circulation could reach the cervical trachea. However, these data do not demonstrate a physiological role for M_2 inhibitory receptors in limiting tracheal constriction in cat trachea *in vivo*.

The electromechanical tracheal caliper attached to the outer surface of tracheal ring 4 provides a very sensitive method to quantify isotonic forces developed by contracting smooth muscle *in situ* and is unaffected by airway wall oedema and mucus accumulation. This technique allows the smooth muscle to be studied with minimal surgical intervention without transecting tracheal rings and at physiological length without stretching to obtain maximal contraction. Use of the tracheal caliper also avoids passing a tracheal cannula through the study segment, thereby averting epithelial disruption that could alter tracheal responses. If gallamine had augmented constriction to the same degree in both the trachea and bronchi, calculations show that the enhanced contraction should have been measured easily by the tracheal caliper. Assuming the length of the airways does not change during vagal stimulation or gallamine administration, resistance is inversely proportional to radius to the fourth power. The augmentation in tracheal contraction following the highest dose of gallamine predicted by the change in R_L should be greater than 1000 μm. A change of this magnitude is well within the measurement capabilities of the tracheal caliper.

Failure to demonstrate a physiological role for M_2 receptors in the trachea could be due to an absence of presynaptic M_2 receptors on parasympathetic nerves in this region of the airways. The *in vitro* data do not support this idea. The responses of both the isolated tracheal and bronchial tissues to EFS were similarly augmented by gallamine in a dose-dependent manner. Hexamethonium had no effect on tension development, but both atropine and tetrodotoxin completely inhibited the increased response to EFS following gallamine. Gallamine did not affect the ACh dose-response curve *in vitro* or alter the *in vivo* measurements of R_L or C_{dyn} provoked by ACh. These observations suggest that the augmented contraction induced by gallamine was due to blockade of M_2 receptors on postganglionic neurones.

In order to obtain 8 tracheal strips from each cat for the *in vitro* studies, the strips were obtained from the cervical and thoracic trachea. The fact that tracheal strips originated from both the intra- and extra-thoracic airways for *in vitro* studies may account for the discrepancy with the *in vivo* studies. Differences in neural input to the cervical trachea compared to intrathoracic airways have been reported in other *in vivo* cat studies, as well as discrepancies between *in vivo* and *in vitro* data. The presence of nonadrenergic nonchloinergic (NANC) inhibitory neural pathways in the intrathoracic airways of cats has been demonstrated *in vivo* using tantalum bronchography (Matsumoto *et al.*, 1985) and measurements of lung resistance and dynamic compliance (Diamond & O'Donnell, 1980; Don *et al.*, 1988). However, the NANC inhibitory system has not been demonstrated *in vivo* in cat cervical trachea (Don *et al.*, 1988). In contrast, the presence of NANC inhibitory pathways has been demonstrated *in vitro* in both the cervical (Ito & Takeda, 1982) and thoracic trachea of the cat (Altiere *et al.*, 1984). These differences in NANC neural pathways of extra- and intra-thoracic cat airways and between the *in vivo* and *in vitro* data are comparable to the differences in M_2 inhibitory receptor distribution and function detected in our studies. Similar to our conclusion regarding a lack of M_2 receptor function in the cervical trachea, Don *et al.* (1988) stated that the most plausible conclusion was that *in vivo* in the cat, NANC pathways are not activated in the cervical trachea.

The presence of M_2 inhibitory receptors has also been demonstrated in canine and guinea-pig trachea *in vitro* (Minette & Barnes, 1988; Brichant *et al.*, 1990), but the significance of M_2 autoreceptors in tracheal caliber regulation *in vivo* remains unanswered. Results of *in vitro* studies do not always correlate well with *in vivo* studies due to the use of very different experimental techniques (Vincenc *et al.*, 1983; Armour *et al.*, 1984; Woolcock & Permutt, 1986). If airway smooth muscle shortened *in vivo* to the extent that it does *in vitro*, maximal bronchoconstriction would result in complete closure of virtually all airways (Macklem, 1971). Investigators have hypothesized that there must be inhibitory mechanisms, perhaps mechanical in nature, that operate to limit maximal airway smooth muscle shortening *in vivo* (Macklem, 1985; Bates & Martin, 1990).

In conclusion, although we have demonstrated a functional role of muscarinic M_2 receptors *in vitro*, we were unable to establish an autoinhibitory role for these receptors in cat trachea *in vivo*. This was despite repeated demonstration of M_2 receptor-mediated limitation of airway constriction of intrathoracic airways. It is likely that cartilaginous rings limit tracheal constriction, but cartilaginous rings do not exist throughout the intrathoracic airways. Hence, whereas M_2 inhibitory receptors could play an important role in limiting intrathoracic airway constriction where cartilaginous rings do not exist, *in vivo* they may not have a functional influence in the trachea.

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Characterization of receptors involved in the direct and indirect actions of prostaglandins E and I on the guinea-pig ileum

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1 A study of the effects of prostaglandin E_2 (PGE_2) and eleven synthetic analogues on the guinea-pig isolated ileum preparation has revealed three distinct contractile actions, each associated with a different prostaglandin E (EP-) receptor subtype. In addition, PGI_2 (prostacyclin) and its stable analogues can activate prostaglandin I (IP-) receptors to elicit both contraction and relaxation of the ileum.

2 Two of the PGE actions involve direct stimulation of the smooth muscle, being unaffected by 1 μM morphine treatment. One action is blocked by AH 6809 at micromolar concentrations and ICI 80205 and 16,16-dimethyl PGE_2 are particularly potent agonists. Activation of EP_1-receptors appears to be involved. The second action is unaffected by AH 6809; sulprostone and MB 28767 are potent agonists. Comparison with agonist potency rankings on the guinea-pig vas deferens indicates that EP_3-receptors may be involved.

3 The third PGE effect and the stimulant PGI effect are blocked by morphine, indicating enteric neurones and/or sensory nerve terminals as sites of action. EP_2-receptors may be involved in the PGE action, in view of the marked effect of morphine on the contractile actions of misoprostol, 11-deoxy PGE_2-1-alcohol, 11-deoxy PGE_1 and butaprost, all of which show some selectivity for EP_2-receptors. The PGI action is most easily studied with cicaprost (EC_{25} = 1.3 nM), since iloprost, carbacyclin and to a lesser extent PGI_2 also have agonist activity at EP_1-receptors.

4 The contractile action of 17-phenyl-ω-trinor PGE_2 on the ileum is unaffected by morphine. Since this analogue shows only weak agonist activity on the rabbit jugular vein (EP_2 preparation) and guinea-pig vas deferens (EP_3), it may be a more useful standard agonist than PGE_2 in EP_1-receptor studies.

5 In the presence of morphine and AH 6809, cicaprost inhibits histamine-induced contractions (IC_{25} = 22 nM). PGI_2 and iloprost show mixed inhibitory/potentiating actions, whereas carbacyclin only potentiates histamine contractions. This IP-receptor-mediated inhibition may account for the bell-shaped log concentration-response curve of cicaprost (no inhibitors present) and the very marked block of iloprost-induced contractions by AH 6809.

6 We have found no evidence for either IP-receptors mediating direct contraction or EP-receptors mediating inhibition of the ileum longitudinal smooth muscle, as has been suggested in the literature.

7 In view of the complexity of prostanoid action on the guinea-pig ileum we feel that the preparation must be used with caution to ascertain the EP_1 agonist or antagonist potencies of novel compounds.

Keywords: Prostaglandins E; prostacyclin analogues; prostaglandin antagonists; morphine; smooth muscle

Introduction

Contractions of the longitudinal muscle of the guinea-pig isolated ileum to prostaglandin E_1 (PGE_1) and PGE_2 are unaffected by hexamethonium but are partially inhibited by tetrodotoxin (TTX) and atropine (Horton, 1965; Bennett *et al.*, 1968). It has been suggested that two excitatory actions are involved, one on smooth muscle cells, the other on intrinsic cholinergic nerves (Bennett *et al.*, 1968). Further evidence for a neuronal component of PGE action stems from the finding that morphine inhibits the contractile action of PGE_1 by 60% and PGE_2 by 41% (Sanner, 1971).

In later studies directed towards characterizing prostaglandin E (EP-) receptors, Kennedy *et al.* (1982) showed that PGE_2 was the most potent of the natural prostanoids on the guinea-pig ileum, in the presence of atropine, indomethacin and phenoxybenzamine. They also found that the dibenzoxazepine SC 19220 caused parallel rightward shifts in the log concentration-response curves of PGE_1, PGE_2 and $PGF_{2\alpha}$ (pA_2 = 5.4, 5.6 and 5.3 respectively). On the basis of these results they defined the guinea-pig ileum as an EP_1-selective preparation. More recently, the xanthone carboxylic acid AH 6809 has been shown to have a similar profile of activity to SC 19220 in the guinea-pig ileum (Coleman *et al.*, 1985). Its higher potency (pA_2 = 6.8, PGE_2 as agonist) and good water solubility are distinct advantages over SC 19920.

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The neuronal EP-receptor in the ileum has never been fully characterized. Recent work by Poll *et al.* (1988a, b), in which potentiation of electrical field stimulation responses was measured, suggests that this receptor is unlikely to be the EP_1 subtype.

Finally, Gardiner (1986) has reported that the PGE analogue, butaprost, at high concentrations (3–10 μM) produces an unsurmountable inhibition of histamine-induced contractions of the guinea-pig ileum. Gardiner (1986) has proposed that butaprost is a selective EP_2-receptor agonist (EP_2-receptors are associated with the smooth muscle relaxant actions of prostaglandins E) and consequently that EP_2 receptors mediate smooth muscle relaxation in the ileum.

In this study we have examined the actions of PGE_2 and 11 of its analogues (Figure 1) on the guinea-pig isolated ileum with a view to determining the number and nature of the EP-receptors present. At the start of the study, ICI 80205 and 16,16-dimethyl PGE_2 were known to be highly potent EP_1-receptor agonists, with some selectivity for EP_1- as opposed to EP_2-receptors (Dong *et al.*, 1986). Sulprostone was known to be a highly potent EP_3-receptor agonist (inhibition of the twitch response of guinea-pig vas deferens to electrical field stimulation), with moderate EP_1 agonist potency, but negligible EP_2 agonist activity (Coleman *et al.*, 1987). Misoprostol was claimed to be an EP_1-selective agonist (Eglen & Whiting, 1988), but concurrent work indicated potent EP_2 agonist activity (relaxation of cat trachea) (Coleman *et al.*, 1988).

In general, PGI_2 relaxes smooth muscle preparations (see Gryglewski, 1987). However, Gaion & Trento (1983) have

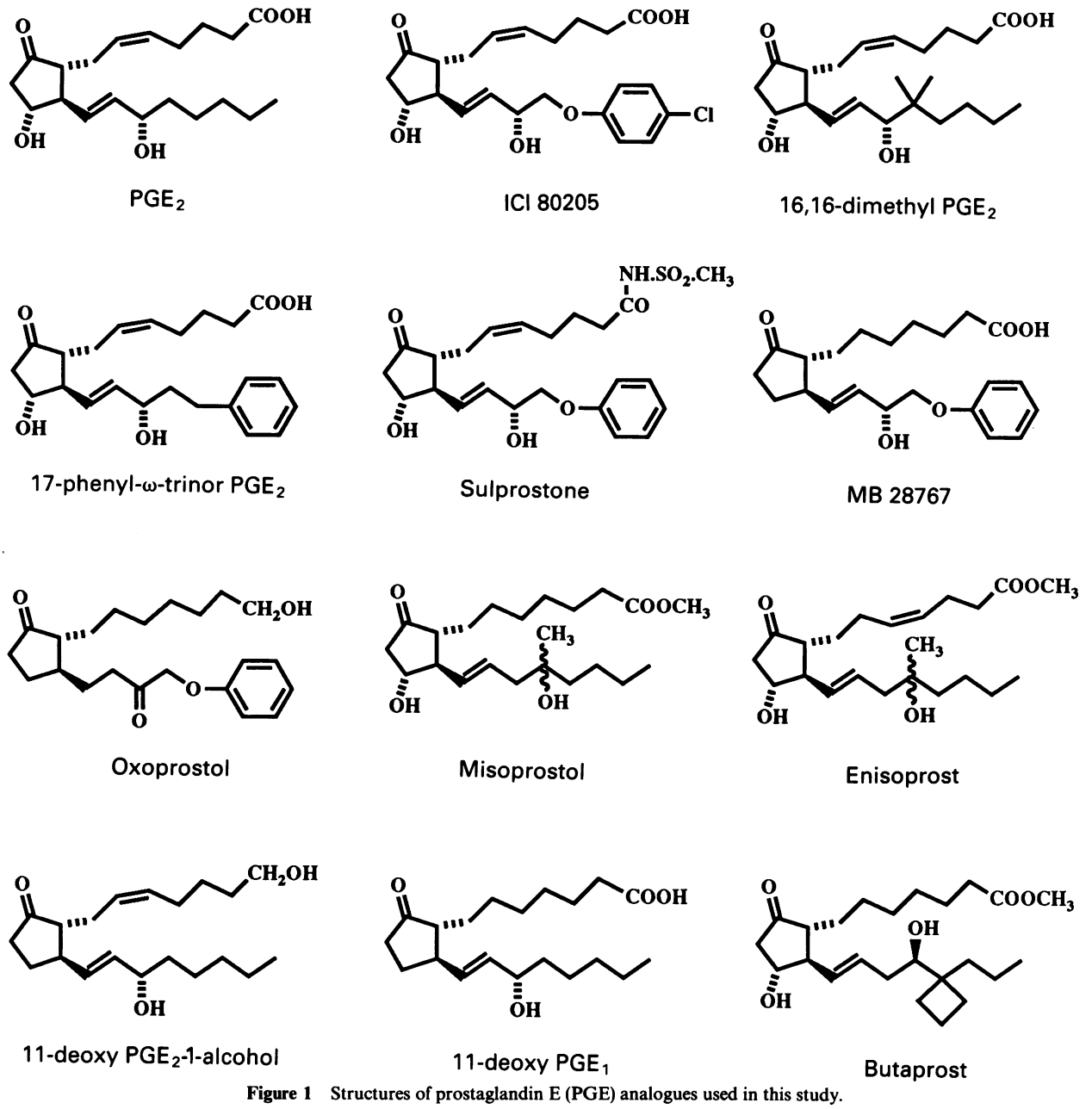


Figure 1 Structures of prostaglandin E (PGE) analogues used in this study.

shown that PGI_2 contracts the longitudinal muscle of the guinea-pig ileum and at a concentration of 20 nM ($\sim EC_{50}$) these contractions are abolished by TTX, and markedly inhibited by atropine and hemicholinium-3. In addition, PGI_2 potentiates electrically-induced contractions whilst having no effect on responses to exogenous acetylcholine (ACh). On the basis of these results it was proposed that PGI_2-induced contractions are mediated by ACh released from cholinergic neurones as a consequence of increased excitability of the cell bodies. Further evidence for this mechanism of action derives from the observation (Gaion & Trento, 1984) that the contractile action of PGI_2 is inhibited by noradrenaline, morphine and the purinergic receptor agonist N^6-phenylisopropyl adenosine (PIA). Gaion & Gambaratto (1987) later showed that TTX or atropine only partially inhibit contractions to higher concentrations of PGI_2 (1 μM). The authors suggested that part of the contractile action of PGI_2 at high concentrations is due to stimulation of prostaglandin I (IP-) receptors present on the smooth muscle cells.

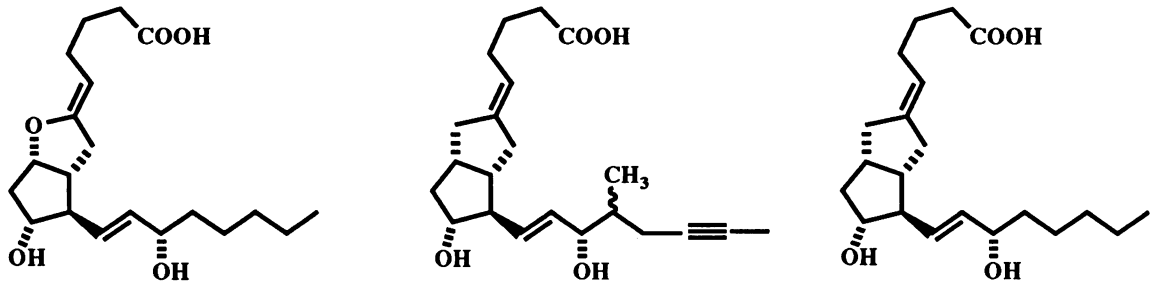
There have been no reports of the actions of stable analogues of PGI_2 on the neuronal IP-receptor in the guinea-pig ileum. We wished to investigate the action of cicaprost (Figure 2) since we have shown it to be a highly specific agonist for IP-receptors (Dong *et al.*, 1986), in contrast to iloprost which has unexpectedly high EP_1-agonist potency (Dong & Jones, 1982; Dong *et al.*, 1986; Sheldrick *et al.*, 1988). We have also examined carbacyclin and 10,10-difluoro-13,14-didehydro PGI_2; the latter has greater aqueous stability than PGI_2 due to the electron-withdrawing effect of the fluoro substituents on the enol ether unit (Fried *et al.*, 1980).

Methods

Isolated preparations

Male guinea-pigs (300–500 g) were killed by a blow to the head followed by exsanguination. Preparations were mounted in 10 ml organ baths for recording of tension with Grass FT03 isometric transducers connected to a Grass Polygraph recorder. The bathing solution was aerated with 95% O_2 and 5% CO_2, maintained at 37°C and changed by upward displacement and overflow.

Guinea-pig ileum The terminal portion of the ileum was excised after discarding the 8–10 cm portion nearest the ileo-



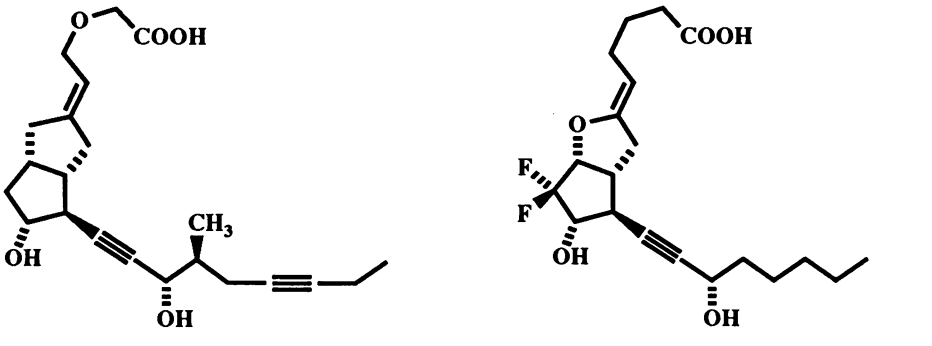


Figure 2 Structures of prostaglandin I (PGI) analogues used in this study.

caecal junction. Segments about 2 cm long were bathed in Tyrode (composition, mM: NaCl 136, KCl 2.7, $CaCl_2$ 1.4, $MgCl_2$ 0.49, NaH_2PO_4 0.32, $NaHCO_3$ 12 and glucose 5). The tissues were allowed to equilibrate for about 1 h prior to testing with a priming dose of PGE_2 (14 nM).

Agonist doses were added non-cumulatively in a 1, 3, 10, 30 or 1, 5, 10, 50 sequence. Sufficient time was allowed for the response to each dose to reach a maximum and a minimum of 15 min was allowed between additions. Responses were calculated as a percentage of the ACh maximum obtained in each preparation at the end of the experiment.

Concentration-response relationships for a particular prostanoid were established simultaneously in two preparations from the same animal, one being continuously exposed to the inhibitor, starting 20 min prior to the first agonist dose. Log concentration-response curves were constructed with results from five different animals. Mean pD_{25} values ($-\log EC_{25}$) in the presence and absence of the inhibitor were calculated and compared by an unpaired Student's t test. A mean dose-ratio at the EC_{25} level was calculated from the five individual experiments.

For the estimation of inhibitory potency, preparations were bathed in Tyrode solution containing 1 μM morphine and 2 μM AH 6809. The tissue maximum was established initially with 1.5 μM histamine and subsequently, each preparation was exposed repeatedly at 2 min intervals to a dose of histamine producing a contraction 40–50% of the maximum. Inhibitors were added immediately after wash-out of histamine. Log concentration-inhibition curves were obtained and pIC_{50} values calculated.

Guinea-pig trachea Tracheal rings were set up as described previously by us (Tymkewycz *et al.*, 1991). The Krebs solution (composition, mM : NaCl 118, KCl 5.4, $MgSO_4$ 1.0, $CaCl_2$ 2.5, $NaHPO_4$ 1.1, $NaHCO_3$ 25 and glucose 10) contained indomethacin (1 μM) and atropine (20 nM); EP 092 (1 μM) was added 15 min before each cumulative agonist sequence. 17-Phenyl-ω-trinor PGE_2 was used as the standard agonist. Schild analysis of the antagonism of 17-phenyl-ω-trinor PGE_2 by AH 6809 (15 min pre-incubation) was carried out as described previously (Tymkewycz *et al.*, 1991).

Guinea-pig vas deferens Lengths (20 mm) of vas deferens were suspended in Krebs solution (no additions) between stainless steel electrodes for supra-maximal electrical field stimulation (60 V, 1 ms, 10 Hz for 1 s every 32 s). Prostanoid doses were added cumulatively and PGE_2 was used as the standard agonist. Log concentration-inhibition curves were constructed.

Compounds

11-Deoxy PGE_2-1-alcohol (starting material *nat* PGA_2) and EP 092 were prepared in our laboratory. The following compounds were gifts which we gratefully acknowledge: 10,10-difluoro-13,14-didehydro PGI_2 sodium salt from Prof. J. Fried, University of Chicago, U.S.A.; sulprostone, PGI_2 sodium salt, iloprost, carbacyclin and cicaprost from Prof. H. Vorbruggen, Schering AG, Berlin; ICI 80205 (racemic) from Dr K. Gibson, ICI Pharmaceuticals, U.K.; MB 28767 and oxoprostol (both racemic) from Dr M. Caton, Rhone-Poulenc, U.K.; misoprostol and enisoprost from Dr P. Collins, G.D. Searle, U.S.A.; butaprost from Dr P. Gardiner, Bayer, U.K.; AH 6809 from Dr R.A. Coleman, Glaxo, U.K. PGE_2, $PGF_{2\alpha}$, 16,16-dimethyl PGE_2, 17-phenyl-ω-trinor PGE_2, 11-deoxy PGE_1 and U-46619 were purchased from Cayman Chemicals, U.S.A.

Ethanolic stock solutions of the prostanoids (10^{-2}–3×10^{-2} M) were stored at -20°C and diluted with 0.9% NaCl solution for use. In the case of PGI_2 and 10,10-difluoro-13,14-didehydro PGI_2, the sodium salt was dissolved in 50 mM Tris-HCl buffer pH 9.0. Dilutions of the PGI_2 stock were kept on ice and used for one dose sequence only, whereas with the more stable 10,10-difluoro analogue dilutions were frozen to -20°C after use and re-used for several experiments.

Results

Contractile actions of prostaglandin E analogues on the guinea-pig ileum

No inhibitors present Several different types of contractile response were elicited by the PGE analogues tested. ICI 80205, 16,16-dimethyl PGE_2, PGE_2 and 17-phenyl-ω-trinor PGE_2 produced rapidly developing increases in tension with minimal increase in rhythmic activity. On washout of the organ bath the return to resting tension was rapid. In contrast, MB 28767, oxoprostol, misoprostol, enisoprost, 11-deoxy PGE_2-1-alcohol, 11-deoxy PGE_1 and butaprost produced more slowly developing responses which typically showed a large degree of spiking. These responses were slower to wane on washout.

Log concentration-response curves for all the analogues are shown in Figure 3. Those for MB 28767, oxoprostol, enisoprost, 11-deoxy PGE_2-1-alcohol, 11-deoxy PGE_1 and butaprost were shallower than those of the other analogues and in the case of MB 28767, enisoprost and 11-deoxy PGE_2-1-alcohol maximum responses were lower. Control pD_{25} values (−log of molar concentration required to elicit a contraction 25% of the ACh maximum) are given in Table 1, in order that the effects of the inhibitors on all analogues can be compared.

Effect of morphine Using parallel preparations from the same guinea-pigs, log concentration-response curves for the PGE analogues in the presence of 1 μM morphine were obtained

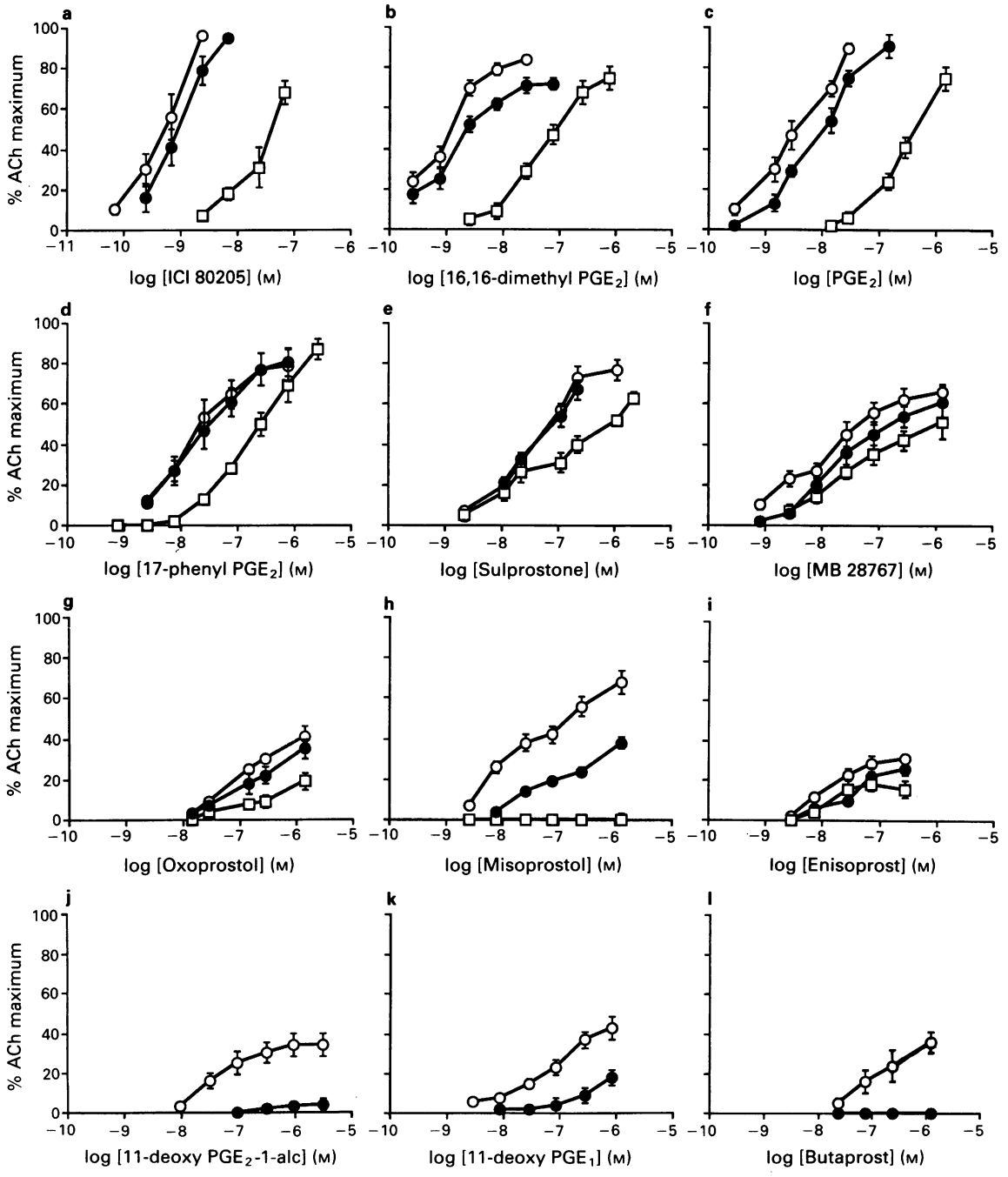


Figure 3 Log concentration-response curves for contraction of the guinea-pig ileum by prostaglandin E (PGE) analogues, with no inhibitors (○), 1 μM morphine (●) and 1 μM morphine and 2 μM AH 6809 (□) present. (a) ICI 80205, (b) 16,16-dimethyl PGE_2, (c) PGE_2, (d) 17-phenyl-ω-trinor PGE_2, (e) sulprostone, (f) MB 28767, (g) oxoprostol, (h) misoprostol, (i) enisoprost, (j) 11-deoxy PGE_2-1-alcohol, (k) 11-deoxy PGE_1, (l) butaprost. Each point is the mean of 4–6 experiments, with s.e.mean shown by vertical bars. Note the different concentration scale for ICI 80205.

Table 1 Agonist potencies of prostaglandin E (PGE) and prostaglandin I (PGI) analogues on the guinea-pig ileum, trachea and vas deferens

	Ileum				
		Dose-ratio		*Trachea*	*Vas deferens*
Prostanoid	*pD_{25} for contraction (no inhibitors)*	*Morphine (1 μM) vs no inhibitors*	*Morphine 1 μM/ AH 6808 (2 μM) vs Morphine (1 μM)*	*pD_{50} for contraction*	*pIC_{50} for inhibition*
PGE analogues					
ICI 80205	9.54 ± 0.27	1.96 ± 0.19	12.6 ± 1***	10.08 ± 0.10	9.02 ± 0.10
16,16-Dimethyl PGE_2	9.38 ± 0.11	1.62 ± 0.24	24.2 ± 5***	9.82 ± 0.03	9.62 ± 0.08
PGE_2	8.96 ± 0.17	3.58 ± 0.38*	31.2 ± 7***	(8.0)↓	8.76 ± 0.03
17-Phenyl-ω-trinor PGE_2	8.10 ± 0.10	0.94 ± 0.04	11.5 ± 2***	8.66 ± 0.15	7.96 ± 0.06
Sulprostone	7.92 ± 0.07	1.15 ± 0.03	3.5 ± 2	8.16 ± 0.08	9.69 ± 0.03
MB 28767	8.50 ± 0.11	5.2 ± 1.4*	1.8 ± 0.5	<6.0	8.97 ± 0.10
Oxoprostol	7.03 ± 0.16	4.0 ± 1.2	3.9 ± 1*	5.85 ± 0.16	8.35 ± 0.11
Misoprostol	8.10 ± 0.09	42 ± 8***	10 ± 4*	↓	9.12 ± 0.07
Enisoprost	7.37 ± 0.13	3.7 ± 1.0	4.1 ± 1.1	↓	9.22 ± 0.13
11-Deoxy PGE_2-1-alcohol	7.11 ± 0.14	>50	—	↓	7.78 ± 0.13
11-Deoxy PGE_1	6.87 ± 0.22	>20	—	↓	—
Butaprost	6.67 ± 0.28	>10	—	↓	<6.0
PGI analogues					
Iloprost	8.62 ± 0.12	3.5 ± 1.2*	245 ± 57***	8.09 ± 0.13	6.79 ± 0.09
Carbacyclin	7.72 ± 0.08	1.6 ± 0.5	24.9 ± 8***	7.53 ± 0.04	6.96 ± 0.12
PGI_2	8.12 ± 0.08	18.9 ± 0.5***	—	6.45 ± 0.09	—
Cicaprost	8.89 ± 0.11	>100	—	<6.0	<6.0
10,10-Difluoro-13,14-didehydro PGI_2	8.05 ± 0.18	>100	—	—	—

Values are means ± s.e.mean (n = 4–6).
*** $P < 0.001$; * $P < 0.05$: significant rightward shift of the log concentration-response curve at the 25% maximum response level.
↓ Indicates that relaxant activity was observed.

(Figure 3). It had been established in preliminary experiments that 1 μM morphine was sufficient to abolish the neuronal component of prostaglandin action on the ileum. The control and morphine curves for both 17-phenyl-ω-trinor PGE_2 and sulprostone were virtually superimposible. The corresponding curves for ICI 80205, 16,16-dimethyl PGE_2, oxoprostol, PGE_2 and MB 28767 were shifted to the right to a small extent, but only with the latter two agonists were the pD_{25} values statistically significant. More marked shifts were seen for misoprostol, 11-deoxy PGE_2-1-alcohol, 11-deoxy PGE_1 and butaprost. Indeed butaprost showed no contractile activity in the presence of morphine.

Effect of morphine/AH 6809 Our original intention was to determine the effects of both AH 6809 and morphine/AH 6809 on the contractile actions of the PGE analogues. However we found that continuous exposure of the ileum preparations to AH 6809 alone often resulted in increased spontaneous activity; this was not seen when morphine was also present. Therefore in a separate series of experiments, log concentration-response curves in the presence of 1 μM morphine/2 μM AH 6809 were obtained (Figure 3) and compared with contemporaneous 1 μM morphine curves (Table 1). For all analogues, morphine pD_{25} values in the two series of experiments were not significantly different ($P > 0.05$). AH 6809 caused a parallel rightward shift in the log concentration-response curve of PGE_2 (dose-ratio = 31.2), consistent with a pA_2 of 7.08 ± 0.14 (95% confidence limits, n = 5). Similar shifts were found for 16,16-dimethyl PGE_2, ICI 80205, and 17-phenyl-ω-trinor PGE_2, while a smaller but significant shift was seen for oxoprostol. Misoprostol showed no contractile action in the presence of morphine/AH 6809. Over the 10–30% response level the curve for sulprostone was little affected by AH 6809 but at concentrations giving responses of 40–70% of maximum the shift was similar to that produced in the PGE_2 curve. The log concentration-response curve for MB 28767 was unaffected by AH 6809. In two experiments, 10 μM AH 6809 caused a greater shift in the log concentration-response curve for PGE_2 (mean dose ratio = 52, pA_2 = 6.71). However, the curve for MB 28767 was not shifted.

Contractile actions of prostacyclin analogues on the guinea-pig ileum

No inhibitors present All the PGI analogues tested contracted the ileum, the rank order of potency at the 25% response level being: cicaprost ≥ iloprost > PGI_2 = 10,10-difluoro-13,14-didehydro PGI_2 ≥ carbacyclin (Figure 4 and Table 1). The log concentration-response curve for cicaprost was bell-shaped and the maximum response was lower than those of PGI_2 and iloprost (Figure 4). 10,10-Difluoro-13, 14-didehydro PGI_2 showed a shallow log concentration-response curve, with threshold response at 2.5 nM and 60% maximal response at 800 nM (Figure 4).

Effects of morphine The contractile actions of cicaprost and 10,10-difluoro-13,14-didehydro PGI_2 were abolished by morphine treatment (Figure 4). Indeed the higher concentrations of these two analogues produced an inhibitory response in those preparations which exhibited resting spontaneous activity. The log concentration-response curves for PGI_2 and iloprost were subject to a significant rightward shift in the presence of 1 μM morphine. However the carbacyclin curve was virtually unaffected at the 25% response level and moved slightly to the right at the 60% level.

Effects of morphine/AH 6809 In the presence of morphine, AH 6809 (2 μM) produced a roughly parallel shift to the right of the log concentration-response curve to carbacyclin, the dose ratio of 25 (Table 1) being similar to that for PGE_2. However, the inhibitory effect of AH 6809 on iloprost contractions was much greater (Figure 4).

Relaxant actions on the guinea-pig ileum

In the presence of 1 μM morphine and 2 μM AH 6809, butaprost (15–1500 nM) slightly potentiated (0–10%) submaximal contractile response to histamine in two preparations. In a further two preparations the histamine response was unaffected. More marked potentiation was seen with PGE_2 (14–1000 nM), often associated with small contractile responses to PGE_2 itself.

Cicaprost consistently inhibited histamine-induced contractions of the ileum; IC_{25} = 22 ± 3 nM (mean ± s.e.mean, n = 5)

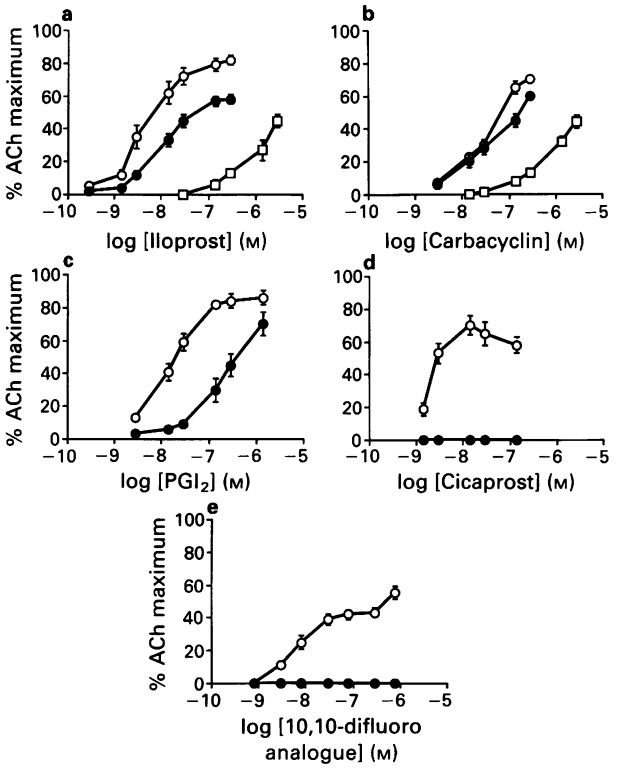


Figure 4 Log concentration-response curves for contraction of the guinea-pig ileum by prostaglandin I (PGI) analogues, with no inhibitors (○), 1 μM morphine (●) and 1 μM morphine and 2 μM AH 6809 (□) present. (a) Iloprost, (b) carbacyclin, (c) PGI_2, (d) cicaprost, (e) 10,10-difluoro-13,14-didehydro PGI_2. Each point is the mean of 4–6 experiments, with s.e.mean shown by vertical bars.

(Figure 5). PGI_2 and iloprost (100–1000 nM) also inhibited histamine responses, but they also produced an initial contraction of the tissue prior to inhibition and the inhibitory effect did not reach a maximum until two or three histamine doses had been added. In some tissues (2/6 for iloprost and 2/5 for PGI_2) no inhibition was observed. In contrast, carbacyclin (10–1000 nM) did not inhibit histamine contractions but instead potentiated these responses.

Actions of other prostanoids on the guinea-pig ileum

The thromboxane A_2-mimetic, U-46619, did not contract the ileum at concentrations up to 3 μM. $PGF_{2\alpha}$ contracted the preparation ($EC_{25} \sim 200$ nM); however, this prostanoid retained little activity in the presence of 2 μM AH 6809. The $PGF_{2\alpha}$-mimetic, ICI 81008, was inactive as a contractile agent at concentrations up to 2 μM.

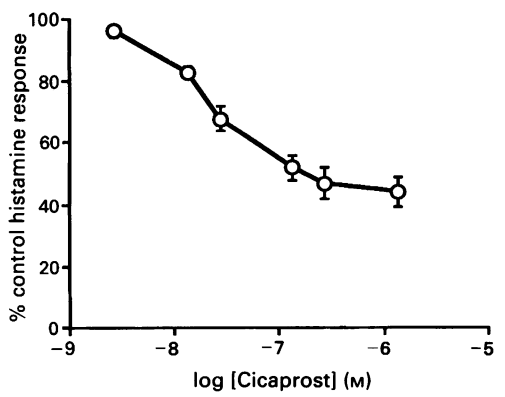


Figure 5 Log concentration-response curve for cicaprost (○) as an inhibitor of histamine-induced contraction of the guinea-pig ileum (1 μM morphine and 2 μM AH 6809 present). Each point is the mean of 5 experiments, with the s.e.mean shown by vertical bars.

Agonist potencies on EP-receptor systems in the guinea-pig trachea and vas deferens

pD_{50} values for contraction of guinea-pig tracheal rings are shown in Table 1; the TP-receptor antagonist EP 092 (1 μM) was present to block potential TP-agonist actions. Several of the PGE analogues (including PGE_2) relax the tracheal muscle and consequently estimates of their contractile potency cannot be obtained. The regression of guinea-pig ileum pD_{25} values (morphine present) on guinea-pig trachea pD_{50} values for ICI 80205, 16,16-dimethyl PGE_2, 17-phenyl-ω-trinor PGE_2, sulprostone, iloprost, carbacyclin and PGI_2 shows a highly significant dependence: slope = 1.46, $r = 0.989$, $P < 0.001$.

On the guinea-pig trachea, AH 6809 (0.2–5 μM) shifted the log concentration-response curve to 17-phenyl-ω-trinor PGE_2 to the right in a parallel manner. The slope of the Schild plot (1.13) was not significantly different from unity and a pA_2 of 7.35 ± 0.15 (95% confidence limits) was obtained. AH 6809 also shifted the log concentration-response curves to sulprostone, iloprost and carbacyclin to the right in a parallel manner and its blocking potency was similar.

pIC_{50} values for inhibition of the twitch response to electrical field stimulation of the guinea-pig vas deferens are also shown in Table 1. All the analogues with pIC_{50} values greater than 7.0 were able to inhibit completely the twitch response. Comparison of guinea-pig ileum pD_{25} values (morphine present) with guinea-pig vas deferens pIC_{50} values for the above analogues, but substituting PGE_2 for PGI_2, does not result in a statistically significant correlation: $r = 0.57$, $P > 0.05$.

Discussion

The agonist specificities of the PGE and PGI analogues for the three EP-receptor subtypes and the IP-receptor are given in Table 2. The rankings are compiled from the present and previously published studies (see Table 2 legend). To simplify the analysis of our results we have assumed that PGE_2 and its analogues interact only with EP-receptors in the ileum. On human platelets, which are very sensitive to the inhibitory action of IP-receptor agonists (see Armstrong *et al.*, 1989), only PGE_2 and 11-deoxy PGE_1 show evidence of inhibitory activity; they are about 300 and 1000 times less potent than iloprost respectively (Jones unpublished observations). Furthermore, there is no evidence from these or other studies for DP-, FP-, or TP-receptors in the ileum (Welburn & Jones, 1978; Kennedy *et al.*, 1982).

Our antagonism experiments with AH 6809 confirm the existence of an EP_1-receptor mediating contraction of the ileum longitudinal smooth muscle. AH 6809 at 2 μM significantly shifted the log concentration-response curves of ICI 80205, 16,16-dimethyl PGE_2, PGE_2 and 17-phenyl-ω-trinor PGE_2 to the right in a roughly parallel manner: the pA_2 values agree with previously published data on the guinea-pig ileum ($pA_2 = 6.8$, PGE_2 as agonist, Coleman *et al.*, 1985) and the present measurements on the guinea-pig trachea ($pA_2 = 7.35$, 17-phenyl-ω-trinor PGE_2 as agonist). Of the PGI analogues investigated, iloprost and carbacyclin showed potent contractile actions which were blocked by AH 6809. A similar profile was seen on the guinea-pig trachea. This extends our original finding that the contractile actions of iloprost and $\Delta^{6,6a}$-6a-carba PGI_1 (an isomer of carbacyclin) on the guinea-pig trachea and bullock iris sphincter were blocked by SC 19920 and its analogue SC 25191 (Dong *et al.*, 1986). The very pronounced block by AH 6809 of the contractile action of iloprost on the ileum is probably due to a combination of EP_1-receptor antagonism and the IP-receptor-mediated inhib-

Table 2 Specificity of prostaglandin E (PGE) and prostaglandin I (PGI) analogues as agonists at EP- and IP-receptors

Prostanoid	*EP_1*	*EP_2*	*EP_3*	*IP*
PGE analogues				
ICI 80205	++++	+	+++	
16,16-Dimethyl PGE_2	++++	++(+)	++++	
PGE_2	+++	+++	+++	
17-Phenyl-ω-trinor PGE_2	+++	+	++	
Sulprostone	++	0	++++	
MB 28767	(+)	(+)	+++	
Oxoprostol	(+)	0	++	
Misoprostol	+	++(+)	+++	
Enisoprost	+	+(+)	+++	
11-Deoxy PGE_2-1-alcohol	(+)	++	++	
11-Deoxy PGE_1	+	++(+)	++	
Butaprost	(+)	++*	0	
PGI analogues				
Iloprost	+++	(+)	+	++++
Carbacyclin	++		+	++
PGI_2	+	+	+	+++(+)
Cicaprost	(+)	(+)	0	++++

One + corresponds to a potency difference of approximately one order of magnitude. EP_1 ranking from measurements on guinea-pig fundus (Coleman *et al.*, 1988) and guinea-pig trachea and ileum (this study); EP_2 ranking from cat trachea (Coleman *et al.*, 1988), guinea-pig trachea (Dong *et al.*, 1986) and rabbit jugular vein (Lawrence *et al.*, 1989); EP_3 rankings from guinea-pig vas deferens (this study); IP rankings from human platelets (Armstrong *et al.*, 1989).
* Potency of butaprost is low on rabbit jugular vein.

itory action of iloprost (see later) becoming more effective as its concentration is increased.

There is a very good correlation between contractile potencies on the guinea-pig ileum (morphine present) and guinea-pig trachea (analogues with relaxant activity excluded), consistent with EP_1-receptor activation in both preparations. Furthermore, there is no significant correlation between agonist potencies on guinea-pig ileum (morphine present) and guinea-pig vas deferens, the latter being the standard EP_3 preparation. Both ICI 80205 and 17-phenyl-ω-trinor PGE_2 show some selectivity for EP_1- as opposed to EP_3- (this study) and EP_2-receptors (Dong *et al.*, 1986; Lawrence *et al.*, 1989). However, 17-phenyl-ω-trinor PGE_2 would have a potential advantage over ICI 80205 in studies to characterize EP_1-receptors owing to its very low TP agonist potency. 17-Phenyl-ω-trinor PGE_2 may be a useful lead compound in the development of more selective EP_1-receptor agonists.

In our earlier studies the potent prostacyclinmimetic, cicaprost, showed only very weak EP_1-receptor agonist activity (Dong *et al.*, 1986). This also appears to be the case in the ileum. However, it is possible (cf. iloprost) that an inhibitory action operating through an IP-receptor is masking a weak EP_1 contractile component. The 10,10-difluoro13,14-didehydro analogue of PGI_2, again a potent prostacyclin mimetic, also appeared to have low EP_1 agonist activity. The presence of the 13,14-acetylenic bond in cicaprost and the difluoro analogue extends the rigidity of the C4–C13 structure to C15 and this could prevent a favourable interaction of the 15α-hydroxyl with the agonist binding site of the EP_1-receptor.

The finding of a morphine/AH 6809-resistant contractile effect for sulprostone, MB 28767, oxoprostol and enisoprost suggests the existence of a second EP-receptor on the smooth muscle cells. Whether the more potent EP_1-receptor agonists also exhibit this type of activity is impossible to determine without a considerably greater block of EP_1-receptors than that achieved with 2 μM AH 6809; higher concentrations of AH 6809 produce low antagonist specificity in other systems (Keery & Lumley, 1988). It is unlikely that this second direct contractile component is mediated via an EP_2-receptor since both sulprostone (Coleman *et al.*, 1987) and MB 28767 (Lawrence *et al.*, 1989) have very weak activity in this respect. They are however highly potent EP_3-agonists as judged by their ability to inhibit the twitch response of the guinea-pig vas deferens to electrical field stimulation (inhibition of transmitter release) (Coleman *et al.*, 1987). Thus the ileum receptor may be an EP_3 subtype. Against this proposal are the low potencies of enisoprost and 11-deoxy PGE_2-1-alcohol and the complete inactivity of misoprostol. At this stage it is tempting to suggest yet a further EP-receptor subtype. However, it must be remembered that the sensitivity of the vas deferens to sulprostone is about two orders of magnitude greater than that of the AH 6909-resistant contractile system of the ileum. Differences in receptor density and/or efficiency of receptor-effector coupling between the two systems could dramatically alter relative agonist potencies.

In relation to the morphine-sensitive contractile actions of prostanoids on the ileum, it would seem sensible to postulate the presence of both EP- and IP-receptors on enteric neurones and/or sensory nerve terminals. From the previous argument, the lack of effect of morphine on the contractile actions of 17-phenyl-ω-trinor PGE_2 and sulprostone would suggest that the EP-receptor is neither the EP_1 nor EP_3 subtype. A large component of the actions of misoprostol, 11-deoxy PGE_2-1-alcohol, 11-deoxy PGE_1 and butaprost is due to activation of the neuronal receptor. Since these analogues show some selectivity for EP_2-receptors which mediate relaxation of vascular and respiratory smooth muscle (Table 2), it seems likely that the neuronal receptor may be of the EP_2 subtype. The pD_{25} values for these analogues in the absence of inhibitors give rough estimates of potency at the neuronal EP-receptor (Table 1). The ranking is misoprostol > 11-deoxy PGE_2-1-alcohol $\geq$ 11-deoxy PGE_1 $\geq$ butaprost: PGE_2 is likely to be at least as potent as misoprostol. However, the activity of MB 28767 is difficult to reconcile with the presence of an EP_2-receptor subtype and further studies are required. The actions of butaprost and 11-deoxy PGE_2-1-alcohol are entirely or almost entirely due to activation of the neuronal EP-receptor and it is of interest that the maximum contraction induced by these analogues is only about 40% of the maximum contraction produced by PGE_2, which probably activates all three EP-receptor-mediated systems. EP_1-selective analogues elicit contractions of the ileum which are more rapid in onset and less prone to spiking than those elicited by analogues with pronounced EP_2/EP_3 activity. The difference is similar to that usually seen with ACh and nicotine on the ileum and probably reflects direct (EP_1) as opposed to neuronal (EP_2) activation of the ileum smooth muscle; why slow, spiky contractions should also be typical of EP_3-receptor activation is not clear however.

The neuronal excitatory (morphine-sensitive) action of PGI_2 and its analogues on the guinea-pig ileum is unusual since IP-receptor activation is typically associated with inhibition of smooth muscle tone, including rhythmic activity of intestinal smooth muscle (see Skuballa *et al.*, 1987). With the exception of carbacyclin, most of the 25% maximum contractile response appears to be due to activation of neuronal IP-receptors and hence the pD_{25} values in the absence of inhibitors give estimates of agonist potency: cicaprost $\geq$ iloprost > PGI_2 = 10,10-difluoro-13,14-didehydro PGI_2. Carbacyclin would appear to have the lowest potency. This ranking is similar to those found for other PGI-sensitive systems in smooth muscle and platelets (Armstrong *et al.*, 1989).

In the presence of morphine and AH 6809, PGI_2, cicaprost and iloprost, but not carbacyclin, inhibited contractile responses to histamine. However, only cicaprost was devoid of accompanying contractile activity. This stimulant action may be due to surmountability of the EP_1-receptor block and/or an agonist action at the AH 6809-resistant EP-receptor. The ileum is more sensitive to the neuronal contractile action of cicaprost (pD_{25} = 8.89) than the direct inhibitory action (pIC_{25} = 7.66) and this probably accounts for the bell-shaped nature of the cicaprost log concentration-response curve.

Gardiner (1986) found that the log concentration-response curve for butaprost on the guinea-pig ileum was bell-shaped, with the maximum response being <20% of the tissue maximum at 1 μM. Gardiner also reported that butaprost inhibited histamine-induced contractions at concentrations of 3 and 10 μM. In contrast, we found a linear log concentration-response curve (36% response at 1 μM) and no inhibition of histamine. It is impossible to prepare concentrated aqueous solutions of butaprost (a methyl ester) without initial dissolution in an organic solvent. We therefore suspect a depression of smooth muscle contractility due to the organic solvent (nature unknown) used in Gardiner's experiments. In our experiments, ethanol was used, resulting in a maximum concentration in the bathing fluid of 20 mM.

In conclusion, the data presented demonstrate that the guinea-pig longitudinal muscle/enteric neurone system contains EP- and IP-receptors additional to the EP_1 subtype. Thus, claims about the EP_1-selectivity of a compound based on its contractile potency on this preparation should be interpreted with caution. In order to determine unequivocally the receptor subtype involved the effect of both morphine and AH 6809 should be investigated. With the use of these inhibitors, however, the guinea-pig ileum remains a useful preparation for assessing the receptor selectivity of PGE and PGI analogues.

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Endothelium-dependent and -independent effects of exogenous ATP, adenosine, GTP and guanosine on vascular tone and cyclic nucleotide accumulation of rat mesenteric artery

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1 The effects of exogenous guanosine 5'-triphosphate (GTP) and guanosine on vascular tone and cyclic nucleotide accumulation of noradrenaline-precontracted endothelium-intact and endothelium-denuded rat mesenteric artery rings were compared with the effects of the known purinoceptor agonists adenosine 5'-triphosphate (ATP) and adenosine.

2 GTP (10 μM–1 mM) dose-dependently relaxed endothelium-intact mesenteric artery rings by producing a rapid initial response followed by sustained relaxation resembling the relaxant response to acetylcholine. GTP also slightly relaxed endothelium-denuded artery rings. The acetylcholine- and GTP-induced relaxations of endothelium-intact rings were attenuated by N^G-nitro L-arginine methyl ester (L-NAME, 330 μM) which attenuation was reversed with L-arginine (1 mM).

3 Guanosine (10 μM–1 mM) relaxed both endothelium-intact and -denuded artery rings in a dose-dependent manner. The relaxations were more pronounced in endothelium-intact preparations and were only slightly attenuated by L-NAME (330 μM).

4 ATP (1 μM–1 mM) and adenosine (10 μM–1 mM) dose-dependently relaxed endothelium-intact and -denuded artery rings. The responses were more pronounced in endothelium-intact vascular preparations.

5 GTP (100 μM) and guanosine (100 μM) increased guanosine 3':5'-cyclic monophosphate (cyclic GMP) accumulation in both endothelium-intact and -denuded artery rings corresponding to the relaxations observed. The concentrations of adenosine 3':5'-cyclic monophosphate (cyclic AMP) were not affected.

6 ATP (100 μM) increased cyclic GMP concentration of endothelium-intact artery rings. The concentrations of cyclic AMP were not affected by ATP (100 μM) and adenosine (100 μM) in endothelium-intact and -denuded vascular preparations.

7 These results provide evidence that exogenous GTP and guanosine relax precontracted endothelium-intact and -denuded rat mesenteric artery rings by increasing cyclic GMP accumulation. The response to GTP of endothelium-intact rings can mainly be explained by the release of endothelium-derived relaxing factor (EDRF), but that of guanosine is only partly due to EDRF, and is a combination of endothelium-dependent and -independent effects. The endothelium-independent response of GTP and guanosine is a direct, unknown effect on smooth muscle and guanylate cyclase.

Keywords: Endothelium; vascular tone; ATP; adenosine; GTP; guanosine; cyclic nucleotides

Introduction

Furchgott & Zawadski (1980) were the first to discover that acetylcholine relaxed vascular smooth muscle only in the presence of an intact endothelium. Several other agents, including adenine nucleotides, calcium ionophore A23187, bradykinin, substance P and related peptides, unsaturated fatty acids, histamine, 5-hydroxytryptamine, noradrenaline and thrombin have also been shown to relax vascular smooth muscle endothelium-dependently (Rapoport & Murad, 1983). The factor released from endothelium has been termed the endothelium-derived relaxing factor (EDRF). EDRF is believed to relax smooth muscle by activating soluble guanylate cyclase and increasing intracellular levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Rapoport & Murad, 1983; Waldman & Murad 1987; Ignarro, 1989). The chemical nature of EDRF has been the subject of intense investigation and is proposed to be identical or closely related to nitric oxide synthesized from L-arginine (Palmer *et al.*, 1987; 1988; Moncada *et al.*, 1989; Myers *et al.*, 1990). The synthesis of EDRF from L-arginine in endothelium can be inhibited by N^G-nitro L-arginine methyl ester (L-NAME) (Rees *et al.*, 1990).

Extracellular adenosine and adenine nucleotides modulate vascular tone and platelet function by interacting with specific receptors on the cell surface (Burnstock, 1978; Burnstock & Kennedy, 1985; Gordon, 1986). ATP and ADP can be liberated into the extracellular space as a consequence of vessel wall damage and local platelet aggregation (Gordon, 1986), after which they are rapidly sequentially dephosphorylated to adenosine by ectonucleotidases of endothelium and circulating blood cells (Pearson & Gordon, 1985; Coade & Pearson, 1989). The endothelium-independent effects of ATP can be either vasodilatation or vasoconstriction, depending on the subclass of the P_2 purinoceptor in question (Kennedy *et al.*, 1985; Houston *et al.*, 1987; Pearson, 1988). The endothelium-dependent effect of ATP is vasodilatation due to its action at P_{2Y} purinoceptors which leads to release of EDRF and/or prostacyclin (De Mey & Vanhoutte, 1981; Gordon & Martin, 1983; Needham *et al.*, 1987; Boeynaems & Pearson, 1990). The vasodilator action of adenosine is mediated by the A_2 subclass of the P_1 purinoceptor, which stimulates adenylate cyclase (Collis & Brown, 1983; Ramagopal *et al.*, 1988). The location of adenosine receptors has not been well defined and both endothelium-dependent (Gordon & Martin, 1983; Rubanyi & Vanhoutte, 1985) and -independent (Kennedy *et al.*, 1985; White & Angus, 1987) responses have been described.

Although the effects of extracellular adenine nucleotides are well known, there is little evidence about the effects of extracellular guanine nucleotides on vascular tone. We have recently reported that exogenous GTP augments sodium

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nitrite-induced cyclic GMP accumulation in endothelium-denuded rat mesenteric artery rings and human platelets. This increase in cyclic GMP is accompanied by corresponding physiological changes, i.e. increased vascular relaxation and decreased platelet aggregation (Laustiola *et al.*, 1991). The present work was carried out to evaluate in more detail the endothelium-dependent and -independent effects of exogenous GTP and guanosine on vascular tone and cyclic nucleotide accumulation, and to compare them to the effects of the known purinoceptor agonists, ATP and adenosine.

Preliminary results of part of this work were presented at the IUPHAR Satellite Symposium on EDRF and EDRF-related Substances, at Antwerp, in 1990 (Pörsti *et al.*, 1990) and the International Symposium on Pharmacology of Purinergic Receptors, at Noordwijk, in 1990 (Laustiola & Vuorinen, 1990).

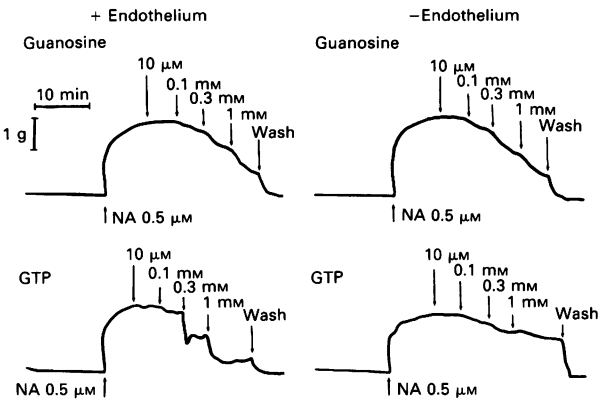

Figure 1 Record showing the cumulative effects of guanosine (upper panel) and GTP (lower panel) on noradrenaline (NA) precontracted endothelium-intact and -denuded rat mesenteric artery rings.

Methods

Relaxation of rat mesenteric arteries

Non-fasted male Sprague-Dawley rats weighing about 350 g (age 10–12 weeks) were decapitated. The mesenteric artery was excised and cut into 3 mm long rings. Six rings were usually obtained from one artery. When endothelium-dependent effects were studied the endothelium was left intact, but for studies of endothelium-denuded rings the endothelium was removed by rubbing it gently with a scuffed injection needle. The rings were placed between two stainless steel hooks and mounted in an organ bath chamber in Krebs bicarbonate buffer solution (pH 7.4) of the following composition (mM): NaCl 118.0, $NaHCO_3$ 25.0, glucose 11.1, $CaCl_2$ 2.5, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2. The preparations were maintained at +37°C and aerated with 95% O_2:5% CO_2. They were equilibrated for 1 h with a resting tension of 1.5 g. The force of contraction was measured with an isometric force-displacement transducer (Grass FT03) and registered on a Grass Polygraph (Model 7 E Polygraph; Grass Instrument Co., Quincy, MA, U.S.A.).

Successful removal and the integrity of the endothelium were confirmed by adding acetylcholine (1 μM, final concentration) to 0.5 μM noradrenaline-precontracted vascular rings. If any relaxation of the denuded preparation was observed, the endothelium was further rubbed. For studies with endothelium-intact vascular rings, the relaxation with acetylcholine had to be nearly 100%.

After rinsing four times with Krebs buffer the rings were stabilized for an additional 30 min; cumulative vascular relaxations after precontraction with noradrenaline (0.5 μM) were then elicited in response to acetylcholine (1 nM–1 μM), ATP (1 μM–1 mM), adenosine (10 μM–1 mM), GTP (10 μM–1 mM) and guanosine (10 μM–1 mM) (Figures 1 and 2). The relaxation time for each dose was 5 min, but for the highest dose it was 10 min. In some experiments indomethacin (10 μM) was incubated with endothelium-intact rings before the addition of GTP. The relaxations induced by acetylcholine, GTP and guanosine in endothelium-intact artery rings were also studied in the presence of 330 μM L-NAME or L-NAME plus L-arginine (1 mM). In these studies, L-NAME or L-NAME plus L-arginine were added 15 min before precontraction with noradrenaline and then the cumulative dose-response relaxations were elicited. In control studies, 0.5 μM noradrenaline was found to produce a steady contraction lasting throughout the experimental procedure without any relaxation.

Measurement of cyclic nucleotides

In parallel experiments with identical conditions to the studies of physiological responses, samples for cyclic nucleotide determinations were taken. The control samples for endothelium-intact and -denuded rings were taken after a 6 min precontraction with noradrenaline. After 4 min noradrenaline precontraction, the rings were incubated with acetylcholine (0.1 μM), ATP (100 μM), adenosine (100 μM), GTP (100 μM) and guanosine (100 μM). The incubation time for acetylcholine was 30 s and for the other compounds 2 min. The artery rings were taken from the organ baths after exposure to the test agents and immediately frozen in liquid nitrogen. They were homogenized in 0.5 ml ice cold 6% trichloroacetic acid (TCA) in small glass homogenizers and centrifuged at 2000 *g* for 20 min. Supernatant fractions were washed four times with water-saturated ether and aliquots of extracts were diluted with 100 mM sodium acetate buffer, pH 5.8, and then acetylated. Adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and cyclic GMP concentrations were determined by radioimmunoassay with a commercial kit (Amersham International Plc, U.K.). The pellets of TCA extracts were dissolved in 0.5 ml of 1 M NaOH and used for measurements of protein content (Lowry *et al.*, 1951).

Drugs

Acetylcholine chloride, adenosine, adenosine 5′-triphosphate, guanosine, guanosine 5′-triphosphate, indomethacin, L-arginine and N^G-nitro L-arginine methyl ester were obtained from Sigma Chemical Company (St Louis, Mo, U.S.A.), and (−)-noradrenaline-L-hydrogen tartrate was from Fluka

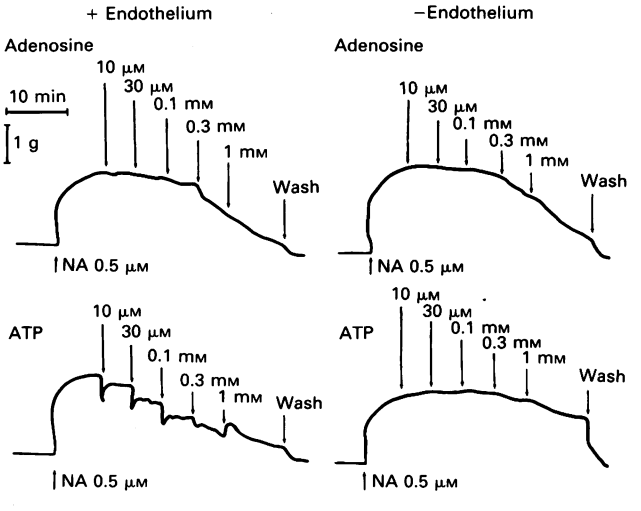

Figure 2 Record showing the cumulative effects of adenosine (upper panel) and ATP (lower panel) on noradrenaline (NA) precontracted endothelium-intact and -denuded rat mesenteric artery rings.

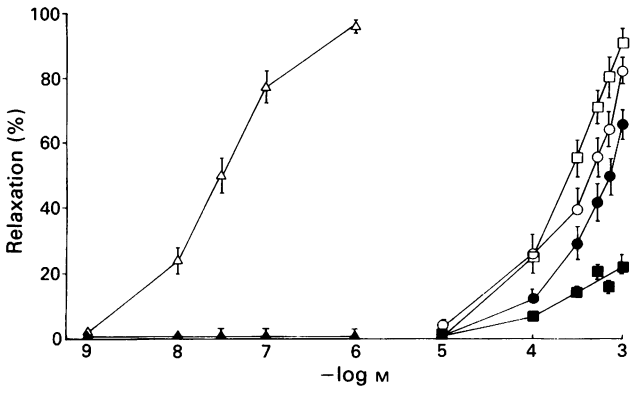

Figure 3 Concentration-response curves for the relaxation induced by acetylcholine (△), GTP (□) and guanosine (○) in endothelium-intact (open symbols) and endothelium-denuded (closed symbols) noradrenaline (0.5 μM)-precontracted rat mesenteric artery rings. Each point represents the mean, and vertical lines show s.e.mean (n = 10–12 rings of 6–8 rats).

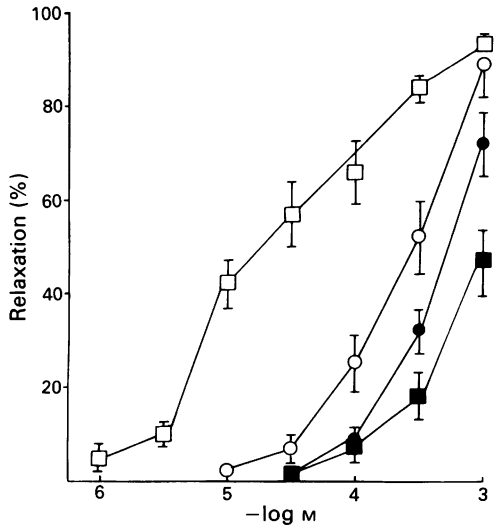

Figure 4 Concentration-response curves for the relaxation induced by ATP (□) and adenosine (○) in endothelium-intact (open symbols) and endothelium-denuded (closed symbols) noradrenaline (0.5 μM) precontracted rat mesenteric artery rings. Each point represents the mean, and vertical lines show s.e.mean (n = 8–10 rings of 6–8 rats).

Chemie AG (Buchs, Switzerland). The test agents were prepared in Krebs buffer on the day of use.

Statistical analysis

Data are presented as mean ± s.e.mean. Statistical comparisons were performed by Student's t test. P values of less than 0.05 were considered to be statistically significant.

Results

The effects of acetylcholine, GTP and guanosine on vascular tone

In the noradrenaline-precontracted endothelium-intact rings acetylcholine (1 nM–1 μM) produced a dose-dependent relaxation. The maximal relaxation at 1 μM acetylcholine was 96 ± 1%. In the absence of endothelium this relaxation was completely abolished (Figure 3).

GTP (10 μM–1 mM) relaxed endothelium-intact artery rings with a fast initial relaxation response (Figures 1 and 3). The relaxation at 1 mM GTP was 91 ± 4% in endothelium-intact rings. Pretreatment of endothelium-intact artery rings with 10 μM indomethacin for 30 min before inducing contraction did not attenuate the relaxant effect of GTP (data not shown). GTP also slightly relaxed endothelium-denuded rings, the relaxation at 1 mM GTP being 23 ± 3% (Figures 1 and 3).

Guanosine (10 μM–1 mM) relaxed both endothelium-intact and -denuded artery rings in a concentration-dependent manner, the response being slightly more pronounced in intact preparations (Figures 1 and 3). The relaxation at 1 mM guanosine was 82 ± 4% in intact and 65 ± 5% in denuded artery rings (Figure 3).

The effects of ATP and adenosine on vascular tone

ATP (1 μM–1 mM) relaxed endothelium-intact rings with a fast initial response at lower concentrations, while higher concentrations of ATP produced a transient contraction followed by a maintained relaxation (Figures 2 and 4). ATP (1 mM) relaxed endothelium-intact rings by 93 ± 2% (Figure 4). ATP also relaxed endothelium-denuded rings, the response being smaller than that in intact rings (Figures 2 and 4). The relaxation produced by 1 mM ATP in denuded rings was 46 ± 7% (Figure 4).

Adenosine (10 μM–1 mM) produced concentration-dependent relaxations of the endothelium-intact and denuded-artery

Table 1 The effects of acetylcholine, GTP and guanosine on cyclic GMP and cyclic AMP content of noradrenaline precontracted endothelium-intact (+E) and -denuded (−E) rat mesenteric artery rings

	Cyclic GMP (pmol mg^{-1} prot)		*Cyclic AMP* (pmol mg^{-1} prot)	
	+E	−E	+E	−E
Control	1.18 ± 0.08	0.82 ± 0.08	2.30 ± 0.18	1.31 ± 0.15
Acetylcholine (0.1 μM)	1.88 ± 0.12**	0.66 ± 0.08	NM	NM
GTP (100 μM)	1.85 ± 0.09**	1.13 ± 0.06**	1.94 ± 0.15	1.55 ± 0.10
Guanosine (100 μM)	1.98 ± 0.20*	1.22 ± 0.12*	2.48 ± 0.10	1.73 ± 0.16

Data represent the means ± s.e.mean (n = 8–12 rings). Differences from corresponding control values (precontracted with 0.5 μM noradrenaline) are denoted by * P < 0.05 and ** P < 0.01 (NM = not measured).

Table 2 The effects of ATP and adenosine on cyclic GMP and cyclic AMP content of noradrenaline precontracted endothelium-intact (+E) and -denuded (−E) rat mesenteric artery rings

	Cyclic GMP (pmol mg^{-1} prot)		*Cyclic AMP* (pmol mg^{-1} prot)	
	+E	−E	+E	−E
Control	0.47 ± 0.05	0.22 ± 0.05	2.51 ± 0.18	1.31 ± 0.15
ATP (100 μM)	0.99 ± 0.08**	0.19 ± 0.02	2.74 ± 0.28	1.59 ± 0.13
Adenosine (100 μM)	0.59 ± 0.06	0.15 ± 0.03	2.71 ± 0.17	1.42 ± 0.18

Data represent the means ± s.e.mean (n = 8–12 rings). Difference from corresponding control value (precontracted with 0.5 μM noradrenaline) is denoted by ** P < 0.01.

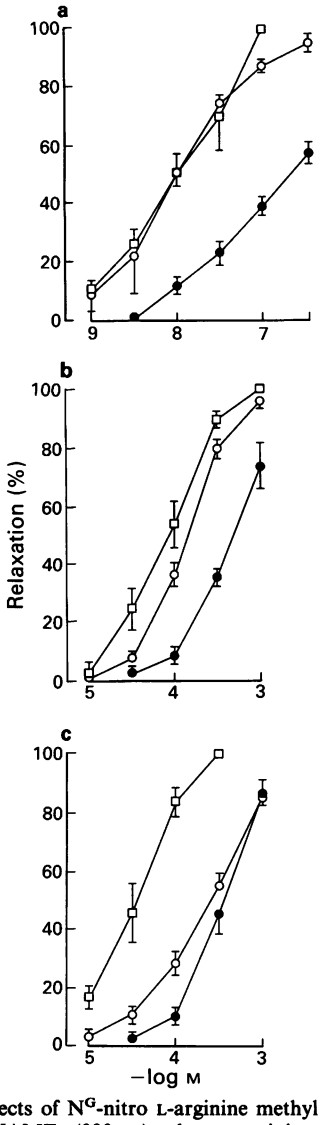

Figure 5 The effects of N^G-nitro L-arginine methyl ester (L-NAME, 330 μM) and L-NAME (330 μM) plus L-arginine (1 mM) on the concentration-response curves for the relaxation induced by (a) acetylcholine, (b) GTP and (c) guanosine in endothelium-intact noradrenaline (0.5 μM)-precontracted rat mesenteric artery rings. In (a) (○) acetylcholine; (●) acetylcholine + L-NAME; (□) acetylcholine + L-NAME + L-arginine. In (b) (○) GTP; (●) GTP + L-NAME; (□) GTP + L-NAME + L-arginine. In (c) (○) guanosine; (●) guanosine + L-NAME; (□) guanosine + L-NAME + L-arginine. Each point represents the mean, and vertical lines show s.e.mean (n = 6–8 rings of 4–6 rats).

rings, the relaxation response again being more pronounced in intact preparations (Figures 2 and 4). Adenosine at 1 mM caused 89 ± 5% relaxation in endothelium-intact rings and 72 ± 7% relaxation in denuded rings (Figure 4).

The effects of acetylcholine, ATP, adenosine, GTP and guanosine on cyclic nucleotides

Acetylcholine (0.1 μM, $P < 0.01$), GTP (100 μM, $P < 0.01$) and guanosine (100 μM, $P < 0.05$) all increased cyclic GMP levels in endothelium-intact artery rings when compared to the noradrenaline-contracted control (Table 1). When the endothelium was removed acetylcholine did not affect cyclic GMP, but GTP ($P < 0.01$) and guanosine ($P < 0.05$) still significantly increased it (Table 1). Cyclic AMP levels were not affected by these compounds (Table 1).

ATP (100 μM, $P < 0.01$) increased the cyclic GMP concentration of endothelium-intact rings (Table 2). ATP and adenosine had no effects on the cyclic GMP of endothelium-denuded rings. Neither ATP (100 μM) nor adenosine (100 μM) had any effects on cyclic AMP in endothelium-intact and -denuded rat mesenteric artery rings (Table 2).

The effects of L-nitroarginine on relaxation of endothelium-intact rings

L-NAME (330 μM) shifted the concentration-response curve of acetylcholine-induced relaxation to the right (Figure 5a). The relaxation of 0.33 μM acetylcholine was 95 ± 3% and in the presence of L-NAME 57 ± 4% ($P < 0.001$) (Figure 5a). L-Arginine (1 mM) totally reversed the inhibitory action of L-NAME (Figure 5a).

The concentration-response curve for GTP-induced relaxation of endothelium-intact rings was also shifted to the right by L-NAME. GTP (1 mM) relaxed the rings by 96 ± 2%, and in the presence of L-NAME by 73 ± 8% ($P < 0.001$) (Figure 5b). The inhibitor action of L-NAME was reversed by L-arginine and the GTP-induced relaxation was even slightly improved (Figure 5b).

L-NAME inhibited guanosine-induced relaxation only at low guanosine concentrations. At 0.1 mM guanosine, the relaxation was decreased from 24 ± 4% to 10 ± 2% ($P < 0.001$) in the presence of L-NAME (Figure 5c). At higher guanosine concentrations the inhibitory action of L-NAME was not seen. The combination of L-NAME and L-arginine clearly enhanced the guanosine-induced relaxation of endothelium-intact artery rings (Figure 5c).

Discussion

The relationship between the increase in cellular cyclic GMP and vascular smooth muscle relaxation has become widely accepted (Waldman & Murad, 1987; Lincoln, 1989). The actions of nitrovasodilators are mediated by the release of nitric oxide, which interacts with the heme prosthetic group of soluble guanylate cyclase and activates the enzyme (Ignarro *et al.*, 1986; Waldman & Murad, 1987). The release of nitric oxide or a closely related compound and the subsequent activation of soluble guanylate cyclase is also believed to account for the biological actions of the endothelium-derived relaxing factor (Palmer *et al.*, 1987; Myers *et al.*, 1990).

The present results show that ATP and GTP efficiently relaxed endothelium-intact mesenteric artery rings and increased cyclic GMP concentration without an effect on cyclic AMP, thus resembling the endothelium-dependent effects of acetylcholine. ATP acts at P_{2y} receptors on endothelial cells releasing both EDRF and prostacyclin (De Mey & Vanhoutte, 1981; Needham *et al.*, 1987). The release of EDRF and the subsequent increase in cyclic GMP are considered to be the main mediators of ATP-induced relaxation (Houston *et al.*, 1987; Rose'meyer & Hope, 1990). GTP has been described as mediating an endothelium-dependent relaxation of pig aorta, but when compared to ATP, it was found to be a very weak relaxant (Martin *et al.*, 1985). Our results with rat mesenteric artery indicate that GTP is an efficient endothelium-dependent relaxant. Indomethacin did not impair the endothelium-dependent relaxation of GTP, and GTP did not increase cyclic AMP in mesenteric rings, suggesting that prostacyclin does not mediate these effects. This is further supported by the finding that GTP does not induce prostacyclin production in pig aortic endothelial cells (Needham *et al.*, 1987). The L-arginine analogue, L-NAME, attenuated acetylcholine-induced relaxation of endothelium-intact artery rings as previously reported (Rees *et al.*, 1990), the inhibitor action being reversed by L-arginine. L-NAME also inhibited GTP-induced relaxation, this attenuation could be reversed by L-arginine, although the inhibitory action was not as marked as in the case of acetylcholine relaxation. Thus the increase in cyclic GMP and the relaxation of endothelium-intact rat mesenteric artery rings by GTP are probably mediated by the release of EDRF.

ATP relaxed precontracted endothelium-denuded rat mesenteric artery rings without any effects on cyclic nucleotides.

Higher concentrations of ATP sometimes first produced a transient contraction of both intact and denuded preparations which was followed by relaxation. The transient contraction can be explained by the activation of P_{2x} receptors on smooth muscle (Pearson, 1988). The relaxation to ATP observed in denuded preparations could result from a direct P_{2y} receptor action on smooth muscle or the production of adenosine from ATP by ectonucleotidases during incubation.

GTP also increased cyclic GMP and relaxed endothelium-denuded artery rings. Since the removal of the endothelium completely abolished the effect of acetylcholine on relaxation and cyclic GMP, the effect of GTP on endothelium-denuded rings cannot be explained by an EDRF-mediated mechanism.

Adenosine relaxed both endothelium-intact and -denuded mesenteric artery rings, in accordance with earlier results (Furchgott, 1984). The relaxant effect of adenosine was slightly more pronounced in endothelium-intact preparations, although no significant changes were observed in cyclic nucleotide levels of either endothelium-intact or -denuded artery rings. It has been suggested that adenosine A_2 receptors which stimulate adenylate cyclase mediate vasodilatation in major vessels (Collis & Brown, 1983; Ramagopal *et al.*, 1988; Olsson & Pearson, 1990). However, no clear correlation between adenosine-induced relaxation and accumulation of cyclic AMP has been found in smooth muscle cells (Olsson & Pearson, 1990). In rat aorta, adenosine has been reported to induce the production of EDRF via endothelial A_2 receptors and thus stimulate smooth muscle soluble guanylate cyclase. This adenosine-induced EDRF-release was found to decrease with increasing age and to be absent in the aorta of 12 week-old rats. The aortic cyclic AMP levels were not affected by adenosine (Moritoki *et al.*, 1990). In cultured vascular smooth muscle cells adenosine has been shown to stimulate particulate guanylate cyclase (Kurtz, 1987). However, in the present study, adenosine did not affect cyclic GMP levels in either endothelium-intact or -denuded preparations.

The relaxant effects of guanosine on endothelium-intact and -denuded rat artery rings were almost identical to the effects of adenosine, but guanosine produced an increase in cyclic GMP in both vascular preparations. The guanosine-induced relaxation of intact rings was inhibited by L-NAME only at low guanosine concentrations, and L-arginine not only reversed the action of L-NAME, but clearly enhanced the relaxation to guanosine. This indicates that the endothelium-dependent relaxation by guanosine is only partly due to EDRF, and suggests that guanosine has a direct effect on smooth muscle which is more pronounced than that of GTP. Thus, the response to guanosine in endothelium-intact rings is a combination of endothelium-dependent and -independent effects.

The present results show that exogenous ATP and adenosine can cause both endothelium-dependent and -independent relaxations of precontracted rat mesenteric artery rings, in confirmation of previous studies (Furchgott, 1984; White & Angus, 1987; Olsson & Pearson, 1990; Moritoki *et al.*, 1990; Rose'meyer & Hope, 1990). ATP increased the cyclic GMP of endothelium-intact mesenteric rings, but adenosine did not affect cyclic nucleotide levels of intact and denuded preparations. On the other hand, exogenous GTP and guanosine increased cyclic GMP accumulation and caused concentration-related relaxation of both endothelium-intact and -denuded rat mesenteric artery rings. The mechanisms underlying these effects of GTP and guanosine are not known. GTP is also the substrate for producing cyclic GMP by guanylate cyclase. To enter an intact cell, GTP must first undergo sequential hydrolysis to guanosine by ectonucleotidases. In guinea-pig urinary bladder, GTP and other nucleotide triphosphates all lost their terminal phosphate groups by ectonucleotidase action at the same half-life rate of about 15 min, which means that the production of guanosine from GTP is an even slower processs (Welford *et al.*, 1987). Extracellular guanosine, taken up by cardiac myocytes was found to be phosphorylated slowly to guanine nucleotides, the majority of guanosine (98%) still existing free after 1 min (Geisbuhler *et al.*, 1987). In the present study, GTP and guanosine increased cyclic GMP in both intact and denuded artery rings after a 2 min exposure. Thus these results are unlikely to be explained by a pure substrate effect of GTP on guanylate cyclase. Extracellular guanosine 5'-(β-thio)diphosphate, GTP and GDP have all been reported to inhibit agonist-induced platelet aggregation (Krishnamurthi *et al.*, 1988), in accordance with our recent work on human platelets (Laustiola *et al.*, 1991). This suggests an extracellular site of action for these nucleotides mediating the inhibition of platelet aggregation. Our earlier results show that GTP can increase cyclic GMP and potentiate cyclic GMP accumulation caused by sodium nitrite both in platelets and in endothelium-denuded rat mesenteric arteries (Laustiola *et al.*, 1991). Since guanylate cyclase activity in platelets is almost totally in the soluble fraction (Tremplay *et al.*, 1988) and sodium nitrite is an activator of soluble guanylate cyclase (Waldman & Murad, 1987), it seems evident that the soluble guanylate cyclase is stimulated.

Recently it has been shown that neurally-mediated vasodilatation in the sheep middle cerebral artery is mediated by vasoactive intestinal polypeptide through a cyclic GMP-mediated mechanism that appears to involve synthesis of nitric oxide from L-arginine (Gaw *et al.*, 1991). Interleukin-1β induces the production of a transferrable factor from cultured smooth muscle cells which relaxes vascular smooth muscle and stimulates the production of cyclic GMP (Schini *et al.*, 1991). The stimulation of membrane receptors in NG 108-15 cells also increases cyclic GMP accumulation, an effect inhibited by pertussis toxin (Kurose & Ui, 1985) and a cell surface receptor activates Mg^{2+}-dependent guanylate cyclase in the cellular slime mold *Dictyostelium discoidenum* (Janssens *et al.*, 1989).

The results presented here raise the possibility of a cell membrane site of action for GTP and guanosine which mediates the activation of soluble guanylate cyclase and leads to increased cyclic GMP accumulation and relaxation of arterial smooth muscle.

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The effects of chronic treatment with the dihydropyridine, Bay K 8644, on hyperexcitability due to ethanol withdrawal, *in vivo* and *in vitro*

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1 The effects of chronic treatment with the dihydropyridine, Bay K 8644, were studied on the ethanol withdrawal syndrome, *in vivo* and *in vitro*.

2 Addition of racemic Bay K 8644 to the drinking mixture, throughout the chronic ethanol treatment, decreased the behavioural excitability seen during ethanol withdrawal *in vivo*.

3 All the signs of hyperexcitability in field potentials in the isolated hippocampal slice, caused by ethanol withdrawal, were decreased by the chronic administration of Bay K 8644.

4 These effects resembled those previously reported for chronic administration of calcium channel antagonists; racemic Bay K 8644 has both calcium channel activating and antagonist properties.

5 Measurement of brain levels of Bay K 8644 at the end of the chronic treatment showed that the compound reached micromolar concentrations during the treatment, but none could be detected in the tissues at the time of the above measurements.

6 It is possible that the results might be explained by predominance of the calcium channel antagonist properties of this compound, owing to the high central concentrations achieved during the treatment. Tolerance to the calcium channel activating properties of Bay K 8644 may also have occurred during the chronic treatment.

Keywords: Ethanol; withdrawal syndrome; calcium channels; dihydropyridine; hippocampus

Introduction

Ethanol has acute actions on many systems, but the basis of the dependence that develops on chronic treatment is not certain. There has been much interest recently in the actions of ethanol on calcium-mediated control mechanisms in neurones. Calcium spikes in cultured neurones were decreased by ethanol (Stokes & Harris, 1982). Ethanol decreased the fast phase of depolarization-induced calcium flux into neurones and tolerance developed to this effect on chronic treatment (Harris & Hood, 1980; Friedman *et al.*, 1980).

Chronic ethanol treatment has been found to increase the number of dihydropyridine-sensitive high affinity binding sites in the CNS (Dolin *et al.*, 1987). These sites are thought to correspond to voltage-sensitive calcium channels, of the high-voltage activated subtype. They do not appear to play a large part in normal neuronal activity, although effects have been reported in neurones under experimental conditions leading to prolonged depolarization (Docherty & Brown, 1986a; Louvel *et al.*, 1986; Jones & Heinemann, 1987; Takahashi *et al.*, 1989; Grover & Teyler, 1990).

Dihydropyridine calcium channel antagonists were found to have a protective effect against the ethanol withdrawal syndrome *in vivo*; this effect was stereospecific (Littleton *et al.*, 1990). We have also found that the dihydropyridine calcium channel antagonist, PN 200-110, stereospecifically prevented all the measured signs of hyperexcitability in the isolated hippocampal slice caused by withdrawal from chronic ethanol treatment *in vivo* (Whittington & Little, 1991a). The changes in the field potentials, caused by ethanol withdrawal, included decreases in thresholds for production of population spikes, following both orthodromic and antidromic stimulation, increases in paired pulse potentiation and shifts to the left of the input/output curves (Whittington & Little, 1990a; 1991a,b). They followed different time courses during the withdrawal period, suggesting different origins of the forms of hyperexcitability.

Adaptive responses to chronic ethanol administration can be modulated by concurrent chronic treatment with a dihydropyridine calcium channel antagonist. Nitrendipine, given concurrently with ethanol in chronic treatment, prevented the development of tolerance to ethanol (Wu *et al.*, 1987; Little & Dolin, 1987; Dolin & Little, 1989). Nitrendipine, given chronically with ethanol, also prevented the ethanol withdrawal syndrome (Whittington *et al.*, 1991) and decreased the electrophysiological manifestations of withdrawal described above (Whittington & Little, 1991b). These effects were considered to be responses to the continued presence of nitrendipine, rather than acute actions, as the CNS concentrations at the time of testing were too low to produce acute effects in either the tolerance studies or on the withdrawal measurements (Dolin & Little, 1989; Whittington *et al.*, 1991). The increase in the number of dihydropyridine binding sites, measured after chronic ethanol administration, was also prevented, suggesting a possible causal relationship between the changes at these sites and development of ethanol tolerance and dependence (Dolin *et al.*, 1988a; Dolin & Little, 1989). Long-term administration of a dihydropyridine calcium channel antagonist causes down-regulation of the high affinity binding sites (Panza *et al.*, 1985). We have put forward the theory that the effects of concurrently administered calcium channel antagonists in preventing ethanol dependence were due to the prevention of the upregulation of voltage-sensitive calcium channels (Littleton & Little, 1989; Whittington *et al.*, 1991; Little, 1991).

The dihydropyridine, Bay K 8644, has been shown to increase the opening of voltage-sensitive calcium channels (Schramm *et al.*, 1983; Brown *et al.*, 1984; Nowycky *et al.*, 1985) and this effect of Bay K 8644 has been shown to be the property of the (−)-isomer (Hof *et al.*, 1985). In the present study we have measured the effects of chronic treatment with Bay K 8644, on the ethanol withdrawal syndrome and on the changes in field potentials in the hippocampal slice following chronic ethanol treatment. Bay K 8644 was given in the drinking fluid with the ethanol, throughout the chronic treatment. It was necessary to use the racemate in this chronic

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treatment study, because insufficient of the isomers was available.

Methods

Chronic drug treatment

Male mice, C57 strain, 25–30 g, were given ethanol, 24% v/v, as sole fluid, for 18 weeks. Racemic Bay K 8644 was dissolved in the ethanol and the solution then added to the drinking fluid. The dihydropyridine was given with the ethanol, at 71 μM, throughout the drinking period. Bay K 8644 was removed from the drinking fluid 24 h before the experiments (the ethanol remaining) so that the effects of chronic Bay K 8644 treatment, rather than any acute action, could be studied. Controls drank tap water only. Owing to the low aqueous solubility of Bay K 8644 it was not possible to treat animals by this method with the compound in the absence of ethanol.

The addition of Bay K 8644 did not alter the ethanol intake; the mean intakes of ethanol were $16.0 \pm 1.7\,g\,kg^{-1}$ daily ($n = 15$ mice) for the ethanol alone treated group, and $15.3 \pm 2.7\,g\,kg^{-1}$ daily ($n = 16$ mice) for the ethanol plus Bay K 8644 treated group. The intake of Bay K 8644 was $1.9 \pm 0.3\,mg\,kg^{-1}$, daily ($n = 16$ mice) during the 18 week treatment period. Mice were taken at random from the treatment groups for preparation of the hippocampal slices.

Bottles containing Bay K 8644 were protected from light, as the compound is light-sensitive. The mice were weighed at regular intervals during the chronic treatment and there were no significant differences between the weights of any of the treatment groups. Bay K 8644 (Bayer AG) is methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethyl)phenyl-pyridine-5-carboxylate.

The ethanol withdrawal syndrome

The ethanol withdrawal syndrome was measured by ratings of convulsive behaviour (Goldstein & Pal, 1971; Little *et al.*, 1990). This method measures both tremor and clonic convulsions on a continuous scale. The mice were removed from the cages containing the ethanol drinking mixture between 09 h 00 min and 09 h 30 min, 24 h after removal of Bay K 8644 from the drinking solution. Behavioural ratings were made once an hour on all mice for the next 12 h, by an observer who was unaware of the prior drug treatment. The treatment groups were tested concurrently. The numbers in each group in this experiment were 8 for those drinking ethanol alone, and 10 for those receiving ethanol plus Bay K 8644.

For comparison, control (drug-naive) C57 mice were given intraperitoneal injections of (±)-Bay K 8644, $2\,mg\,kg^{-1}$, suspended in Tween 80, 0.5%, or the vehicle, $n = 8$ per treatment group. Ratings were made of convulsive behaviour on handling, as above, hourly for the next 12 h.

Statistical analysis of withdrawal ratings

The ratings of the withdrawal syndrome, expressed as median and interquartile range, were compared by nonparametric analysis of variance, designed for repeat measurements on the same samples and multiple comparisons with controls (Meddis, 1984). Comparisons were made on the ratings over the whole 12 h period from the start of withdrawal. During the first 2 h the withdrawal syndrome was beginning, but was not seen clearly until about 3 h.

Electrophysiological recordings

Hippocampal slices were prepared between 09 h 00 min and 10 h 00 min immediately on removal of the ethanol drinking solution (i.e. 24 h after removal of Bay K 8644 from the drinking solution), and perfused with standard Ringer solution. The animals were not withdrawn from the ethanol before preparation of the slices, so ethanol would have been washed out of the slices during the first part of the recording period. Hippocampal slices, 400 μm thick, were prepared as described previously, (Whittington & Little, 1990a), and maintained in Krebs solution at 30°C ± 0.5°C, flow rate $1.5\,ml\,min^{-1}$. Extracellular recordings from the stratum pyramidale in area CA1 were started 30 min after the slices were placed in the perfusion bath, that is 45 min from removal of tissues. The times give in the results sections are all from removal of tissues, i.e. from withdrawal of ethanol.

Schaffer collateral/commissural fibres were stimulated using paired pulses, constant current, 50 μs duration, every 10 s throughout the 7 h recording period. The stimulating electrodes consisted of two silver insulated wires, 0.2 mm in diameter, passed through 1 mm diameter silicon tubing. Recording electrodes were made from 1.2 mm diameter electrode glass and filled with 2 M KCl; the resistances were between 5 and 15 MΩ. A 70 ms interval was used for the paired pulses because preliminary experiments showed that it gave the maximum paired pulse potentiation of the population spike in normal mouse hippocampal slices. A stimulation level of 1.25 times the threshold for eliciting a single population spike was used in the studies on paired pulse potentiation.

The thresholds for the production of population spikes, and levels of paired pulse potentiation of these responses, were measured every 15 min. Measurements were made of the thresholds for eliciting single population spikes and multiple population spikes as described previously (Whittington & Little, 1990a). Input/output curves were determined at 1, 3, 5, and 7 h from the preparation of tissues. These were made for the population spikes and for the slopes of the field e.p.s.ps (excitatory postsynaptic potentials). The field e.p.s.ps were recorded from the cell body area, so the measurements were made at some distance from the actual origin of the dendritic e.p.s.ps.

Certain precautions were taken to ensure that valid comparisons could be made between the field potentials from tissues from different treatment groups (Whittington & Little, 1990a). The order of testing and the distribution of the various treatments between the two recording chambers were carefully balanced. The positions of the stimulating and recording electrodes were determined in every tissue by using the ends of the arc of granular cells in the dentate gyrus and the apex of the CA3 region above the fimbria as common reference points between slices.

The composition of the perfusion fluid was (mM): NaCl 124, KCl 3.25, NaH_2PO_4 1.25, $NaHCO_3$ 20.0, $MgSO_4$ 2.0, $CaCl_2$ 2.0, and D-glucose 10.0. The pH was 7.2 at 30°C. At the completion of every day's experiments the perfusion chamber was washed through with distilled water for at least 30 min, and washed through every 5 days with sodium hydroxide followed by citric acid.

Statistical analysis of electrophysiological results

The areas of the population spikes were measured by Acorn computer analysis. In every case a minimum of three responses was averaged for each stimulus. The results in the figures are expressed as mean ± s.e.mean for each of the different treatments. A minimum of five tissues, all from different animals, were studied in each treatment group. Comparisons were made between results from tissues following the different treatments by two-way analysis of variance. The times given in the results section are all from the removal of the tissues. This corresponded with the withdrawal of ethanol.

Input/output data, derived from population spike area and field e.p.s.p. slope measurements, were fitted to a logistic equation by non-linear regression. Analysis of the resulting sigmoidal curves allowed for calculation of maximum responses, the stimulus required to produce half-maximal responses (S_{50}), and the slope of the stimulus/response relationship. Comparisons of data from these curves were

made between different tissues, following the various treatments, by Student's nonpaired t test.

Measurement of central Bay K 8644 concentrations

Measurements were made of the concentrations of Bay K 8644 in brains removed from separate groups of animals receiving the chronic treatment, to determine how much Bay K 8644 was present during the treatment and at the time of testing. The brains were removed, following cervical dislocation, from C57 mice, that had been drinking the ethanol and Bay K 8644 solution for 18 weeks as described above. The tissues were taken at the end of the chronic treatment at 10 h 00 min, and 8 h after withdrawal of ethanol (i.e. before and 32 h after removal of Bay K 8644 from the drinking solution). The 8 h timepoint was chosen to correspond with the peak drug effect in the withdrawal testing. Six tissues were taken per treatment group. For comparison, brains were also taken from control mice ($n = 6$) 1 h after i.p. injection of racemic Bay K 8644, 2 mg kg^{-1}.

Whole brain Bay K 8644 concentrations were measured by gas/liquid chromatography. Electron capture detection was used to analyse samples separated with a 25 meter capillary column coated with SE30. The temperature programming for the analysis was as follows: injector 280°C, detector 320°C, oven started at 180°C for 2 min then ramped up to 290°C at 16°C min^{-1} and left at this temperature for 10 min. Helium carrier gas at 6 p.s.i. was used to ensure the flow of sample along the column at the optimum speed for separation. The sample was flushed from the end of the capillary column into the detector with nitrogen make-up gas at 20 p.s.i.

The brains were homogenized in 10 M NaOH, 1 ml per 50 mg tissue wet weight, and residual proteins precipitated with sodium tungstate and sulphuric acid. The samples were centrifuged at 4000 r.p.m. for 10 min, then the Bay K 8644 was extracted by shaking with the same volume of toluene. The organic layer was removed and dried with anhydrous $MgSO_4$. Samples were evaporated to dryness and reconstituted in 100 μl toluene containing 1 μM nimodipine as internal standard.

Dihydropyridine binding

Central dihydropyridine receptor binding was measured by the method of Glossman & Ferry (1985), using [^{3}H]-nimodipine (specific activity 130 Ci mmol^{-1}). Brains were removed 8 h after cessation of ethanol drinking (i.e. 32 h after removal of Bay K 8644 from the drinking solution), and the cerebral cortices dissected out. The tissues were homogenized in Tris buffer, 50 mmol and centrifuged (45,000 g, 15 min) and washed with the buffer three times. Tissue aliquots were incubated (25°C, 45 min) with [^{3}H]-nimodipine, 0.125 nM to 4 nM, then filtered (Whatman GF/C glass filters) and counted. All measurements were made in triplicate. Nonspecific binding was estimated with 1 μM nitrendipine. The protein content of the tissues was measured with Folin's reagent. The results were subjected to Scatchard analysis to give the receptor density, B_{max}, in fmol mg^{-1} protein and the dissociation constant, K_d, in nM.

Results

Behavioural studies

The ethanol withdrawal syndrome The addition of Bay K 8644 to the drinking mixture significantly decreased the ethanol withdrawal syndrome (Figure 1). The ratings for the animals that drank ethanol alone were significantly higher than those that received ethanol plus Bay K 8644, when compared over the time period 1 h to 12 h from ethanol withdrawal ($P < 0.001$). The protection against the withdrawal syndrome was complete, as the values after treatment with ethanol plus Bay K 8644 were not significantly different from controls ($P > 0.05$).

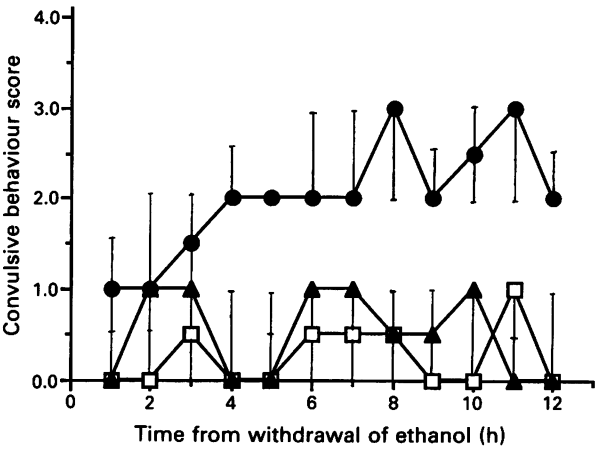

Figure 1 Convulsive behaviour on handling measured after withdrawal from chronic treatment with ethanol or ethanol plus Bay K 8644. The dihydropyridine was removed from the drinking fluid 24 h before ethanol withdrawal. Values after treatment with ethanol alone were significantly higher than control values or those after treatment with ethanol plus Bay K 8644, $P < 0.001$, over the whole test period. Values are median ± interquartile ranges. Controls (□); ethanol (●); ethanol plus Bay K 8644 (▲).

The effects of acute Bay K 8644 When an acute injection of Bay K 8644 was given to control animals the measurements of convulsive behaviour were increased (Figure 2). This effect lasted for 2 h, then declined over the next 4 h. The difference from control values was significant over the period 1 h to 6 h from injection ($P < 0.001$).

Electrophysiological recordings

Thresholds for eliciting population spikes As shown previously (Whittington & Little, 1990a), chronic ethanol treatment lowered the thresholds for eliciting both single and multiple population spikes in the CA1 area. These effects were significant over the time period 2 h to 7 h from ethanol withdrawal ($P < 0.001$). Chronic administration of Bay K 8644 significantly decreased the effects of chronic ethanol on both these thresholds (Figures 3 and 4). The difference between the thresholds after treatment with ethanol alone and ethanol plus Bay K 8644 was significant from 3 h to 7 h ($P < 0.001$). An example of the form of multiple spiking seen during ethanol withdrawal, and the absence of this form of spiking is shown in Figure 5.

Paired pulse potentiation The paired pulse potentiation of the population spike was increased after ethanol treatment

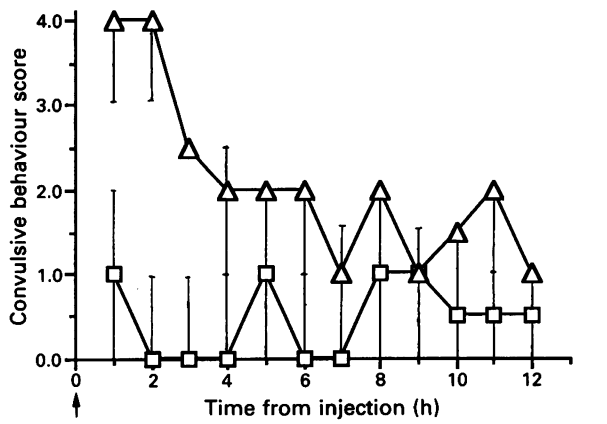

Figure 2 Convulsive behaviour on handling measured after injection of control mice with Bay K 8644, 2 mg kg^{-1}, or its Tween vehicle. Values after Bay K 8644 were significantly higher than control values, $P < 0.001$, between 1 h and 6 h from injection. Values are median ± interquartile ranges. Controls (□); Bay K 8644 (△).

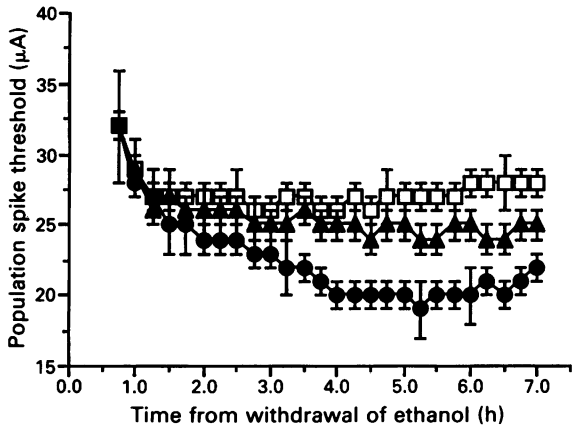

Figure 3 Thresholds for production of single population spikes in isolated hippocampal slices prepared after chronic treatment *in vivo* with ethanol or ethanol plus Bay K 8644. (The dihydropyridine was removed from the drinking fluid 24 h before slice preparation.) Values after treatment with ethanol alone were significantly lower than control values or those after treatment with ethanol plus Bay K 8644, $P < 0.001$, over the whole test period. Values are mean with s.e.mean shown by vertical bars. Controls (□); ethanol (●); ethanol plus Bay K 8644 (▲).

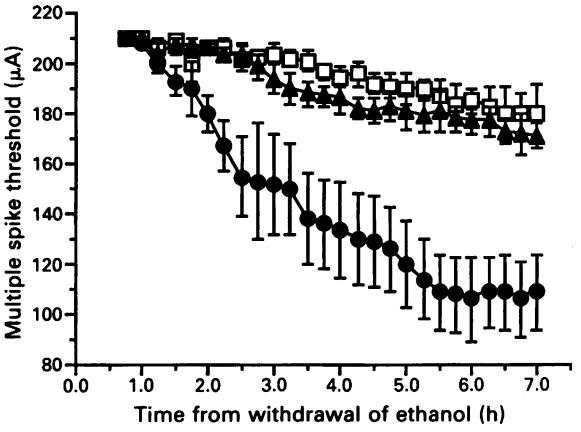

Figure 4 Thresholds for production of multiple population spikes in isolated hippocampal slices prepared after chronic treatment *in vivo* with ethanol or ethanol plus Bay K 8644. Values after treatment with ethanol alone were significantly lower than control values or those after treatment with ethanol plus Bay K 8644, $P < 0.001$, over the whole test period. Values are mean with s.e.mean shown by vertical bars. Controls (□); ethanol (●); ethanol plus Bay K 8644 (▲).

(Figure 6), but, as we have demonstrated previously (Whittington & Little, 1990a; 1991a,b), this change followed a different time course from that of the changes in thresholds, being maximal at 2 h from withdrawal, and returning to control values by between 3.5 h and 4 h. The chronic Bay K 8644 treatment considerably, and significantly, reduced the effects of chronic ethanol on paired pulse potentiation. The difference between the results after ethanol alone and after ethanol plus Bay K 8644 was significant ($P < 0.001$) when compared over the time period 1 h to 4 h from ethanol withdrawal.

Input/output curves The input/output curves for the population spike areas showed a shift to the left after chronic ethanol treatment, measured as a decrease in the minimum stimulus intensity required to evoke half-maximal population spikes (S_{50}). A significant decrease in the gradients after chronic ethanol treatment was also seen between 3 h and 7 h into withdrawal (see Table 1). These changes were significantly decreased by the addition of Bay K 8644 to the drinking mixture ($P < 0.05$). The input/output curves for the field e.p.s.p. slopes did not show any significant changes after the chronic ethanol alone, or ethanol and Bay K 8644 treatment schedules ($P > 0.05$, for comparison with control data) (Table 1). An example of the input/output curves obtained is shown in Figure 7, drawn from data obtained 7 h after ethanol withdrawal.

Table 1 Measurements from recordings of field potentials made from hippocampal slices prepared after chronic treatment *in vivo* with either ethanol or ethanol plus Bay K 8644, compared with control values

Time (h)	1	3	5	7
Slopes of input/output curves for population spike areas				
Control	4.7 ± 0.7	8.0 ± 0.5	7.0 ± 0.4	7.1 ± 0.3
Ethanol	6.1 ± 0.6	4.2 ± 0.4*	5.2 ± 0.3*	4.8 ± 0.3*
Ethanol plus Bay K 8644	5.4 ± 0.5	6.1 ± 0.6	8.0 ± 0.7	7.2 ± 0.5
S_{50} *values for population spike areas* (μA)				
Control	36.5 ± 0.9	35.5 ± 0.3	35.7 ± 0.3	35.9 ± 0.3
Ethanol	38.0 ± 0.6	30.1 ± 0.8*	19.2 ± 0.4*	27.8 ± 0.4*
Ethanol plus Bay K 8644	37.2 ± 0.7	34.1 ± 0.5	32.4 ± 0.4	33.0 ± 0.3
Maximum values for population spike areas (μVs)				
Control	10.9 ± 0.4	11.5 ± 0.3	11.8 ± 0.4	12.0 ± 0.5
Ethanol	10.3 ± 0.3	10.4 ± 0.6	10.5 ± 0.3	10.2 ± 0.4
Ethanol plus Bay K 8644	10.9 ± 0.3	10.9 ± 0.2	11.2 ± 0.2	10.5 ± 0.2
Slopes of input/output curves for field e.p.s.ps				
Control	1.9 ± 0.8	3.5 ± 0.6	3.1 ± 0.5	3.7 ± 0.6
Ethanol	3.7 ± 0.3	4.0 ± 0.4	4.0 ± 0.7	4.0 ± 0.6
Ethanol plus Bay K 8644	3.4 ± 0.4	3.1 ± 0.5	3.2 ± 0.5	3.7 ± 0.4
S_{50} *values for field e.p.s.ps* (μA)				
Control	67.6 ± 2.7	41.2 ± 3.2	38.2 ± 2.1	39.7 ± 2.7
Ethanol	44.7 ± 2.0	35.0 ± 3.1	35.5 ± 2.8	39.0 ± 1.9
Ethanol plus Bay K 8644	38.2 ± 6.7	39.8 ± 4.0	45.0 ± 5.7	41.3 ± 3.2
Maximum field e.p.s.p. slope ($V s^{-1}$)				
Control	5.3 ± 1.9	3.4 ± 1.2	3.1 ± 0.3	3.0 ± 0.2
Ethanol	3.0 ± 1.4	3.0 ± 0.5	3.2 ± 0.3	2.7 ± 0.2
Ethanol plus Bay K 8644	2.6 ± 0.7	3.0 ± 0.3	3.8 ± 0.4	3.3 ± 0.3

All results are expressed as mean ± s.e.mean. Times (h) are from withdrawal of ethanol.
* $P < 0.05$, Student's non-paired t test; comparison with control slices.

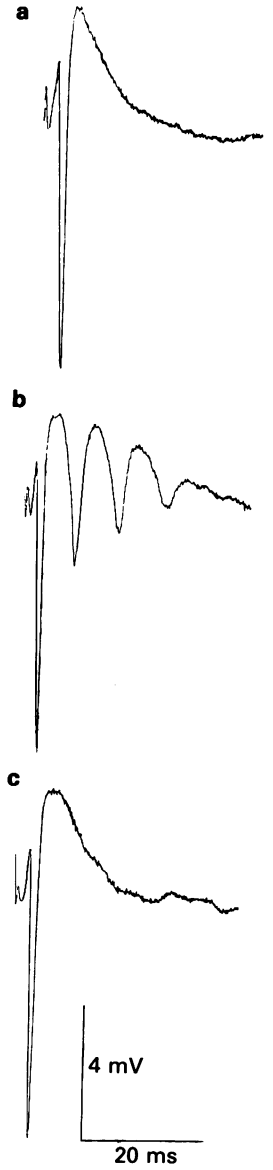

Figure 5 Example of traces showing the type of multiple spiking seen after chronic treatment with ethanol alone. Traces were recorded 7 h after cessation of chronic ethanol treatment. The stimulus intensity was 150 μA for each of the three recordings. (a) Response from slices from control mice; (b) response from slices from mice treated with ethanol alone; (c) response from slices from mice treated with ethanol and Bay K 8644.

Brain concentrations of Bay K 8644

The mean value ($\pm$ s.e.mean) for the central concentrations of Bay K 8644 at the end of the chronic treatment period was $1159 \pm 179\,ng\,g^{-1}$ wet tissue weight. This corresponded to $3.26 \pm 0.5\,\mu M$, assuming equal distribution throughout the brain. During the withdrawal period, 32 h from removal of Bay K 8644 from the drinking fluid and 8 h from withdrawal of ethanol, the concentrations were not detectable. The lowest concentration that could be detected with our assay was $53\,ng\,g^{-1}$ or 150 nM. One hour after acute administration of $2\,mg\,kg^{-1}$ Bay K 8644, in naive mice, the mean central concentration was $2150 \pm 432\,ng\,g^{-1}$ wet tissue weight, corresponding to $6.07 \pm 1.22\,\mu M$.

Dihydropyridine binding

The data from the receptor binding studies (Table 2) suggested that addition of Bay K 8644 to the chronic ethanol treatment may prevent the increase in the number of cortical dihydropyridine binding sites. However, in this study, there was a high degree of variability in the measurements after ethanol administration, and the increase did not reach statistical significance ($P > 0.05$). Measurement of the K_d for dihydropyridine binding showed a significant increase after chronic ethanol treatment ($P < 0.05$), but not after chronic ethanol and Bay K

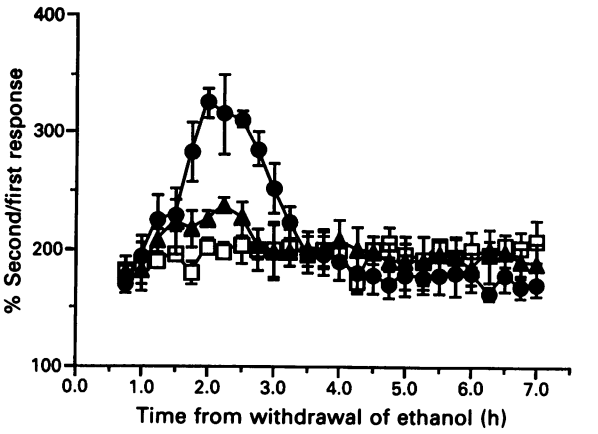

Figure 6 Paired pulse potentiation in isolated hippocampal slices prepared after chronic treatment *in vivo* with ethanol or ethanol plus Bay K 8644. Values after treatment with ethanol alone were significantly higher between 1 h and 4 h from ethanol withdrawal, than control values or those after treatment with ethanol plus Bay K 8644, $P < 0.001$. Values are mean with s.e.mean shown by vertical bars. Controls (□); ethanol (●); ethanol plus Bay K 8644 (▲).

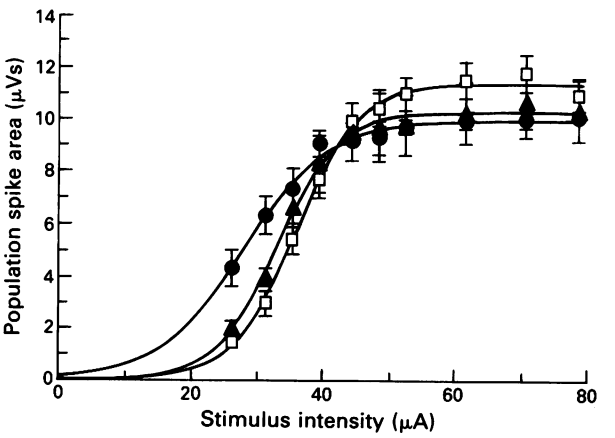

Figure 7 Changes in input/output relationships derived from population spike data taken 7 h after preparation of slices. Values of curve slope and S_{50} after treatment with ethanol alone were significantly lower than after treatment with ethanol and Bay K 8644 ($P < 0.05$). Values are mean with s.e.mean shown by vertical bars. Controls (□); ethanol (●); ethanol plus Bay K 8644 (▲).

Table 2 B_{max} and K_d values for [^{3}H]-nimodipine binding after chronic treatment *in vivo* with either ethanol or ethanol plus Bay K 8644, compared with control values

B_{max} (fmol mg^{-1})	
Controls	174 ± 14 (8)
Ethanol	227 ± 32 (7)
Ethanol plus Bay K	208 ± 8 (7)
K_d (nM)	
Control	1.62 ± 0.27
Ethanol	3.01 ± 0.98*
Ethanol plus Bay K	2.12 ± 0.98

All results are expressed as mean ± s.e.mean. The values were derived from cerebrocortical tissue, taken 8 h from the cessation of ethanol drinking.
* $P = 0.04$, Student's t test for comparison with control data.

8644 treatment ($P > 0.05$), when comparisons were made with control values (Table 2).

Discussion

The addition of Bay K 8644 to the chronic ethanol treatment completely prevented the ethanol withdrawal syndrome in mice *in vivo* and significantly decreased the electrophysiological signs of withdrawal in the isolated hippocampal slices. These actions closely resembled the effects previously reported for chronic treatment with the calcium channel antagonist, nitrendipine (Whittington *et al.*, 1991; Whittington & Little, 1991b). Bay K 8644 is normally considered to increase calcium channel opening (Brown *et al.*, 1984; Nowycky *et al.*, 1985), but it may have other actions (see below).

In the present study, after removal of Bay K 8644 from the drinking fluid for the last 24 h of ethanol intake, the central concentrations fell to levels below the detection of the assay, that is below about 150 nM. This is lower than would have been required to cause behavioural changes by an acute action. We conclude, therefore, that the effects seen were due to the presence of Bay K 8644 in the CNS during the ethanol chronic treatment, rather than to any acute actions at the time of testing. The concentration of any residual Bay K 8644 in the hippocampal slices during recording would also have been lower than those required to produce acute effects on the withdrawal hyperexcitability (see below). A dose of $2\,mg\,kg^{-1}$ was chosen to illustrate the acute effects, and this produced central concentrations of around $6\,\mu M$ at a time when the excitatory action was maximal. This action is lost at doses below about $1\,mg\,kg^{-1}$ (our unpublished results and O'Neill & Bolger, 1988). The central concentrations measured in the present study after the acute dosing are similar to those reported by O'Neill & Bolger (1988), who found a level of approximately $2\,\mu M$ in mouse brain 15 min after $2\,mg\,kg^{-1}$ i.p.

When given acutely to control mice, racemic Bay K 8644 caused a pattern of behaviour that resembled in some respects that seen during ethanol withdrawal. The ratings of convulsive behaviour on handling were increased. Bolger *et al.* (1985) have reported that single doses of the racemate produced signs of hyperexcitability in naive mice. We found previously that a dose of $2\,mg\,kg^{-1}$ of the racemate did not significantly affect the ethanol withdrawal syndrome when given acutely on withdrawal, but did prevent the protective action of the calcium channel antagonist, nimodipine (Littleton *et al.*, 1990). However this was not a simple interaction, as the withdrawal syndrome was increased by the combination of nimodipine plus Bay K 8644. We have also shown that the (−)-isomer of Bay K 8644 increased the ratings of convulsive behaviour during ethanol withdrawal (unpublished results).

The results from the receptor binding studies appeared to follow the same pattern as that seen in the behavioural and electrophysiological studies, but the interpretation of these results is complicated by the lack of a significant change in receptor density after the chronic treatment with ethanol alone. The reason for the large standard error in these measurements and the increase in K_d values was not clear, as we have previously demonstrated consistent increases in B_{max}, with no change in K_d after such treatment (Dolin *et al.*, 1987; Whittington *et al.*, 1991). The effects of Bay K 8644 on the upregulation of calcium channels in cultured cells caused by growth in ethanol have been studied by two groups. Marks *et al.* (1989) showed that cultured PC12 cells, grown in medium containing (±)-Bay K 8644 (3 nM) showed an increase in dihydropyridine binding site number, although this change was not additive with the increase in such binding produced by ethanol. Brennan *et al.* (1989) grew PC12 cells in a higher concentration of (±)-Bay K 8644 (50 nM) and found the increase in dihydropyridine binding caused by addition of ethanol to the growth medium was prevented by the presence of the Bay K 8644. These results suggest that adaptations to the calcium channel activating and antagonist properties (according to the concentration used) of Bay K 8644 may be demonstrated after chronic treatment, when the interactions with ethanol are studied. Direct comparisons of the absolute Bay K 8644 concentrations used in these studies and in the present work, however, are not necessarily valid, because cultured cells are known to be more sensitive to dihydropyridines than mammalian tissues *in vivo* or *ex vivo*.

We have previously shown that when applied to hippocampal slices in the bathing medium at 500 nM or $2\,\mu M$, (−)-Bay K 8644 considerably potentiated the signs of hyperexcitability in the isolated hippocampal slice after chronic ethanol treatment, while the (+)-isomer had protective effects (Whittington & Little, 1990b). The brain concentrations of each of the isomers during the chronic treatment in the present study would have been in the same range as the concentration that increased (minus isomer) or decreased (plus isomer) the effects of ethanol withdrawal when added *in vitro*.

The demonstration of similar effects with a calcium channel activator and a calcium channel antagonist seem contradictory, but the results may have been due to the fact that racemic Bay K 8644 is not a pure calcium channel activator. It is well established that the effects of Bay K 8644 are stereospecific, the calcium channel activating properties of Bay K 8644 residing in the (−)-isomer, while the (+)-isomer is a calcium channel antagonist, both *in vitro* (Schramm *et al.*, 1983; Hof *et al.*, 1985; Franckowiak *et al.*, 1985) and *in vivo* (O'Neill & Bolger, 1988). In addition, *in vitro* studies have shown loss of effects and possible antagonist actions at high concentrations of Bay K 8644, as the dose-response curves in many studies showed a bell-shaped pattern. Concentrations over 100 nM of the (−)-isomer were found, by Franckowiak *et al.* (1985), to have less effect on cardiac tissues than lower concentrations. Biphasic dose-response curves have been demonstrated for the racemate in cardiac preparations (Dube *et al.*, 1985). Hess *et al.* (1984) did not report biphasic effects on cardiac cells at concentrations of the racemate up to $10\,\mu M$, but these authors mentioned that a 'partial antagonist' action was seen when strong depolarizations were applied. Few studies of the effects of Bay K 8644 on neuronal preparations have investigated a range of concentrations, but White & Bradford (1986) demonstrated biphasic effects of Bay K 8644 on the stimulation of calcium uptake by synaptosomes. The potentiating effect of Bay K 8644 was maximal at 1–10 nM and disappeared as the concentration was raised to $1–10\,\mu M$. The stimulation of inositol phospholipid hydrolysis by Bay K 8644 was half-maximal at $0.3\,\mu M$ and declined at concentrations over $10\,\mu M$ (Kendall & Nahorski, 1985).

The effects of Bay K 8644 may also be influenced by the membrane potential. In patch-clamp studies on ventricular myocytes, the (−)-isomer and the racemate of Bay K 8644 have been shown to increase calcium currents evoked from negative, non-depolarizing, potentials, but to possess calcium channel antagonist properties on currents evoked from more positive holding potentials (Sanguinetti & Kass, 1984; Sanguinetti *et al.*, 1986; Kass, 1987). Brown *et al.* (1984), however, did not find this pattern. It is possible that the some of the reported *in vitro* effects of Bay K 8644 may have been influenced by the solvent used. In many studies the drug was dissolved in ethanol and interactions between ethanol and dihydropyridines are well established (see Introduction). Even if control experiments showed no solvent effect, this does not exclude the occurrence of interactions when the two drugs are applied together.

There is some evidence that the application of Bay K 8644 may produce effects at sites other than the L-subtype of calcium channel. In hypothalamic neurones, Akaike *et al.* (1989) demonstrated a blocking action on the low threshold, T-subtype, of calcium channel, at Bay K 8644 concentrations above $10\,\mu M$. Docherty & Brown (1986a) found increases in the transient calcium current in hippocampal CA1 neurones at $1–5\,\mu M$ Bay K 8644, but this effect was reported to be very variable. Docherty & Brown (1986b) found that $5\,\mu M$ racemic Bay K 8644 caused a slow increase in membrane resistance

and suppression of outward current. It was suggested that this was due to accumulation of intracellular calcium, as a result of the agonist action of Bay K 8644, leading to inactivation of potassium channels.

Evidence of a biphasic action of Bay K 8644 was found *in vivo*, when ethanol and (±)-Bay K 8644 were given together, acutely, to mice. The general anaesthetic actions of ethanol were antagonized by $1\,mg\,kg^{-1}$ racemic Bay K 8644, but potentiated by 5 and $10\,mg\,kg^{-1}$ of the dihydropyridine (Dolin *et al.*, 1988b). The central concentrations produced by these doses were $1\,\mu M$ for the $1\,mg\,kg^{-1}$ dose and 4.6 and $11.8\,\mu M$, respectively, for the two higher doses. In the present study, no overt behavioural changes were seen during the chronic treatment.

The central concentrations of Bay K 8644 during the chronic treatment were in the low micromolar range. Calcium channel antagonist actions have been reported at concentrations of the racemate from 500 nM to $5\,\mu M$ in various tissues (see above), while the calcium channel activating effects have been seen at nanomolar concentrations, and a similar pattern was seen in the *in vivo* interactions with ethanol (Dolin *et al.*, 1988b). It is possible, therefore, that because the central concentrations during the chronic treatment were in this range, calcium channel antagonist actions were produced, rather than calcium channel activation. However, this does not provide a complete explanation, as the behavioural effects of single doses of the racemate, sufficient to produce these concentrations, are excitatory when the compound is given alone.

One possible explanation for the effects of the chronic treatment may be that tolerance to the excitatory effects may have occurred. O'Neill & Bolger (1988) demonstrated tolerance to the excitatory behavioural effects of racemic Bay K 8644 after four, once daily, injections of a dose that produced central concentrations of $2\,\mu g\,g^{-1}$. Although the incidence of convulsions was not decreased by this short course of repeated administration, their latency was increased and other signs of hyperexcitability were reduced. The brain concentrations in this study were similar to those in the present work; both were in the low micromolar range. The present authors are unaware of any reports of tolerance to the calcium channel antagonist effects of the (+)-isomer of Bay K 8644. If such tolerance occcurred to the calcium channel activating actions of the (−)-isomer of Bay K 8644 during the more prolonged chronic treatment in the present study, the resultant action on the CNS would have been that of the (+), calcium channel antagonist, isomer.

One possible explanation for the results in this study, therefore, is that the protective action of chronic administration of racemic Bay K 8644 on the ethanol withdrawal syndrome *in vivo* and *in vitro*, may have been due to a combination of the calcium channel antagonist properties of this compound, caused by the high concentrations in the CNS, plus tolerance to the calcium channel activating actions. Studies using lower doses of Bay K 8644, and if possible, the stereoisomers, are needed to investigate these theories. It was apparent throughout the studies that the patterns of results seen in the behavioural, receptor binding and electrophysiological experiments all followed the same pattern, the only exception being the effects of the Bay K 8644 on paired pulse potentiation in the hippocampal slices.

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Bradykinin receptors in the guinea-pig taenia caeci are similar to proposed BK_3 receptors in the guinea-pig trachea, and are blocked by HOE 140

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1 Bradykinin (BK) receptors of the guinea-pig taenia caeci were compared with those of the guinea-pig trachea, a preparation proposed to possess novel BK_3 receptors.

2 Bradykinin-evoked contractile responses were unaffected in both preparations by the selective BK_1 receptor antagonist [des-Arg^9,Leu^8]-BK (1 μM–10 μM). The BK_2 receptor antagonists, D-Arg-[Hyp^3,D-Phe^7]-BK and D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-BK, both had low affinities (apparent pK_B estimates < 6) which did not differ significantly between the two preparations ($P > 0.05$). In contrast, the novel bradykinin receptor antagonist D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK (HOE 140) potently antagonized responses to bradykinin with relatively high affinity (apparent $pK_B = 8.42 \pm 0.15$ and 8.94 ± 0.16 in the taenia caeci, and trachea, respectively).

3 We conclude that the bradykinin receptors in the guinea-pig taenia caeci have similar recognition properties to those present in the guinea-pig trachea, and in this respect the taenia caeci represents a useful preparation for the further study of proposed novel BK_3 receptors.

Keywords: Bradykinin; BK_3-receptors; trachea (guinea-pig); taenia caeci (guinea-pig); HOE 140; D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK; bradykinin antagonist; bradykinin receptors

Introduction

Bradykinin (BK) receptors were originally divided into BK_1 and BK_2 subtypes (see Regoli & Barabé, 1980). Recently, Farmer and colleagues reported that both the selective BK_1 receptor antagonist [des-Arg^9,Leu^8]-BK (Regoli & Barabé, 1980), and also certain [D-Phe^7]-BK substituted analogues (Vavrek & Stewart, 1985) are rather inactive against BK-evoked contraction of the epithelium-denuded guinea-pig trachea, and did not displace [^{3}H]-BK binding in tracheal smooth muscle membrane preparations (Farmer *et al.*, 1989). These observations led to the proposal by this group, of a novel BK receptor subtype which they termed BK_3. To date, the guinea-pig trachea is still the only convenient *in vitro* assay preparation for the study of proposed BK_3 receptors. However, this preparation suffers from a number of disadvantages in that responses vary considerably depending on the involvement of epithelium-dependent factors and prostaglandins (Bramley *et al.*, 1990), contractions are slow and small in tension, and this preparation is rather insensitive to BK so that determination of the maximum response is difficult.

Our preliminary studies (Field *et al.*, 1988) have shown that a number of [D-Phe^7]-BK substituted antagonist analogues, as well as [des-Arg^9,Leu^8]-BK, have low affinity also in the guinea-pig taenia caeci as compared to a number of other preparations, thereby suggesting similarities between the bradykinin receptors of the guinea-pig taenia caeci with those proposed as 'BK_3' receptors in the guinea-pig trachea. In contrast to the guinea-pig trachea, contractile responses to bradykinin in the guinea-pig taenia caeci have the advantage that they appear to be largely due to a direct action on these strips of pure longitudinal smooth muscle and responses are large in tension, with multiple replications possible within individual preparations.

The aim of this study, therefore, was to compare the recognition properties of the BK receptors mediating contraction of the guinea-pig taenia caeci, with the proposed BK_3 receptors in the guinea-pig trachea, by functional studies in isolated preparations. The effect of BK receptor antagonists, including the novel analogue D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK (HOE 140), the most potent of a series recently described (Hock *et al.*, 1991; Lembeck *et al.*, 1991), were used to compare receptor recognition characteristics.

A preliminary account of these results has been communicated to the British Pharmacological Society (Hall *et al.*, 1991).

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Methods

General

Male Dunkin Hartley guinea-pigs (450–800 g) were killed by cervical dislocation, and the taenia caeci and trachea removed and cleared of superficial blood vessels and connective tissue. The trachea was opened longitudinally, the epithelium was removed by gentle rubbing with a cotton wool bud (see, Goldie *et al.*, 1986) and three rings were separated in the cartilage ring to yield preparations for isometric recording in the radial direction. An absence of relaxant responses to carbachol and BK was taken as indicating that the preparations had been functionally denuded of epithelium (Goldie *et al.*, 1986; Farmer *et al.*, 1987; Bramley *et al.*, 1990). Of the three possible taenia strips, the two without mesenteric attachment were separated from underlying circular muscle with fine scissors, and mounted longitudinally. Both preparations were mounted on a micrometer-controlled isometric assembly that allowed precise length adjustments. An initial resting tension of 500 mg was applied, and tissues allowed to develop spontaneous tone. In the taenia caeci, preparations that developed additional tone ('high tone preparations') to BK tended to produce a biphasic response, whereas in those that did not, contractile responses only were observed. Tension was measured by means of Grass FT03B force-displacement transducers coupled to JJ Instruments Cr342 potentiometric flat-bed recorders.

Experiments were carried out in Krebs solution (composition mM: Na^+ 140, K^+ 5.9, Cl^- 104.8, $H_2PO_4^-$ 1.2, HCO_3^- 24.9, Ca^{2+} 2.6, Mg^{2+} 1.15, SO_4^- 1.15, glucose 10), maintained at 37°C and oxygenated with 95%O_2:5%CO_2. The Krebs solution contained atropine, mepyramine, cimetidine, guanethidine, (all 1 μM) and hexamethonium (10 μM).

Antagonist pK_B estimates

In preparations of taenia caeci or trachea, concentration-response curves for BK were obtained using non-cumulative doses in a randomised block design, with responses obtained in the presence of BK receptor antagonist (test) or no antagonist (concurrent control). A 12 min BK dose-cycle was used in conjunction with a 10 min antagonist pre-test incubation time. Other experiments were carried out to test for any effect on pK_B estimates for D-Arg-[Hyp^3,D-Phe^7]-BK of peptidase inhibitors. The kininase I inhibitor DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MERGETPA), the kininase II inhibitor enalaprilat and the neutral endopeptidase EC 3.4.24.11 inhibitor phosphoramidon (all 1 μM) were used. A similar protocol to that described above was used, but with matched preparations bathed in Krebs solution containing the peptidase inhibitors (present from the start of tissue equilibration), or control.

Specificity of antagonism by D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK

The effect of D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK (1 or 10 μM) was tested against responses to submaximal concentrations of substance P, neurokinin A, angiotensin II and carbachol. Control responses were obtained and then repeated following a 10 min incubation period with antagonist.

Source of drugs

Agents were obtained as follows: carbamylcholine chloride, atropine sulphate and hexamethonium bromide (Sigma, UK), mepyramine maleate (May and Baker, U.K.), cimetidine (Smith, Kline and French, U.K.), guanethidine sulphate (Ciba, U.K.), enalaprilat (Merck, Sharp & Dohme, New Jersey, U.S.A.), phosphoramidon, angiotensin, neurokinin A, substance P (Peninsula) bradykinin, D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-BK, D-Arg-[Hyp^3,D-Phe^7]-BK, (Bachem, U.K.). D-Arg-[Hyp^3, Thi^5,D-Tic^7,Oic^8]-BK (HOE 140) was a kind gift from Dr A. Hallett, Sandoz Institute for Medical Research, London. All salts used were of analytical grade and were obtained from B.D.H.

All agents were dissolved in distilled water and peptides were stored deep frozen under N_2.

Expression of results and statistical analysis

Contractile responses were normalised in each preparation in terms of maximal carbachol contractions, and the estimates are shown as means ± s.e.mean. Tests for significance were made with Student's t test for two independent samples. The apparent pK_B estimates and their s.e.mean were obtained from individual dose-ratio estimates (x) between test and control preparations, by calculation from the Gaddum-Schild equation, $pK_B = \log_{10}(x - 1) - \log_{10}[A]$, where [A] is the antagonist concentration. In the guinea-pig trachea, lateral shifts of the lower half of the log concentration-response curve only were used for calculation of apparent pK_B values (see Results).

Results

Isolated tissue studies

Both preparations contracted in response to BK. 'High tone' taenia caeci preparations showed an initial relaxant response, but this was never seen in the tracheal preparations (see Figure 1). In both preparations, the threshold concentration of BK causing contraction was *ca.* 0.3 nM. The log concentration-response curve to BK for contraction of the taenia caeci appeared in individual preparations to be monophasic, with a clearly defined maximal response obtained at concentrations 10 μM or lower. In contrast, in the trachea, two phases of contraction were evident on individual and meaned log concentration-response curves for BK (see control curve of Figures 2 and 3). With regard to the main purpose of the present study, the second phase begins above the highest concentration used by Farmer *et al.* (1989) (1 μM) in their analysis of the 'BK_3' receptor. For present purposes, therefore, apparent pK_B estimates for the antagonists in the tracheal preparation were determined only for the lower portion of the curve that corresponds to that portion of the BK response curve studied by Farmer's group, rather than the upper portion of the curve where greater shifts with D-Arg-[Hyp^3, Thi^5,D-Tic^7,Oic^8]-BK were seen.

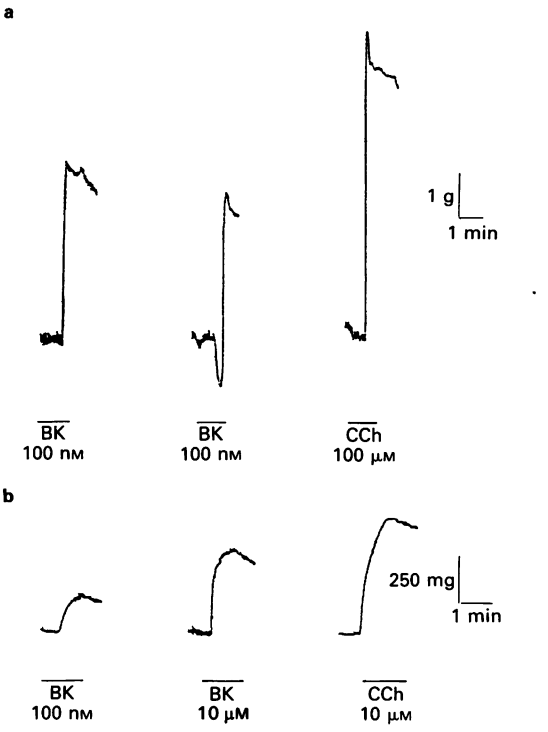

Figure 1 Typical traces of isometric tension recordings in (a) the guinea-pig taenia caeci, and (b) the epithelium-denuded trachea, in response to bradykinin (BK) and carbachol (CCh). Note the initial relaxant response which is sometimes seen in high-tone taenia caeci preparations. Bars indicate the periods of drug application.

None of the BK_2/BK_3 antagonists had partial agonist activity. The selective BK_1 receptor agonist [des-Arg^9]-BK was inactive (data not shown) and the BK_1 receptor antagonist [des-Arg^9,Leu^8]-BK (1–10 μM) did not antagonize responses to BK in either the guinea-pig trachea or taenia caeci (Table 1) and showed partial agonist activity at higher concentrations ($\geq$ 10 μM). The BK analogues D-Arg-[Hyp^3,D-Phe^7]-BK and D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-BK, though having no appreciable activity at 3 μM, at 10 and 30 μM produced shifts to the right of the BK log concentration-response curves in both preparations (Figure 2). The apparent pK_B values estimated from these shifts are shown in Table 1. For all antagonists the range of concentrations that could be used was not wide enough to warrant full Schild plot analysis, but since calculated individual pK_B estimates did not differ significantly ($P > 0.05$) with antagonist concentration, these values were pooled, and are described in the text and Table 1 as apparent pK_B estimates. The apparent pK_B estimates for D-Arg-[Hyp^3, D-Phe^7]-BK were not significantly different when estimated in the presence of the carboxypeptidase inhibitor MERGETPA, the kininase II inhibitor enalaprilat and the neutral endopeptidase inhibitor phosphoramidon (each 1 μM: $n = 4$; $P > 0.05$, data not shown).

D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK (30 nM–300 nM) potently antagonized contractile responses to BK in the guinea-pig taenia caeci and trachea (see Figure 3). At 300 nM D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK, depression of the maximal

Table 1 Affiinity estimates of bradykinin (BK) receptor antagonists in the guinea-pig trachea and taenia caeci

	Apparent pK_B		
Antagonist	*Taenia caeci* (± s.e.mean; *n*)	*Trachea* (± s.e.mean; *n*)	*Difference* (± s.e.mean)
[des-Arg^9,Leu^8]-BK	<5.0 (9)	<5.0 (9)	
D-Arg-[Hyp^3,D-Phe^7]-BK	5.89 (0.32; 11)	5.94 (0.31; 10)	0.05† (0.45)
D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-BK	5.81 (0.35; 8)	5.87 (0.22; 7)	0.06† (0.36)
D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK	8.42 (0.15; 12)	8.94 (0.16; 14)	0.52* (0.21)

Apparent affinities were calculated from individual dose-ratios (x) using the Gaddum-Schild equation $pK_B = \log_{10}(x - 1) - \log_{10}[A]$, where [A] is the antagonist concentration. Abbreviations: n = number of estimates; <5.0 denotes inactive at 10 μM.
† $P > 0.05$; * $P < 0.05$.

response obtained to BK was evident, so the apparent pK_B values shown in Table 1 were estimated only from individual dose-ratios obtained at 30 nM and 100 nM antagonist. The initial relaxation seen with BK in high-tone taenia caeci preparations was found to be antagonized to an equivalent extent (data not shown).

D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK (1 μM) appeared selective in its action since it was inactive against responses to submaximal concentrations of substance P, neurokinin A, carbachol and angiotensin II in both preparations ($n = 4$; data not shown).

Discussion

Results from this study show that BK receptors mediating contraction of the guinea-pig taenia caeci have recognition properties very similar to those mediating contraction of the guinea-pig trachea. In the present study, log concentration-response curves to BK in the taenia caeci were monophasic, whereas those in the guinea-pig trachea were evidently biphasic. Whether in the trachea these phases represent the existence of two receptor subtypes, or coupling mechanisms, within this preparation will be the subject of further investigation.

The bradykinin receptors in these two preparations are certainly not of the BK_1 subtype in view of the lack of activity of the BK_1-selective ligands [des-Arg^9]-BK and [des-Arg^9,Leu^8]-BK. Furthermore, antagonists of the [D-Phe^7]-BK series had low affinity ($pK_B < 6$) as compared to that measured in a wide variety of preparations regarded as expressing BK_2 receptors ($pK_B > 7.0$) (Griesbacher *et al.*, 1987; Field *et al.*, 1988; Hall & Morton, 1991a). The low pK_B values obtained with the [D-

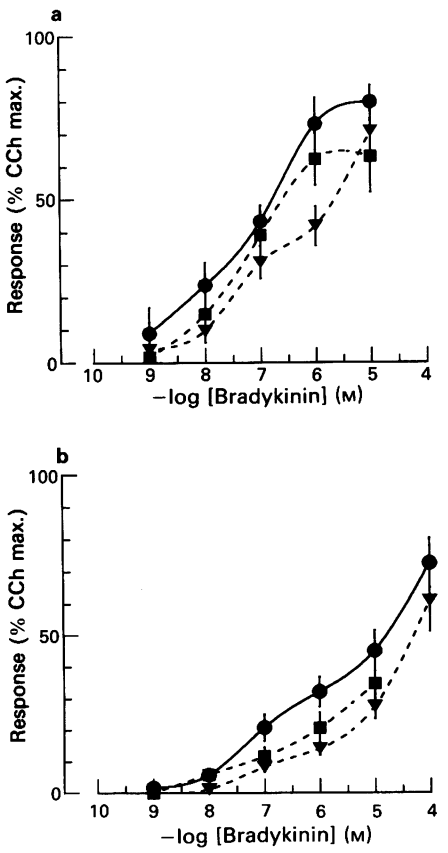

Figure 2 Antagonism by D-Arg-[Hyp^3,D-Phe^7]-BK of the contractile responses to bradykinin in (a) guinea-pig taenia caeci and (b) the guinea-pig trachea. Curves shown are control (●), or in the presence of antagonist at 3 μM (■) and 10 μM (▼). Each point is the mean taken from 9–12 preparations; s.e.mean shown by vertical lines.

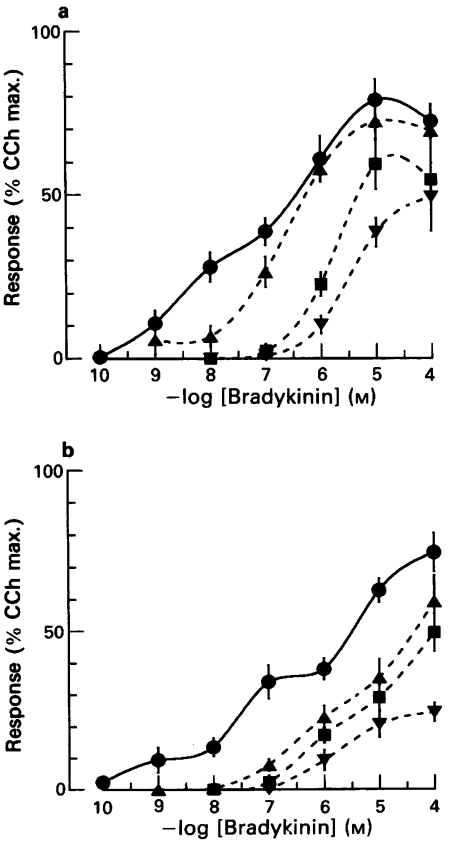

Figure 3 Antagonism by D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK of the contractile responses to bradykinin in (a) guinea-pig taenia caeci and (b) the guinea-pig trachea. Curves shown are control (●), or in the presence of antagonist at 30 nM (▲), 100 nM (■) and 300 nM (▼). Each point is the mean taken from 9–12 preparations; s.e.mean shown by vertical lines.

Phe^7]-BK substituted analogues are unlikely to be due to degradation by peptidases since the pK_B for D-Arg-[Hyp^3,D-Phe^7]- BK was unaffected by the presence of peptidase inhibitors.

In contrast to the [D-Phe^7]-BK substituted analogues, potent antagonism of responses to BK by D-Arg-[Hyp^3,Thi^5, D-Tic^7,Oic^8]-BK was seen in the trachea and taenia caeci (apparent $pK_B = 8.9$ and 8.4 respectively). This finding with D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK in the guinea-pig trachea confirms a preliminary report by Perkins *et al.* (1991). Though these apparent pK_B estimates for D-Arg-[Hyp^3,Thi^5,D-Tic^7, Oic^8]-BK differed statistically ($P < 0.05$), it should be pointed out that strict competition at equilibrium was not established with this compound in the present experiments, and further, it is evident that a second receptor site (or mechanism) is present in the trachea. In pharmacological terms, therefore, we do not consider the difference in apparent pK_Bs as being particularly significant. It is also interesting to note that the pK_B values are approximately one log unit lower than the pA_2 value for D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK described in typical BK_2 preparations, the rat uterus ($pA_2 = 9.74$; Perkins *et al.*, 1991), and the rat duodenum ($pA_2 = 10.0$; unpublished data) and the rabbit iris spincter pupillae ($pA_2 = 10.5$; Everett *et al.*, 1991).

Given that [D-Phe^7]-BK antagonists and D-Arg-[Hyp^3, Thi^5,D-Tic^7,Oic^8]-BK have lower affinities in the taenia and trachea as compared with typical BK_2 preparations, these results would support the proposal by Farmer *et al.* (1989) that BK receptors with properties differing from typical BK_2 receptors do exist. It may be noted however, that similar pK_B values for D-Arg-[Hyp^3,D-Phe^7]-BK, D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-BK and D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK have been reported in further preparations taken from the guinea-pig: the ileum (Birch *et al.*, 1991; Perkins *et al.*, 1991), and the urinary bladder (Maggi *et al.*, 1989). Thus both series of antagonists give affinity data suggesting a common difference between BK receptor of the guinea-pig preparations as compared to preparations from other species. These observations highlight the possibility that differences in antagonist affinities may reflect species-related differences in bradykinin receptor subtypes.

In conclusion, the predominant receptor type in the guinea-pig taenia caeci is very similar to the novel receptor type in the guinea-pig trachea which has been described as a BK_3 subtype (Farmer *et al.*, 1989). Whether the properties of these receptors justify the creation of a distinct receptor class should, however, perhaps depend on evidence from other studies, such as cloning, coupling studies or the development of BK_3 selective antagonists. It is of great interest that these novel receptors, both in guinea-pig airways and gastrointestinal smooth muscle, are effectively blocked by D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK a potent example of a recently developed analogue series (see also Farmer *et al.*, 1991). For further study of the 'BK_3' receptor, the taenia would seem to have a number of advantages over the tracheal preparation. There appears to be only one receptor type, there are good responses in terms of tension developed and replication of responses within individual preparations. Further since the taenia consists of pure smooth muscle it is very suitable for mechanistic studies (Hall & Morton, 1991b).

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Hepoxilins sensitize blood vessels to noradrenaline—stereospecificity of action

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1 The vascular activity of two stereoisomers of hepoxilin A_3 (HxA_3) (**8R** and **8S**) and of its glutathione conjugate, hepoxilin A_3-C (HxA_3-C) (**8R** and **8S**), was investigated on rat helicoidal strips of thoracic aorta and longitudinal strips of portal vein.

2 Neither of the hepoxilins tested had a direct effect on the tone of the aortic strip or on the spontaneous contractions of the portal vein. However, the noradrenaline (NA)-induced response of these vessels, as expressed by the dose required for half maximal contraction, (EC_{50}) was greater in HxA_3 (**8S**)- and HxA_3-C (**8R**)-treated aorta. Increased frequency and strength of spontaneous contractions of the portal vein were detected at lower concentrations of NA in the presence of hepoxilins.

3 The threshold dose for both hepoxilins was 10^{-8} M and their effect was not dose-related beyond 10^{-8} M. The effect of hepoxilin appeared after a 45 min incubation period and could be observed even if the compounds were washed out after 15 min.

4 Stereochemical specificity was observed. The **8S** isomer of HxA_3 was active in potentiating the NA-induced contraction of these vessels while the **8R** isomer was inactive. In contrast, the **8R** isomer of HxA_3-C was active while the **8S** isomer was inactive. In both tissues, HxA_3 (**8S**) was more potent than its glutathione conjugate, HxA_3-C (**8R**).

5 In calcium-free buffer or in the presence of a calcium channel blocker (nifedipine 1 μM), no potentiation of NA-induced contraction by hepoxilins could be observed, suggesting the involvement of extracellular calcium in the actions of hepoxilins.

6 These experiments suggest that hepoxilins may be involved in the modulation of vascular tone and contractility.

Keywords: Hepoxilin A_3; hepoxilin A_3-C; trioxilin A_3; aorta; portal vein; potentiation; contraction; calcium; noradrenaline

Introduction

Alterations in the reactivity of blood vessels to neurotransmitters and circulating hormones may cause cardiovascular disorders such as hypertension (Mulvany *et al.*, 1980). Different mechanisms have been proposed to explain the enhanced responsiveness of arteries from hypertensive rats to an endogenous vasoconstrictor, noradrenaline. Several hypotheses have been proposed to explain this increase in sensitivity to involve a generalized increase in the contractility of the blood vessels or a reduced relaxation to vasodilators (Winquist & Bohr, 1983; Cohen & Berkowitz, 1976). Other studies have suggested that the increased sensitivity of hypertensive blood vessels results from an alteration in the regulation of calcium influx in vascular muscle (Shibata *et al.*, 1975; Zsoter *et al.*, 1977). The contractile machinery in muscles is activated by a rise in intracellular calcium. Two sources of calcium may be involved (Bohr, 1963). The release of calcium from intracellular stores, believed to be responsible for the initial phasic component and the influx of extracellular calcium contributes to the tonic component of the contraction (Godfraind *et al.*, 1982; Cauvin & Malik, 1984). The concentration of intracellular free (cytosolic) calcium in vascular smooth muscle cells determines the degree of tension (Bohr, 1963) and is the trigger for muscle contraction (Filo *et al.*, 1965). Elevated intracellular free cytosolic calcium in vascular smooth muscle cells has been suggested in the pathophysiology of hypertension (Kwan, 1985).

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Blood vessels respond to various stimuli by releasing free arachidonic acid from membrane phospholipids. The free fatty acid is then rapidly converted by the cyclo-oxygenase pathway to predominantly prostacyclin in endothelial cells and smooth muscle cells (Weksler *et al.*, 1977; Moncada *et al.*, 1977). Alternative pathways for arachidonic acid metabolism have been described in vascular tissue. Lipoxygenases and cytochrome P_{450} epoxygenase metabolize arachidonic acid to various biologically active hydroperoxy-, hydroxy-, dihydroxyeicosatetraenoic acids and leukotrienes (Juchau *et al.*, 1976; Greenwald *et al.*, 1979; Larrue *et al.*, 1983). More recently we have described the release of hydroxy epoxide metabolites of arachidonic acid named hepoxilins by vascular tissue (Laneuville *et al.*, 1991a). Hepoxilins are formed through the 12-lipoxygenase pathway via a haemoglobin or other haemoprotein-assisted intramolecular rearrangement of 12(S)-HPETE (Pace-Asciak *et al.*, 1983; Pace-Asciak, 1984a,b; Pace-Asciak & Martin, 1984). Intact pieces of the rat thoracic aorta stimulated by exogenous arachidonic acid release hepoxilin A_3 (HxA_3) (Laneuville *et al.*, 1991a). Homogenates of the rat aorta metabolize HxA_3 via two competing pathways; one involves hydrolysis of the epoxide group to form a trihydroxy metabolite, trioxilin A_3 ($TrXA_3$), and the second pathway involves conjugation of HxA_3 with glutathione via glutathione S-transferase to form a glutathione conjugate, hepoxilin A_3-C (HxA_3-C) (Laneuville *et al.*, 1991a). The formation of HxA_3-C in blood vessels is dependent on the presence of reduced glutathione and is greatly enhanced in the presence of trichloropropene oxide (TCPO), an epoxide hydrolase inhibitor which blocks utilization of the substrate, HxA_3, via the heoxilin epoxide hydrolase. We have reported that the glutathione conjugation pathway of HxA_3 was more

evident in veins than arteries as quantified by HxA_3-C formation (Laneuville *et al.*, 1991a). The aorta from spontaneous hypertensive rats (SHR) was similar to aorta from local normotensive Wistar rats in HxA_3-C formation although the aorta from the normotensive Wistar Kyoto rats (WKy) was much more active than aortae from either of the two other rat types (Laneuville *et al.*, 1991a).

Knowing that hepoxilins are formed by vascular tissue, it was of interest to determine whether they act on this tissue. In this study we report the actions of hepoxilins on isolated helicoidal strips of the thoracic aorta and longitudinal strips of portal vein of the rat.

The following abbreviations are used in the text: hepoxilin A_3, HxA_3, 8(**R**) and 8(S)-hydroxy, 11(S), 12(S)-epoxy-eicosa-5Z, 9E, 14Z-trienoic acid; trioxilin A_3, $TrXA_3$, 8(**R**) and 8(S), 11(**R**), 12(S)-trihydroxy-eicosa-5Z, 9E, 14Z-trienoic acid; hepoxilin A_3-C, HxA_3-C, 8(**R**) and 8(S), 12(S)-dihydroxy-11(**R**)-glutathionyl-eicosa-5Z, 9E, 14Z-trienoic acid; noradrenaline, NA; trichloropropene oxide (TCPO); dimethyl sulphoxide, DMSO; hydroperoxyeicosatetraenoic acid, HPETE; hydroxy-eicosatetraenoic acid, HETE; ethylene glycol-*bis* (β-amino ethyl ether) N,N,N′,N′-tetraacetic acid, EGTA; 12-O-tetradecanoylphorbol-13-acetate, TPA.

Methods

Blood vessel preparation

Experiments were performed on male Wistar rats, 200–300 g, purchased from Charles River, St-Constant, Quebec, Canada. Animals were maintained on a standard rat chow diet (Prolab rat chow, Agway, Syracuse, NY, U.S.A.) and tap water *ad libitum*. Animals were killed, thoracic aorta and portal veins were rapidly removed and placed in Krebs solution (composition in mM: NaCl 119.00, KCl 4.70, KH_2PO_4 0.40, $NaHCO_3$ 14.90, $MgSO_4$ 1.17, $CaCl_2$ 2.50 and glucose 5.50) kept at 37°C and continuously gassed with 95% O_2, 5% CO_2 mixture. Aortae were cleaned of connective tissue and cut helically by the method of Furchgott & Bhadrakom (1953). From each animal, two strips of the following dimensions, 2.0 to 4.0 mm wide and 2.0 to 2.5 cm long, were cut. Portal veins, dissected free from connective tissue, were cut longitudinally by the method of Couture *et al.* (1978) and one entire vessel (0.5 to 1.0 cm) was used per experiment. Tissues were mounted vertically in organ bath containing 10 ml of Krebs buffer maintained at 37°C and equilibrated with a 95% O_2:5% CO_2 gas mixture. A force of 1 g and 0.25 g was applied to the aortae and the portal veins respectively. An equilibration period of 60 min was allowed during which time the bath solutions were replaced every 15 min. Isometric contractions were recorded as changes in grams of tension on a Grass polygraph (model 79D) with a Grass FT03 force displacement transducer.

Effects of hepoxilins on noradrenaline-induced contractile response in the aorta and portal vein

The contractile capacity of the aorta was tested three times on each preparation by constructing cumulative concentration-response curves to NA. Wash out periods of 5 min were allowed between each curve to return to basal tension. The third curve was used for calculations and was identical to the second. When this was not the case, concentration-response curves were repeated until a reproducible response was obtained for both the aortae and the portal veins. The interval time between each cumulative dose-response curve was 15 min. Hepoxilins (at different concentrations) were applied 45 min before the cumulative addition of NA was repeated. In a series of experiments, concentration-response curves to NA were carried out 5 min after the addition of hepoxilins and were repeated for 1 h every 15 min. In control experiments, DMSO was added instead of hepoxilins and concentration-response curves by successive cumulative addition of NA were obtained over the same period of experimentation as for the hepoxilins.

When the effect of calcium was investigated, a calcium channel blocker (1 μM nifedipine) was added 20 min before the addition of hepoxilins. Experiments were also performed in a calcium-free Krebs buffer containing 1 mM EGTA. Tissues were dissected and incubated in the calcium free buffer. Calcium (2.5 mM) was added at the end of the experiment and a cumulative concentration-curve to NA was performed.

Expression of results and statistical analysis

Contraction is expressed as the percentage of the maximal response to NA obtained before the addition of hepoxilins. Sensitivity to NA in the aorta was expressed as an EC_{50} value, EC_{50} being the concentration of agonist required to give half-maximal response of the vessels. EC_{50} values were obtained by logit/log regression analysis of each individual preparation and averaged. Contraction of the portal vein was characterized as the frequency of the spontaneous contractions, the basal level of tension and the amplitude of the contractions. All results are expressed as mean ± s.e.mean of six experiments. The differences between the values, obtained in the same strip, were tested for significance by use of Student's *t* test for paired observations. Unpaired Student's *t* test was used to compare the effect of hepoxilins obtained in different preparations. Probabilities (*P*) smaller than 0.05 were considered to be significant.

Drugs

Noradrenaline (Sigma, MO, U.S.A.) was dissolved in deionized water, containing 0.01% ascorbic acid (Fisher Scientific, Toronto, Canada), to give a stock solution of 10^{-2} M. Authentic hepoxilins prepared chemically as described (Corey & Su, 1990) were kindly provided by Prof. E.J. Corey (Harvard University, Cambridge, MA, U.S.A.), HxA_3 and $TrXA_3$ were dissolved in DMSO (Calbiochem, Toronto, Canada) and HxA_3-C was dissolved in a mixture of phosphate buffer and ethanol (4 to 1). Hepoxilins were diluted with Krebs buffer to a final concentration of DMSO or ethanol not higher than 0.02%. Nifedipine (Sigma) was dissolved in absolute ethanol to give a 5 mM stock solution which was kept in the dark and frozen until use.

Results

Time-related response of the aorta to hepoxilin

NA at a concentration of 0.98 nM weakly contracts the rat aorta and reaches a plateau which persists until drug washout (Figure 1, upper trace). HxA_3 (8S) added at a concentration of 10^{-8} M to the bath containing NA had no immediate effect on

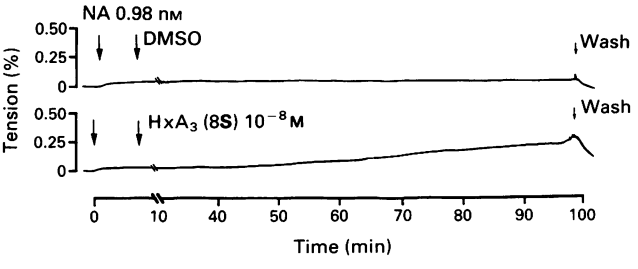

Figure 1 Time related induction of noradrenaline (NA) contraction (g tension) of helicoidal strips of rat aorta by hepoxilin A_3 (HxA_3) (8S). NA (0.98 nM) was added at time zero, followed 8 min later by dimethyl sulphoxide (DMSO) vehicle (top panel) or HxA_3 (8S) (lower panel). Note the slow onset but gradual rise in tension in the HxA_3-treated strip as compared to the strip exposed only to vehicle.

the NA-precontracted tissue (Figure 1, lower trace). However, upon leaving the tissue in contact with both NA and HxA_3, a contraction began to develop around 45 min after the initial addition of the hepoxilin. This contraction continued to develop further with time up to 100 min whereupon the bathing fluid was replaced. The tone of the vessel rapidly returned to pre-drug levels after drug washout. In contrast, the NA-precontracted tissue in the absence of hepoxilin did not contract further with vehicle addition only.

Potentiation by hepoxilins of the noradrenaline-induced contraction of the aorta

Isolated helicoidal strips of the rat contracted in a dose-dependent fashion to increased concentrations of NA (Figure 2). None of the hepoxilins tested had any direct effect on the tone of the aortic strip, but some acted to potentiate the contraction by NA. Figure 2 shows tracings of dose-dependent contractions of the aortic strips to NA before and during the administration of vehicle (DMSO) and 10^{-8} M concentrations of HxA_3 (8R), HxA_3 (8S) and $TrXA_3$ (8S). HxA_3 (8S) significantly potentiated the threshold concentration at which NA caused a contraction (compare Figure 2a for vehicle with 2c for HxA_3 (8S); compare 0.48 nM NA in the presence of HxA_3 (8S) with the corresponding response with DMSO vehicle and with HxA_3 (8R) (Figure 2b) and $TrXA_3$ (8S) (Figure 2d)).

The dose-response curves to NA were quite reproducible throughout the experiment. Results of a series of experiments are shown in Figure 3. Here, the contractions of the aorta to increasing concentrations of NA are shown in the presence of vehicle, and in the presence of HxA_3 (8S) at two concentrations, 10^{-9} and 10^{-8} M (Figure 3a). Higher concentrations of HxA_3 were also tested but the effects were not significantly different from that observed at 10^{-8} M (Table 1). The stable trihydroxy metabolite of HxA_3, was inactive in modulating the vascular reactivity to NA (Figure 2d and Table 1). The EC_{50} for various compounds is shown in Table 1.

Table 1 EC_{50} values for noradrenaline (NA) in the presence and absence of the 8-enantiomers of hepoxilin A_3 (HxA_3), trioxilin A_3 ($TrXA_3$) and hepoxilin A_3-C (HxA_3-C) in rat thoracic aorta

Treatment	EC_{50} (nM) ± s.e.mean
Control	13.1 ± 2.4
HxA_3 (8S) 10^{-9} M	12.7 ± 3.7
10^{-8} M	2.4 ± 0.9***
10^{-7} M	3.3 ± 0.9***
10^{-6} M	3.9 ± 0.8***
Control	10.4 ± 0.6
HxA_3 (8R) 10^{-6} M	10.5 ± 0.5
Control	8.7 ± 1.5
HxA_3-C (8S) 10^{-8} M	9.3 ± 0.9
10^{-6} M	7.5 ± 0.6
Control	11.7 ± 0.5
HxA_3-C (8R) 10^{-9} M	12.5 ± 0.3
10^{-8} M	6.4 ± 0.3**
10^{-6} M	7.6 ± 0.3*
Control	9.8 ± 1.5
$TrXA_3$ (8S) 10^{-6} M	8.8 ± 1.8

Values are means ± s.e.mean for six separate tissue preparations. Each treatment was compared to the control value which corresponds to the pretreatment value for each tissue by using Student's t test for paired samples. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.005$ with respect to corresponding control value.

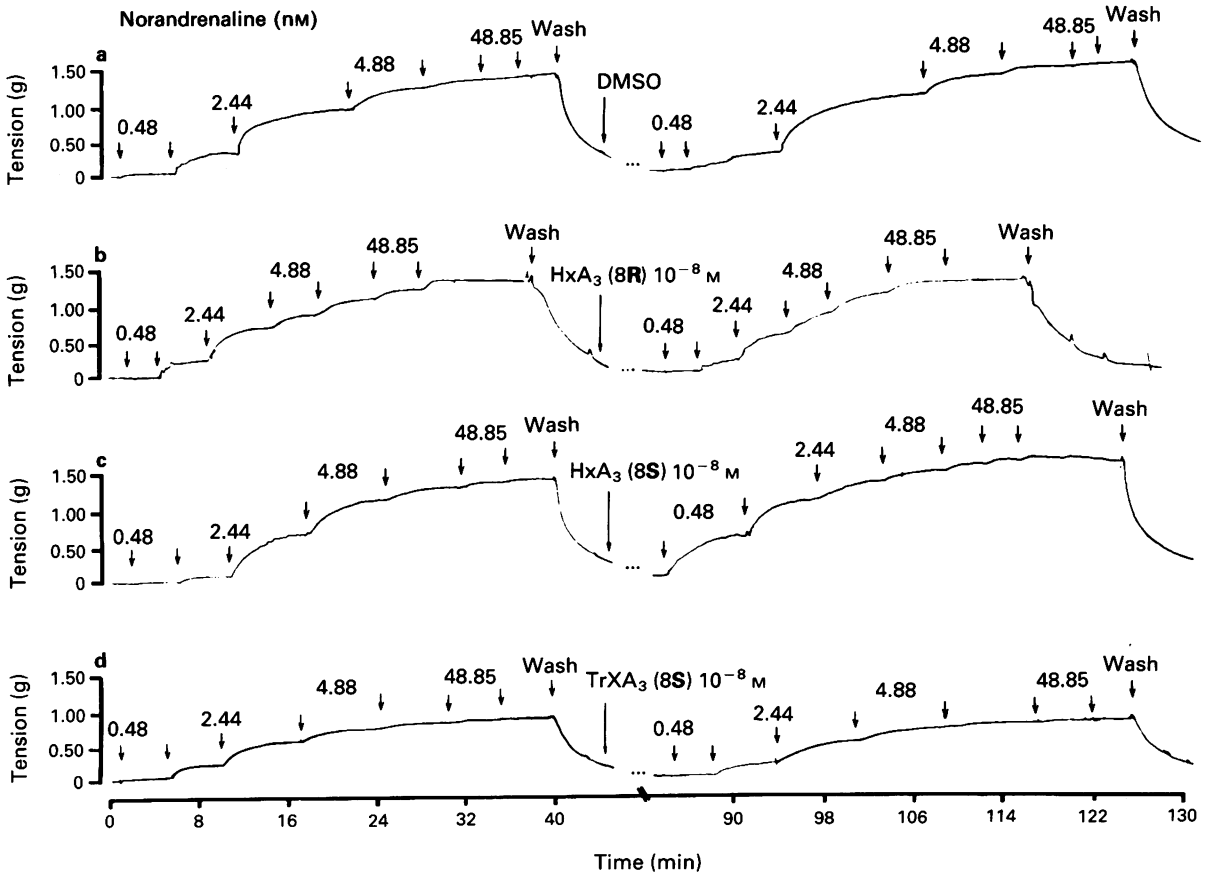

Figure 2 Effect of hepoxilins on contractions of strips of rat thoracic aorta induced by cumulative doses of noradrenaline (NA). Three successive complete dose-response curves to NA (0.48 to 48.85 nM) were obtained (the last one is shown here in each panel) followed by tissue washout. Subsequent application was made of dimethyl sulphoxide (DMSO) vehicle (a), hepoxilin A_3 (HxA_3) (8R) (b), HxA_3 (8S) (c) or trioxilin A_3 ($TrXA_3$) (8S) (d), followed 45 min later by another cumulative dose-response curve to NA. Note that the presence of HxA_3 (8S) (c) potentiates the contraction of the tissue to NA so that significant contraction takes place at 0.48 nM NA.

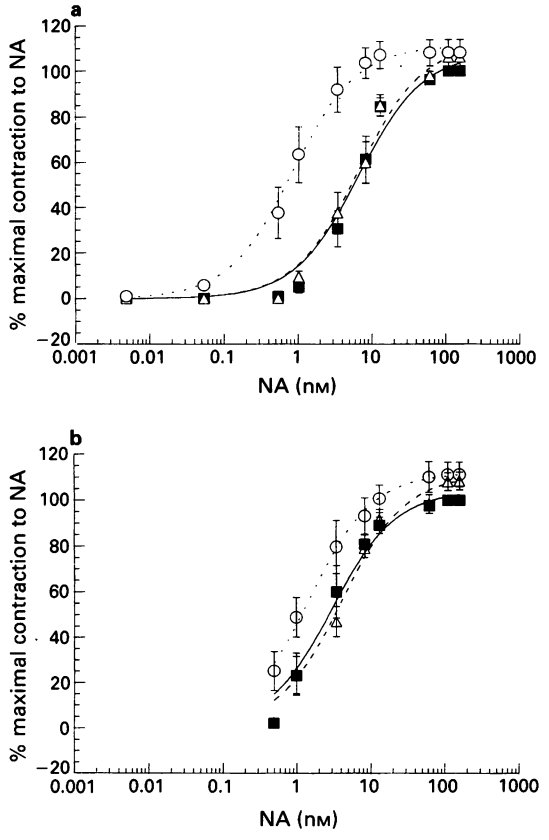

Figure 3 Dose-response curves of aortae ($n = 6$) to noradrenaline (NA) in the presnce of (a) hepoxilin A_3 (HxA_3) (8S) and (b) HxA_3-C (8R) at two concentrations in normal Krebs solution. Note the left shift of the response curve to NA in the presence of 10^{-8} M concentration of hepoxilins. Symbols: (■), vehicle control (dimethyl sulphoxide); (△) HxA_3 (8S) 10^{-9} M for (a) and HxA_3-C (8R) 10^{-9} M for (b); (○) HxA_3 (8S) 10^{-8} M for (a) and HxA_3-C (8R) 10^{-8} M for (b).

Stereospecificity of the hepoxilin-induced potentiation in the contraction of the aorta to noradrenaline

The above results are shown for the 8S isomer of HxA_3. The 8R isomer is essentially inactive in potentiating the NA-induced contraction of the aorta at the concentrations used here (10^{-9} to 10^{-6} M) (Table 1 and Figure 2b). In contrast, the 8R isomer of the glutathione conjugate of HxA_3, i.e. HxA_3-C, was active (Figure 3b) but not the 8S isomer (Table 1). In separate experiments we tested whether the inactive isomer interfered with the potentiating response of the tissue to the active hepoxilin isomer. After a cumulative dose-response curve to NA was generated, tissues were exposed first to different concentrations of the inactive isomers, HxA_3 (8R) or HxA_3-C (8S) (10^{-9} to 10^{-6} M), and then exposed to 10^{-8} M of HxA_3 (8S) or HxA_3-C (8R) respectively, and after 45 min another cumulative dose-response to NA was generated. In both cases, with either HxA_3 or HxA_3-C, the inactive isomer neither affected the threshold nor the extent of potentiation of NA contraction by the active isomer.

Effect of hepoxilins on noradrenaline-induced contraction of the portal vein

The portal vein displayed spontaneous contractions when stretched with 0.25 g tension (Figure 4a). The tissue returned to the same basal tension between each contraction as opposed to the aorta which kept a plateau of contraction after the tension was applied or after the addition of NA (Figures 1 and 2). NA induced an increase in the frequency of contractions and of the basal tension of the tissue in a dose-dependent fashion (Figure 4 and Table 2). HxA_3, (8S) 10^{-8} M increased the sensitivity of the tissue to NA by increasing the frequency of the spontaneous contractions as well as the basal level of tension of the portal vein to doses of NA that were not active before the addition of hepoxilins (Figure 4b). The strength of the contractions as reflected by the height of the peaks, was not significantly altered by any of the hepoxilins tested. However, the threshold dose of NA required to produce a flurry of spontaneous contractions was lowered when 10^{-8} M hepoxilin was added. As observed with the aorta, the hepoxilin effect was not dose-dependent beyond the threshold of 10^{-8} M (Table 2) although it was also time-dependent. Only the 8S isomer of HxA_3 was active while its trihydroxy metabolite ($TrXA_3$ (8S)), the 8R isomer of HxA_3 and the 8S isomer of HxA_3-C were all inactive at a concentration of 10^{-6} M. HxA_3-C (8R) was also active in potentiating the NA-induced increase of frequency as well as the basal level of tension (Table 2). All the hepoxilins tested on the portal vein preparation were inactive on their own at all the doses used (10^{-9} to 10^{-6} M). Control preparations were run in parallel to demonstrate the sensitivity of the portal vein to NA remains unchanged during the time of the experiments (3 h) and the frequency, the basal level of tension and the strength of the contractions obtained after cumulative addition of NA when tested every 30 min were not significantly different from the pre-administration ones (data not shown).

Calcium dependence of the hepoxilin A_3 (8S) effect

Contractile responses to cumulative concentrations of NA were measured following incubation of the aorta in Ca^{2+}-free Krebs solution containing 1 mM EGTA. This resulted in a slow phasic contraction of the aortic strip, smaller in magnitude when compared to contractions obtained in a calcium-containing buffer. The EC_{50} value for NA under these conditions was: 93.1 ± 6.2 nM ($n = 6$) in a Ca^{2+}-free buffer and 13.3 ± 3.3 nM ($n = 6$) in a Ca^{2+}-containing buffer. The threshold dose of NA required to cause a contraction was 0.49 mM in the Ca^{2+}-containing buffer and 8.30 nM in Ca^{2+}-free buffer. The amplitude of the maximal contraction was much lower in the Ca^{2+}-free buffer, 0.2 g, compared to that observed in the Ca^{2+}-containing buffer, 1.05 g. This reflects the importance of calcium influx that takes place during the tonic phase of the smooth muscle contraction. Under Ca^{2+}-free conditions, HxA_3 (8S) 10^{-8} M did not significantly reduce the threshold dose of NA (8.30 nM) required to elicit a contraction (Figure 5). The dose-response curve for NA remained unchanged in the presence of HxA_3 (8S) (10^{-8} M). The magnitude of the maximal contractile response obtained with 110.9 nM of NA in the presence of HxA_3 (8S) 10^{-8} M was not significantly different than that observed before administration of hepoxilin. Following readdition of Ca^{2+} (2.5 mM), the contractile response of the aorta to NA was comparable to that obtained in Ca^{2+}-containing Krebs solution and a leftward shift (recovery) of the dose-response curve to NA induced by HxA_3 (8S) could be observed ($EC_{50} = 2.6$ ± 2.0 nM).

The possible role of calcium influx caused by HxA_3 (8S) to explain its role in inducing potentiation of NA was further investigated by using a calcium channel blocker, nifedipine. Experiments were performed in a calcium containing medium and tissues were exposed to nifedipine (1 μM) or its vehicle ethanol (5 μl), 20 min before the addition of HxA_3 (8S) 10^{-8} M. As before, a dose-response curve to NA was generated 45 min after the addition of the hepoxilin. Nifedipine caused a shift of the NA curve to the right when compared to the ethanol control (Figure 5b). The EC_{50} for NA in the nifedipine plus hepoxilin-treated tissue (18.2 ± 1.5 nM) was significantly higher than the value observed prior to administration of ethanol plus hepoxilin (10.1 ± 1.0 nM) ($P < 0.005$, $n = 6$). In the presence of HxA_3 (8S) 10^{-8} M, only the ethanol-treated tissue displayed a lower EC_{50} (1.7 ± 0.3 nM) compared to the pre-administration value (9.4 ± 1.6 nM) ($P < 0.005$, $n = 6$).

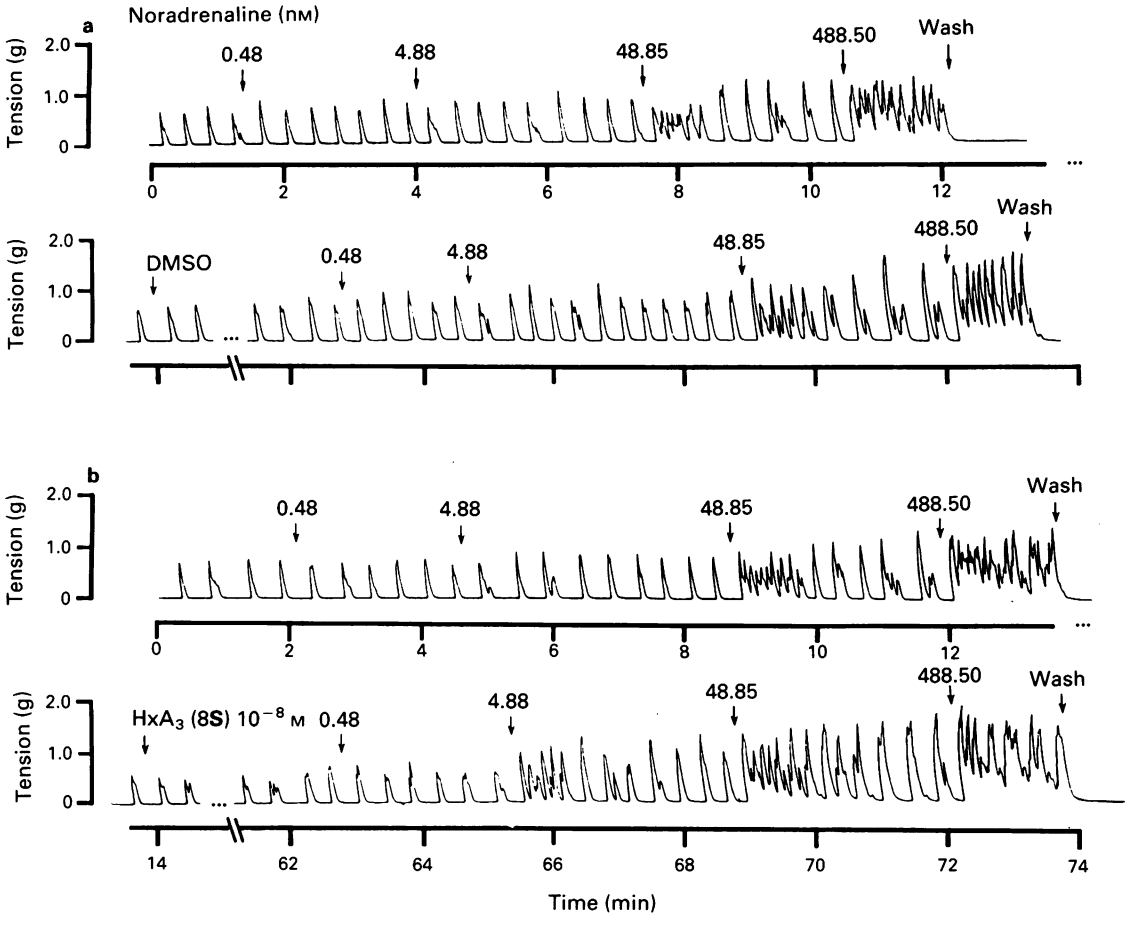

Figure 4 Effects of hepoxilin A_3 (HxA_3) (8S) on the frequency and strength of contractions of strips of rat portal vein to noradrenaline (NA). Cumulative applications of NA are shown before and after addition of (a) dimethylsulphoxide (DMSO) vehicle and (b) HxA_3 (8S). Note the effect of hepoxilin in inducing the contraction to NA at 4.88 nM compared to the control at 48.85 nM. Also note that hepoxilin alone fails to alter the strength or the frequency of contraction.

Discussion

In this paper we show that hepoxilins cause an increase in response of the aorta and of the portal vein to contractions induced by NA. The hepoxilins do not display any contractile activity of their own, but potentiate the contractile activity of NA in both vascular tissues. We do not know whether the hepoxilin effect is general to other vasoconstrictors or whether the hepoxilins affect only the NA-induced contraction. An interesting observation lies in the finding that the action of the hepoxilins on the NA-induced contraction is not an immediate response but is delayed in time. It requires about 45 min of contact with the tissue for the hepoxilin-induced effect to start. The reason for this delayed action is unknown but we may speculate that the hepoxilins 'prime' the cell for NA-contraction. This 'priming' may result from a calcium change in the smooth muscle cells or from the release of some hitherto undefined second messenger. Indeed, hepoxilins have been reported to raise intracellular calcium in the neutrophil and this occurs through a mobilization of calcium from intracellular stores as well as through influx mechanisms (Dho *et al.*, 1990). In support of the influx mechanism, hepoxilins have been shown to increase the transport of calcium across membranes (Derewlany *et al.*, 1984). Hence a calcium-related mechanism may be used in the priming of the smooth muscle cells for NA-contraction. However, this may not be entirely correct because HxA_3-C, while causing a potentiation of NA-vasoconstriction, as shown in the present study, does not raise intracellular calcium in the neutrophil (unpublished observations). Assuming that similar mechanisms hold for both types of cells (neutrophils and muscle cells), then the hepoxilin 'priming' may not be exclusively through a calcium-mediated mechanism.

An alternate mechanism for hepoxilin action as shown in this study, may be due to the release by hepoxilins of some second messenger. Indeed, hepoxilins have been shown to induce the release of diacylglycerol and arachidonic acid from the human neutrophil (Nigam *et al.*, 1990), but these second messengers are released very quickly (within 5 min). The time-delayed action (onset around 45 min) of the hepoxilins seen here must employ some other mechanism. Rasmussen *et al.* (1984) and Forder *et al.* (1985) demonstrated that TPA caused a time-delayed contraction (onset around 60 min) of the rabbit aorta, and they speculated that this action was due to the activation of protein kinase C (Hockin, 1985). Hence our findings with hepoxilins may suggest that their action is mediated through a diacylglycerol-induced activation of protein kinase C.

The stereospecificity of hepoxilin action warrants further comment. We find in the present study that only the 8S enantiomer of HxA_3 is active in potentiating the contraction by NA of both the aorta and the portal vein. This contrasts with our recent findings on the effect of hepoxilins on vascular permeability in the rat skin (Laneuville *et al.*, 1991b). HxA_3 (both enantiomers) are weakly active on their own, but only the 8R enantiomer potentiates at very low doses (threshold 1 ng) the bradykinin-induced response (Laneuville *et al.*, 1991b). With regard to HxA_3-C, the active isomer in potentiating the NA-induced contraction of the aorta and the portal vein in the present study is the 8R isomer, while this isomer is inactive in the vascular permeability studies (unpublished observations). The reasons for the observed differences in isomer specificity

Table 2 Effects of hepoxilin A_3 (HxA_3) (8S and 8R), hepoxilin A_3-C (HxA_3-C) (8S and 8R) and trioxilin A_3 ($TrXA_3$) (8S) on noradrenaline (NA)-induced increase in frequency and strength of spontaneous contractions and basal tone of the rat portal vein strip

Treatment	*NA* (nM)	*Frequency* (contractions min^{-1})	*Basal tone* (g)	*Strength* (g)
A				
Control	0	3.00 ± 0	0.25 ± 0	0.68 ± 0.04
	0.48	3.80 ± 0.30	0.25 ± 0	0.71 ± 0.04
	5.36	4.30 ± 0.30	0.28 ± 0.03	0.78 ± 0.07
	54.21	8.83 ± 1.20	0.43 ± 0.06	0.90 ± 0.09
	542.71	17.67 ± 2.10	0.98 ± 0.09	1.07 ± 0.09
HxA_3 (8S)	0	3.20 ± 0.20	0.25 ± 0	0.62 ± 0.06
10^{-9} M	0.48	4.33 ± 0.50	0.25 ± 0	0.61 ± 0.06
	5.36	4.67 ± 0.70	0.27 ± 0.02	0.66 ± 0.07
	54.21	9.33 ± 1.00	0.47 ± 0.04	0.92 ± 0.10
	542.71	19.30 ± 2.10	1.09 ± 0.11	1.11 ± 0.13
HxA_3 (8S)	0	2.83 ± 0.20	0.25 ± 0	0.48 ± 0.07
10^{-8} M	0.48	3.67 ± 0.40	0.25 ± 0	0.52 ± 0.07
	5.36	11.83 ± 0.80***	0.46 ± 0.04***	0.76 ± 0.13
	54.21	14.17 ± 1.10***	0.68 ± 0.05**	1.01 ± 0.10
	542.71	19.83 ± 2.20	1.04 ± 0.12	1.25 ± 0.10
HxA_3 (8S)	0	3.00 ± 0.30	0.25 ± 0	0.43 ± 0.07
10^{-6} M	0.48	4.50 ± 0.70	0.25 ± 0	0.45 ± 0.07
	5.36	9.80 ± 1.40***	0.41 ± 0.07**	0.76 ± 0.12
	54.21	11.50 ± 1.50**	0.58 ± 0.09*	1.08 ± 0.16
	542.71	17.50 ± 0.09	0.97 ± 0.09	1.16 ± 0.15
B				
Control	0	3.00 ± 0	0.25 ± 0	0.62 ± 0.03
	0.48	3.00 ± 0	0.25 ± 0	0.68 ± 0.02
	5.36	3.10 ± 0.10	0.25 ± 0	0.65 ± 0.04
	54.21	5.50 ± 0.22	0.40 ± 0.05	0.84 ± 0.04
	542.71	15.30 ± 1.31	0.85 ± 0.06	1.29 ± 0.04
HxA_3 (8R)	0	3.00 ± 0	0.25 ± 0	0.63 ± 0.03
10^{-6} M	0.48	3.00 ± 0	0.25 ± 0	0.65 ± 0.04
	5.36	3.70 ± 0.30	0.25 ± 0	0.65 ± 0.04
	54.21	5.50 ± 0.22	0.39 ± 0.03	0.83 ± 0.04
	542.71	15.20 ± 0.07	0.83 ± 0.04	1.33 ± 0.04
C				
Control	0	3.00 ± 0	0.25 ± 0	0.45 ± 0.02
	0.48	3.20 ± 0.17	0.25 ± 0	0.45 ± 0.05
	5.36	5.80 ± 0.30	0.35 ± 0.03	0.60 ± 0.02
	54.21	6.50 ± 0.60	0.43 ± 0.04	0.79 ± 0.05
	542.71	10.60 ± 0.90	0.71 ± 0.05	0.88 ± 0.05
HxA_3-C (8S)	0	3.00 ± 0	0.25 ± 0	0.50 ± 0.04
10^{-6} M	0.48	3.00 ± 0	0.25 ± 0	0.61 ± 0.03
	5.36	5.20 ± 0.30	0.35 ± 0.02	0.62 ± 0.03
	54.21	6.80 ± 0.70	0.56 ± 0.07	0.78 ± 0.04
	542.71	11.00 ± 1.20	0.74 ± 0.05	0.90 ± 0.05
D				
Control	0	3.00 ± 0	0.25 ± 0	0.70 ± 0.03
	0.48	3.00 ± 0	0.25 ± 0	0.90 ± 0.03
	5.36	6.50 ± 0	0.33 ± 0.05	1.04 ± 0.05
	54.21	9.00 ± 0.70	0.50 ± 0.04	1.19 ± 0.04
	542.71	14.22 ± 0.10	0.97 ± 0.10	1.42 ± 0.04
HxA_3-C (8R)	0	3.00 ± 0	0.25 ± 0	0.69 ± 0.05
10^{-6} M	0.48	3.00 ± 0	0.25 ± 0	0.89 ± 0.21
	5.36	9.50 ± 0.60***	0.53 ± 0.08*	1.01 ± 0.02
	54.21	12.30 ± 0.04***	0.68 ± 0.06*	1.28 ± 0.03
	542.71	14.30 ± 1.00	1.01 ± 0.08	1.45 ± 0.06
E				
Control	0	3.00 ± 0	0.25 ± 0	0.51 ± 0.03
	0.48	3.00 ± 0	0.25 ± 0	0.57 ± 0.03
	5.36	4.00 ± 0.30	0.25 ± 0	0.77 ± 0.09
	54.21	6.00 ± 0.40	0.55 ± 0.07	0.99 ± 0.05
	542.71	14.80 ± 1.10	0.95 ± 0.14	1.33 ± 0.04
$TrXA_3$ (8S)	0	3.00 ± 0	0.25 ± 0	0.53 ± 0.04
10^{-6} M	0.48	3.00 ± 0	0.25 ± 0	0.51 ± 0.03
	5.36	4.00 ± 0.30	0.25 ± 0	0.77 ± 0.07
	54.21	6.30 ± 0.20	0.58 ± 0.08	0.93 ± 0.05
	542.71	15.30 ± 1.30	0.94 ± 0.11	1.27 ± 0.03

Values are means ± s.e.mean for six separate tissues. Each treatment was compared to the control value which corresponds to the pretreatment value by using Student's *t* test for paired samples. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.005$ with respect to corresponding control value.

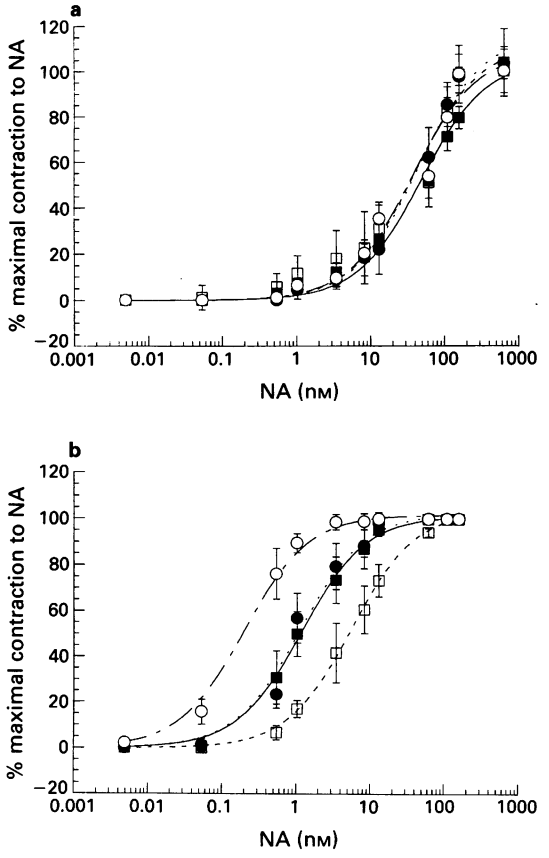

Figure 5 Dose-response curves to noradrenaline (NA) ($n = 6$) showing the requirement of calcium for the hepoxilin-induced potentiation of NA contraction. (a) Calcium-free medium, (b) normal Krebs solution in the presence of nifedipine. Note that the hepoxilins are ineffective in potentiating the NA effect in a calcium-free medium (a), and that nifedipine in normal Krebs solution causes an inhibition of the hepoxilin effect. When nifedipine is washed out, hepoxilin potentiation returns in the presence of the vehicle for nifedipine (ethanol). Symbols: (a) (■) control pre-hepoxilin A_3 (HxA_3); (□) HxA_3 (8S) 10^{-8} M; (●) control pre-dimethylsulphoxide (DMSO); (○) DMSO vehicle for HxA_3; (b) (■) control, pre-nifedipine and HxA_3; (□) nifedipine (1 μM) + HxA_3 (8S) (10^{-8} M); (●) control, pre-ethanol and HxA_3; (○) ethanol + HxA_3 (8S) 10^{-8} M.

are not yet known. We have shown in neutrophils that the hepoxilin-mediated actions on calcium and second messenger release are inhibited by pertussis toxin (Dho *et al.*, 1990), suggesting that hepoxilin action may be under receptor control coupled to G-protein activation. Further work needs to be done to explore whether the vasculature has receptors for the hepoxilins.

It has been shown that 12-HETE potentiates the vasoconstrictor effect of phenylephrine in rabbit thoracic aortic rings (Masferrer & Mullane, 1988). These authors further showed that the effect of HETE was stereospecific in that only the 12**R**-isomer was active. It should be noted that the hepoxilins used in this study possess either an epoxide group with a 12**S** stereochemistry (HxA_3), or a 12S-hydroxyl group (HxA_3-C). Hence the stereochemistry at carbon 12 of the hepoxilins is opposite to that in the active enantiomer of 12-HETE, i.e. 12**R**. It is interesting that while 12S-HETE was found to be inactive in modulating vascular contraction (Masferrer & Mullane, 1988), the hepoxilins with the 12**S** stereochemistry were indeed active as long as the hydroxyl group at carbon 8 was in the proper stereochemistry, i.e. in the HxA_3 series, the 8**S** enantiomer is the active compound while in the HxA_3-C series in which a 12S-hydroxyl group is present, the 8**R** isomer is the active species. Further correlation of the responses between 12**R**-HETE and the hepoxilins will be derived by comparing the activity of the hepoxilins with the 12**R** stereochemistry. This would be worth investigating to determine which part of the molecule is responsible for the biological activity shown in this study.

Hepoxilin action requires calcium. This was shown in the aorta by the lack of effect of hepoxilins in potentiating the NA-induced contraction in calcium-free medium as well as blockade of the hepoxilin-induced potentiation of the NA-induced contraction in the presence of nifedipine. This supports our concept that the hepoxilins affect the smooth muscle cells through a calcium-mediated mechanism, either making calcium available in stores for mobilization by vasoconstrictors or through the compartmentalization and/or release of second messengers for immediate activation by vasoconstrictors. Further studies are in progress to investigate these possibilities.

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Endothelial-dependent sexual dimorphism in vascular smooth muscle: role of Mg^{2+} and Na^+

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1 In isolated aortae of the male rat $[Mg^{2+}]_o$ withdrawal and concomitant reduction in $[Na^+]_o$ (to 84 mM) induced significant increases of basal tone, but, surprisingly, this did not occur in intact aortae removed from female rats. Such tension development, however, was observed in endothelium-denuded aortic preparations from both sexes. These observed gender-related differences were not dependent on animal strain or types of tissue preparations.

2 No tension development was observed in aortae obtained from castrated males treated with oestradiol. Aortic tissues of sexually-immature male and female rats exhibited marked tension development when exposed to 0 mM $[Mg^{2+}]_o$ and low $[Na^+]_o$.

3 Tension development in Mg^{2+}-free, low-Na^+ media was not tachyphylactic and completely dependent on extracellular Ca^{2+}; addition of 1.2 mM Mg^{2+} to the Mg^{2+} and Na^+-deficient incubation media relaxed the increase in tension to a normal basal level.

4 Two known endothelial-derived relaxant factor (EDRF) inhibitors, methylene blue and haemoglobin, induced tension development in female aortae with intact endothelium exposed to Mg^{2+}-Na^+ deficient media, while use of a specific inhibitor of EDRF-derived nitric oxide, *viz.*, N^G-monomethyl-L-arginine (L-NMMA), resulted in potentiation of tension development in male, but not in female, aortae. This effect of L-NMMA was antagonized by L-arginine.

5 The Ca ionophore, A23187, partially relaxed contractile responses in male aortae (with intact endothelium) which were followed by potentiated contractions. Endothelium-dependent vasodilator responses to A23187 (10^{-10}–10^{-6} M) of aortic rings from male or female rats in normal Krebs-Ringer bicarbonate solution were not different.

6 These results suggest that: (a) as in vascular smooth muscle cells, Mg^{2+} plays an important role in Ca^{2+} homeostasis in endothelial cells, probably via Na^+-Ca^{2+} exchange; and (b) sex steroid hormones, probably the female sex hormone, 17-β-oestradiol, may regulate contractile responses of intact vascular smooth muscle by modifying endothelium functions through such Mg^{2+}-regulated internal Na^+-dependent Ca^{2+} entry. These data may help to explain why female subjects, despite Mg deficiency, unlike male subjects, are protected against ischaemic heart disease and cerebrovascular disease until menopause.

Keywords: Gender differences; magnesium regulated Na^+-Ca^{2+} exchange; endothelial-derived factors; vascular smooth muscle

Introduction

Gender-related differences in haemodynamic characteristics have received considerable scientific attention because women, prior to menopause, are known to be less susceptible to numerous cardiovascular disorders when compared to men (for reviews, see Altura & Altura, 1977; Caplan *et al.*, 1986; Lerner & Kannel, 1986). The precise mechanism(s) of sex steroids in the modulation of cardiovascular function, however, remains to be elucidated.

Recently, Maddox *et al.* (1987) reported that there is an endothelium-dependent gender difference in responses of rat aortae to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). *In vivo* and *in vitro* studies have demonstrated that female hormones potentiate endothelium-dependent relaxations to acetylcholine (Williams *et al.*, 1990; Gisclard *et al.*, 1988). Sex steroid hormones may influence vascular reactivity (Altura & Altura, 1977) via this previously unrecognized property of endothelial cells which generates and releases endothelium-derived relaxant factor(s) (EDRF) (Furchgott & Vanhoutte, 1989).

Intracellular, free calcium ions ($[Ca^{2+}]_i$) are typically thought of as playing a critical role in synthesis and/or release of EDRF from endothelial cells (Long & Stone, 1985). It has been suggested that, in addition to Ca channels, Na^+-Ca^{2+} exchange may participate in the ion transport mechanisms involved in Ca^{2+} homeostasis in both endothelial and vascular smooth muscle cells (Adams *et al.*, 1989).

It has been demonstrated that magnesium (Mg^{2+}) can exert antagonistic effects on Na^+-Ca^{2+} exchange in cardiac and vascular smooth muscle cells (Wakabayashi & Goshima, 1981; Smith *et al.*, 1987), but such data have not been reported for endothelial cells. However, removal of extracellular Mg^{2+} ($[Mg^{2+}]_o$) has been shown to induce $[Ca^{2+}]_o$-dependent vasodilatation by releasing EDRF from endothelial cells (Ku & Ann, 1987; Gold *et al.*, 1990). Such relaxation of vascular smooth muscle is inhibited by dichlorobenzamil (DCB), an amiloride analogue and inhibitor of Na^+-Ca^{2+} exchange (Siegel *et al.*, 1984). This led us to the suggestion that Mg^{2+} may also modulate activity and function of Na^+-Ca^{2+} exchange in endothelial cells. Mg^{2+}-regulated Na^+-Ca^{2+} exchange may have opposite effects on vascular tone: (1) a release of EDRF from endothelium, which leads to relaxation, and (2) activation of contraction in vascular smooth muscle (either of which may be modulated by the presence of sex steroids).

With this in mind, we conducted experiments to examine possible gender differences in contractile responses of vascular tissues to alteration of $[Mg^{2+}]_o$ and $[Na^+]_o$ ions. It was anticipated that such experiments would provide new information on how hormones act on Ca^{2+} translocation pathways involved in the expression of endothelium-dependent relaxation. The data presented here, suggest that the female sex hormone, 17-β-oestradiol, regulates contraction and tone of intact blood vessels by modulating endothelial cell-derived factors through Mg^{2+}-regulated Na^+-dependent Ca^{2+} entry.

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Methods

Animals, preparations and general procedure

The experiments were performed on isolated aortae taken from: (1) adult Wistar and Sprague-Dawley male and female rats (16–24 weeks old and weighing 275–400 g); (2) young (sexually-immature) Wistar male and female rats (2–4 weeks old and weighing 80–100 g); (3) adult Wistar male and female rats treated by castration and replacement of sex steroid hormones. In these experiments, 8-week-old male and female rats were castrated or ovariectomized bilaterally under pentobarbitone sodium anaesthesia (Anthony Products Co, Arcadia, California; 30 mg kg^{-1}, i.p.). After 3 days of recovery, these male and female animals were treated with 17-β-oestradiol benzoate (Squibb and Sons, Inc., Princeton, New Jersey, U.S.A.; 1.5 mg kg^{-1}, i.m.) or testosterone (Schein Pharmaceutical, Inc., Phoenix, Arizona, U.S.A.; 2.5 mg kg^{-1}, i.m.), respectively, every three days for 4 weeks.

All animals were killed by decapitation and exsanguinated. Thoracic aortae were excised and immediately placed in normal Krebs-Ringer bicarbonate (NKRB) solution at room temperature and cleaned of blood, loose connective tissue and fat. Aortic strips were cut helically (2 mm in width by 25 mm in length) for adult animals (Altura & Altura, 1974), and 1 mm in width by 20 mm in length for the sexually-immature animals. Some aortae isolated from adult Wistar rats were cut into rings about 2 mm long. For intact tissue preparations, extreme care was taken to avoid damage of endothelial cells. In every other ring, the endothelium was removed gently with small forceps according to the method of De Mey & Vanhoutte (1982).

The composition of the NKRB was (in mM): NaCl 118, KCl 4.7, $KHPO_4$ 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 10 and $NaHCO_3$ 25. When Mg^{2+}-free, low-Na^+ solutions (Mg-free low-Na KRB) were used, 59 mM NaCl was replaced by an isosmolar amount of sucrose. The residual Na ion concentration in the substituted solutions was 84 mM.

Measurement of vascular reactivities of aortic rings and strips

Thoracic aortic rings, with and without endothelium, and strips isolated from adult rats were arranged isometrically, under resting tensions of 4.0 or 1.5 g, respectively, while 0.5 g of loading tension was used in aortic strip tissues from younger (sexually-immature) Wistar rats. All tissues were initially equilibrated for 2 h in chambers containing 20 ml of NKRB at 37°C and gassed continuously with a 95%O_2:5%CO_2 mixture. The loading tensions were adjusted periodically and maintained throughout the equilibration period. The incubation media were routinely changed every 10–15 min as a precaution against interfering metabolites (Altura & Altura, 1970). The tissues were attached to force-displacement transducers (Grass Model FT 03) connected to Grass Model 7 polygraphs, and isometric tensions of the vascular smooth muscle preparations were recorded. The stable level of tension developed in response to the addition of 80 mM KCl was always measured prior to the collection of data. To examine the functional viability of an intact endothelium, aortic rings were precontracted by ED_{50} doses of $PGF_{2\alpha}$ as described below, and the presence and absence of endothelium was confirmed by testing for relaxation to acetylcholine (5×10^{-7} M) (Furchgott & Vanhoutte, 1989), which generally resulted in 90% relaxation in rat aortae with intact endothelium.

After equilibration in NKRB, the tissues were exposed to Mg^{2+}-free low-Na^+ KRB for variable periods, of from 30 to 120 min, and then observed for tension development (Altura & Altura, 1974). In order to determine if Mg^{2+} and Ca^{2+} were important in low-Na^+-induced alterations of tension development, $MgSO_4$ (1.2 mM) was reintroduced to the Mg^{2+}-free low-Na^+ KRB (to restore normal $[Mg^{2+}]_o$) or $CaCl_2$ was withdrawn from Mg^{2+}-free low-Na^+ KRB, respectively. In these experiments, all observations were repeated at least twice in the same tissue, and each tissue was returned to NKRB after the incubation in modified KRB solutions for at least 30 min to re-establish normal vascular reactivity and tone.

Role of endogenous vasoactive substances

To determine whether the gender-related differences in responses in Mg^{2+}-free, low-Na^+ KRB could be attributed to differences in the endogenous release of specific types of vasoactive amines from the blood vessels (e.g., noradrenaline, acetylcholine, histamine and 5-hydroxytryptamine (5-HT)), adenine nucleotide (ATP), peptides (substance P) or prostaglandins) these vasoactive substances (10^{-6} M) were examined in the modified KRB solutions. Other experiments were conducted by treating tissues with a variety of specific pharmacological antagonists as well as a cyclo-oxygenase inhibitor (i.e., indomethacin) before and during incubation in modified KRB solutions. These antagonists were used in concentrations which produced specific antagonism to their respective agonists and cyclo-oxygenase in rat aortic tissue (i.e., 10^{-7} to 10^{-5} M) (Altura *et al.*, 1976). The drugs used for these studies included noradrenaline bitartrate (Sigma Chem. Co., St. Louis, Missouri, U.S.A.), acetylcholine chloride (Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A.), 5-HT creatinine sulphate (Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A.), histamine hydrochloride and substance P (Sigma Chem. Co., St. Louis, Missouri, U.S.A.), atropine sulphate (Mann Res. Labs, New York, U.S.A.), phentolamine methanesulphonate (Regitine, Ciba-Geigy, Summit, New Jersey, U.S.A.), diphenhydramine HCl (Benadryl, Parke Davis Co; Ann Arbor, Michigan, U.S.A.), propranolol HCl and ATP (Sigma Chem. Co., St. Louis, Missouri, U.S.A.), methysergide maleate (Sandoz Ltd; Basel, Switzerland), and indomethacin (Merck, Rahway, New Jersey, U.S.A.).

Role of endothelium-derived relaxing factor

To examine whether activation or release of EDRF from endothelial cells was involved in the vascular responses to Mg^{2+}-free, low-Na^+ media, three known EDRF inhibitors, i.e., methylene blue (10^{-5} M, Sigma Chem. Co., St. Louis, Missouri, U.S.A.), oxyhaemoglobulin (10^{-5} M, kindly provided by Dr R.F. Furchgott) and N^G-monomethyl-L-arginine (L-NMMA, 3×10^{-4} M, Calbiochem Co., La Jolla, California, U.S.A.), as well as the Ca ionophore A23187 (5×10^{-7} M, Calbiochem Co., La Jolla, California, U.S.A.) (Furchgott & Vanhoutte, 1989), were tested in aortic ring preparations incubated in Mg^{2+}-free, low-Na^+ KRB solutions. In the experiments dealing with L-NMMA, L-arginine (3×10^{-4} M, Calbiochem Co., La Jolla, California, U.S.A.) was used to test whether the effects of L-NMMA could be reversed.

In order to study endothelium-dependent responses in NKRB solutions, the intact and denuded ring preparations of male or female rat aortae were initially precontracted with an ED_{50} dose of $PGF_{2\alpha}$, i.e., $2–4 \times 10^{-7}$ M or $1–3 \times 10^{-6}$ M, respectively, followed by challenge with the Ca ionophore A23187 (10^{-10}–10^{-6} M); concentration-dependent relaxation curves were obtained. The observed differences in ED_{50}s for $PGF_{2\alpha}$ used here resulted from sexual differences in sensitivity to the agent (Maddox *et al.*, 1987). However, concentrations chosen for $PGF_{2\alpha}$ were intended to produce identical levels of contractile force, which is important for comparing vasodilator effectiveness (Winquist *et al.*, 1984), in both male and female animals. Care was taken to contract each ring tested, herein, to similar levels of force (i.e., 800–900 mg) before challenging with A23187.

Statistical analyses

Where appropriate, means ± s.e.mean were calculated and compared for statistical significance by means of Student's *t*

tests, paired t tests or ANOVA using Scheffe's contrast test. P values less than 0.05 were considered significant.

Results

Tension development in response to lowering of $[Mg^{2+}]_o$ and $[Na^+]_o$ with and without castration and/or replacement of sex hormones

Figure 1 shows recordings of typical changes in resting tension of isolated aortic rings from male and female rats when placed in Mg^{2+}-free low-Na^+ KRB solution. Simultaneous $[Mg^{2+}]_o$ withdrawal and lowering the $[Na^+]_o$ concentration from 143 to 84 mM induced significant increases of basal tone in aortic rings isolated from male Wistar rats, but not in tissues from female Wistar rats. Surprisingly, such tension development induced by lowering $[Mg^{2+}]_o$ and $[Na^+]_o$ was observed in endothelial-denuded aortic rings isolated from either sex, in which male tissues clearly exerted greater responses than female tissues (Figure 1). Similarly, sex differences in responsiveness to Mg^{2+}-free low-Na^+ KRB solution were also observed in aortic strip preparations from both adult Wistar and Sprague-Dawley rats as shown in Figure 2. It should be noted that about 14% of intact male aortae (7 out of 51 preparations) did not show significant contractile responses, i.e., the tone induced by $[Mg^{2+}]_o$- and $[Na^+]_o$-deficient media was below 30% maximal K^+-induced contraction in these tissues, and therefore these results were not included in Figure 2. However, little in the way of tension development on lowering $[Mg^{2+}]_o$ and $[Na^+]_o$ ions was observed in aortae from male Wistar rats castrated and treated with 17-β-oestradiol, while castration and treatment with testosterone failed to induce contraction in female Wistar rats exposed to Mg^{2+}-free low-Na^+ KRB solution (Figure 2). Castration without replacement of sex steroids failed to exert any effect on basal tone (data not shown). In contrast to adults, no gender-related differences were observed in the young sexually-immature Wistar rats; relatively greater contractile responses in terms of %K^+-induced maximal contractions (to lowering of $[Mg^{2+}]_o$ and $[Na^+]_o$ ions) were observed compared to the adult rats (Figure 2). Such mechanical changes in base-line tension were not tachyphylactic and

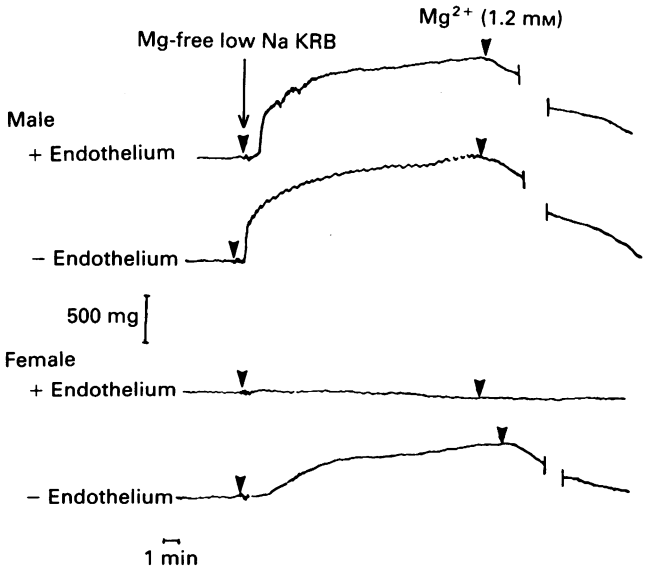

Figure 1 Effects of extracellular magnesium removal and readdition on basal tone of aortae isolated from adult Wistar male and female rats in low-Na containing medium (84 mM NaCl). Intact (+ Endothelium) and endothelium-denuded (− Endothelium) aortic rings were mounted and equilibrated as described in the text. Subsequent replacements of NKRB medium with Mg-free, low-Na KRB medium are indicated by the arrows. Mg^{2+} (1.2 mM) denotes readdition of 1.2 mM $MgSO_4$ to the bathing medium.

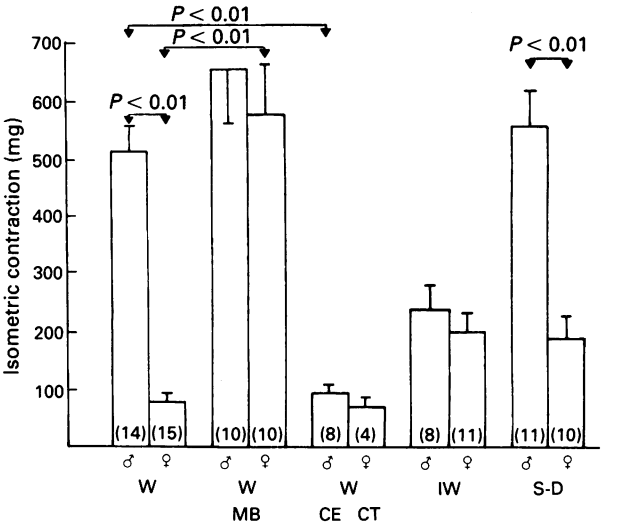

Figure 2 Effects of $[Mg^{2+}]_o$ removal from low-Na KRB medium on basal tone of rat aortic strips. Strip tissues were mounted and equilibrated as described in the text. Columns represent the means with s.e.mean shown by vertical bars; numbers of animals in parentheses; W = adult Wistar rats; MB = methylene blue-treated; CE = castrated and oestradiol-treated; CT = castrated and testosterone-treated; IW = sexually-immature Wistar rats; S-D = adult Sprague-Dawley rats.

could be maintained for at least 2 h. Addition of 1.2 mM $MgSO_4$ to the incubation media relaxed the increase in tension observed to normal resting levels in all of these intact or denuded tissues (Figure 1). Removal of $[Ca^{2+}]_o$ from Mg^{2+}-free low-Na^+ KRB completely abolished tension development in all tissues tested (data not shown, $n = 12$). Lowering of only $[Na^+]_o$ to 84 mM (in the presence of 1.2 mM $[Mg^{2+}]_o$) failed to produce any changes in basal tone in either male or female tissues ($n = 16$).

Failure of specific neurotransmitters and hormones to induce relaxation, and failure of specific pharmacological antagonists as well as cyclo-oxygenase inhibitor to interfere with tonic responses of rat aortae

None of the relaxants when tested over wide concentration ranges (i.e., acetylcholine, substance P, 5-HT, histamine or ATP) (10^{-9}–10^{-6} M), induced relaxation of male aortic tissues incubated in Mg^{2+}-free low-Na^+ KRB solution. Experiments with antiadrenoceptor, anticholinoceptor and antihistamine agents, as well as a cyclo-oxygenase inhibitor (phentolamine, propranolol, atropine, diphenhydramine, and indomethacin, respectively) revealed an inability of these specific pharmacological antagonists and cyclo-oxygenase inhibitor to interfere with the contractile responses observed on lowering of $[Mg^{2+}]_o$ and $[Na^+]_o$ ions (six to eight experiments were performed with each agent). Methysergide, an antagonist of 5-HT, in a concentration of 10^{-6} M, induced slight contractions (8% K^+-induced maximal contractions) followed by relaxation in aortic tissues incubated in Mg^{2+}-free low-Na^+ KRB solution.

Effects of methylene blue, oxyhaemoglobulin and N^G-monomethyl-L-arginine, as well as A23187, on tonic responses of rat aortae

Figures 3 and 4 illustrate recordings of typical changes of resting tension in aortic rings obtained with Mg^{2+}-free, low-Na^+ KRB before and after the addition of methylene blue and A23187. Methylene blue (MB, 10^{-5} M) enhanced tension development in both intact and denuded aortic rings from both male and female rats in Mg^{2+}-free, low-Na^+ KRB solution (Figure 3). In aortic strips, MB also significantly potentiated contractile responses as seen in the ring preparations

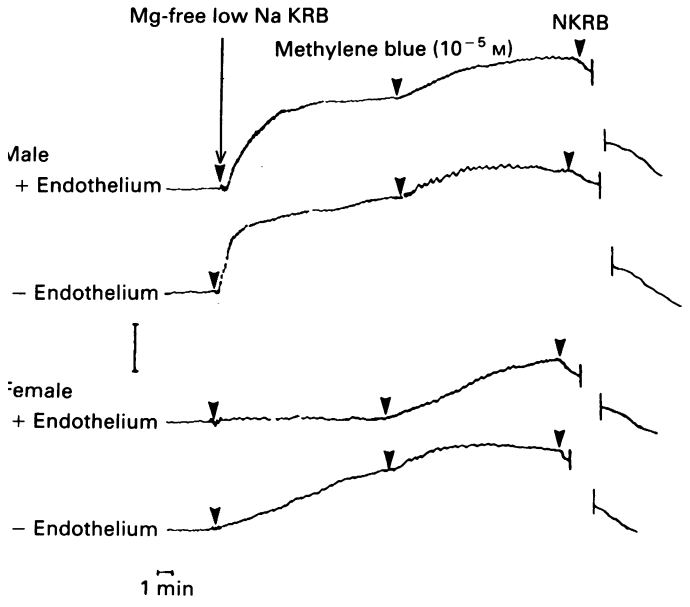

Figure 3 Influence of methylene blue on alterations in tone elicited by reduction $[Mg^{2+}]_o$ and $[Na^+]_o$ in rat aortae. Intact and denuded aortic rings were mounted and equilibrated as described in the text. Conventions similar to these used in Figure 1. At peak tension in Mg-free low-Na KRB, methylene blue (10^{-5} M) was added as indicated by the second set of arrows. This tracing is representative of six different tissues.

(Figure 2). In contrast to MB, L-NMMA treatment (3×10^{-4} M) exerted only potentiating effects on contractile responses of male aortae, and failed to interfere with responses of female aortae (data not shown, $n = 6$). The effects of L-NMMA were completely antagonized by the same concentration of L-arginine (3×10^{-4} M). A23187 (10^{-6} M) produced initial relaxation (about 50% of tonic amplitude) followed by enhanced contraction in intact male tissues; however, no effects of this agent were observed in aortic rings from female rats, or in denuded tissues (Figure 4). Neither methylene blue nor A23187 was found to exert such effects in NKRB solution ($n = 6$). As with methylene blue, addition of oxyhaemoglobulin (10^{-5} M) resulted in a furtherance of contractile amplitude

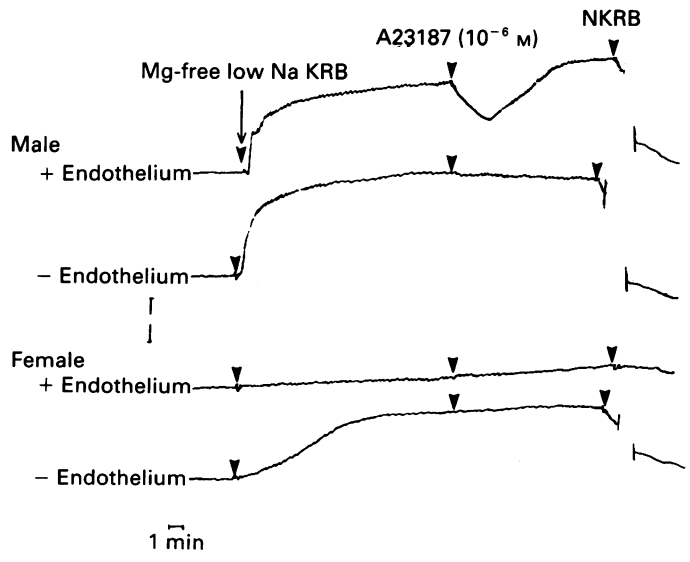

Figure 4 Influence of the Ca ionophore, A23187, on alterations in basal tone elicited by reduction $[Mg^{2+}]_o$ and $[Na^+]_o$ in rat aortae. Intact and denuded aortic rings were mounted and equilibrated as described in the text. Conventions similar to those in Figure 1. At peak tension in Mg-free, low-Na KRB, A23187 (10^{-6} M) was added as indicated by the second set of arrows. This tracing is representative of eight different tissues.

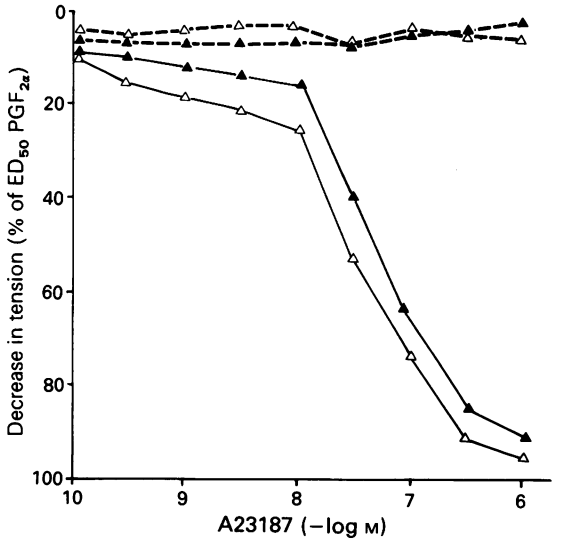

Figure 5 Lack of a gender difference observed on cumulative concentration-response curves to A23187-induced relaxation in aortic rings isolated from adult male and female rats, which were precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (ED_{50}). Data are presented as a percentage of the contractile response to $PGF_{2\alpha}$ and expressed as means with s.e.means shown by vertical bars. $n = 6$ in each gender group. Denuded tissues from both gender groups exerted little or no relaxation response to acetylcholine. Female: ▲——▲ intact rings; ▲---▲ denuded rings; Male: △——△ intact rings; △---△ denuded rings.

(160–190% of K^+-induced maximal contraction) in aortae from both male and female rats in $[Mg^{2+}]_o$- and $[Na^+]_o$-deficient medium (data not shown, $n = 4$).

A23187-induced relaxation in normal Krebs Ringer solution

In both male and female rats, A23187 (10^{-10} to 10^{-6} M) produced cumulative concentration-dependent relaxations in the $PGF_{2\alpha}$-precontracted aortic rings with endothelium; no relaxations were observed in rings without endothelium (Figure 5). The observed differences in magnitudes of relaxation to A23187 between male and female rats were not found to be significant in the present studies ($n = 6$).

Discussion

The results described here with acute removal of Mg^{2+} ions in low-Na^+ media demonstrate two contrasting aortic vascular effects of Mg^{2+} in male and female animals. A marked increase in resting tension occurs in isolated intact aortae of males but not in intact aortae from females when Mg^{2+} and Na^+ concentrations are reduced in the incubation medium. Such contractions are observed in tissues from both male and female rats, when denuded. This gender-related difference is evident in both Wistar and Sprague-Dawley rats in both helical-cut strips and rings of aorta. Therefore, the data suggest a strain- and tissue-type-independence. Endothelial cells in female animals seem to exert predominant effects on the modulation of vascular responses to a lowering of $[Mg^{2+}]_o$ and $[Na^+]_o$, probably by a release of EDRF, because contractile responses were observed in tissues without endothelium in either sex. Indomethacin failed to interfere with such events, suggesting that prostacyclin and other dilator prostanoids are not involved. Two different inhibitors of EDRF, viz., methylene blue and oxyhaemoglobulin, however, potentiated tension in male and female aortae supporting the hypothesis that EDRF released from endothelial cells mediates the gender-related differences observed in intact rat aortae.

The mechanism whereby endothelial function in females is dominant, as observed in the present studies, is not known.

We cannot find any difference between young, sexually-immature male and female rats, suggesting that the unusual change of vascular reactivity and basal tone noted herein is associated with sexual maturity. Since castration and treatment with oestradiol inhibited the cation-related increments in basal tension of male rat aortae, while aortae from castrated-testosterone-treated female rats did not produce contraction, it seems that female sex hormones (probably 17-β-oestradiol) exert predominant effects on the modification of vascular responses to reduction of $[Mg^{2+}]_o$ and $[Na^+]_o$ ions. Several explanations and sites of action of sex steroid hormones seem plausible.

The lack of effect of amine antagonists in male or female tissues in our studies, suggest that sex-related differences of vascular responsiveness in Mg^{2+}-free, low-Na^+ media cannot be attributed to the release of neurotransmitters, from autonomic nerve terminals or endothelial cells (Altura & Altura, 1977; Burnstock, 1987).

Since no difference of vasorelaxation to the Ca ionophore A23187 in NKRB solution was found between male and female animals, the passive mechanical properties of vascular tissues (Fischer & Swain, 1977; Cox & Fischer, 1978) also cannot explain our results. The Ca ionophore A23187 is believed to increase $[Ca^{2+}]_i$ by enhancement of Ca^{2+} influx (Reed & Lardy, 1972), independent of membrane Ca channels and antiporter systems. Thus, our results of endothelium-dependent relaxation to A23187 in NKRB solution suggest further that, after the initial triggering step of Ca^{2+} influx into the vascular endothelial cells, there are no differences in the expression of endothelium-dependent relaxation, including synthesis and/or release of EDRF as well as sensitivity of the vascular smooth muscle to EDRF, between male and female vascular tissues. Since oestrogen has been demonstrated to have no effect on the level of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in vascular smooth muscle (Kishi & Numano, 1982) such a direct mechanism of action is unlikely.

We reported recently, that reductions in $[Na^+]_o$, which cause no contractile effects in normal $[Mg^{2+}]_o$ media, caused a Ca^{2+}-dependent tension development when $[Mg^{2+}]_o$ was simultaneously withdrawn from isolated aortic smooth muscle of male rats (Altura *et al.*, 1990). These studies, concomitant with observations in cultured vascular smooth muscle cells (Smith *et al.*, 1987; 1989), support the idea that there are Mg^{2+}-regulated Na^+-Ca^{2+} exchanges in the plasma membrane in vascular smooth muscle (Altura & Altura, 1982; Smith *et al.*, 1987). Thus, it is not unreasonable to postulate that there is facilitation of Na^+-Ca^{2+} exchange when endothelial cells are exposed to Mg^{2+}-free, low-Na^+ media.

There is substantial evidence that both endothelial cells and vascular smooth muscle cells possess specific oestrogen receptors (Colburn & Buonassisi, 1978; Harder & Coulson, 1979). Female steroid hormones may, in some unknown way, exert direct influences on Mg^{2+}-regulated Na^+-Ca^{2+} exchange to control the intracellular Ca^{2+} concentration of endothelial cells. Since potentiation of tension development of male rat aortae always occurred with removal of endothelial cells, a relatively lower but definite activity of endothelium seems to operate normally also in these tissues. Support for this hypothesis can be derived from the present studies performed with the Ca ionophore, A23187, which results in endothelium-dependent vasodilatation followed by contraction.

Nitric oxide (NO) is an important EDRF, which is formed from L-arginine in endothelial cells (Palmer *et al.*, 1988; Furchgott & Vanhoutte, 1989). L-Arginine metabolism in endothelium can be reversibly inhibited by competition of the NO-synthesizing enzyme using an analogue of L-arginine, L-NMMA (Furchgott & Vanhoutte, 1989; Rees *et al.*, 1989). It is noteworthy, in our present studies, that there was gender dimorphism of vascular responses to L-NMMA when aortic tissues were incubated in Mg^{2+}- and Na^+-deficient media, i.e., L-NMMA potentiated tension development in male, but not in female, aortic tissue. Since such potentiating effects of L-NMMA were completely reversed by L-arginine, the data are compatible with the notion that a certain amount of NO was indeed produced and released from endothelial cells in male aortae by lowering $[Mg^{2+}]_o$ and $[Na^+]_o$, and may be a reason why a small number of the male aortic tissues failed to undergo tension development. It is puzzling as to why addition of L-NMMA to the female aortic smooth muscle, exposed to low $[Mg^{2+}]_o$ and $[Na^+]_o$, does not result in tension development.

Although considerable evidence now exists that low dietary intake of Mg is associated with increased incidence of sudden death ischaemic heart disease (SDIHD) in men below the age of 50 (Seelig, 1980; Turlapaty & Altura, 1980; Altura & Altura, 1985), which is thought to be attributed to coronary vasospasm (for reviews, see Altura & Altura, 1985; 1990), it has not been possible to explain why women ingesting similar, low levels of Mg do not exhibit such an incidence until after the age of 50 (Seelig, 1980). If our results pertain to human coronary vessels, then the loss of oestrogenic hormones in postmenopausal women together with deficits in Mg dietary intake would result in a similar incidence of SDIHD, exactly as is noted clinically.

In conclusion, the results presented here represent the first demonstration of an endothelium-dependent gender-related difference of vascular responsiveness to activation of Na^+-Ca^{2+} exchange, which is regulated by Mg^{2+}, in isolated arteries. Irrespective of the exact mechanism(s) whereby female steroid hormones modulate Mg^{2+}-regulated Na^+-Ca^{2+} exchange, our data could prove valuable in elucidating the precise control mechanisms for sexual dimorphism of vascular responsiveness. These observations may be of importance in explaining why premenopausal females are much less susceptible to cardiovascular disease processes than are males.

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Decreased inhibition by gravidin of arachidonate release from transformed compared to nontransformed cells

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1 Gravidin (a phospholipase A_2 inhibitor) reduced the release of arachidonic acid from human lymphocytes by 51% at 10^{-8} M.

2 Under normal culture conditions, nanomolar gravidin caused a significant reduction in the release of free arachidonic acid from human lymphocytes or nontransformed fibroblasts but in transformed cells, nanomolar gravidin was ineffective.

3 Inhibition of arachidonate release appeared to be related to rate of growth as inhibitory effects of gravidin on Jurkat cells and HL-29 cells could be observed if the cells were cultured under conditions where DNA synthesis was low.

4 The reported disparate effects of lipocortin on cell phospholipase A_2 activity may be reconciled if DNA synthesis is investigated.

Keywords: Gravidin; phospholipase A_2; DNA synthesis; arachidonic acid; cell growth; lymphocytes; lymphoma

Introduction

Regulation of phospholipase A_2 (PLA_2) (EC3.1.1.4) activity holds the key to the control of the arachidonic acid (AA) cascade and therefore inflammation and other disorders. Glucocorticoids are well characterized anti-inflammatory agents that are reported to inhibit PLA_2 through induction and release of lipocortins (Hirata *et al.*, 1980; Blackwell *et al.*, 1982). However, the antiphospholipase activity of lipocortins is controversial as in some systems, synthesis of lipocortins is coincidental with no change or an increase in phospholipid hydrolysis (Duval *et al.*, 1986; Northup *et al.*, 1988; Mitchell *et al.*, 1988). We isolated and characterized a PLA_2 inhibitor, gravidin (Wilson & Christie, 1991) that has been linked with maintenance of human pregnancy (Wilson *et al.*, 1989). At low concentrations gravidin inhibited human endometrial cell prostaglandin synthesis and decidual cell and lymphocyte [^{3}H]-AA release (Wilson *et al.*, 1985; Wilson & Christie, 1991) but it was noted that the active concentration varied according to the cell type used, suggesting that cells may differ in sensitivity to gravidin as for lipocortin. We have now extended these studies to human cells from other tissues and confirm that the effect of gravidin is selective in that it differs according to the type of cell tested and is markedly reduced in cells derived from tumours. Furthermore, for a given cell type, the inhibitory response to gravidin only occurs when cells are synthesizing DNA slowly.

Methods

Cell culture

Lymphocytes Lymphocytes were obtained with informed consent from healthy volunteers and one lymphoma patient diagnosed with a malignant lymphoma (nodular, poorly differentiated). Blood was collected in citrate anticoagulant and centrifuged in Sepalymph at 1000 *g* for 30 min. The layer of lymphocytes was removed carefully, washed twice in medium G199 with Earle's salts pre-equilibrated with 5% CO_2 and immediately labelled with [^{3}H]-AA (180 Ci mmol^{-1}) as described below.

Fibroblasts Human foreskin fibroblasts were obtained from primary cell cultures that had been stored in liquid N_2 (−196°C) and grown before treatment for at least two cell cycles in RPMI 1640 medium containing 5% foetal calf serum (FCS), 50 mg ml^{-1} penicillin and 50 u ml^{-1} streptomycin at 37°C in a humidified atmosphere containing 5%CO_2. Cells were detached from their dishes by treatment with 0.05% trypsin for 2 min before labelling with [^{3}H]-AA.

Measurement of [^{3}H]-arachidonic acid release

Radiolabelled AA in ethanol was dissolved in G199 media; the ethanol solvent constituted less than 0.1% of the final volume. Cells washed free from FCS were incubated for 45 min in 0.5 ml medium containing [^{3}H]-AA (1 μCi/10^6 cells). The radiolabelled cells were washed twice in G199 medium containing 1 mg ml^{-1} BSA and resuspended in G199 medium to a final concentration of approximately 4×10^6 cells per ml. Aliquots (50 μl) of the cell suspension were placed in Eppendorf tubes containing test substances in a final volume of 350 μl and incubated at 37°C in an atmosphere of 5% CO_2. Each treatment was performed in triplicate.

Following incubation, 100 μl of the cell suspension was removed from each incubation tube and the cells centrifuged lightly into a pellet before 75 μl of the supernatant was removed and its associated radioactivity was measured in a liquid scintillation spectrometer [^{3}H]-AA release which was linear over 5 h, was measured at time 0, 3 and 4 h. The mean of the 3 and 4 h values was taken for all data. Released radioactivity in the presence of gravidin was expressed as a percentage of the radioactivity released in the absence of gravidin. The average amount of [^{3}H]-AA incorporated by an aliquot containing 10^5 cells was 6118 ± 1010 d.p.m. During incubation, 3280 ± 390 d.p.m. were released and the d.p.m. in the supernatant at zero time averaged 29.5% of the total d.p.m. released during the experiment (mean ± s.e., $n = 27$ for all data above).

Preparation of gravidin

Gravidin was extracted from amniotic fluid as previously described (Wilson & Christie, 1991) and stored at −20°C in HEPES buffer (100 mM, pH 7.0) containing 50% glycerol. Purity was judged to be 98% on a Coomassie Blue stained SDS gel. Before its addition to test solutions, gravidin was incubated with 1 mM dithiothreitol (DTT) for 15 min at room temperature. Neither glycerol nor DTT affected the release of

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[^{3}H]-AA from the cells at the concentrations in the test solutions.

Thymidine incorporation

Cells were washed free of FCS and incubated with [^{3}H]-methyl-thymidine (1 μCi/0.5 ml cells; 25 Ci mmol^{-1}) for 3.5 h. The cells were pelleted and washed with SSC (0.15 M NaCl, 0.017 M Na citrate pH 7.00) resuspended in SSC containing 0.3 mg ml^{-1} BSA and left on ice for 30 min. DNA was precipitated with HCl-sodium pyrophosphate (1 ml 12% w/v in 1.5 M HCl) at 0°C for 15 min and then washed three times by centrifugation in 0.5% w/v Na pyrophosphate in 0.5 M HCl before radioactivity in the precipitate was measured.

Results

The effect of different doses of gravidin on [^{3}H]-AA release from normal unstimulated lymphocytes is shown in Figure 1. There was no change in [^{3}H]-AA release at 10^{-11} M gravidin but a statistically insignificant increase in PLA_2 activity occurred at 10^{-10} M. Inhibition reached a maximum of 51% between 3 and 10 nM gravidin.

To determine whether gravidin inhibits [^{3}H]-AA release in other cells, investigations were extended to confluent cell lines and cells from a variety of other sources. Gravidin was used at 10^{-9} M because at this concentration half the maximum response was obtained with lymphocytes. A comparison of cell types showed that gravidin was substantially more inhibitory to nontransformed than transformed cells (Table 1). Gravidin at a higher concentration (5×10^{-9} M) caused insignificant inhibition of arachidonate release from HT-29 or HL-60 cells (data not shown).

To determine if the different response of transformed and nontransformed cells could be attributed to differences in rate of growth, DNA synthesis was measured in cells of the same type from different sources (Table 2) and it was observed that DNA synthesis was significantly lower in normal lymphocytes that were inhibited by gravidin compared to lymphoma lymphocytes that were not.

To test further the hypothesis that the action of gravidin was related to the rate of cell growth, cultures of normal fibroblast, Jurkat and HL-60 cells were harvested and assayed for gravidin effects at intervals after subculture. DNA synthesis

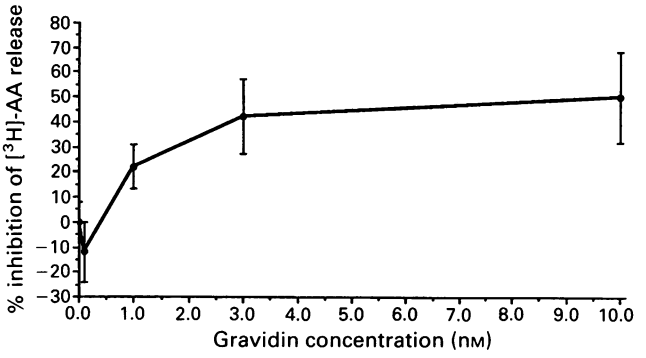

Figure 1 The effect of increasing gravidin concentration on lymphocyte [^{3}H]-arachidonic acid ([^{3}H]-AA) release. Lymphocytes were collected from healthy volunteers, washed and preincubated with [^{3}H]-AA as described in the Methods section. Nonstimulated release of [^{3}H]-AA was measured in the absence and presence of gravidin at different concentrations. Values given are the means and range of two experimental results and are expressed as a percentage of the control value in the absence of gravidin.

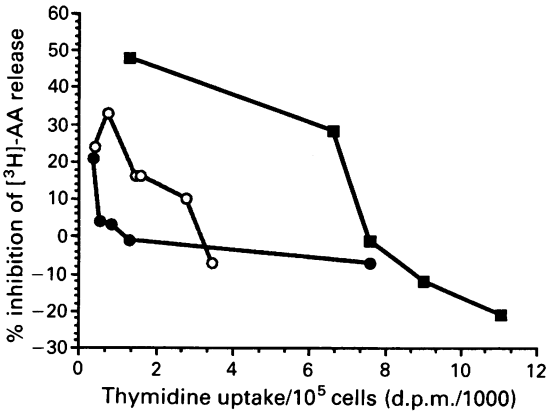

Figure 2 Effect of gravidin with cells with different rates of growth. Cells were treated with or without gravidin (10^{-9} M) at different stages of growth and thymidine incorporation/10^5 cells measured at the same time as inhibition of [^{3}H]-AA release by gravidin. Values shown are pooled from at least two subcultures and are expressed as a percentage of the control value in the absence of gravidin: (■) fibroblasts; (●) HT-29 cells; (○) Jurkat cells.

Table 1 The effect of gravidin on [^{3}H]-arachidonic acid ([^{3}H]-AA) release from different cultured human cell types

Cell type	*Source*	*Normal or transformed*	*% Inhib*	*s.e.mean*	n
HCT-8	Ileocaecal cancer	Transformed	−4	3.5	3
HT-29	Colon cancer	Transformed	−4	3.5	3
HL-60	Leukemia	Transformed	2	1.0	3
Jurkat	T cell	Transformed	11	5.7	3
Lymphoma	Lymphocytes	Transformed	2	6.4	3
	Lymphocytes	Normal	27	6.4	9
Fibroblasts	Foreskin	Normal	24	5	3

Cells were treated with gravidin (10^{-9} M) as described in Methods. Non-stimulated release of [^{3}H]-AA was measured in the absence and presence of gravidin at different concentrations. The lymphoma patient donated cells three times; 9 healthy donors contributed cells. Values given are the means of the experimental results and are expressed as a percentage of the control value in the absence of gravidin.

Table 2 Incorporation of thymidine by lymphocytes from different sources

Cell source	*Normal or transformed*	*Thymidine* (d.p.m. incorporated/ 10^5 cells)	*s.e.mean*	n
Jurkat	Transformed	1221	257	3
Lymphoma cells	Transformed	971	281	2
Lymphocytes	Normal	247	107	3

Lymphocytes were obtained from healthy donors, one lymphoma patient and T cells established in culture and assayed for thymidine incorporation as described in the Methods section.

was measured at the same times and the assays were made every two or three days until the cell number no longer increased and thymidine incorporation fell to below 1500 d.p.m./10^5 cells. Both thymidine incorporation and gravidin inhibition of arachidonate release were measured from the same cultures so that the two parameters could be correlated. The inhibitory effect of gravidin was found to be inversely related to the amount of DNA synthesized by the cells (Figure 2) with measurable inhibition only in cells with low rates of DNA synthesis. Gravidin stimulated [^{3}H]-AA release from fibroblasts with very high rates of DNA synthesis, while at all rates of DNA synthesis there was reduced sensitivity to gravidin in HT-29 cells and Jurkat cells compared to normal fibroblasts.

Discussion

Gravidin, an inhibitor of PLA_2 has recently been identified as secretory component of IgA (Wilson & Christie, 1991). Although gravidin is a potent inhibitor of PLA_2 in human endometrial cells, (Wilson *et al.*, 1985), it was found to have variable or little effect on transformed cells investigated in this study whereas [^{3}H]-AA release from normal lymphocytes and fibroblasts was significantly inhibited by gravidin.

A comparison of normal versus transformed lymphocytes from different sources showed that they responded differently to gravidin. Transformation appeared to make the cells much less sensitive to gravidin as [^{3}H]-AA release from both Jurkat cells (a T cell line) and lymphocytes obtained from an untreated lymphoma patient was inhibited less than [^{3}H]-AA release from normal cells.

To test whether the effect of gravidin on cells varied with their rate of growth, thymidine incorporation into DNA was compared with gravidin effect on [^{3}H]-AA release (Figure 2). With both normal and transformed cells, gravidin inhibition was greatest when thymidine incorporation was low indicating that the effect of gravidin depended on the rate of cell growth. However, the rate of DNA synthesis at which the cells began to respond to gravidin varied according to the cell type and the effects on transformed cells occurred only at very low rates of growth.

Another phospholipase inhibitor, lipocortin, is synthesized in response to glucocorticoid or dexamethasone treatment of cells (Hirata *et al.*, 1980; Blackwell *et al.*, 1982) and dexamethasone also has different effects on confluent and growing cells. In one study, Mitchell *et al.* (1988) demonstrated enhanced lipocortin and prostaglandin synthesis in cultured cells from human amnion. However, Gibb & Lavoie (1990) in a later study found that the inhibitory effect of dexamethasone on prostaglandin synthesis by human amnion cells depended on whether or not the cells were freshly cultured or had been cultured for several days. Glucocorticoids inhibited freshly cultured cells but became stimulatory after several days of culture. Such inconsistencies in the anti-phospholipase effect of lipocortin have impeded its acceptance as an inhibitor of PLA_2 (Duval *et al.*, 1986; Northup *et al.*, 1988). From our data and the other results, we suggest that the susceptibility of phospholipase to inhibitors changes with growth rate.

The reason for the decreased sensitivity of transformed cells to gravidin is not known. The lack of effect was not due to the protease treatment used to detach the cells from their dishes as cultured fibroblasts which responded to gravidin were detached from the dishes in the same way as the transformed cell lines (Figure 2). Increased PLA_2 activity has been associated with cell transformation (Alonso & Santos, 1990) and appears to be necessary for high growth rates (Palombella & Vicek, 1989). Possibly, acquired resistance to inhibition of phospholipase is an early event in cell transformation.

A selective effect of gravidin could be advantageous under some circumstances. Gravidin has been shown to inhibit decidual cell prostaglandin synthesis and thus may play a role in the maintenance of human pregnancy (Wilson *et al.*, 1985). Indomethacin, a cyclo-oxygenase inhibitor is sometimes used to delay preterm labour but may have adverse effects due to inhibition of prostaglandin synthesis in the foetus (Moise *et al.*, 1988; Mari *et al.*, 1989; Eronen *et al.*, 1991). If gravidin inhibits phospholipase only in non-growing cells, it would have an advantage over indomethacin in that foetal prostaglandin production would not be compromised.

In summary, our results show that cells have different sensitivity to gravidin depending on their stage of growth; actively growing cells are less affected by gravidin than freshly cultured or confluent cells. An *in vivo* consequence of this finding could be a selective effect of gravidin on cell types at different stages of growth (e.g. foetal vs. maternal cells during pregnancy). Transformed cells may be less sensitive to gravidin than growth controlled cells because of altered control of PLA_2. It seems possible that reported variable responses of other cells to lipocortins may also be explained by different rates of cell growth.

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Influence of N^G-nitro-L-arginine methyl ester on vagally induced gastric relaxation in the anaesthetized rat

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1 The influence of the nitric oxide (NO) biosynthesis inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) on the gastric relaxation induced by peripheral vagal stimulation was investigated in the anaesthetized rat.

2 Peripheral vagal stimulation (10 Hz, 10 V, 1 ms for 20 s) induced a reproducible biphasic response: a short-lasting increase followed by a more pronounced decrease in intragastric pressure. This response also occurred in reserpinized animals (5 mg kg^{-1}, i.p., 24 h before the experiment) while atropine (1 mg kg^{-1}, i.v.) abolished the initial increase in intragastric pressure.

3 L-NAME (1–30 mg kg^{-1}, i.v.) induced an increase in arterial blood pressure. L-NAME (1 mg kg^{-1}, i.v.) had no influence on the vagally induced gastric response while L-NAME (10 and 30 mg kg^{-1} i.v.) significantly changed it: the initial increase in intragastric pressure was enhanced while the decrease in intragastric pressure was reduced or abolished. N^G-nitro-L-arginine (L-NNA, 10 mg kg^{-1}, i.v.) had the same effect.

4 An i.v. infusion of phenylephrine (10 μg kg^{-1} min^{-1}) inducing a pressor response similar to that produced by L-NAME (30 mg kg^{-1}, i.v.) did not influence the vagal gastric response. Infusion of L-arginine (300 mg kg^{-1} bolus, then 100 mg kg^{-1} h^{-1}) starting 30 min beforehand, reduced the pressor effect and prevented the influence of L-NAME (10 mg kg^{-1}, i.v.) on the vagal gastric response. After injection of both atropine (1 mg kg^{-1}, i.v.) and L-NAME (30 mg kg^{-1}, i.v.), the vagally induced decrease in intragastric pressure was similar to that obtained under control conditions.

5 These results are consistent with NO being released and inducing gastric relaxation during peripheral vagal stimulation. In addition to NO, another inhibitory non-adrenergic non-cholinergic neurotransmitter is released.

Keywords: Rat stomach; gastric relaxation; non-adrenergic non-cholinergic; vagal stimulation; N^G-nitro-L-arginine methyl ester (L-NAME); nitric oxide

Introduction

Nitric oxide (NO) has been recognized as an endothelium-derived relaxing factor (Palmer *et al.*, 1987; Ignarro, 1990). Inhibition of NO biosynthesis by L-arginine analogues such as N^G-mono-methyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME) upon intravenous administration of these compounds induces pressor responses in rats (Rees *et al.*, 1990; Gardiner *et al.*, 1990), guinea-pigs (Aisaka *et al.*, 1989) and rabbits (Rees *et al.*, 1989). These results suggest that the formation of NO has an important role in the regulation of blood pressure. Recent *in vitro* data suggest that NO is also a neurotransmitter of peripheral inhibitory non-adrenergic non-cholinergic (NANC) neurones. Indeed, in the anococcygeus muscle of the rat (Gillespie *et al.*, 1989) and the mouse (Gibson *et al.*, 1990), the ileocolonic junction of the dog (Boeckxstaens *et al.*, 1990), and the guinea-pig trachea (Li & Rand, 1991), the NO synthesis inhibitors L-NMMA, L-NAME and/or N^G-nitro-L-arginine (L-NNA) inhibited the relaxation, induced by electrical stimulation of the NANC neurones. Furthermore, in a superfusion bioassay, the release of a vasorelaxant factor with NO characteristics was shown upon stimulation of the NANC nerves in the canine ileocolonic junction (Bult *et al.*, 1990) and the presence of NO synthase has been shown in myenteric plexus neurones of the rat intestine (Bredt *et al.*, 1990).

In the rat gastric fundus, vasoactive intestinal polypeptide (VIP) has been proposed as inhibitory NANC neurotransmitter (De Beurme & Lefebvre, 1987; Kamata *et al.*, 1988) but the non-blockade of the initial relaxation, induced by electrical stimulation of the NANC neurones, by VIP-antiserum suggested the involvement of a non-VIP component (De Beurme & Lefebvre, 1988). There is now evidence that NO might be the co-transmitter with VIP in this preparation, as L-NMMA reduced NANC relaxations elicited by short periods of field stimulation (Li & Rand, 1990; Boeckxstaens *et al.*, 1991). The inhibitory NANC neurones of the gastric fundus represent the final step of the vagal inhibitory pathway involved in gastric relaxation (Abrahamsson, 1986). In studies *in vivo*, stimulation of the peripheral cut end of the vagus, especially after atropine treatment, induces NANC gastric relaxation in the guinea-pig (Ohta *et al.*, 1985), the cat (Martinson, 1964) and the dog (Jahnberg, 1977). In the present study, we investigated the influence of L-NAME on gastric relaxation induced by efferent vagal stimulation in the anaesthetized rat. Our results provide *in vivo* evidence for the involvement of NO in the vagal inhibitory NANC pathway to the stomach. A preliminary account of the results has been given to the British Pharmacological Society (Lefebvre *et al.*, 1991).

Methods

Preparation of animals

Male Wistar rats (230–460 g) were fasted for 24 h with water available *ad libitum*. The animals were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.); anaesthesia was maintained by bolus administration (3 mg kg^{-1}, i.v.) when required. A tracheotomy was performed and a tracheal tube inserted, through which the animals breathed room air spontaneously. Catheters containing heparinized (50 units ml^{-1}) saline were inserted into the right carotid artery and the right external jugular vein for blood pressure measurement and intravenous administration of drugs respectively. The arterial catheter was connected to a PDCR 75 S/N 1684 or a Statham P23AA pressure transducer and mean arterial blood pressure was derived from the direct measurement. Intragastric pressure was measured by use of a rubber balloon inserted into the stomach via the mouth. The balloon was connected to a

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Statham P23BB or a PDCR 75/1 S/N 430 pressure transducer and filled with water at 37°C (1.1–1.6 ml). This volume was determined before the use of each new balloon as the quantity of water, that just did not induce pressure in the balloon, outside the body. After each experiment, the exact localization of the balloon was verified. Blood pressure and intragastric pressure were registered on a Beckman Dynograph recorder, type R.

The cervical vagal nerves were carefully isolated and bilaterally sectioned. The peripheral cut end of the left cervical vagus was placed on a bipolar electrode and covered with liquid paraffin. The electrode was connected to an electric stimulator (Braun Type I, HSE). In a few animals, the peripheral cut end of the right cervical vagus was stimulated, as no response was obtained upon stimulation of the left vagus. Except for the preliminary experiments, stimulation was with square wave pulses of 1 ms, 10 V and 10 Hz for 20 s.

Experimental protocols

After section of the vagal nerves, a stabilization period of 20 min was allowed. In a first series of experiments ($n = 24$), vagal stimluation was performed 3 times at an interval of about 1 h. Before the second vagal stimulation, an i.v. bolus injection of saline or L-NAME (1, 10 or 30 mg kg^{-1}) was given. The interval between the saline injection and the second vagal stimulation was 10 min; when L-NAME was injected, the second vagal stimulation was performed at the time of the maximal blood pressure increase induced by L-NAME. The gastric response to vagal stimulation was also studied before and after injection of L-NNA (10 mg kg^{-1}, i.v.; $n = 6$).

The influence of vagal stimulation on intragastric pressure was also studied before and 10 min after i.v. bolus injection of pentolinium (2 mg kg^{-1}; Howe *et al.*, 1986; $n = 8$) and before and during i.v. infusion of phenylephrine (10 μg kg^{-1} min^{-1}; $n = 8$); vagal stimulation was performed when the phenylephrine-induced pressor response was maximal. The influence of L-NAME (30 mg kg^{-1}) was also studied in atropinized ($n = 8$) and reserpinized ($n = 5$) rats. Atropine (1 mg kg^{-1}, i.v.) was administered 10 min before the second vagal stimulation. Fifty min after the second vagal stimulation, the atropine injection was repeated, followed after 10 min by the i.v. injection of L-NAME (30 mg kg^{-1}). When the blood pressure increase induced by L-NAME was maximal, a third vagal stimulation was performed. Reserpine (5 mg kg^{-1}) was administered i.p. 24 h before the experiment.

In the final series of experiments, the influence of pretreatment with L-arginine on the effect of L-NAME was investigated. Vagal stimulation was performed 3 times with an interval of at least 1 h between each stimulation. Fifty min after the first vagal stimulation, an i.v. bolus injection of L-arginine (300 mg kg^{-1}) was given, followed after 10 min by L-NAME (30 mg kg^{-1}) as an i.v. bolus ($n = 6$). In another group of rats, L-arginine was infused (300 mg kg^{-1} bolus, 100 mg kg^{-1} h^{-1} infusion) for 30 min before i.v. bolus administration of 10 ($n = 6$) or 30 mg kg^{-1} ($n = 3$) L-NAME. In a similar way, the influence of D-arginine (300 mg kg^{-1} bolus, 100 mg kg^{-1} h^{-1} infusion for 30 min) was tested versus L-NAME (10 mg kg^{-1}; $n = 8$).

Drugs

The following drugs were used: atropine sulphate (Boehringer Ingelheim, Germany), D-arginine hydrochloride (Sigma, St. Louis, Mo, U.S.A.), L-arginine hydrochloride (Sigma), N^G-nitro-L-arginine (Sigma), N^G-nitro-L-arginine methyl ester hydrochloride (Sigma), pentolinium tartrate (Janssen Chimica, Geel, Belgium), phenylephrine (Winthrop, Brussels, Belgium), reserpine (Aldrich Chemie, Brussels, Belgium).

Drugs were dissolved or diluted with sterile saline. For phenylephrine, commercially available ampoules were used. A stock solution of reserpine was prepared from powder (5 mg ml^{-1} dissolved in 10% ascorbic acid). The substances were injected in volumes of 0.1 ml/100 g, flushed in with 0.2 ml saline. Infusions were given at a rate of 0.1 ml min^{-1}.

Statistical analysis

Results are given as mean ± s.e.mean. Responses to vagal stimulation after a given treatment were compared to those before by means of the signed-ranks test. $P < 0.05$ was taken as statistically significant.

Results

During preliminary experiments ($n = 5$), the vagal nerve was stimulated at 5 min intervals for 20 s at a frequency of 2 or 10 Hz; the voltage was stepwise increased from 10 to 50 V. Stimulation at 2 Hz tended to increase intragastric pressure (corresponding to gastric contraction), while stimulation at 10 Hz yielded a decrease in intragastric pressure (corresponding to gastric relaxation) or a biphasic response, i.e. a small increase followed by a more pronounced decrease in intragastric pressure. From these results, vagal stimulation at 10 Hz, 10 V, 1 ms for 20 s was chosen for further experiments.

Influence of L-NAME and L-NNA on the vagally induced gastric response

Vagal nerve stimulation generally induced a decrease in mean arterial blood pressure (Figure 1). In the first series of experiments ($n = 24$), the response of intragastric pressure to vagal stimulation was biphasic, i.e. increase followed by decrease, in 23 rats (Figure 1) while in one rat only a decrease occurred. Except for 2 rats, the decrease in intragastric pressure was always clearly more pronounced than the increase. In 14 experiments out of 24, stopping vagal stimulation was followed by a rebound contraction (Figure 1). Even when a rebound contraction occurred, intragastric pressure quickly decreased to a lower level than before vagal stimulation. Within the 1 h before the following vagal stimulation, intragastric pressure slowly returned to its original level. Bolus injection of saline did not influence mean blood pressure nor intragastric pressure; the gastric response to vagal stimulation performed 10 and 70 min after the saline injection was the same as that to the first vagal stimulation (Figure 2).

Bolus administration of L-NAME (1, 10 and 30 mg kg^{-1}) significantly increased mean blood pressure from 105 ± 6 to 121 ± 5 mmHg ($n = 6$), from 110 ± 8 to 126 ± 12 mmHg ($n = 6$) and from 123 ± 10 to 148 ± 9 mmHg ($n = 6$) respectively. Intragastric pressure was not influenced by the administration of L-NAME or tended to decrease (−0.1 ± 0.3, −0.4 ± 0.1 and −0.3 ± 0.1 cmH_2O for 1, 10 and 30 mg kg^{-1} L-NAME respectively). L-NAME (1 mg kg^{-1}) had no influence on the gastric response to vagal stimulation but the higher doses markedly changed this response (Figures 1 and 2). The vagally induced increase in intragastric pressure was increased in 5 rats out of 6 in each group, while the vagally induced decrease in intragastric pressure was abolished or greatly reduced in all rats. Although the relaxation during vagal stimulation was reduced or abolished, intragastric pressure quickly decreased after stopping the stimulation similar to that observed before administration of L-NAME. The effect of L-NAME on the vagally induced gastric response persisted for more than 1 h as can be seen from the response to the third vagal stimulation in Figure 2.

L-NNA (10 mg kg^{-1} i.v.) increased blood pressure from 110 ± 9 to 140 ± 9 mmHg ($n = 6$); intragastric pressure slightly decreased from 7.1 ± 0.8 to 6.8 ± 0.8 cmH_2O. L-NNA (10 mg kg^{-1}, i.v.) had the same influence on the vagally induced gastric response as L-NAME (10 and 30 mg kg^{-1}, i.v.): the vagally induced increase in intragastric pressure was

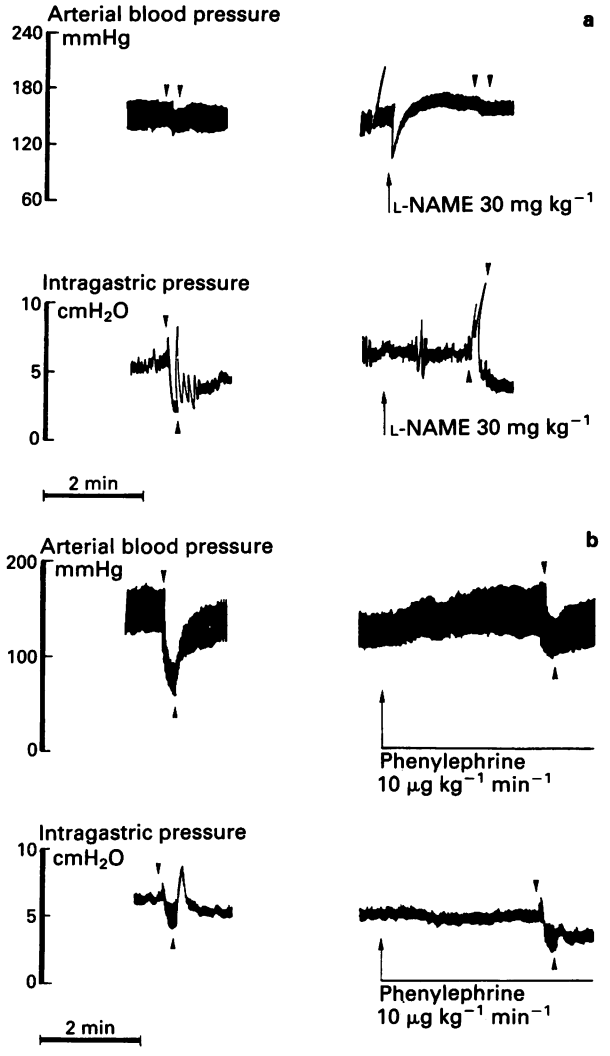

Figure 1 Original recordings showing the influence of an i.v. bolus injection of 30 mg kg^{-1} N^G-nitro-L-arginine methyl ester (L-NAME) (a) and an i.v. infusion of 10 μg kg^{-1} min^{-1} phenylephrine (b) on the gastric response induced by vagal stimulation (10 Hz, 10 V, 1 ms, 20 s) in 2 different rats. The arrows indicate the beginning and the end of the stimulation.

enhanced from 3.5 ± 0.7 to 9.0 ± 0.4 cmH_2O ($n = 6$, $P < 0.05$) while the decrease (−5.0 ± 0.8 cmH_2O) was abolished ($P < 0.05$).

In 6 experiments out of 8, a single i.v. administration of pentolinium (2 mg kg^{-1}) abolished or greatly reduced the gastric response to vagal stimulation. The initial increase in intragastric pressure was reduced from 0.4 ± 0.3 to 0.04 ± 0.04 cmH_2O while the vagally induced decrease in intragastric pressure was reduced from 2.7 ± 0.4 cmH_2O before administration of pentolinium to 0.3 ± 0.1 cmH_2O in its presence ($n = 6$, $P < 0.05$). The injection of pentolinium reduced blood pressure by 55 ± 5 mmHg from 113 ± 8 to 58 ± 5 mmHg ($n = 6$); it did not manifestly change basal intragastric pressure (−0.3 ± 0.1 cmH_2O, $n = 6$). In the 2 other experiments, the dose of pentolinium needed to be increased to 4 and 7 mg kg^{-1} respectively before the vagally induced responses were clearly reduced. These doses of pentolinium reduced blood pressure by 60 and 52 mmHg.

Infusion of phenylephrine (10 μg kg^{-1} min^{-1}) increased blood pressure by 25 ± 3 mmHg from 127 ± 6 to 152 ± 5 mmHg ($n = 8$). This pressor response did not influence the gastric response to vagal stimulation (Figure 1). The vagally induced increase and decrease in intragastric pressure was 0.4 ± 0.1 and 1.8 ± 0.3 cmH_2O before and 0.5 ± 0.1 and 1.6 ± 0.1 cmH_2O during the phenylephrine infusion ($n = 8$).

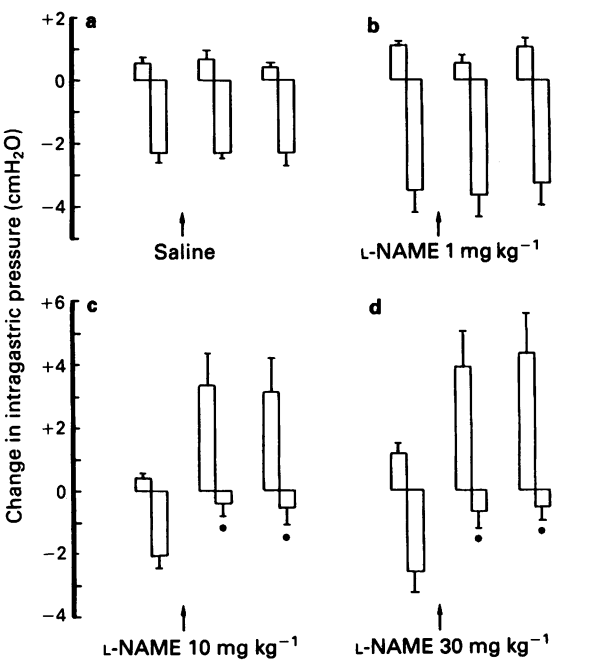

Figure 2 Influence of vagal stimulation (10 Hz, 10 V, 1 ms, 20 s) on intragastric pressure in 4 groups of rats (a, b, c, d). Vagal stimulation was performed 3 times; before the second stimulation, saline (a) or N^G-nitro-L-arginine methyl ester (L-NAME) 1 (b), 10 (c) or 30 (d) mg kg^{-1} was administered i.v. The columns show mean values with the s.e.mean (vertical bars) of $n = 6$, except for the third vagal stimulation in group (b) where $n = 4$. * $P < 0.05$, significantly different versus the response during the first vagal stimulation. The enhancement of the vagally induced increase in intragastric pressure after i.v. administration of 10 and 30 mg kg^{-1} L-NAME did not reach statistical significance at the two tail level because it only occurred in 5 of the 6 rats.

Influence of L-NAME in atropinized and reserpinized rats

Before administration of atropine, vagal stimulation induced the usual biphasic response although the mean decrease in intragastric pressure in this series was less pronounced than in the other series (Figure 3). The i.v. injection of atropine did not manifestly change blood pressure or intragastric pressure. In 5 rats out of 6, intragastric pressure decreased somewhat giving a mean reduction of intragastric pressure from 6.3 ± 0.7 to 5.6 ± 0.5 cmH_2O ($n = 6$). After administration of atropine, vagal stimulation no longer induced an increase in intragastric pressure while the vagally induced decrease in intragastric pressure was not significantly influenced (Figure 3). In all rats of this group, a rebound contraction occurred after the first vagal stimulation but this rebound contraction was absent after the injection of atropine.

The i.v. injection of L-NAME (30 mg kg^{-1}), after atropine had been administered again increased blood pressure by 26 ± 6 mmHg from 101 ± 11 till 127 ± 15 mmHg ($n = 6$). The decrease in intragastric pressure, induced by vagal stimulation, was not significantly reduced by L-NAME (Figure 3). Also in reserpinized animals, vagal stimulation induced an initial short-lasting gastric contraction, followed by a more pronounced gastric relaxation. The amplitude of the gastric relaxation was similar to that induced by the first vagal stimulation in the group where atropine was administered (Figure 3). In the reserpinized rats, L-NAME (30 mg kg^{-1}, i.v.) increased the blood pressure from 91 ± 8 to 134 ± 4 mmHg ($n = 5$) and changed the gastric response to vagal stimulation in a similar way to non-treated rats i.e. the vagally induced increase in intragastric pressure was greatly enhanced while the decrease in intragastric pressure was completely abolished (Figure 3). Ten min before a third vagal stimulation, atropine

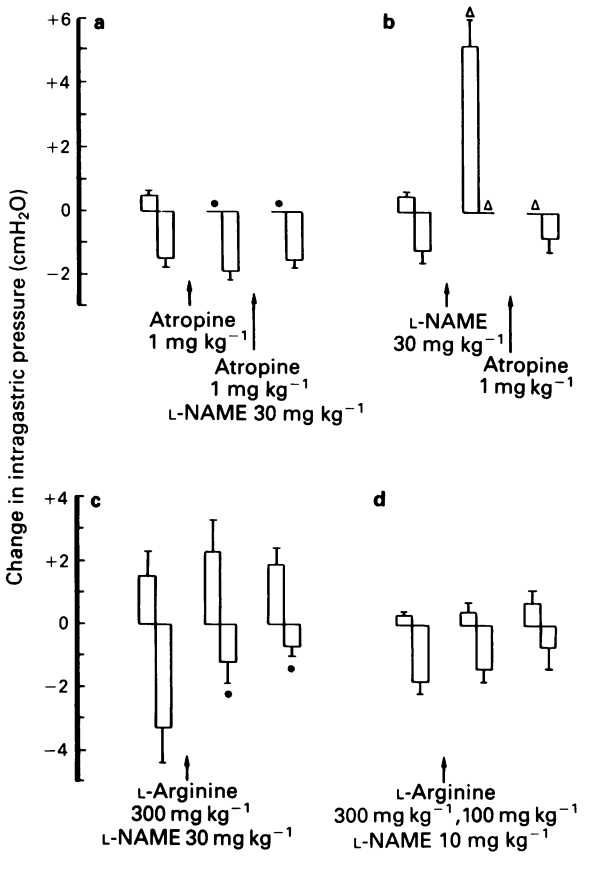

Figure 3 Influence of vagal stimulation (10 Hz, 10 V, 1 ms, 20 s) on intragastric pressure in 4 groups of rats (a, b, c, d). Gr (b) was reserpinized (5 mg kg^{-1}, i.p., 24 h before the experiment). Substances administered in between the vagal stimulation are indicated. The columns show mean values with s.e.mean (vertical bars) of $n = 6$ except for the reserpinized group where $n = 5$. $^{*}P < 0.05$; $^{\Delta}P < 0.05$ (one tail), significantly different versus the response during the first vagal stimulation.

(1 mg kg^{-1}) was injected i.v. Vagal stimulation no longer induced an increase in intragastric pressure but in 3 rats a small decrease and in 2 rats a more pronounced decrease in intragastric pressure occurred (mean decrease = 0.8 ± 0.4 cmH_2O, $n = 5$, Figure 3).

Influence of L-arginine and D-arginine on the effect of L-NAME

Preliminary experiments showed that an i.v. bolus injection of arginine (100 mg kg^{-1}) did not prevent the pressor effect of L-NAME (30 mg kg^{-1}) nor its influence on the vagally induced change in intragastric pressure. Also when the dose of L-arginine was increased to 300 mg kg^{-1}, the effect of L-NAME (30 mg kg^{-1}) was not reversed since blood pressure increased from 119 ± 7 to 145 ± 9 mmHg ($n = 6$). The vagally induced gastric response after administration of L-arginine and L-NAME is shown in Figure 3. The increase in intragastric pressure was enhanced although to a smaller extent than in other groups. The vagally induced decrease in intragastric pressure was reduced and remained reduced after 1 h.

In another group of 9 rats, L-arginine was given as an i.v. bolus of 300 mg kg^{-1} followed by an infusion of 100 mg kg^{-1} h^{-1} for 30 min. In 3 of these rats, L-NAME (30 mg kg^{-1}) was then injected. Only in one of these rats, was the influence of L-NAME on the vagally induced gastric response inhibited. In contrast, the effect of L-NAME (10 mg kg^{-1}) was prevented (Figure 3). During the infusion of L-arginine, blood pressure was not manifestly influenced (110 ± 6 mmHg before the infusion, 106 ± 10 mmHg just before the injection of 10 mg kg^{-1} L-NAME, $n = 6$), while intragastric pressure tended to decrease (from 5.0 ± 0.7 to 4.2 ± 0.5 cmH_2O, $n = 6$). L-NAME (10 mg kg^{-1}, i.v.) moderately increased blood pressure (from 106 ± 10 to 114 ± 11 mmHg, $n = 6$) and did not influence intragastric pressure (maintained at 4.2 ± 0.5 cmH_2O). The injection of L-NAME (10 mg kg^{-1}) did not influence the vagally induced gastric response in 5 rats out of 6, while the vagally induced decrease in intragastric pressure was abolished in one rat. However, during another vagal stimulation 60 min later, the inhibitory effect of L-NAME (10 mg kg^{-1}) was no longer prevented by L-arginine as the vagally induced decrease in intragastric pressure was abolished in 4 and greatly reduced in one of the 6 rats studied.

During the infusion of D-arginine, the blood pressure increased from 111 ± 10 to 117 ± 10 mmHg while the intragastric pressure decreased from 8.4 ± 1.0 to 7.5 ± 1.1 cmH_2O ($n = 8$). The i.v. injection of L-NAME (10 mg kg^{-1}) increased blood pressure by 30 ± 5 mmHg; intragastric pressure showed a moderate increase (0.3 ± 0.3 cmH_2O). After administration of D-arginine and L-NAME, the vagally induced increase in intragastric pressure was consistently enhanced (from 1.9 ± 0.5 to 2.9 ± 0.5 cmH_2O, $n = 8$, $P < 0.01$). The vagally induced relaxation was decreased in 6 experiments out of 8 but slightly increased in the 2 others, yielding a mean response of 2.6 ± 0.8 cmH_2O (versus 4.1 ± 0.5 cmH_2O before D-arginine and L-NAME, $n = 8$, $P < 0.05$, one tail).

Discussion

Recent *in vitro* data have provided evidence that NO and VIP are co-transmitters of the inhibitory NANC neurones of the rat gastric fundus (Li & Rand, 1990; Boeckxstaens *et al.*, 1991). In different mammals such as the guinea-pig (Ohta *et al.*, 1985), the cat (Martinson, 1964) and the dog (Jahnberg, 1977), it has been established that the vagal nerve carries 2 types of preganglionic fibres i.e. those that synapse with postganglionic cholinergic neurones and induce an increase in gastric tone upon stimulation and those that synapse with postganglionic NANC neurones and induce a reduction in gastric tone upon stimulation. The presence of both pathways has also been shown in the rat (Delbro, 1989). The aim of the present study was therefore to investigate *in vivo* the influence of the NO biosynthesis inhibitor L-NMMA on the vagally induced NANC relaxation of the rat stomach.

The inhibitory vagal fibres have a higher excitation threshold than the excitatory ones (Martinson & Muren, 1963; Jansson & Martinson, 1965). Our preliminary experiments revealed that peripheral vagal stimulation at 10 Hz, 10 V and 1 ms induced a clear reduction of intragastric pressure even in the absence of atropine. The train duration of stimulation was limited to 20 s as *in vitro* studies in the rat gastric fundus showed that the short-lasting relaxation induced by this type of stimulation of the inhibitory NANC neurones was greatly reduced by the NO synthesis inhibitors (Boeckxstaens *et al.*, 1991). The major part of our experiments was done in the absence of atropine to avoid the pronounced decrease in gastric tone, that has been observed in other species and that can interfere with the registration of inhibitory responses (Martinson, 1964). However, the experiments with atropine later showed that it only moderately decreased intragastric pressure, suggesting that the intrinsic cholinergic neurones contribute only moderately to the maintenance of rat gastric tone in our experimental conditions. Atropine blocked the initial increase in intragastric pressure, induced by vagal stimulation, illustrating the cholinergic nature of this component of the response, but did not influence the vagally induced decrease in intragastric pressure; it also blocked the rebound contraction. The relaxation also occurred in reserpinized animals (Lefebvre, 1986), confirming the NANC nature of the vagally induced gastric relaxation. The ganglion-blocking agent, pentolinium (Taylor, 1990) greatly reduced or abolished the vagally induced decrease in intragastric pressure, confirming the presence of nicotinic synapses between

the preganglionic vagal fibres and the inhibitory NANC neurones (Roman & Gonella, 1987). The gastric response to vagal stimulation was perfectly reproducible after i.v. administration of saline.

The i.v. injection of L-NAME increased the mean arterial blood pressure as expected, although there was no clear dose-dependency as reported by Rees *et al.* (1990) for L-NAME in anaesthetized rats. The two higher doses of L-NAME (10 and 30 mg kg^{-1}) reduced or abolished the vagally induced decrease in intragastric pressure, suggesting that NO release is essential for this response. This was confirmed by the study of another inhibitor of NO-synthesis, L-NNA. When NO release is blocked, the cholinergic contractile response becomes predominant during vagal stimulation. The fact that cholinergic neurones are activated during the whole course of stimulation at our parameters but are overruled by the simultaneously released relaxant agent is also illustrated by the cholinergic rebound contraction that occurred in many animals after stimulation. The increase in mean arterial blood pressure is not the mechanism by which L-NAME influences the vagally induced gastric response, as the same pressor response as for the highest dose of L-NAME evoked by phenylephrine infusion had no such effect. Although L-NAME (1 mg kg^{-1}) also increased blood pressure, it had no influence on the vagal gastric response. Similarly, it was reported that a lower dose of L-NMMA increased arterial blood pressure but did not reduce gastric mucosal blood flow while higher doses did (Pique *et al.*, 1989). The finding that pretreatment with an L-arginine infusion reversed the action of L-NAME (10 mg kg^{-1}), both on blood pressure and on the vagally induced gastric relaxation, provides further evidence that NO is involved in the latter response. Neither bolus injections nor infusion of L-arginine, however, prevented the pressor effect and the reduction of the vagal gastric relaxation by 30 mg kg^{-1} L-NAME. It might be difficult to reverse the effect of this high dose of L-NAME, which was shown to be the most potent in increasing blood pressure amongst the NO synthesis inhibitors available (Rees *et al.*, 1990). In our hands, a 300 mg kg^{-1} bolus plus a 100 mg kg^{-1} h^{-1} infusion of L-arginine prevented the effect of 10 mg kg^{-1} L-NAME but 1 h after stopping the L-arginine infusion, the inhibitory effect of 10 mg kg^{-1} L-NAME was again observed. We have no explanation as to why D-arginine prevented the influence of L-NAME on vagally induced relaxation in 2 experiments. Recently, it has been shown that the D-enantiomer of N^G-nitro-arginine can also interfere with the L-arginine/NO pathway, although this effect was not prevented by D-arginine (Wang *et al.*, 1991).

L-NAME did not reduce the vagally induced gastric relaxation after injection of atropine. This seems to indicate that another relaxant transmitter besides NO is released during vagal stimulation. This seems also corroborated by the observation that in the absence as well as in the presence of L-NAME, intragastric pressure stayed decreased after stopping vagal stimulation. Vagal stimulation at our parameters would thus yield release of acetylcholine, NO and the second relaxant transmitter, the overall response being relaxation. When NO release is prevented, the cholinergic contraction becomes predominant; when the effect of acetylcholine is antagonized, a relaxation due to the second relaxant transmitter occurs. This transmitter is certainly not noradrenaline as L-NAME also abolished the vagal gastric relaxation in reserpinized rats, but after injection of atropine a vagally induced gastric relaxation again occurred. A suitable candidate for the second NANC neurotransmitter is VIP, which was proposed as co-transmitter of NO in studies *in vitro* in the rat gastric fundus (Li & Rand, 1990; Boeckxstaens, 1991). In those studies in atropinized conditions, the relaxation of rat gastric fundus strips induced by transmural stimulation at low frequencies was completely abolished by NO synthesis inhibitors, while the response to higher frequency stimulation (5 Hz, Li & Rand, 1990; 16 Hz, Boeckxstaens, 1991) was only partially reduced and evidence for the involvement of VIP was provided.

Twenty four h after reserpinization, the arterial blood pressure of the rats was lower than in the non-reserpinized animals confirming previous reports (Kisin & Yuzhakov, 1976). The pressor effect of L-NAME (30 mg kg^{-1}) tended to be higher (43 mmHg) than in non-treated rats (25 mmHg). This contrasts with the report of Vargas *et al.* (1990) that the pressor effect of L-NMMA was attenuated in rats devoid of sympathetic tone by pithing or ganglionic blockade. We have no explanation for this difference although it should be kept in mind that the experiments of Vargas *et al.* (1990) were conducted acutely, while ours were performed 24 h after reserpinization.

The administration of L-NAME and L-NNA did not increase intragastric pressure; it even tended to decrease it. L-NMMA and L-NNA *in vitro* increase the resting tension of rat gastric fundus strips, suggesting a tonic release of NO; however, non-neurogenic sources of NO or a direct action of L-NMMA and L-NNA at the smooth muscle cells could not be excluded (Li & Rand, 1990; Boeckxstaens *et al.*, 1991).

In conclusion, the present results are consistent with NO being released and inducing gastric relaxation during vagal stimulation: the NO synthesis inhibitor L-NAME reduced the vagally induced gastric relaxation, and this effect of L-NAME was prevented by pre-administration of L-arginine. This finding provides *in vivo* evidence for NO being an inhibitory NANC neurotransmitter at this site in the gastrointestinal tract. The pathway might be involved in adaptive relaxation of the stomach; it was recently shown in the guinea-pig isolated stomach that adaptive relaxation, induced by stimulation of ganglionic nicotinic receptors, is NO-dependent (Desai *et al.*, 1991).

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Effect of dihydropyridines on calcium channels in isolated smooth muscle cells from rat vena cava

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1 Whole-cell patch-clamp method was applied to single smooth muscle cells freshly isolated from the rat inferior vena cava.

2 Depolarizing pulses, applied from a holding potential of −90 mV, activated both Na^+ and Ca^{2+} channels. The fast Na^+ current was inhibited by nanomolar concentrations of tetrodotoxin (TTX). The slow Ba^{2+} current (measured in 5 mM Ba^{2+} solution) was inhibited by Cd^{2+} and modulated by dihydropyridine derivatives. When the cells were held at a holding potential of −80 mV, racemic Bay K 8644 increased the Ba^{2+} current (ED_{50} = 10 nM) while racemic isradipine inhibited the current (IC_{50} = 21 nM).

3 The voltage-dependency of isradipine blockade was assessed by determining the steady-state availability of the Ca^{2+} channels. From the shift of the inactivation curve in the presence of isradipine, we calculated a dissociation constant of 1.11 nM for inactivated Ca^{2+} channels. Scatchard plots of the specific binding of (+)-[^{3}H]-isradipine obtained in intact strips incubated in 5.6 mM or 135 mM K^+ solutions confirmed the voltage-dependency of isradipine binding.

4 Specific binding of (+)-[^{3}H]-isradipine was completely displaced by unlabelled (±)-isradipine, with an IC_{50} of 15.1 nM. This value is similar to the IC_{50} for inhibition of the Ba^{2+} current (21 nM) in cells maintained at a holding potential of −80 mV.

5 Bay K 8644 had no effects on the Ba^{2+} current kinetics during a depolarizing test pulse. The steady-state inactivation-activation curves of Ba^{2+} current were not significantly shifted along the voltage axis.

6 The present data suggest the existence of two distinct dihydropyridine binding sites which can be bound preferentially by agonist or antagonist derivatives.

Keywords: Vena cava; isolated smooth muscle cells; Ca^{2+} channel; Na^+ channel; dihydropyridines

Introduction

There is reported evidence of two types of Ca^{2+} channels in vascular smooth muscle cells (Bean *et al.*, 1986; Friedman *et al.*, 1986; Loirand *et al.*, 1986; Yatani *et al.*, 1987; Benham *et al.*, 1987). However, some reports showed that the dihydropyridine-sensitive, high-threshold Ca^{2+} channel or L-type channel was predominant in several smooth muscles (Inoue *et al.*, 1989; Honoré *et al.*, 1989). More recently, tetrodotoxin-sensitive Na^+ currents have been described in dissociated cells from visceral and vascular smooth muscles (Amédée *et al.*, 1986; Sturek & Hermsmeyer, 1986; Okabe *et al.*, 1988; Ohya & Sperelakis, 1989; Mironneau *et al.*, 1990; Martin *et al.*, 1990).

Little is known about the electrophysiological properties of smooth muscle cells from vena cava. Mekata & Nagatsu (1982) have shown that there is no difference in the electrical properties of outer and inner muscles of the dog inferior vena cava, and that the resting membrane potential was approximately −55 and −60 mV in circular and longitudinal layers, respectively. We used the whole-cell patch-clamp method to identify the Na^+ and Ca^{2+} channel currents in freshly isolated smooth muscle cells of the rat inferior vena cava. The Ba^{2+} current was enhanced by Bay K 8644 and inhibited by isradipine. The isradipine blockade of Ca^{2+} channels was potential-dependent as shown by both electrophysiological and biochemical data. In contrast, the agonistic action of Bay K 8644 showed a weaker sensitivity to voltage. The results suggest the existence of two distinct dihydropyridine binding sites, an activator and a blocker site, which can be bound preferentially by Bay K 8644 and isradipine, respectively.

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Methods

Single cells isolation procedure

The enzymatic dispersion procedure for isolating single cells from rat vena cava was identical to that previously described (Loirand *et al.*, 1986) except that in this study, experiments were carried out on freshly isolated cells within 10 h of preparing the cells.

Electrophysiological recordings

Cells attached to collagen-coated coverslips were placed in a small experimental chamber which was mounted on the stage of an inverted microscope. The whole-cell recordings (Hamill *et al.*, 1981) were performed with patch pipettes (resistance 2–3 MΩ) connected to the headstage of a patch-clamp amplifier (List LM/EPC7, Darmstadt, Germany). The data were filtered with a 8-pole Bessel filter (Frequency Devices, Haverhill, MA, U.S.A.) at 1 kHz and analyzed with an IBM PC microcomputer. Currents were digitally corrected for leakage and capacitive currents by subtraction of scaled current traces obtained when hyperpolarizing or small depolarizing pulses from the holding potential were applied to the cells. The external physiological solution contained (mM): NaCl 130, KCl, 5.6, $CaCl_2$ 2, $MgCl_2$ 0.24, glucose 11, HEPES 8.3 (pH 7.4 with NaOH). In order to block outward currents the pipettes were filled with (mM): CsCl 130, Cs-pyruvate 5, Cs-succinate 5, Cs-oxalate 5, EGTA 10, HEPES 10 (pH 7.3 with CsOH). In some experiments, 30 mM CsCl was substituted by NaCl in order to calculate a theoretical equilibrium potential for Na^+ ions. In the external solution KCl and $CaCl_2$ were substituted by 5.6 mM CsCl and 5 mM $BaCl_2$, respectively. To isolate the

inward currents, the fast Na^+ current was recorded in the presence of 50 μM Cd^{2+} while the slow Ca^{2+} current was recorded in the presence of 10 nM tetrodotoxin (TTX). The bath solution was maintained at 30 ± 1°C. The series resistances measured at 10 kHz was 4–6 MΩ. At the peak of the inward current (600 pA), errors in potential measurements were between 2.4 and 3.6 mV due to series resistance.

Measurements of (+)-[^{3}H]-isradipine binding to intact strips

Studies of binding to intact strips of rat vena cava (0.5–1 mg wet weight) were performed under conditions similar to those described previously (Dacquet *et al.*, 1989; Rakotoarisoa *et al.*, 1990). Strips were incubated for 60 min at 37°C with various concentrations of ligand for equilibrium studies or for varying periods of time for kinetic studies. At the end of the incubation period each strip was dried on filter paper, and then weighed. Radioactivity was measured by dissolving the vein strips in 100 μl of a mixture of perchloric acid and H_2O_2 (1:1) and counting by liquid scintillation with an efficiency of 55%. Nonspecific binding was determined in the presence of 2 μM nifedipine and subtracted from the total binding to give the specific binding. The strips were incubated either in physiological solution (normally polarized veins) or in 135 mM K^+ solution obtained by equimolar substitution of NaCl with KCl (depolarized veins). In the latter case, the radioligand was added after a 10 min period in elevated K^+ solution in order to allow a stable membrane potential. Nonspecific binding was unaffected by increasing the external K^+ concentration from 5.6 to 135 mM.

Chemicals

(+)-[^{3}H]-isradipine (specific activity, 80–85 Ci mmol^{-1}) was from Amersham (Les Ulis, Franc). (±)-, (+)-and (−)-Isradipine were from Sandoz (Rueil-Malmaison, France). Nifedipine and Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) were from Bayer (Puteaux, France). Tetrodotoxin was from Calbiochem (Meudon, France).

Statistical analysis

Radioligand binding data were analyzed by the nonlinear least-square LIGAND programme for multiple binding sites (Munson & Rodbard, 1980), with the assistance of an IBM PC microcomputer. The experimental results were expressed as means ± s.e.mean and significance was tested by Student's *t* test; *P* values smaller than 0.05 were estimated to be significant.

Results

Passive properties of isolated vena cava smooth muscle cells

After the enzymatic isolation procedure, approximately 60% of the cells were relaxed and had an elongated appearance in 2 mM Ca^{2+}-solution. When recorded with patch pipettes filled with 130 mM KCl, the resting potential was −61 ± 9 mV (n = 23) in 2 mM Ca^{2+}-solutions. From the electronic potential produced by passing a constant-current pulse through the recording electrode the input resistance and the membrane time constant were estimated. The value of the cell capacitance was calculated by dividing the membrane time constant by the input resistance (2.4 ± 0.4 GΩ, n = 17). Assuming a specific membrane capacitance of 1 μF cm^{-2}, the cell capacitance and the specific membrane resistance were 19.6 ± 3.1 pF and 48.2 ± 5.2 kΩ cm^{-2} (n = 17), respectively.

Identification of Na^+ and Ba^{2+} inward currents

Two types of inward current (fast and slow) were recorded in single vena cava smooth muscle cells after minimizing outward currents by intracellular Ca^+ loading. Figure 1 shows typical current traces of the fast and the slow inward currents, evoked by command potentials to 0 mV from a holding potential of −90 mV. When 50 μM Cd^{2+} or 0.1 μM isradipine was added to the bathing solution to suppress the Ca^{2+} channel current, only the fast current was observed. Removal of external Na^+ as well as addition of 10 nM TTX suppressed the fast (Na^+) current. Peak amplitude of the isolated fast and slow currents was plotted against the command voltage in Figure 2a. The Na^+ current had a threshold of activation at −47 ± 5 mV, peaked at −10 ± 3 mV with a maximal amplitude of 600 pA and had an apparent reversal potential at +31 ± 3 mV (with 30 mM Na^+ in the pipette solution, n = 7). In contrast, the Ba^{2+} current had a threshold potential at −32 ± 4 mV, peaked at +10 ± 3 mV with a maximal amplitude of 300 pA, and had an apparent reversal potential at +55 ± 6 mV (n = 11). To characterize further the Na^+ and Ba^{2+} currents, steady-state inactivation curves were obtained by the double-pulse protocol (Figure 2b). The steady-state inactivation curve for the Na^+ current (in the presence of 50 μM Cd^{2+}) was shifted in a negative direction, compared with that for the Ba^{2+} current (in the presence of 10 nM TTX). The potential for half-inactivation of the Na^+ current was −54.5 ± 3.5 mV with a Boltzmann coefficient, k = 5.6 mV (n = 4). For the Ba^{2+} current, the potential for half-inactivation was −32.4 ± 3.7 mV with k = 1.

A single type of Ca^{2+} channel current

Two types of Ca^{2+} current have been shown to coexist in a variety of smooth muscle cells (Hirst *et al.*, 1986; Loirand *et*

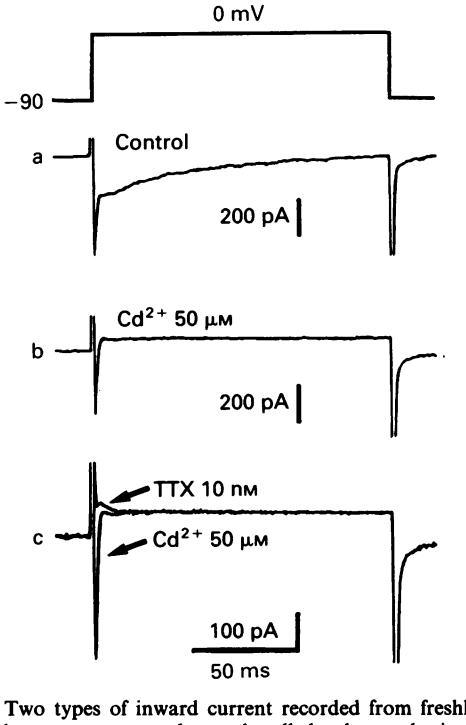

Figure 1 Two types of inward current recorded from freshly dissociated single vena cava smooth muscle cells by the patch pipette technique. Depolarizing steps (0 mV) were applied from a holding potential of −90 mV in reference solution (a) and after adding 50 μM Cd^{2+} to block Ca^{2+} channels (b–c). Tetrodotoxin (TTX, 10 nM) inhibited the fast inward Na^+ current (c). The pipette solution contained 100 mM CsCl and 30 mM NaCl instead of KCl. In this experiment inward currents are not corrected for leakage and capacitive currents. It should be noted that the current scale was 200 pA in (a) and (b), and 100 pA in (c). Cell capacitance = 28 pF. The external Ba^{2+} concentration was 5 mM.

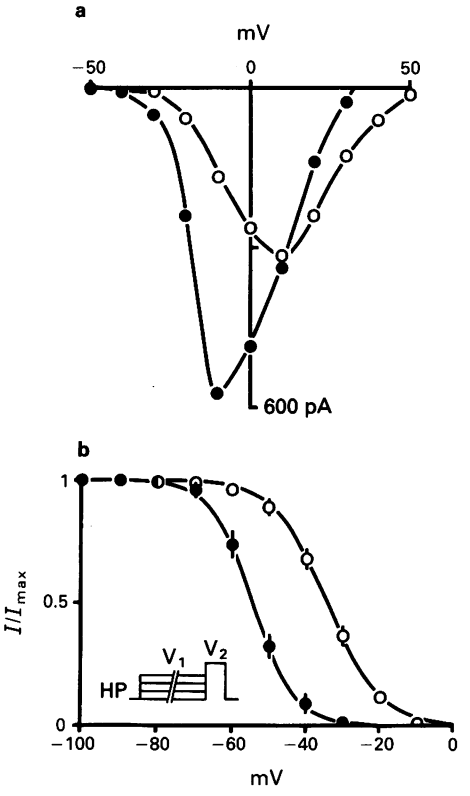

Figure 2 (a) Current-voltage relationships obtained for the fast Na^+ current in the presence of 50 μM Cd^{2+} (●) and the slow Ba^{2+} current in the presence of 10 nM tetrodotoxin (○). Peak currents elicited from a holding potential of −80 mV were plotted against command potentials. Data were obtained from the same cell. Cell capacitance = 21 pF. (b) Steady-state inactivation curves for isolated Na^+ (●) and Ba^{2+} (○) currents obtained as shown in inset. Conditioning pulses (V_1) for 30 s of various amplitudes were applied before a test pulse (V_2) to −10 mV (for Na^+ current) and +10 mV (for Ba^{2+} current) was applied from a holding potential of −100 mV. The amplitude of the test current was normalized by its value in the absence of a conditioning pulse (I/I_{max}). Two curves were obtained by fitting data to Boltzmann distribution equation, $I/I_{max} = 1/[1 + \exp(Vm - Vh)/k]$ where Vm is the membrane potential, Vh is the mid-potential and k is the Boltzmann coefficient. Each point is the mean of 4–9 experiments with s.e.mean shown by vertical lines. The external Ba^{2+} concentration was 5 mM.

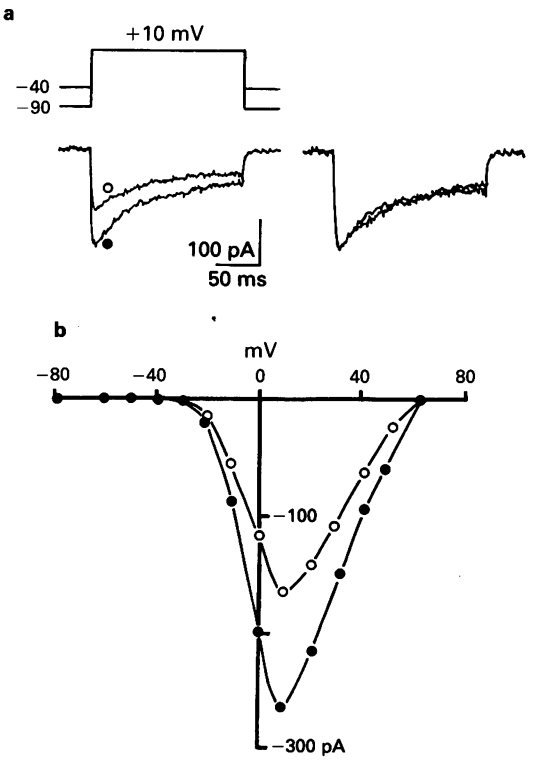

Figure 3 Comparison of Ba^{2+} currents elicited from holding potentials of −90 mV (●) and −40 mV (○) in the presence of 10 nM (tetrodotoxin). (a) The currents did not superimpose at the end of the pulse. Current trace obtained at −40 mV was superimposed by appropriate magnification so as to match the peak with the current obtained at −90 mV. No difference was found in inactivation kinetics. Cell capacitance = 18 pF. (b) Current-voltage relationships for the peak current elicited from −90 mV (●) and from −40 mV (○). No shift in the maximal peak Ba^{2+} current was observed. The external Ba^{2+} concentration was 5 mM.

al., 1986; Yatani *et al.*, 1987). They are thought to arise from two separate populations of Ca^{2+} channels which can be distinguished in terms of their conductance, kinetics, voltage-dependencies and sensitivities to pharmacological agents.

We examined whether the Ba^{2+} current (in the presence of 10 nM TTX) in single vena cava cells might be composed of two components by applying depolarizing pulses to various test potentials from two different holding potentials (−90 and −40 mV). As illustrated in Figure 3, neither change in kinetics of inactivation nor shift in the activation threshold, maximal peak current and apparent reversal potential was observed between the two families of currents ($n = 12$).

These results suggest that vena cava cells are unlikely to possess many of the channels responsible for the low-threshold, rapidly-inactivating Ca^{2+} current reported in some, but not all, smooth muscle so far examined (Loirand *et al.*, 1986; Honoré *et al.*, 1989; Marthan *et al.*, 1989), unless they have a virtually identical voltage sensitivity to the slowly inactivating, dihydropyridine-sensitive (see below) Ca^{2+} channel currents recorded.

Effects of (±)-isradipine on the Ca^{2+} channel current

The effects of one of the most potent dihydropyridine inhibitors, isradipine, were tested on the Ba^{2+} current. Figure 4a illustrates the effect of 50 nM (±)-isradipine on the current-voltage relationship for the Ba^{2+} current. No significant change in the peak current against voltage as well as in the inactivation kinetics (not shown) was recorded with 50 nM isradipine ($n = 7$). When the cell was held at −80 mV, the concentration of (±)-isradipine required to produce 50% inhibition of the current (IC_{50}) was 21 ± 3 nM ($n = 4$). In order to investigate whether isradipine bound with a higher affinity to depolarized cells, we studied the effects of 20 nM (±)-isradipine on the voltage-dependence of the Ba^{2+} current (Figure 4b). The inactivation curves were normalized to currents measured with the most negative conditioning potential whose duration was 30 s. In the presence of isradipine the inactivation curve was shifted to more negative membrane potentials by 16 ± 3 mV ($n = 5$). Thus, it is clear that depolarization intensified the isradipine blockade of the Ba^{2+} current. From the mean shift in the inactivation curve, it is possible to estimate the dissociation constant for isradipine binding in the resting and inactivated state by using an approach described by Bean *et al.* (1983) assuming one-to-one binding of drug to the resting and inactivated states. The dissociation constant for binding to the inactivated state (K_I) can be calculated using the equation:

$$\Delta Vh = k \ln [(1 + [N]/K_I)/(1 + [N]/K_R)]$$

where ΔVh is the shift of the mid point of the steady-state inactivation curve, k is the slope factor of the inactivation curve, [N] is the isradipine concentration used and K_R is determined as the potency of isradipine for the resting channel (measured at a holding potential of −80 mV). With ΔVh = 16 mV, k = 7.1 mV, K_R = 21 nM and [N] = 20 nM, we found K_I = 1.11 nM. The mean K_I value was 0.95 ± 0.25 nM

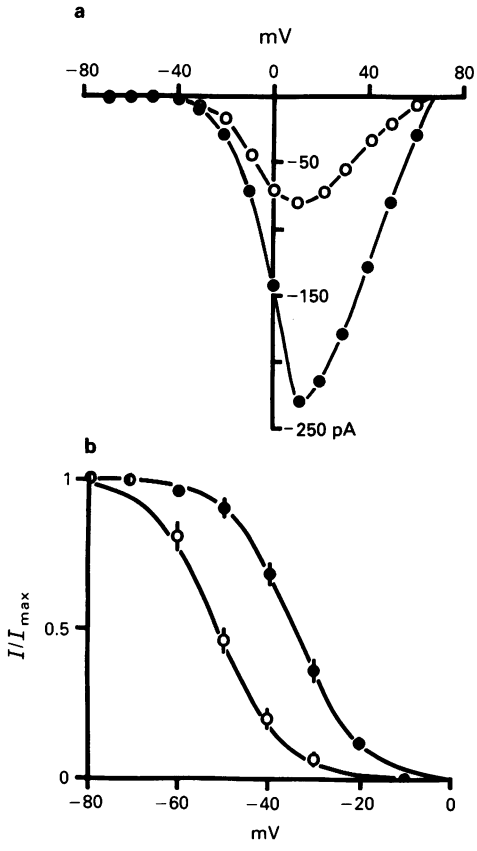

Figure 4 Effects of (±)-isradipine on the Ba^{2+} current in the presence of 10 nM tetrodotoxin. (a) Current-voltage relationships for the peak current obtained from a holding potential of −80 mV in control (●) and after addition of 50 nM isradipine (○). No shift in activation threshold, maximal peak current and apparent reversal potential was observed. Cell capacitance = 26 pF. (b) Steady-state inactivation of the Ba^{2+} current in control (●) and after addition of 20 nM isradipine (○). The amplitude of test current was normalized by its value in the absence of a conditioning pulse (I/I_{max}). The solid lines were drawn as in Figure 2. Each point is the mean of 3–4 experiments with s.e.mean shown by vertical lines. The external Ba^{2+} concentration was 5 mM.

($n = 5$). When Ca^{2+} channels were opened by a conditioning depolarizing pulse sequence (applied at 0.05 Hz), the inhibitory effect of isradipine was similar to that obtained in the absence of stimulation (not shown). This observation is similar to data previously obtained on the effects of dihydropyridines on Ca^{2+} channels (Terada *et al.*, 1987; Honoré *et al.*, 1989).

Binding characteristics of (+)-[^{3}H]-isradipine in intact vena cava strips

Figure 5 illustrates the equilibrium binding of (+)-[^{3}H]-isradipine to intact vena cava strips incubated in 5.6 and 135.6 mM external K^+ solutions, at various concentrations of labelled isradipine. Specific bindings were hyperbolic functions of (+)-[^{3}H]-isradipine concentration and Scatchard plots of the data were linear. The K_D and B_{max} values were 0.26 ± 0.02 nM and 5.9 ± 0.4 fmol mg^{-1} wet weight in 5.6 mM K^+ solution ($n = 5$) and 0.083 ± 0.009 nM and 6.0 ± 0.3 fmol mg^{-1} wet weight in 135.6 mM K^+ solution ($n = 4$). Thus, the B_{max} value was not significantly affected by a high K^+ concentration in the medium while the K_D value was significantly decreased.

Specific binding of (+)-[^{3}H]-isradipine was also determined as a function of the incubation time in 5.6 mM K^+ solution. Association reached a plateau after a 60 min incubation at 37°C (Figure 6a). The data for association were plotted

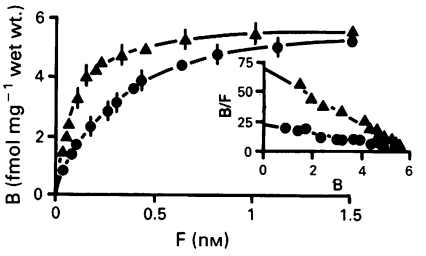

Figure 5 Specific binding of (+)-[^{3}H]-isradipine to rat vena cava strips. Saturation binding experiments were carried out by incubating strips of vena cava with increasing concentrations of (+)-[^{3}H]-isradipine in 5.6 mM (●) and 135.6 mM (▲) K^+-containing solutions for 60 min at 37°C. Specific binding was defined as the binding displaceable by 2 μM nifedipine and accounted for 55 to 60% of the total binding at a concentration close to the K_D value. Each point represents the mean response in 5 experiments with the s.e.mean shown by vertical lines. Inset, Scatchard analysis of specific binding values was done with the non linear least-square LIGAND programme (Munson & Rodbard, 1980). B/F, bound/free.

according to a first-order equation (Weiland & Molinoff, 1981):

$$\ln [LR_E]/[LR_E] - [LR] = k_1 t. [L_T][R_T]/[LR_E]$$

were $[L_T]$ is the total concentration of (+)-[^{3}H]-isradipine, $[R_T]$ is the total concentration of specific binding sites, $[LR_E]$ is the concentration of the complex at equilibrium and [LR] is the concentration of the complex at time t. The slope of this plot, k_{obs}, was estimated in 3 different experiments to be 0.055 ± 0.010 min^{-1}. The binding of labelled isradipine to intact strips of vena cava was a reversible process. After equilibrium was reached, dissociation of the complex was initiated by a 50 fold dilution of the medium with the solution (Figure 6b). The data were plotted according to a first-order kinetics: $\ln [LR]/[LR_E] = -k_{-1} t$. The rate constant for dissociation

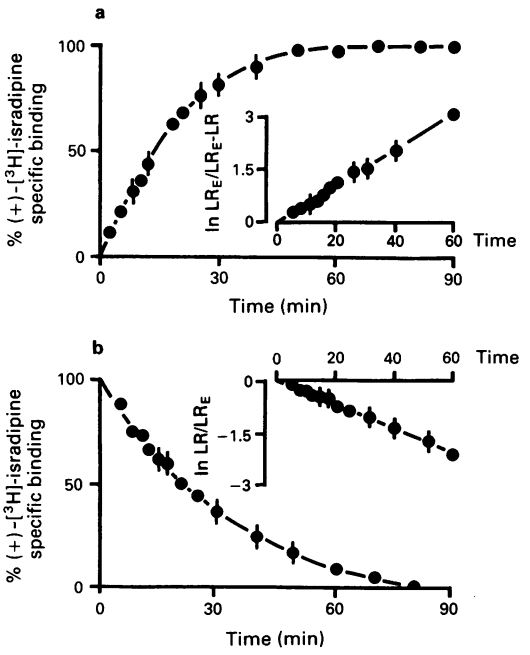

Figure 6 Association and dissociation kinetics of (+)-[^{3}H]-isradipine in intact vena cava strips incubated in 5.6 mM K^+-containing solution. (a) Association was initiated by adding 0.15 nM (+)-[^{3}H]-isradipine to intact strips. Specific binding was determined at the times indicated. Inset, semilogarithmic plot of association data. (b) Dissociation was initiated, after equilibrium had been reached, by a 50 fold dilution of the medium. Inset, semilogarithmic plot of dissociation data. Non-specific binding was determined in the presence of 2 μM nifedipine. Each point represents the mean response of 3 experiments with the s.e.mean shown by vertical lines.

was calculated to be $0.035 \pm 0.002\,min^{-1}$ ($n = 3$) Using the equation $k_{+1} = (k_{obs} - k_{-1})/[L_T]$, the association rate constant was $0.130 \pm 0.010\,min^{-1}\,M^{-1}$ ($n = 3$). The ratio k_{-1}/k_1 gave a K_D value of 0.27 nM which is similar to that, 0.26 nM, determined from equilibrium binding.

Increasing concentrations of unlabelled (±)-, (+)- and (−)-isradipine inhibited the (+)-[^{3}H]-isradipine binding. The concentration producing half-maximal inhibition (IC_{50}), the inhibition constant (K_i) and the Hill coefficient are listed in Table 1. These results show that the IC_{50} value for (±)-isradipine obtained from ligand experiments (15.10 nM) is similar to that obtained from electrophysiological experiments (21 nM) in normally polarized vena cava cells.

Effects of (±)-Bay K 8644 on Ca^{2+} channel current

In cardiac myocytes changes in Ba^{2+} current induced by dihydropyridine Ca^{2+} channel agonists include an increase in peak current, a faster decay of the current, a slower deactivation and a shift of the current-voltage relationship towards hyperpolarizing potentials (Sanguinetti *et al.*, 1986; Hamilton *et al.*, 1987; Kamp *et al.*, 1989). In smooth muscle cells, an increase in Ca^{2+} channel current with Bay K 8644 is generally reported but there are still unresolved problems concerning the interpretation of the stimulatory effects of Bay K 8644 on whole-cell currents. Shifts in the peak current-voltage relationship to more hyperpolarized potentials have been shown in arterial (Caffrey *et al.*, 1986; Aaronson *et al.*, 1988; Matsuda *et al.*, 1990), tracheal (Hisada *et al.*, 1990) and intestinal myocytes (Yoshino *et al.*, 1988). In some of these papers, the amplitude of the peak Ba^{2+} current in control conditions was rather low (60–80 pA) so that large increases of current induced by Bay K 8644 might produce a significant loss of voltage control. In contrast, there are some reports showing the absence of voltage-dependent modulation of Ca^{2+} channel current by Bay K 8644 in intestinal (Droogmans & Callewaert, 1986) and venous myocytes (Yatani *et al.*, 1987).

In isolated cells of vena cava held at −80 mV, (±)-Bay K 8644 caused a concentration-dependent increase of the Ba^{2+} current through slow Ca^{2+} channels (Figure 7a). The concentration of Bay K 8644 required to produce half-maximal current was 10 ± 3 nM ($n = 5$). In contrast to cardiac cells, Bay K 8644 had no significant effect on the current decay during the test pulse (Figure 7b). The time corresponding to half-maximal amplitude of the Ba^{2+} current was 72 ± 4 ms in control and 71 ± 3 ms in the presence of 1 μM Bay K 8644 ($n = 14$, $P > 0.05$). Similarly, the deactivation of the Ba^{2+} current when the cell was repolarized to −80 mV was unchanged ($n = 14$). In all experiments there were no measurable changes in the time to peak of maximal Ba^{2+} currents. As shown in Figure 8a, 1 μM Bay K 8644 increased the Ba^{2+} current at all potentials tested without significant variation in the apparent reversal potential ($n = 5$). The peak of the current-voltage relationship was only slightly shifted in the hyperpolarizing direction in the presence of Bay K 8644 in this experiment, but the variation was not significant (10.0 ± 3.0 mV in control; 4.5 ± 3.5 mV in Bay K 8644, $n = 11$, $P > 0.05$).

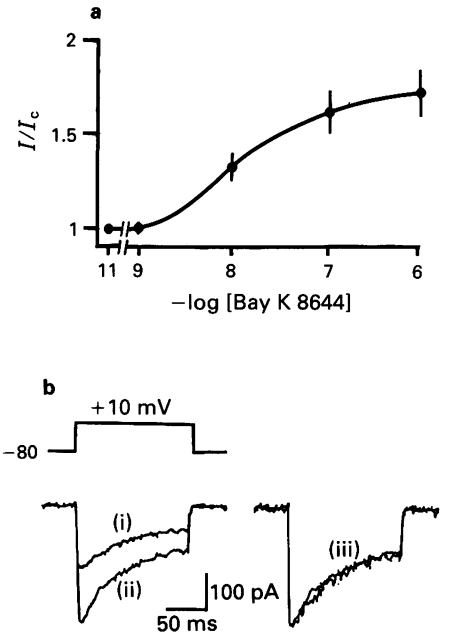

Figure 7 Effects of (±)-Bay K 8644 on the Ba^{2+} current in the presence of 10 nM tetrodotoxin. (a) Concentration-response curve for the stimulatory effect of Bay K 8644. The current elicited from a holding potential of −80 mV to +10 mV was expressed as a fraction of the current in the absence of Bay K 8644 (I/I_c). The concentration producing half-maximal current was 10 ± 3 nM ($n = 5$). Each point is the mean of 5 determinations with s.e.mean shown by vertical lines. (b) Current traces obtained in the absence (i) and presence of 1 μM Bay K 8644 (ii) were superimposed by appropriate magnification so as to match their peaks (iii). No difference was observed in inactivation kinetics. Cell capacitance = 21 pF. The external Ba^{2+} concentration was 5 mM.

The onset of action of Bay K 8644 was examined under two different conditions shown in Figure 8b. First, the onset was examined when the holding potential was held at −80 mV with depolarizing test pulses to +10 mV (150 ms in duration) applied every 20 s. It can be seen that 1 μM Bay K 8644 caused a rapid increase in the peak Ba^{2+} current which reached a steady-state within 2 min. This effect was rapidly reversed after a washing period of 3–4 min. Therefore, we determined whether the presence of depolarizing pulses during exposure of the single cell to Bay K 8644 affected the onset of action. When Bay K 8644 was perfused for 2 min in the absence of stimulation, the steady-state agonist effect was observed at the first post-rest stimulation. Independently of the holding potential (−90 mV to −40 mV) Bay K 8644 produced unequivocal Ba^{2+} current increases ($n = 24$).

In order to examine accurately the effects of voltage on the effects of Bay K 8644 in smooth muscle cells, both activation and inactivation curves were obtained in the absence and presence of 1 μM Bay K 8644. In these experiments leak currents were measured in the presence of both 10 nM TTX and 50 μM

Table 1 Inhibition of (+)-[^{3}H]-isradipine binding to vena cava strips incubated in 5.6 mM K^+ solution

Ligand	IC_{50}	K_i	*n Hill*	*Maximal inhibition*
	nM	nM		%
(+)-Isradipine	0.45 ± 0.01	0.27 ± 0.01	0.90 ± 0.10	100
(±)-Isradipine	15.10 ± 2.00	9.65 ± 1.20	0.95 ± 0.04	100
(−)-Isradipine	127.10 ± 60.00	85.00 ± 20.10	0.93 ± 0.06	100

Each value is mean ± s.e.mean of 3–4 experiments in which each determination is made in duplicate. K_i values were calculated for competitive inhibitors from inhibition curves according to the equation of Cheng & Prusoff (1973).

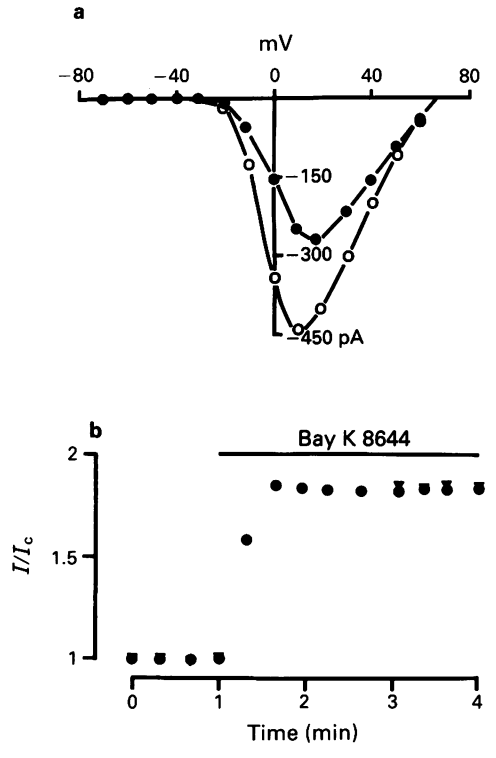

Figure 8 (a) Current-voltage relationships for the peak Ba^{2+} current in the presence of 10 nM tetrodotoxin obtained before (●) and after addition of 1 μM Bay K 8644 (○). Holding potential = −80 mV. The peak current is increased at any membrane potential with no significant shift in maximal peak current against voltage. (b) Effects of Bay K 8644 (1 μM) with (●) and without (▼) repetitive applications of command pulses (20 s intervals). The membrane was stepped to + 10 mV from a holding potential of −80 mV. Steady-state effect of Bay K 8644 was obtained within 1 min. The current was expressed as a fraction of the current in the absence of Bay K 8644 (I/I_c). Similar results were obtained in 4 other cells. The external Ba^{2+} concentration was 5 mM.

Cd^{2+}. The leak corrected Ba^{2+} currents were used to calculate the conductance (G) at each test potential by dividing the peak Ba^{2+} current by the driving force ($V - V_{rev}$). In Figure 9, the normalized conductance (G/G_{max}) is plotted as a function of membrane potential. In control conditions we found a half-

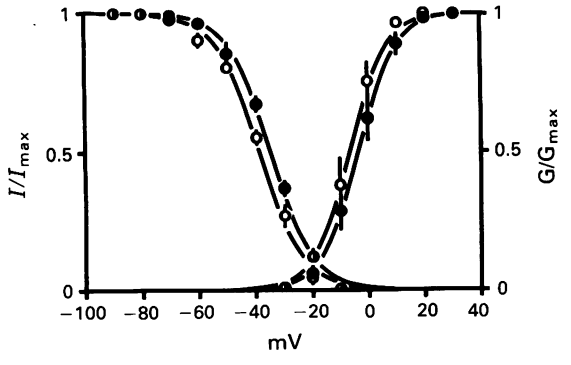

Figure 9 Steady-state activation and inactivation curves for the Ba^{2+} current in the presence of 10 mM tetrodotoxin, before (●) and after addition of 1 μM Bay K 8644 (○). Data were fitted to a Boltzmann distribution equation as indicated in Figure 2. Holding potential = −90 mV. The potentials for half-activation and half-inactivation as well as the Boltzmann coefficients are not significantly modified after addition of Bay K 8644. Each point is the mean of 4–8 experiments with s.e.mean shown by vertical lines. The external Ba^{2+} concentration was 5 mM.

activation potential of -3.5 ± 2.5 mV ($n = 8$). In the presence of 1 μM Bay K 8644, the half-activation potential was not significantly modified (-6.4 ± 3.1 mV, $n = 6$, $P > 0.05$). It is also important to note that no change in the slope of the curve under the two conditions was observed (k = 6.5 mV). The effects of Bay K 8644 were also studied on the inactivation curve of Ca^{2+} channels as described in Figure 2b. In the presence of 1 μM Bay K 8644 the inactivation curve was slightly shifted to more negative membrane potential with a half-inactivation potential of -37.6 ± 4.5 mV ($n = 6$). This was not significantly different from the value obtained in control conditions (-32.4 ± 3.7 mV, $n = 4$, $P > 0.05$). There was no change in the slope of the curves (k = 7.2 mV). These results indicate that in smooth muscle cells Bay K 8644 may increase the Ba^{2+} current without altering the voltage-dependency of the gating parameters.

Discussion

In the present study, we have obtained evidence for the existence of both fast Na^+ and slow Ba^{2+} inward currents in the membrane of freshly isolated smooth muscle cells of rat inferior vena cava. The following data suggest that the permeant ion through the fast channel is mainly Na^+ as (1) the amplitude of the fast current was dependent on the extracellular Na^+ concentration; (2) the fast current was insensitive to Cd^{2+} and isradipine; (3) TTX (10 nM) inhibited the fast current; (4) steady-state inactivation curve had a half-inactivation potential at −55 mV, a value similar to that obtained for Na^+ current in other smooth muscle cells (Okabe *et al.*, 1988; Ohya & Sperelakis, 1989; Mironneau *et al.*, 1990). In the present experiments the peak amplitude of the Na^+ current was observed nearly 1–2 ms after application of the command pulse. As the unsubtracted component of the capacitative current may interfere with accurate measurements of the Na^+ current, the amplitude measured may be an underestimate. This may account for the small discrepancy between the theoretical Na^+ equilibrium potential (39 mV) and the apparent reversal potential measured in these experiments (31 mV). The physiological role of Na^+ channels in smooth muscle is unknown. As about 70% of the Na^+ channels are available at the resting potential (−60 mV), the fast Na^+ current may play an important role in cell-to-cell conduction and in modulation of neuromediator responses.

The major population of Ca^{2+} channels observed in the freshly isolated smooth muscle cells from rat vena cava was, of the slow, L-type, relatively high-threshold Ca^{2+} channel. This conclusion was based on the following observations: (1) failure to separate fast and slow components of current by changing the holding potential between −90 and −40 mV; (2) membrane potential for mid-inactivation at −32 mV; and (3) high sensitivity to dihydropyridines.

The dihydropyridine blockade of L-type Ca^{2+} channels in vascular and visceral cells is enhanced by depolarizing the holding potential. We found that (±)-isradipine not only bound to the resting, available state of Ca^{2+} channels, but also that it had a higher affinity for the inactivated state. These results are in agreement with the classical mechanism of action for dihydropyridines on Ca^{2+} channels (Bean, 1984; Sanguinetti & Kass, 1984; Terada *et al.*, 1987; Loirand *et al.*, 1989). At the resting state (holding potential of −80 mV) where most Ca^{2+} channels were thought to be in the closed, available state the concentration of (±)-isradipine inhibiting 50% of the Ba^{2+} current (IC_{50}) was 21 nM. As isradipine shifted the steady-state inactivation curve of Ca^{2+} channels to more negative membrane potentials, we calculated a dissociation constant for binding to the inactivated state (K_I) of about 1 nM indicating that isradipine inhibition of Ca^{2+} channels was voltage-dependent. In addition, the high-affinity binding site for (+)-isradipine has been identified in intact strips of rat vena cava incubated in either 5.6 mM K^+ or

135 mM K^+ solution. In 5.6 mM K^+ solution the dissociation constant obtained from equilibrium binding data (K_D = 0.26 nM) was identical to that calculated from association and dissociation kinetics (K_D = 0.27 nM). When intact strips were incubated in 135 mM K^+ solution, the K_D value was decreased to 0.08 nM confirming, at the molecular level, the voltage-dependency of isradipine binding to Ca^{2+} channels. Interestingly, the concentration of (±)-isradipine inhibiting 50% of specific (+)-[^{3}H]-isradipine binding in strips incubated in 5.6 mM K^+ solution (15.1 nM) was similar to the IC_{50} value obtained from inhibition of the Ba^{2+} current when the cell was held at −80 mV (21 nM). These results clearly establish that the electrophysiological experiments on the inhibitory effects of (±)-isradipine are comparable to radioligand binding data in intact vena cava strips.

The molecular mechanisms of action of the dihydropyridine Ca^{2+} channel agonist, Bay K 8644, have been studied intensively in myocardial cells and several models have been proposed (Hess *et al.*, 1984; Sanguinetti *et al.*, 1986; Markwardt & Nilius, 1988; Lacerda & Brown, 1989). The effects of Bay K 8644 in vena cava smooth muscle cells differ considerably from those obtained in cardiac cells since: (1) Bay K 8644 does not shift either activation or inactivation of Ba^{2+} current towards hyperpolarizing potentials (2) Bay K 8644 does not affect either time to peak or inactivation kinetics of Ba^{2+} current during a depolarizing test pulse. These results suggest that Bay K 8644 increases the amplitude of Ba^{2+} current in vena cava smooth muscle cells by promoting gate opening in Ca^{2+} channels. In bovine chromaffin cells, a long-lived open state, which is not seen in control records, is obtained in the presence of Bay K 8644 (Hoshi & Smith, 1987). Similarly, in ventricular myocytes, Bay K 8644 promotes mode 2 gating of Ca^{2+} channels, which is characterized by prolonged open times (Hess *et al.*, 1984). However, the mode 2 gating was observed, albeit rarely, in the absence of Bay K 8644. Therefore, it is possible that in vascular smooth muscle cells Bay K 8644 promotes the appearance of an open state of the Ca^{2+} channels which is either not, or only rarely, seen under control conditions. This possibility needs to be explored in single channel recordings.

Recent results have suggested the existence of two dihydropyridine binding sites on the basis of whole-cell and unitary calcium current studies in cardiac and vascular cells. Some authors have demonstrated cooperative interactions between the dihydropyridine binding sites regulating Ca^{2+} channels (Kokubun *et al.*, 1986; Lacerda & Brown, 1989) whilst others have favoured the existence of two independent sites for agonist and antagonist dihydropyridines (Kamp *et al.*, 1989; Hughes *et al.*, 1990). Certain experimental findings observed in the present study are consistent with the presence of two independent dihydropyridine binding sites. For example, the fact that the inhibitory effect of isradipine is potentiated by membrane depolarization while the stimulatory effect of Bay K 8644 is only slightly affected by membrane potential is difficult to explain by a simple single-binding site model. Further radioligand binding data are needed for identifying the different binding sites for agonist and antagonist enantiomers of dihydropyridines. Such experiments are currently a subject of our research.

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M-currents in frog sympathetic ganglion cells: manipulation of membrane phosphorylation

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1 The inward current and the M-current (I_M) suppression produced when muscarine is applied to frog sympathetic ganglion cells was recorded by means of the whole-cell patch-clamp technique. The holding potential was −30 mV and $[K^+]_o$ was 6 mM.

2 The steady-state I_M was maintained for at least 20 min when the patch pipette contained neither adenosine 5'-triphosphate (ATP) nor adenosine 3':5'-cyclic monophosphate (cyclic AMP). Inclusion of these substances or the ATP antagonst, β,γ-methyleneadenosine 5'-triphosphate (β,γ-MethATP; 1 or 2 nM) (failed to alter the rate of I_M 'run down'. By contrast, inclusion of adenosine-5'-O-(3-thiotriphosphate) (ATP-γ-S, 1 or 2 mM) resulted in a 60% reduction of the current within 18 min.

3 Despite the inability of ATP-γ-S to maintain steady-state I_M, it had no effect on the ability of muscarine (2–100 μM) to suppress a constant fraction of the available current. ATP-γ-S and β,γ-MethATP increased the rise time and duration of the response to muscarine.

4 Inclusion of a phosphatase inhibitor, diphosphoglyceric acid (DPG, 1–2.5 mM) or alkaline phosphatase (100 μg ml^{-1}) failed to affect the amplitude of muscarinic responses.

5 These results question the role of the phosphorylation and/or dephosphorylation reactions in the transduction mechanism for muscarine-induced I_M suppression but are consistent with the possibility that M-channels are 'directly coupled' via G-protein to the muscarinic receptor.

Keywords: Adenosine nucleotides; potassium channel; muscarinic receptor; autonomic ganglia; M-current; adenosine-5'-O- (3-thiotriphosphate); protein phosphatase; β,γ-methyleneadenosine 5'-triphosphate; protein kinase; diphosphoglyceric acid

Introduction

Muscarine-induced depolarization of B-cells in amphibian paravertebral sympathetic ganglia results, in part, from the suppression of a voltage-dependent, non-inactivating K^+ current, called the M-current (I_M; Brown & Adams, 1980; Adams *et al.*, 1982a,b; Akasu *et al.*, 1984; Selyanko *et al.*, 1990). Despite extensive investigation, the transduction mechanism which underlies M-channel closure following receptor activation remains to be elucidated (Adams *et al.*, 1986; Hille, 1989; see also Owen *et al.*, 1990). Although experiments with non-hydrolysable guanosine 5'-triphosphate (GTP) analogues support the involvement of a (pertussis toxin-insensitive) G-protein (Pfaffinger, 1988; see also Brown *et al.*, 1989), experiments designed to test the role of known cytosolic second messengers have yielded negative or equivocal results. For example, the involvement of cyclic nucleotides seems unlikely (Busis *et al.*, 1978; Weight *et al.*, 1978; Adams *et al.*, 1982b; Selyanko *et al.*, 1990) and although suppression of I_M can be mimicked by the application of protein kinase C (PKC) activators, e.g. phorbol esters (Brown & Adams, 1987; Pfaffinger *et al.*, 1988; Bosma & Hille, 1989; Selyanko *et al.*, 1990), results with PKC inhibitors (Bosma & Hille, 1989; Selyanko *et al.*, 1990) provide evidence against involvement of the diacyl glycerol/protein kinase C mechanism. In addition, it had been demonstrated that the agonist-induced reduction of I_M is unlikely to be mediated by inositol trisphosphate (Pfaffinger *et al.*, 1988; Hille, 1989; Selyanko *et al.*, 1990; see also Brown *et al.*, 1989) and the possible involvement of changes in intracellular Ca^{2+} concentration remain to be clarified (Kirkwood *et al.*, 1991; Beech *et al.*, 1991; Marrion *et al.*, 1991). The involvement of arachidonic acid metabolites as second messengers for I_M suppression is also unlikely (Hille, 1989; Yu *et al.*, 1991).

Experiments on rat cultured sympathetic ganglion cells with single-channel recording techniques (Owen *et al.*, 1990) have also failed to resolve the question of whether cytosolic second messengers are involved. Had it been possible to record a response in the cell-attached mode when muscarine was applied outside the pipette, this would have been consistent with the involvement of cytosolic second messengers in I_M suppression. If a response had been recorded in an outside-out patch, this would be consistent with 'direct G-protein coupling' as has been suggested for atrial muscarinic receptors (Pfaffinger *et al.*, 1985). Unfortunately, neither type of response has hitherto been reported (Owen *et al.*, 1990).

Many of the effects of the above second messengers are exerted via activation of protein kinases which phosphorylate membrane proteins, such as ion channels (Levitan, 1985 but see also DiFrancesco & Tortora, 1991). The present study was therefore designed to examine the role of phosphorylation and dephosphorylation processes in the transduction mechanism which underlies muscarine-induced I_M suppression. A preliminary report of some of these data has appeared (Zidichouski *et al.*, 1990).

Methods

Medium size leopard frogs (*Rana pipiens* <8 cm 'nose to tail') were purchased from a biological supply house and stored in running water at room temperature (20°C). Each frog was killed by pithing and the VIth to Xth paravertebral sympathetic ganglia removed and dissociated with trypsin and collagenase as described by Selyanko *et al.* (1990). Dissociated cells were left to adhere to the bottom of plastic petri dishes for about 80 min before electrophysiological analysis. Dissociated neurones were observed under a Nikon 'Diaphot' microscope and all experiments were carried out at room temperature (20°C). Some experiments were carried out on small

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bullfrogs (*Rana catesbiana* < 10 cm 'nose to tail') when *Rana pipiens* was unavailable.

Since detailed methods for whole-cell patch-clamp recording from amphibian autonomic neurones have been published (Selyanko *et al.*, 1990), only a brief description will be given here. Recordings were made with an Axopatch 1B amplifier, a Labmaster interface and an IBM-XT computer running 'Pclamp' software (Axon Instruments, Foster City, CA, U.S.A.). Data were stored for off-line analysis on a removable hard disk system and permanent records obtained from an *x-y* plotter. On-line records were obtained with a d.c. rectilinear pen recorder (Gould-Brush 2400; pen rise time < 8 ms). The corner frequency of the filter on the Axopatch amplifier was set to 200 Hz for voltage-ramp experiments and to 500 Hz for voltage-jumps. Current was zeroed at resting membrane potential (r.m.p.) and the holding potential was set to −30 mV. An estimate of the cell size was obtained from the input capacitance (C_{in}) and experiments were only done on the 'large' cells (C_{in} > 30 pF) which exhibited inward current responses to muscarine (at −30 mV with $[K^+]_o$ = 6 mM). Since the currents to be recorded were < 0.4 nA, no corrections were made for the voltage-drop across the series resistance which was always < 10 MΩ (i.e. the maximum voltage error due to series resistance was < 4 mV). Whole-cell M-channel conductance (G_M) was examined using a 5 s ramp command from the holding potential of −30 mV to −110 mV (16 mV s^{-1} see Figure 1). The high conductance part of the resulting *I*-V relationship (i.e. above −75 mV) represents current through M-channels plus leak current (Selyanko *et al.*, 1990). Total G_M at −30 mV was estimated after digitally-subtracting the leak current predicted by the slope between −75 and −90 mV. In order to document the mean change in I_M which occurred with time, I_M in each cell was measured at arbitrary time intervals and mean values for a series of cells at 3 min intervals estimated by extrapolation.

The physiological salt solution contained (mM): NaCl 113, KCl 6, $MgCl_2$ 2, $CaCl_2$ 2, HEPES/NaOH (pH 7.2) 5 and D-glucose 10. Patch pipettes (10–20 MΩ) were pulled from borosilicate glass and coated with Sylgard elastomer. The solution used to fill the pipettes contained (mM): KCl 110, NaCl 10,

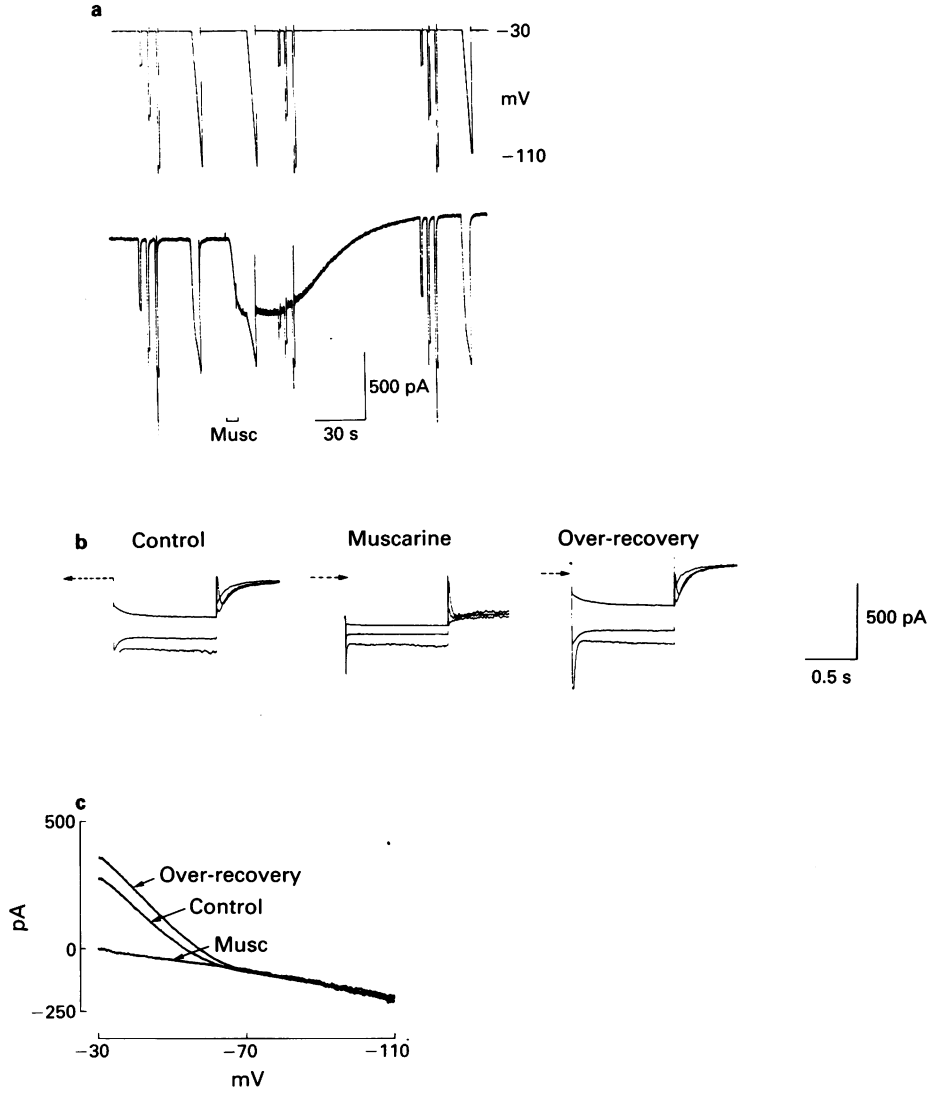

Figure 1 Response of a bullfrog sympathetic ganglion cell to muscarine (10 μM applied via U-tube), pipette contained 1 mM ATP (a) Upper record; voltage commands, comprising steps to −50, −80 and −110 mV or 5 s ramps to −110 mV, from the holding potential of −30 mV. Lower record; steady-state current response, muscarine produces an inward current associated with decreased membrane conductance followed by an outward current (over-recovery). (b) Current responses to voltage steps before, during and after response to muscarine shown on a faster time scale. Arrows represent control current level prior to the application of the drug. I_M relaxations are suppressed during response to muscarine and steady-state outward current is apparent during the 'over-recovery'. (c) Current responses to the ramp commands shown on a faster time scale. Note biphasic nature of the current prior to the application of muscarine and suppression followed by enhancement of conductance in the I_M range in response to muscarine. 500 pA/30 s calibration refers to records in (a) which were from a rectilinear pen recorder. 500 pA/0.5 s calibration refers to records in (b), which like the records in (c) were from an *x* − *y* plotter.

$MgCl_2$ 2, $CaCl_2$ 0.4, EGTA 4.4, HEPES/KOH (pH 6.7) 5 and D-glucose 10. The pCa of this solution, measured with a Ca^{2+}-electrode, was about 7. The Na^+ salts of ATP (1 mM), β,γ-methyleneadenosine 5'-triphosphate (β,γ-MethATP), adenosine 3':5'-cyclic monophosphate (cyclic AMP) or the tetralithium salt of adenosine-5'-O-(3-thiotriphosphate) (ATP-γ-S; 1 or 2 mM) were included in the internal solution. It was assumed that all of these substances would readily enter the cytoplasm because application of ATP analogues via a patch pipette has already been shown to exert pharmacological effects on these neurones (Simmons *et al.*, 1990). Also, we have observed prolongation of agonist-induced responses when GTP-γ-S is applied by this route (Selyanko *et al.*, 1990). Robust and repeatable responses to muscarine were recorded without the inclusion of guanosine triphosphate (GTP) in the patch pipette.

Muscarine was applied either by pressure ejection, using a 'Picospritzer' (General Valve, Fairfield, NJ, USA) or using the U-tube technique (Krishtal & Pidoplitchko, 1980; Selyanko *et al.*, 1990). The fluid exchange time for the U-tube was about 0.2 s. The rise time of the muscarine-induced current was defined as the time taken from the onset of the response to the time when the response had reached 63% of its maximum amplitude. All chemicals and drugs were from Sigma, St. Louis, MO, U.S.A. except for ATP-γ-S which was from Boehringer, Mannheim, Germany. Data are expressed as mean ± s.e.mean and significance of differences were estimated by Student's two-tailed, unpaired *t* test.

Results

The steady-state inward current and the pronounced I_M suppression produced by 'U-tube' application of 10 μM muscarine are illustrated in Figure 1. Prior to the application of the drug, hyperpolarizing voltage commands from the holding potential of −30 mV produce inward relaxations which reflect I_M deactivation. At command potentials negative to the potassium equilibrium potential (−73 mV), I_M deactivation is accelerated and the relaxations are reversed in polarity (Adams *et al.*, 1982a). The current response to a ramp to −110 mV (*I*-V relationship) is biphasic and displays a region of increased conductance due to activation of M-conductance (g_M) at potentials positive to −75 mV (Selyanko *et al.*, 1990). Muscarine evokes a steady-state inward current which is associated with decreased membrane conductance. The amplitude of I_M relaxations are reduced and the high conductance region of the *I*-V relationship is almost completely eliminated. I_M over-recovers following the removal of muscarine (cf. Pfaffinger, 1988). Application of 10 μM muscarine from the 'U-tube' almost completely eliminated I_M in all cells tested and since robust, submaximal responses could be elicited with 2 μM muscarine, this concentration was used for the majority of experiments in the present study. Since 'Picozpritzer' application was less efficient than 'U-tube' application, it was necessary to use 10 or occasionally 100 μM muscarine to produce robust, submaximal responses when this method of drug application was employed.

Dependence of I_M *on intracellular nucleotides*

Although previous work has suggested that I_M tends to 'run down' during whole-cell recording unless ATP or cyclic AMP is included in the patch pipette (Pfaffinger, 1988; Selyanko *et al.*, 1990), the current recorded in the present series of experiments was maintained for at least 20 min in the absence of adenosine nucleotides (Figure 2a and b). The effect of 1 mM ATP on the maintenance of the current was examined in 10 neurones and Figure 2c and d show that I_M was unattenuated during the first 18 min of recording in the presence of the nucleotide but that some slight 'run-down' of the current occurred after 24 min. I_M was also well-maintained when cyclic AMP (100 μM) or lower concentrations of ATP (100 μM) were included in the patch pipette (data not shown). Surprisingly, the ATP-antagonist, β,γ-MethATP (1 or 2 mM) failed to promote 'run-down' of the current (data from 9 cells, Figure 2e and f). By contrast, ATP-γ-S (1 or 2 mM), which is a substrate for protein kinase A and forms stable protein thiophosphates (Eckstein, 1985) was unable to maintain I_M and the current declined to 31.5 ± 7.5% ($n = 8$) of control in 21 min (Figure 2g and h).

Effects of intracellular nucleotides on the response to muscarine

Despite the differing abilities of the various adenosine nucleotides to support steady-state I_M, the ability of muscarine to suppress a constant fraction of the available current was independent of nucleotide content. For example, 2 μM muscarine produced 60.1 ± 10.9% suppression of the available I_M in neurones studied after 3 min with intracellularly applied ATP-γ-S ($n = 5$). After 18 min, the available I_M had decreased to 38.0 ± 6.0% of its control level ($n = 8$) but muscarine still produced about the same fraction suppression (64.4 ± 7.2% $n = 5$, $P > 0.7$) of the remaining I_M. These data are illustrated in Figure 2g and the effects of other nucleotides on the response to muscarine are shown in Figure 2c and e. The rise time of the muscarine response in neurones filled with ATP-γ-S was about 50% greater than in neurones filled with cyclic AMP. The duration of the response was increased even for relatively short periods of recording (i.e. < 15 min) and little or no recovery of the muscarine-evoked inward current was observed in 9 out of 23 cells tested. There was also a progressive increase in duration of successive muscarine responses during recording with intracellularly-applied ATP-γ-S and the amplitude of successive responses decreased with time. These data are summarized in Table 1 and a typical experiment is illustrated in Figure 3.

The rise time of muscarine responses increased about 3 fold when the pipette contained β,γ-MethATP rather than cyclic AMP. The presence of this ATP antagonist also increased response duration by about 50% but failed to exhibit any significant effect on response amplitude (Table 1).

Effect of the phosphatase inhibitor, diphosphoglyceric acid

There is evidence that at least in heart, muscarinic agonists can activate protein phosphatases (Ahmad *et al.*, 1989). If this mechanism were involved in muscarine-induced I_M suppression in frog ganglia, such that channel closure would require dephosphorylation (cf. Pfaffinger, 1988), the response should be blocked by phosphatase inhibitors such as diphosphoglyceric acid (DPG; Downes *et al.*, 1982). If, on the other hand, M-channel closure involved phosphorylation, the action of a protein phosphatase might be required for the termination of the response. If this were the case, irreversible responses to muscarine would be expected in the presence of DPG. However, when DPG (1–2.5 mM plus 100 μM cyclic AMP) was added to the intracellular solution, muscarine responses exhibited normal rise times and amplitudes. Although the responses were always reversible, their duration was increased by about 50% (Table 1).

As a final test for the involvement of phosphorylation and/or dephosphorylation reactions, alkaline phosphatase (100 $\mu g\,ml^{-1}$ plus 100 μM cyclic AMP or 1 mM ATP) was included in the patch pipette. This enzyme, which should dephosphorylate membrane proteins, failed to alter amplitude of responses to muscarine for periods of up to 83 min. The average duration of the response was increased by about 20% (Table 1).

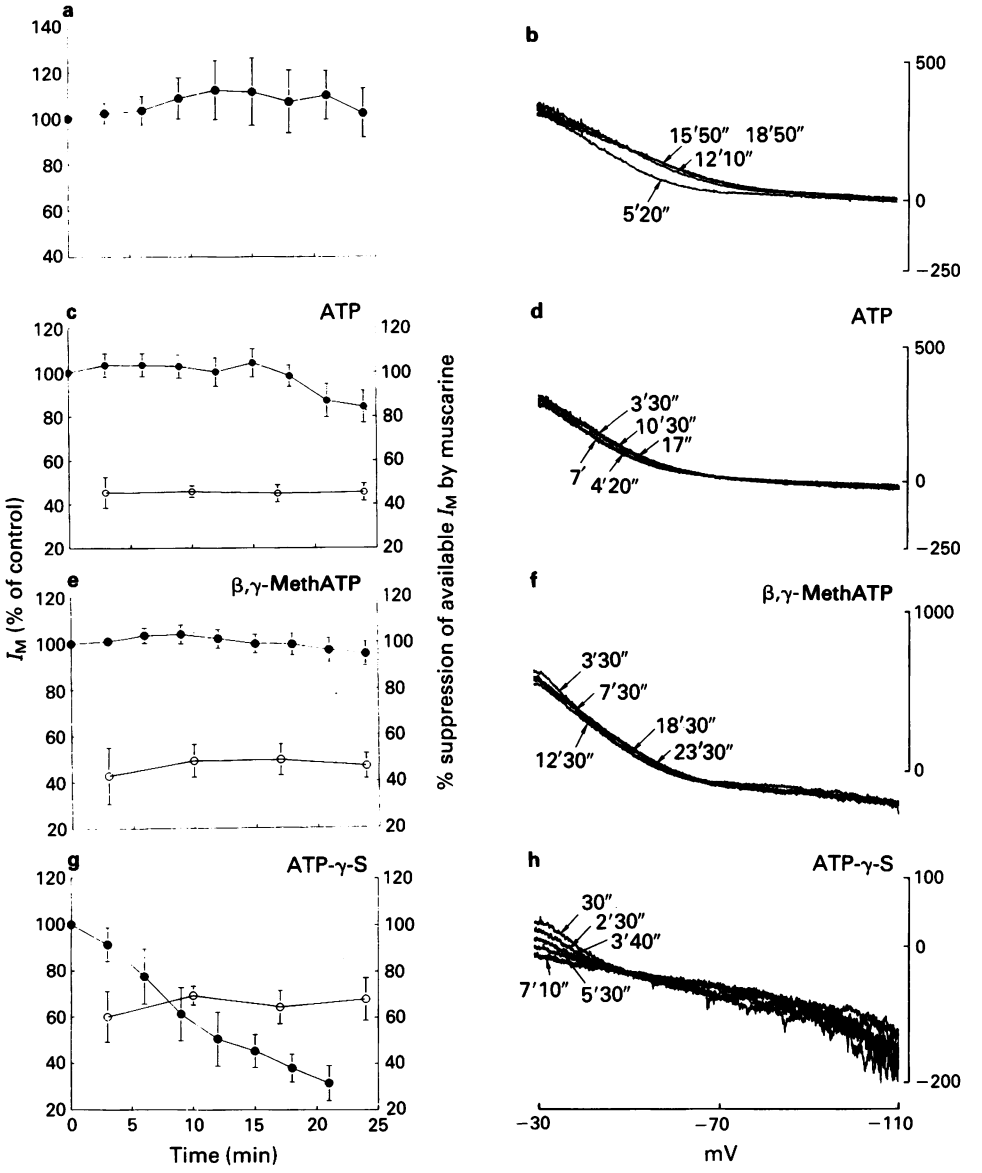

Figure 2 Effects of pipette content on maintenance of I_M and on the ability of muscarine to suppress I_M (Except where otherwise indicated, all data are from *Rana pipiens* cells). (a) Persistence of I_M when no adenosine nucleotides were included in the pipette (data averaged from 7–9 cells; not all cells were studied for the full 25 min period). (b) Original data records to show persistence of I_M. (c) (●) Persistence of I_M when 1 mM ATP was included in the patch-pipette (○). Percentage suppression of the available I_M by muscarine (10 μM, applied from Picospritzer) (data from 10 cells). (d) Original data records of I_M recorded with 1 mM ATP in the patch pipette. (e) (●) Persistence of I_M when 1 or 2 mM β,γ-methyleneadenosine 5'-triphosphate (β,γ-MethATP) was included in the patch-pipette (○). Percentage suppression of the available I_M by muscarine (100 μM, applied from Picospritzer) (Data from 9 cells). (d) Original data records of persistance of I_M in a bullfrog neurone with 2 mM β,γ-MethATP in the patch pipette. (g) (●) Lack of persistence of I_M when adenosine 5'-O-(3-thiotriphosphate) (ATP-γ-S, 1 or 2 mM) was included in the patch-pipette (○). Percentage suppression of the available I_M by muscarine 2 μM, applied from U-tube (Data from 5–8 cells) (h). Original data records of the effect of 2 mM ATP-γ-S on persistance of I_M. Current responses shown in (b), (d), (f) and (h) are evoked from 5 s hyperpolarizing ramp commands as shown in Figure 1. These data are x-y plots of digitally-stored data. Percentage suppression of I_M in (a), (c), (e) and (g) is not the same because different concentrations of muscarine and different methods of application were used in each series of experiments.

Discussion

If a phosphorylation process were involved in the suppression of I_M by muscarine, ATP antagonists such as β,γ-MethATP would be expected to block the response. If a dephosphorylation process were involved (cf. Pfaffinger, 1988), the re-opening of the channels during recovery from muscarine would require phosphorylation so that β,γ-MethATP would be expected to promote irreversible responses. However, responses recorded with intracellular β,γ-MethATP were neither irreversible nor were they any smaller than those recorded with cyclic AMP. Although ATP-γ-S promoted I_M run-down, muscarine still caused the same fractional depression of the remaining current even after 20 min. These results, as well as the lack of blockade by DPG and alkaline phosphatase argue strongly against involvement of phosphorylation and/or dephosphorylation mechanisms in the transduction process for muscarinic responses. This conclusion is supported by previously published data on the lack of effect of kinase inhibitors such as 1-(5-isoquinolinyl-sulphonyl)-2-methyl piperazine (H-7), staurosporine and gold sodium thiomalate (Bosma & Hille, 1989; Selyanko *et al.*, 1990).

Three other observations which may be interpreted to support the involvement of a phosphorylation/dephosphorylation mechanism fail to provide unequivocal evidence for this hypothesis.

Firstly, ATP-γ-S causes progressive reduction and prolongation of responses. If I_M suppression required membrane

Table 1 Effect of pipette contents on muscarinic responses

Pipette content	*Rise time* (s)	*Duration* (s)	*Amplitude* (pA)
U-tube application (Rana pipiens) [Musc] = 2 μM			
Cyclic AMP (0.1 mM) (Control)	10.2 ± 0.8 (n = 26)	125.5 ± 6.3 (n = 26)	127.3 ± 9.3 (n = 26)
ATP-γ-S (<15 min; 1 or 2 mM)	15.8 ± 2.0 (n = 14)	Not determined.	131.1 ± 17.9 (n = 14)
	P < 0.001		P > 0.8
Diphosphoglyceric acid	9.4 ± 0.3 (n = 27)	173.3 ± 11.2 (n = 27)	124.4 ± 11.8 (n = 27)
(1–2.5 mM + 100 μM cyclic AMP)	0.1 > P > 0.05	P < 0.001	P > 0.8
U-tube application (Bullfrog) [Musc] = 2 μM			
Pipette content	*Rise time* (s)	*Duration* (s)	*Amplitude* (pA)
Cyclic AMP (0.1 mM) (Control)	3.7 ± 0.8 (n = 10)	133.8 ± 80 (n = 10)	304.0 ± 24.7 (n = 10)
β,γ-MethATP (1 or 2 mM)	14.2 ± 1.3 (n = 26)	198.1 ± 11.6 (n = 26)	243.1 ± 14.8 (n = 26)
	P < 0.001	P < 0.001	P < 0.05
Picospritzer application (Rana pipiens) [Musc] = 10 μM			
Pipette content	*Rise time* (s)	*Duration* (s)	*Amplitude* (pA)
ATP (1 mM) (Control)	6.5 ± 0.4 (n = 35)	94.3 ± 6.2 (n = 35)	168.6 ± 15.3 (n = 35)
Alkaline phosphatase (100 μg ml^{-1} +	5.9 ± 0.5 (n = 33)	113.0 ± 5.9 (n = 33)	134.2 ± 15.1 (n = 33)
0.1 mM cyclic AMP or 1 mM ATP).	P > 0.2	0.05 > P < 0.025	P > 0.1

Since different methods of drug application, drug concentration and species of frog were used, data are compared to the control situation in each group where the cells contained adenosine 3':5'-cyclic monophosphate (cyclic AMP) or ATP. It was not possible to determine the mean duration of responses recorded with intracellularly-applied adenosine 5'-O-(3-thiotriphosphate) (ATP-γ-S) because the response failed to recover in 7 out of the 20 *Rana pipiens* cells which were tested. All data were collected within the first 15 min of whole-cell recording. Musc = muscarine; β,γ-MethATP = β,γ-methyleneadenosine 5'-triphosphate.

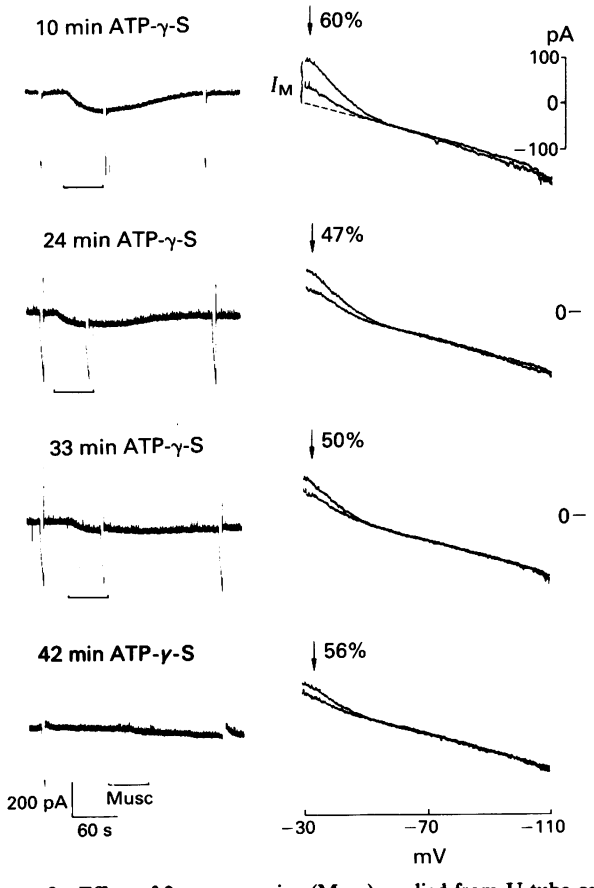

Figure 3 Effect of 2 μM muscarine (Musc) applied from U-tube onto a *Rana pipiens* sympathetic ganglion cell. Pipette contained adenosine 5'-O-(3-thiotriphosphate) (ATP-γ-S, 2 mM). I_M was assessed using 5 s hyperpolarizing ramp commands as in Figures 1 and 2. Note decrease in amplitude and elongation of responses with time. Percentages refer to the percentage depression of the available I_M produced by each test application of muscarine. Note that the percentage depression of the available current changes little with time. 200 pA/60 s calibration refers to left-hand series of traces which were from rectilinear pen recorder. −100/0/+100 pA calibration refers to all right hand traces which are from x-y plotter. Zero current levels are indicated at the extreme right of each trace.

phosphorylation and the recovery of the current following muscarine removal required dephosphorylation, a response recorded in the presence of ATP-γ-S would be expected to be irreversible. This is because ATP-γ-S forms non-hydrolysable protein thiophosphates (Eckstein, 1985). An alternative explanation for the effect of ATP-γ-S is that the thiophosphate moiety is transferred from ADP to GDP resulting in the formation of GTP-γ-S. This type of reaction has been reported to occur both in atria (Otero *et al.*, 1988) and in nodose ganglion cells (Gross *et al.*, 1990). GTP-γ-S would promote persistent G-protein activation (Pfaffinger, 1988; Elmslie *et al.*, 1990) which would explain the progressive prolongation and reduction of muscarine responses. Furthermore, the progressive suppression of I_M as more of the available pool of G-proteins became irreversibly activated would explain the observed run-down of I_M seen only with ATP-γ-S. Another explanation for prolongation of muscarine responses by ATP-γ-S is that it inhibits the desensitization process (Simmons *et al.*, 1990).

Secondly, the prolongation of responses by β,γ-MethATP may imply that recovery requires ATP-dependent phosphorylation so that I_M suppression may have involved dephosphorylation. An alternative explanation for this effect is that it reflects inhibition of the desensitization process in a fashion similar to ATP-γ-S (Simmons *et al.*, 1990).

Thirdly, the prolongation of responses by DPG may imply that I_M recovery requires dephosphorylation so that muscarine-induced M-channel closure would require phosphorylation. If this were the case, it is impossible to explain the inability of the ATP antagonist, β,γ-MethATP to block the response.

Since almost all of the present data support the view that muscarine-induced I_M suppression is independent of phosphorylation and/or dephosphorylation reactions, other possible transduction mechanisms for I_M suppression by muscarine must be considered. One possibility is that a novel or even a conventional second messenger, which exerts its effects independently of protein kinases, could be involved. In fact, it has recently been suggested that cyclic AMP may be able to directly gate ion channels (Di Francesco & Tortora, 1991). This substance is, however, unlikely to be involved in the transduction mechanism for agonist-induced I_M suppression because it fails to mimic the effect of muscarine when applied intracellularly or when membrane permeable ana-

logues are applied extracellularly (Busis *et al.*, 1978; Weight *et al.*, 1978; Adams *et al.*, 1982b; Selyanko *et al.*, 1990). Alternatively, the muscarinic receptor could be 'directly G-protein coupled' to the M-channel. Although it is impossible at present to decide between these possibilities, the latter mechanism is more attractive because several examples of 'direct G-protein coupling' of agonist-binding site to ion channel have now been documented (Pfaffinger *et al.*, 1985; Yatani *et al.*, 1987; North, 1989; Yatani *et al.*, 1990). Confirmation of this hypothesis will only be possible when muscarine-induced I_M suppression can be demonstrated in an outside-out patch (cf. Owen *et al.*, 1990).

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Effects of cromakalim on the contraction and the membrane potential of the circular smooth muscle of guinea-pig stomach

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1 The effects of cromakalim on mechanical and electrical activities of the circular smooth muscles of guinea-pig stomach antrum were observed.

2 Cromakalim ($>1 \times 10^{-7}$ M) decreased the amplitude of spontaneous rhythmic contractions and also the acetylcholine-enhanced spontaneous contractions. Cromakalim was less effective against the 25.9 mM and 35.9 mM K^+-induced tonic contractions.

3 Glibenclamide (1×10^{-6} M) itself caused no detectable change in the spontaneous contractions, those potentiated by acetylcholine or tonic contractions induced by high K^+ solutions, but attenuated the actions of cromakalim. On the other hand, charybdotoxin (3×10^{-8} M) increased the amplitude of spontaneous contractions but failed to affect the actions of cromakalim.

4 Cromakalim ($>1 \times 10^{-6}$ M) decreased the amplitude and duration of slow waves, and hyperpolarized the membrane. These actions of cromakalim were completely antagonized by 1×10^{-6} M glibenclamide, whereas part of the effects of cromakalim on mechanical activity was resistant to glibenclamide.

5 The results suggest that the inhibition by cromakalim of the electrical activity and the hyperpolarization, which may be associated with the opening of glibenclamide-sensitive K^+ channel, are responsible for its inhibitory action on circular smooth muscle of guinea-pig stomach. Further, some effects independent of glibenclamide-sensitive K^+ channel may also be responsible for the mechanical effect.

Keywords: Cromakalim; guinea-pig stomach; membrane potential; contraction; glibenclamide; charybdotoxin; K channel

Introduction

It has been postulated that cromakalim relaxes many types of smooth muscle by hyperpolarizing the membrane through activation of K^+ channels (Hamilton *et al.*, 1986; Allen *et al.*, 1986; Hollingsworth *et al.*, 1987; Standen *et al.*, 1989). The nature of cromakalim as a 'K^+ channel opener' was confirmed not only by electrophysiological experiments but also by $^{86}Rb^+$ efflux experiments (Hamilton *et al.*, 1986; Weir & Weston, 1986; Standen *et al.*, 1989; Masuzawa *et al.*, 1990a,b). Despite its classification as a K^+ channel opener, it is questionable whether its relaxing action on smooth muscles is solely due to membrane hyperpolarization, since the threshold concentration required for muscle relaxation is usually lower than that required for the hyperpolarization or for the stimulation of $^{86}Rb^+$ or $^{42}K^+$ efflux (Hamilton *et al.*, 1986; Hollingsworth *et al.*, 1987; Quast, 1987; Shetty & Weiss, 1987; Gillespie & Sheng, 1988). Such discrepancies are prominent mainly in spontaneously active muscles such as portal vein (Hamilton *et al.*, 1986), uterus (Hollingsworth *et al.*, 1987), trachea (Allen *et al.*, 1986) or urinary bladder (Foster *et al.*, 1989).

We have now investigated the effects of cromakalim on the mechanical and electrical properties of circular smooth muscle of the guinea-pig stomach. This smooth muscle exhibits two types of spontaneous electrical activity, composing slow waves and spike potentials (Tomita, 1981). Tomita & Brading (1990) briefly reported that cromakalim inhibited gastric mechanical activity with no significant effects on the electrical activity of this tissue. The objective of the present study was, therefore, to study further the relationship between electrical and mechanical responses during cromakalim-induced relaxations.

The effects of two types of K^+ channel blocker, glibenclamide and charybdotoxin, on the actions of cromakalim were also investigated, to determine the types of K^+ channel involved in the actions of cromakalim. Putatively glibenclamide inhibits ATP-sensitive K^+ channels (Schmid-Antomarchi *et al.*, 1987; Standen *et al.*, 1989), whereas charybdotoxin blocks the Ca^{2+}-activated K^+ channels (Miller *et al.*, 1985; Talvenheimo *et al.*, 1988; Carl *et al.*, 1990a), although the selectivity of these agents for particular smooth muscle K^+ channels has not been determined.

Methods

Adult male guinea-pigs (200–250 g) were stunned and bled. Small strips of circular muscle were dissected from the antral region of the stomach as described by Ono & Suzuki (1987).

For microelectrode studies the preparation (2 mm wide, 4 mm long) was mounted in a superfusion chamber (fluid volume about 2 ml) with tiny insect pins and superfused with the Krebs solution (35°C) gassed with 95% O_2 and 5% CO_2 at a flow rate of 2 ml min^{-1}. The membrane potential was measured with a glass microelectrode of a tip resistance 40–80 mΩ when filled with 3 M KCl, and the electrode was inserted into a cell from the mucosal side. The signal was amplified by a microelectrode amplifier (Nihon-Kohden MEZ-8101), displayed on an oscilloscope (Nihon-Kohden VC-9) and recorded on a pen-writing oscillograph (NEC-Sa'nei Recti-Horiz) and video cassette tape using a PCM processor (Sony PCM-501 ES). For tension recording the preparation (2 mm wide, 10 mm long) was suspended in a vertical position in a Magnus bath containing 5 ml Krebs solution (37°C) gassed with 95% O_2 and 5% CO_2, with the upper end of the muscle

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connected to an isometric transducer (Nihon-Kohden SB-1T). In some experiments one end of a preparation (2 mm wide, 4 mm long) was fixed in the superfusion chamber for the microelectrode study with insect pins and the other end was connected to a transducer in a horizontal direction. A basal tension of 1 g was maintained throughout the tension experiment.

The Krebs solution used had the following composition (mM): Na^+ 137.4, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134.0, glucose 11.5 (pH 7.4). The high K^+ solution was hypertonic and prepared by adding KCl to the Krebs solution to give a final concentration of 25.9 mM or 35.9 mM K^+. Drugs used were cromakalim (Beecham), glibenclamide (Sigma), charybdotoxin (Peptide Institute), acetylcholine (ACh, Nakarai Chemicals), tetrodotoxin (Sankyo) and atropine (Tokyo Kasei). Cromakalim and glibenclamide were dissolved in ethanol as described elsewhere (Masuzawa *et al.*, 1990a). Other substances were dissolved in distilled water.

Data are expressed as mean ± s.e.mean. To determine the pD_2 value of cromakalim, the EC_{50} was calculated from the log dose-response relationship in each muscle as the concentration required to inhibit a contraction to 50% of the control level and the mean ± s.e.mean of its negative log (pD_2) was calculated. When the antagonism of glibenclamide to cromakalim-induced inhibition of mechanical activity was tested, the control response to cromakalim was observed and after 1 h rinsing of muscles the response was again observed in the presence of glibenclamide. Statistical significance was assessed for responses before and after glibenclamide by Student's *t* test (paired *t* test).

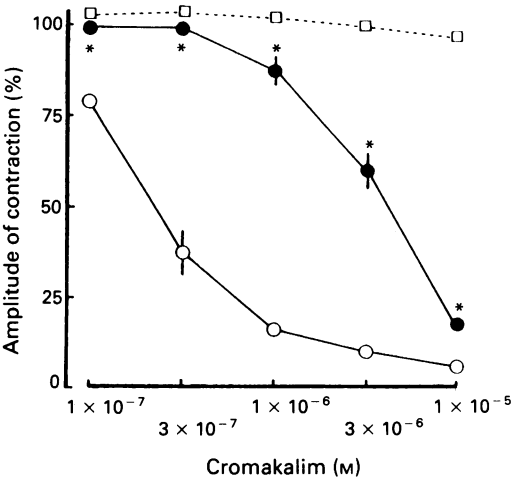

Figure 1 The effect of cromakalim on spontaneous contractions of the circular smooth muscle of guinea-pig stomach in the absence (○) and presence (●) of glibenclamide 1×10^{-6} M. Ordinate scale: 100% = the amplitude of the spontaneous contractions prior to application of the drugs. When used, glibenclamide was applied 10 min before the addition of cromakalim. Broken line represents the effect of vehicle (ethanol), which was added at the same time intervals as cromakalim. Data represent the means with the s.e.means (vertical lines) of 9 experiments. The effect of glibenclamide plus cromakalim was significantly different from cromakalim alone (* $P < 0.05$, paired *t* test).

Results

Effects of cromakalim on the mechanical activity

Circular smooth muscles of the guinea-pig stomach antrum exhibited spontaneous rhythmic contractions under a basal tension of 1 g. When applied cumulatively, cromakalim (1×10^{-7}–1×10^{-5} M) decreased the amplitude of spontaneous contractions in a concentration-dependent manner with a PD_2 of 6.65 ± 0.06 ($n = 9$, Figure 1). However, cromakalim did not significantly affect the frequency of the spontaneous contractions as long as the contractions could be observed, i.e. the frequency was 4.3 ± 1.8 contractions min^{-1} before the application of cromakalim whereas it was 4.5 ± 1.3 contractions min^{-1} in the presence of 3×10^{-6} M cromakalim. In a different group of muscles the effect of ethanol, which was used to dissolve cromakalim and glibenclamide, was observed. Ethanol in concentrations which were the same as those added with cromakalim did not affect the spontaneous contractions (Figure 1). This result also shows that spontaneous contractions remained constant over the time course of the experiment.

When 1×10^{-6} M ACh was applied, the basal tension was elevated (tonic contraction) following a transient large contraction and the spontaneous contractions were augmented to 990 ± 155% ($n = 8$) of the predrug level. Addition of cromakalim decreased the amplitude of enhanced spontaneous contractions and the tonic contraction with a PD_2 of 6.38 ± 0.08 and 6.27 ± 0.20 ($n = 8$), respectively (Figure 2). The vehicle (ethanol) for cromakalim, which was added with the same interval as cromakalim, did not affect the ACh-enhanced contractions over the time course of the experiment. Addition of K^+ (final $[K^+]_o = 25.9$ mM and 35.9 mM) elevated the basal tension which consisted of a transient component followed by a sustained one. The tonic contraction was obviously larger in 35.9 mM $[K^+]_o$ than in 25.9 mM $[K^+]_o$. Often in the presence of 25.9 mM K^+, the augmented spontaneous contractions were superimposed on the tonic response, the frequency of tension waves was not different from that in normal $[K^+]_o$. With

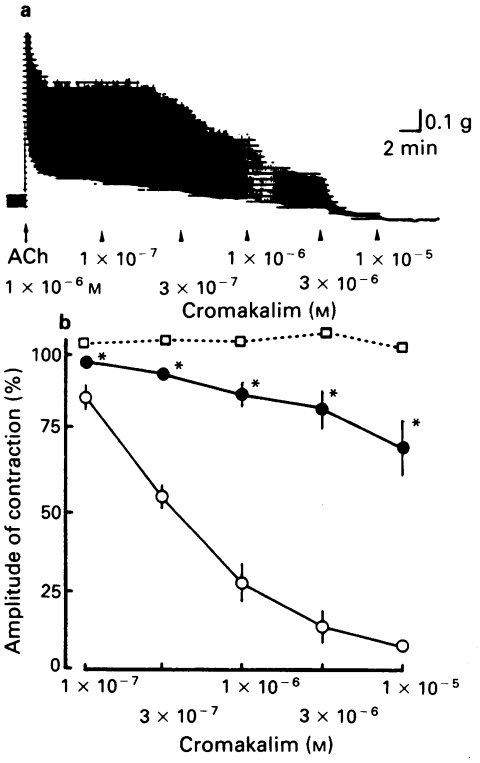

Figure 2 An example of the effect of cumulatively added cromakalim on the acetylcholine (ACh)-enhanced spontaneous contractions of the guinea-pig stomach (a) and the summarized dose-effect relationship of cromakalim in the absence (○) and presence (●) of glibenclamide 1×10^{-6} M (b). When the contraction reached the steady state after the addition of ACh 1×10^{-6} M cromakalim was added. In (b) 100% on the ordinate scale represents the amplitude of the enhanced spontaneous contraction just before the application of cromakalim. Broken line represents the effect of vehicle (ethanol), which was added at the same time intervals as cromakalim. When used, glibenclamide was applied 10 min before the addition of ACh. Data represent the means with the s.e.mean (vertical lines) of 8 experiments. The effect of glibenclamide plus cromakalim was significantly different from cromakalim alone (* $P < 0.05$, paired *t* test).

35.9 mM K^+, the spontaneous contractions were abolished. Cromakalim added during the sustained phase of the tonic contraction relaxed the muscle with a pD_2 of 5.89 ± 0.17 ($n = 8$) and 5.43 ± 0.21 ($n = 7$) at 25.9 mM and 35.9 mM K^+, respectively (Figure 3). These pD_2 values were significantly smaller than the value observed for the spontaneous contractions in normal K^+ medium ($P < 0.01$).

Effects of cromakalim on the electrical activity

Circular smooth muscles of the antrum region generated spontaneous electrical activity, which consisted of slow waves with superimposed spike potentials (Tomita, 1981), events which were accompanied by phasic contractions. Figure 4 shows an example of the change of membrane potential caused by 1×10^{-6} M cromakalim. In this tissue cromakalim inhibited spontaneous electrical and mechanical activities with a relatively slow time course. Two min after superfusion with cromakalim the amplitude of both slow waves and spike potentials decreased, with a substantial hyperpolarization of the membrane. After 3 min exposure, the membrane was further hyperpolarized, the amplitude of slow waves decreased to below 30% of control and spike activity was abolished, with no significant change in slow wave frequency (Figure 4). These effects of cromakalim are summarized in Figure 5.

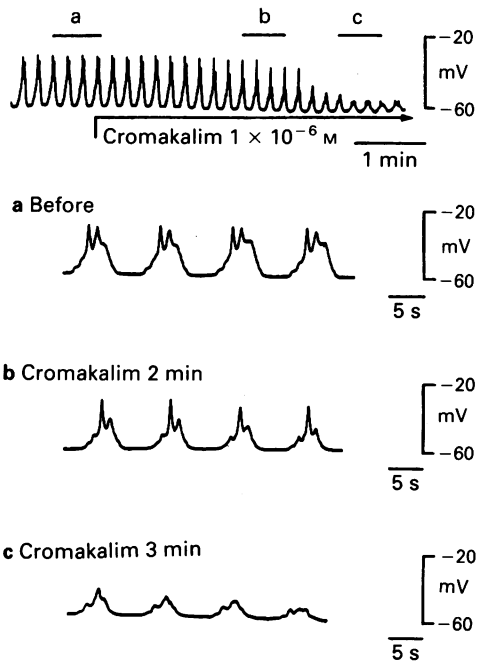

Figure 4 An example of the effect of cromakalim 1×10^{-6} M on the membrane potential of the circular smooth muscle of guinea-pig stomach. Lower three traces; records of membrane potential on an expanded time scale, which correspond to before, 2 min and 3 min after the superfusion of cromakalim shown in the top trace.

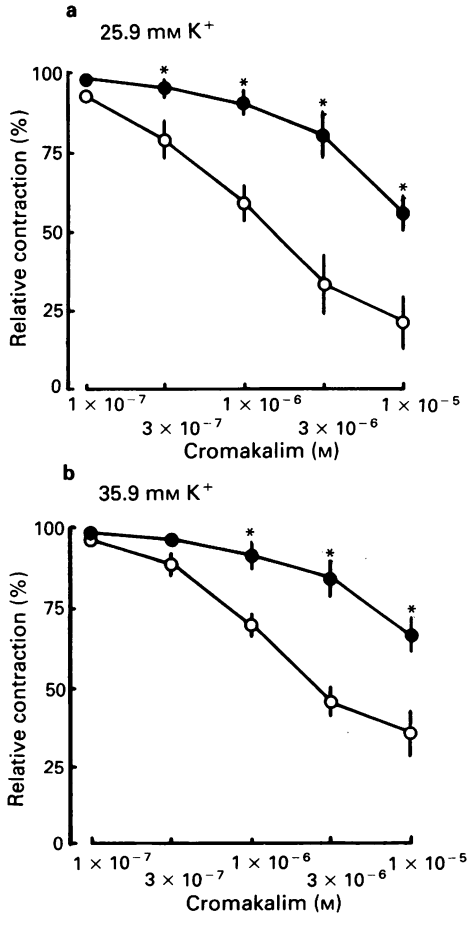

Figure 3 The effects of cromakalim on the high K^+-induced tonic contraction in the guinea-pig stomach in the absence (○) and presence (●) of glibenclamide 1×10^{-6} M. (a) Hypertonically added 20 mM KCl (total 25.9 mM K^+); (b) hypertonically added 30 mM KCl (total 35.9 mM K^+). On the ordinates, 100% = the magnitude of the tonic tension just before the addition of cromakalim. When used, glibenclamide was applied 10 min before the addition of K^+. Data represent the means with the s.e.mean of 8 (a) or 7 (b) experiments. Significantly different from cromakalim alone: $*P < 0.05$, paired t test.

Comparison of the effects of cromakalim on the mechanical (Figure 1) and electrical (Figure 5) activity of stomach antrum indicates that cromakalim ($1–3 \times 10^{-7}$ M) decreased the amplitude of spontaneous contractions without any membrane hyperpolarization. Since these responses were measured separately in different conditions (a Magnus bath and a superfusion bath), we considered the possible involvement of such differences in the observed effects of cromakalim. Experiments were therefore designed to study the effects of cromakalim on spontaneous contractions in the superfusion bath which was used for the microelectrode study. In these experiments, cromakalim at 1×10^{-7} M did not, but 3×10^{-7} M did inhibit the

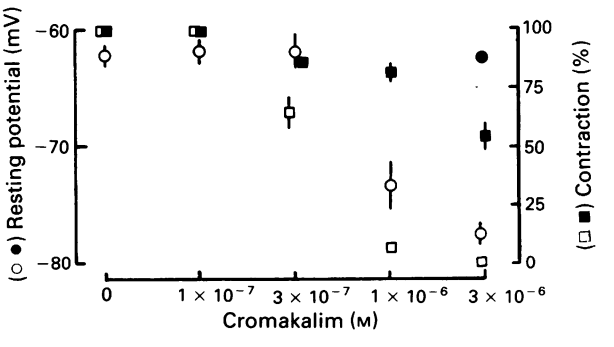

Figure 5 The effect of cromakalim on the resting membrane potential and the spontaneous contraction of the guinea-pig stomach. The contraction and the membrane potential were measured in the same superfusion bath but individually. (○, ●) Resting membrane potential; (□, ■) amplitude of spontaneous contractions; 100% = the amplitude of spontaneous contractions just before superfusion with cromakalim. Both parameters were measured at the peak effects of cromakalim; (■, ●) in the presence of glibenclamide 1×10^{-6} M. The effect of cromakalim on membrane potential in the presence of glibenclamide was tested only in a concentration of 3×10^{-6} M. Data represent the means with s.e.mean (vertical lines) of 6–8 experiments (resting potential) or 6 experiments (contraction).

spontaneous contractions ($pD_2 = 6.27 \pm 0.07$, $n = 6$); the inhibitory potency was slightly less than that observed in the Magnus bath (Figure 5).

Antagonistic effects of glibenclamide on actions of cromakalim

After a control response to cromakalim had been obtained in the absence or presence of stimulants, the muscles were rinsed with Krebs solution for 1 h, and then the response to cromakalim was again observed in the presence of 1×10^{-6} M glibenclamide. When control responses to cromakalim were observed twice with an interval of 1 h between responses, the second inhibitory effect was the same as the first one. Glibenclamide (1×10^{-6} M) alone did not modify the spontaneous contractions, those enhanced by ACh (1×10^{-6} M) or the tonic increase in tension induced by high $[K^+]_o$. However, this agent significantly attenuated the inhibitory actions of cromakalim on these contractions (Figures 1, 2, 3). A concentration above 3×10^{-6} M of cromakalim caused a considerable inhibition of spontaneous contractions even in the presence of glibenclamide (Figure 1) and the effects of cromakalim on spontaneous contractions were similarly resistant to glibenclamide in the superfusion bath (Figure 5).

The resting membrane potential and configurations of spontaneous electrical activities were not detectably affected by glibenclamide (1×10^{-6} M), but the actions of cromakalim on these electrical properties were completely prevented by glibenclamide (Figure 6).

Effects of charybdotoxin on the spontaneous contractions and on the action of cromakalim

Charybdotoxin enhanced the spontaneous contractions. The threshold concentration for this effect was 3×10^{-9} M and the maximum effect was attained at 1×10^{-7} M. With 3×10^{-8} M charybdotoxin, the spontaneous contractions were enhanced to $524 \pm 124\%$ ($n = 7$) with a small elevation of the basal tension (Figure 7a). Even in the presence of both 1×10^{-6} M atropine and 1×10^{-6} M tetrodotoxin, this increase in mechanical activity was observed, suggesting a direct action of the toxin on the gastric smooth muscle. Cromakalim added in the presence of charybdotoxin decreased the amplitude of charybdotoxin-enhanced spontaneous contractions in a dose-dependent manner (Figure 7b) with a pD_2 of 6.57 ± 0.05 ($n = 7$). This value was not significantly different from the pD_2 for the inhibition of the spontaneous contractions by cromakalim in control conditions.

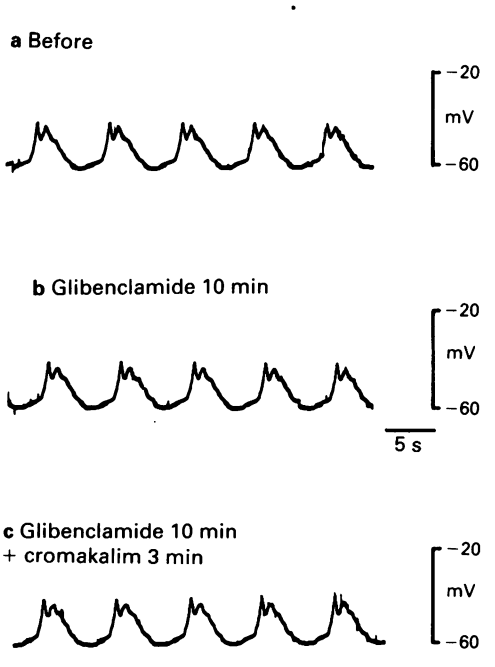

Figure 6 The antagonism of glibenclamide, 1×10^{-6} M, to the effect of cromakalim, 3×10^{-6} M, on the membrane potential of the guinea-pig stomach: (a) just before the addition of the drugs; (b) 10 min after superfusion with glibenclamide, which was just before superfusion with cromakalim; (c) 3 min after superfusion with cromakalim.

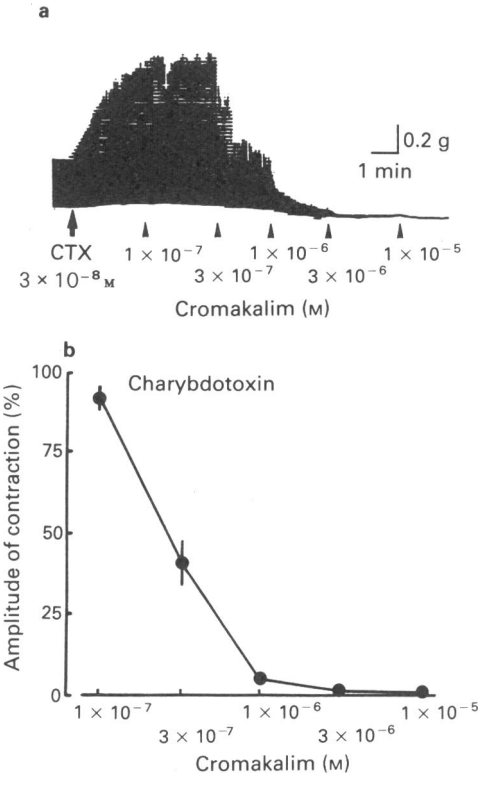

Figure 7 An example of the effect of cumulatively added cromakalim on the charybdotoxin (CTX)-enhanced spontaneous contractions of the guinea-pig stomach (a) and the summarized dose-effect relationship of cromakalim in the presence of charybdotoxin, 3×10^{-8} M (b). In (a) after the spontaneous contractions enhanced by charybdotoxin 3×10^{-8} M, reached the steady level, cromakalim was added cumulatively. In (b) 100% on the ordinate scale = the amplitude of enhanced spontaneous contractions just before the addition of cromakalim. Data represent the means with the s.e.mean (vertical lines) of 7 experiments.

Discussion

In this study cromakalim inhibited spontaneous contractions and those enhanced by ACh, with pD_2 values comparable to those previously observed in other smooth muscles. Cromakalim was less potent in inhibiting contractions produced in high $[K^+]_o$ than in normal $[K^+]_o$. This reduced effectiveness of cromakalim on KCl-induced contractions is in good agreement with other smooth muscle tissues (Allen *et al.*, 1986; Hamilton *et al.*, 1986; Hollingsworth *et al.*, 1987; Masuzawa *et al.*, 1990a,b) and is a first indication of the possible involvement of K^+ channel opening in the inhibitory actions of cromakalim.

In the guinea-pig stomach the threshold concentration of cromakalim required for inhibiting the spontaneous contractions was 1×10^{-7} M in the Magnus bath or 3×10^{-7} M in the superfusion bath, whereas that for the membrane hyperpolarization was 1×10^{-6} M. A typical feature of the cromakalim-induced inhibition of spontaneous contractions

without a change in the membrane potential can be seen at a concentration of 3×10^{-7} M, while higher concentrations of cromakalim inhibited both electrical and mechanical responses. Thus, the inhibitory actions of cromakalim on spontaneous contractions are not necessarily correlated with the membrane hyperpolarization or with inhibition of spontaneously generating electrical activities, as first observed in rat portal vein (Hamilton *et al.*, 1986).

Glibenclamide (1×10^{-6} M) antagonized the inhibitory effects of cromakalim on the spontaneous activities of the guinea-pig stomach. However, the actions of glibenclamide differed between electrical and mechanical responses, as the inhibition by 3×10^{-6} M cromakalim of the electrical activities was completely antagonized, while that of the mechanical activities was only partially blocked by glibenclamide. These results again suggest that the inhibitory actions of cromakalim on contractions are not solely due to membrane hyperpolarization. Some studies have claimed that cromakalim opens a Ca^{2+}-activated K^+ channel in smooth muscle cells of the guinea-pig mesenteric artery and rat portal vein (Nakao *et al.*, 1988; Hu *et al.*, 1990). However, the action of cromakalim on guinea-pig stomach was not affected by charybdotoxin, which is considered to block one type of Ca^{2+}-activated K^+ channel (Talvenheimo *et al.*, 1988; Strong *et al.*, 1989; Carl *et al.*, 1990a). If charybdotoxin is selective for such a channel, this result means that cromakalim does not activate the Ca^{2+}-activated K^+ channel sensitive to charybdotoxin in the guinea-pig stomach. Other studies have postulated that cromakalim (especially at concentrations $>1 \times 10^{-5}$ M) inhibits contractions of vascular smooth muscles and colonic smooth muscles partly through an inhibition of Ca^{2+} channels (Nakao *et al.*, 1988; Okabe *et al.*, 1990; Post *et al.*, 1991). Post *et al.* (1991) found that glibenclamide did not antagonize the Ca^{2+} channels blocking action of cromakalim in canine colonic smooth muscle cells. In the present study, cromakalim did not inhibit spike potentials and slow waves when glibenclamide was present. Therefore, the possible role of a Ca^{2+} channel in the inhibitory effects of cromakalim is unlikely in the guinea-pig stomach.

To explain the cromakalim-induced inhibition of spontaneous activity without hyperpolarization in the rat portal vein and uterus (Hamilton *et al.*, 1986; Hollingsworth *et al.*, 1987), these workers suggest that K^+ channels associated with the pacemaker activity in these tissues were more sensitive to this agent than those responsible for determining the resting membrane potential. This may not be the case in the guinea-pig stomach, because the frequency of the spontaneous slow waves was not changed by cromakalim at concentrations which inhibited spike generation. However, true slow waves are not generated either by the portal vein or the uterus, and further work is clearly required to establish the basis of cromakalim-induced inhibition in the absence of hyperpolarization.

Taking the above discussion into consideration, the lack of any change not only in the resting membrane potential but also in the slow waves and spikes during cromakalim-induced inhibition of contraction in the presence of glibenclamide suggests that a part of the inhibitory action of cromakalim on mechanical activity does not depend on the effects on ionic channels, or is related to the action on channels which cannot be detected by conventional microelectrode technique. The dissociation between electrical effects and mechanical effects of cromakalim was also observed by Tomita & Brading (1990), who showed that cromakalim suppressed the contraction of guinea-pig stomach in spite of the persistance of the slow wave. It was shown that cromakalim does not change the Ca^{2+}-sensitivity of smooth muscles (Allen *et al.*, 1986; Hollingsworth *et al.*, 1987). Therefore, the possible inhibition by cromakalim of an increase in $[Ca^{2+}]_i$ through potential-independent mechanisms, for example inhibition of intracellular Ca^{2+} refilling (Bray *et al.*, 1991), is suggested.

Charybdotoxin considerably potentiated spontaneous contractions. If this potentiation is assumed to be mediated by an action on a Ca^{2+}-activated K^+ channel, this would support the view that a Ca^{2+}-activated K^+ channel plays a role in the slow wave of gastric smooth muscle (Mitra & Morad, 1985; Carl *et al.*, 1990b). The inhibition of such a channel may prolong the open time of voltage-dependent Ca^{2+} channels and thereby augment Ca^{2+} entry. Microelectrode studies, which are now being undertaken, will clarify how this toxin modifies the resting membrane potential, the slow waves and spikes. On the other hand, glibenclamide did not affect spontaneous activity. Although there is disagreement about whether the K^+ channel sensitive to cromakalim and glibenclamide in smooth muscles can be identified as an ATP-sensitive K^+ channel (Beech & Bolton, 1989; Standen *et al.*, 1989; Fujii *et al.*, 1990; Hu *et al.*, 1990), the ineffectiveness of glibenclamide on spontaneous activity suggests that the K^+ channel sensitive to glibenclamide does not play a role under physiological conditions.

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Myocardial hypertrophy, cardiac β-adrenoceptors and adenylate cyclase activity during sinoaortic denervation in dogs

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1 The long-term effects of sinoaortic denervation on the development of left ventricular hypertrophy (assessed by the measurement of the ratio (R): heart weight/total body weight and LVT: left ventricular thickness), myocardial β-adrenergic receptivity (measured by [^{125}I]-cyanopindolol binding and adenylate cyclase activity) and plasma catecholamine levels (measured by h.p.l.c.) were investigated in three groups of dogs: normotensive controls (group 1), dogs made hypertensive by sinoaortic denervation and evaluated 1 (group 2) and 18 months (group 3) later.

2 Noradrenaline (NA) and adrenaline (A) plasma levels were 461 ± 54 and 85 ± 45 pg ml^{-1} in controls, 861 ± 185 and 191 ± 23 pg ml^{-1} in group 2 ($P < 0.05$). They were normal in group 3 (426 ± 132 and 110 ± 16 pg ml^{-1}).

3 R and LVT values were significantly ($P < 0.05$) higher in sinoaortic denervated dogs (R = 7.7 ± 0.1 and 7.8 ± 0.2; LVT = 13.6 ± 1.3 and 14.2 ± 0.9 mm in groups 2 and 3 respectively) than in normotensive dogs (group 1: R = 6.7 ± 0.1, LVT = 9.3 ± 0.8 mm).

4 In group 1, the total number of β-adrenoceptors (B_{max}) was 37 ± 11 and 29 ± 6 fmol mg^{-1} protein in the left ventricle (LV) and right auricle (RA) respectively. In group 2, B_{max} was significantly lower (10 ± 3 in LV and 13 ± 2 fmol mg^{-1} protein in RA, $P < 0.05$) than in group 1. There was no difference between group 1 and group 3 (37 ± 3 fmol mg^{-1} prot in LV and 31 ± 3 fmol mg^{-1} protein in RA).

5 The percentage of β_1-adrenoceptors was 82 ± 4 in LV and 75 ± 5 in RA in group 1. It was significantly lower ($P < 0.05$) in groups 2 (LV: 33 ± 6 and RA: 33 ± 5) and 3 (LV: 59 ± 3 and RA: 55 ± 4).

6 Basal values of adenylate cyclase activity in LV significantly decreased after sinoaortic denervation. Isoprenaline alone or in the presence of a β_2-adrenoceptor antagonist (ICI 118551, 1 μM) failed to stimulate adenylate cyclase activity 1 (but not 18) month after sinoaortic denervation.

7 These data show that sinoaortic denervation is associated with left ventricular hypertrophy which appears early (1 month) and persists until 18 months despite the normalization of plasma catecholamine levels. The total number of myocardial β-adrenoceptors is closely related to catecholamine levels but a selective decrease in β_1-adrenoceptors is observed during cardiac hypertrophy. The fall in basal adenylate cyclase activity suggests that cardiac hypertrophy is associated with an impairment of transmembrane signalling.

Keywords: Sinoaortic denervation; cardiac hypertrophy; catecholamines; β-adrenoceptors; adenylate cyclase

Introduction

In arterial hypertension, pressure overload is associated with left ventricular hypertrophy. The β-adrenergic receptivity plays an important role in myocardial function and its alteration during heart hypertrophy may be involved in the process leading to congestive cardiac failure (Saragoca & Tarazi, 1981). Although many authors have investigated β-adrenergic receptivity during left ventricular hypertrophy due to pressure overload, the data remain controversial. Most of the earlier studies concluded that a decrease in myocardial β-adrenoceptor density occurred with a decrease in the inotropic response to catecholamines in rat pressure overload models (Ayobe & Tarazi, 1983). In contrast, in other species an increased β-adrenoceptor number was reported (Vatner *et al.*, 1985). These conflicting results may be explained by differences in the experimental models as well as in the mechanisms of pressure overload.

However, when investigating β-adrenergic receptivity in heart, it must be kept in mind that β-adrenoceptors are submitted to an homologous regulation by catecholamine plasma levels and sympathetic nervous system activity (Hoffmann & Lefkowitz, 1982). Thus, chronic sinoaortic denervation in the dog may represent an interesting model for the study of β-adrenergic regulation during left ventricular hypertrophy. In fact, we previously found that sinoaortic denervation in the dog induces sustained hypertension and a transient increase in catecholamine plasma levels during the first two months (Valet *et al.*, 1988; 1989). Moreover, in this experimental model, a negative correlation exists between plasma catecholamine levels and β-adrenoceptor number in lymphocytes (Valet *et al.*, 1988; 1989).

Since no data are available on myocardial β-adrenoceptors and cardiac hypertrophy in sinoaortic denervated dogs, the aim of the present study was to investigate plasma catecholamine levels, myocardial hypertrophy, cardiac β-adrenoceptors and adenylate cyclase activity 1 and 18 months after sinoaortic denervation.

Methods

Sinoaortic denervation in dogs

Eighteen beagle dogs of either sex weighing 12–25 kg were studied. Sinoaortic denervation involved two successive surgical procedures as previously described (Valet *et al.*, 1988; 1989). On the day of the first procedure the animals were anaesthetized with alpha-chloralose (80 mg kg^{-1}, intravenously (i.v.)). After intubation of the trachea, they were

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ventilated with a Palmer respirator (London, England). Under sterile surgical conditions, a lateral neck incision was made on the right side. The carotid arteries and sinuses were isolated, the carotid sinus nerve identified and transected with an ophthalmic cauteriser. Moreover, in order to prevent any reinnervation, the carotid bifurcation was destroyed by sectioning the common artery (proximal to the bifurcation) and both internal and external carotids (above their origin). The unilateral surgical procedure did not induce neurological disturbances since, in dogs, the blood supply to the brain mainly comes from the vertebral arteries. During the same experimental procedure, the aortic depressor nerve in the cervical region was also sectioned on the same side: the sheath of the vagus was dissected, the aortic depressor nerve isolated and identified by electrical stimulation and a 4 cm segment of aortic depressor nerve was removed to prevent any reinnervation. During this surgical procedure care was taken to keep intact both the vagal and the sympathetic fibres in the vagus. This procedure was repeated for the left side seven weeks later. The effectiveness of baroreceptor denervation was assessed every month after sinoaortic denervation by the failure of noradrenaline (0·5, 1 and 2 $\mu g\,kg^{-1}$, i.v.) and phenylephrine (0.1, 1 and 10 $\mu g\,kg^{-1}$, i.v.) to induce bradycardia. Moreover, nitroglycerin (1, 3, 10 and 30 $\mu g\,kg^{-1}$, i.v.) induced a dose-dependent decrease in blood pressure without any change in heart rate, thus confirming the absence of baroreceptor pathways. Six sham-operated beagle dogs (group 1) weighing 10 to 25 kg were also prepared: under chloralose anaesthesia, two successive lateral neck incisions without lesion of the aortic depressor or carotid sinus nerves were performed. They were killed 18 months later using an overdose of pentobarbitone. Dogs subjected to sinoaortic denervation were killed under similar experimental conditions 1 month (group 2, $n = 6$) and 18 months (group 3, $n = 6$) after surgery. All investigations described in this paper were conducted in conformity with the guide for the care and use of laboratory animals as adopted and promulgated by the National Institute of Health.

Blood pressure and heart parameters

Blood pressure and heart rate were recorded in conscious animals by means of a catheter introduced into the abdominal aorta via the femoral artery under local anaesthesia (xylocaine 5%) and connected to a Gould P23ID transducer on a Honeywell recorder. Heart rate was obtained by use of a heart period meter triggered by blood pressure.

Heart weight was determined on freshly collected organs. The results are expressed using the ratio (R): heart weight (g)/ total body weight (kg). The left ventricle was then dissected and left ventricular thickness (LVT) was measured with a vernier between the epicardial and the endocardial borders along the left ventricle free wall, below the mitral leaflets and above the mitral pillar.

Plasma catecholamine determination

For plasma catecholamine determinations, 5 ml of fresh blood were collected from conscious dogs on lithium heparin with 10 mM sodium metabisulphite and centrifuged (2000 g, 10 min, 0°C). Plasma was stored at −80°C. Catecholamines were selectively isolated from the sample at 4°C, in the darkness, by adsorption on 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 performance liquid chromatography (h.p.l.c.) 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 C18 Waters column (3.9 × 150 mm) at a constant flow rate of 1 ml min^{-1}. Under these conditions, the detection limit was 10 pg ml^{-1} (Senard *et al.*, 1990).

Preparation of cardiomyocyte membranes

Muscular tissue was obtained from the 4 cardiac cavities after the dogs were killed. After accessory tissues were discarded by dissection, cardiomyocytes were minced and homogenized in an ice-cold lysing buffer containing 5 mM Tris-HCl, 5 mM EDTA (pH 7.5) with an Ultraturrax device. The homogenate was then resuspended in a solution containing 320 mM sucrose, 50 mM Tris-HCl, 0.5 mM $MgCl_2$ (pH 7.5) and centrifuged (100 g, 10 min, 4°C). The supernatant was then carefully collected and centrifuged (31 000 g, 10 min, 4°C). The resultant pellet was resuspended and washed twice in binding buffer (50 mM Tris-HCl, 120 mM NaCl, 0.5 mM $MgCl_2$, pH 7.5). The membranes were frozen at −80°C until analysis. Assays were generally performed within 48 h after freezing.

Binding studies

All experiments were performed in binding buffer (see above) in a total volume of 200 μl. Membranes were incubated under constant shaking in the presence of increasing [^{125}I]-cyanopindolol concentrations ranging from 10 pM to 400 pM for 60 min at 37°C. For determination of total β-adrenoceptor number, non-specific binding was defined in the presence of (−)-adrenaline 10^{-4} M. Determination of β_1- and β_2-adrenoceptor subtypes was obtained through competition experiments. A fixed concentration of [^{125}I]-cyanopindolol (at least twice the K_d) was added to each sample in the presence of increasing concentrations of displacing drug. Two selective antagonists were used: bisoprolol (a β_1-adrenoceptor selective antagonist) and ICI 118551 (a selective β_2-adrenoceptor antagonist). Ritanserin (10^{-5} M) was added to all samples. The reaction was stopped by addition of 4 ml of ice cold buffer. The separation of free and bound ligand was carried out by filtration through GF/C glass fibre filters under vacuum. The filters were washed twice with 10 ml of buffer and then placed in vials. The radioactivity retained on the filters was counted in a Beckmann scintillation counter at an efficiency of 82%.

Computer-assisted analysis of binding data was performed using the EBDA-Ligand programme (McPherson, 1985). Analysis of competition curves was performed by use of a two-site model according to nH value. The protein concentration was determined as described by Bradford (1976) with serum albumin used as standard.

Adenylate cyclase activity

Adenylate cyclase activity was measured in left ventricle membranes by the method of Salomon *et al.* (1974). Briefly, 20 to 30 μg of membrane proteins were incubated in a 100 μl final volume of 40 mM Tris-HCl (pH 7.4) containing 100 μM EGTA, 0.1 μM guanosine 5'-triphosphate (GTP), 1.5 mM $MgCl_2$, 1 mM adenosine 3' : 5'-cyclic monophosphate (cyclic AMP), 0.5 mM isobutylmethylxanthine (IBMX), 0.2 mM adenosine 5'-triphosphase (ATP), 5 mM creatine phosphate, 70 iu ml^{-1} creatine kinase, 0.2% bovine serum albumin and 0.5–1 μCi [α^{32}P]-ATP. The stimulation of adenylate cyclase activity was obtained either with increasing doses of isoprenaline alone or in the presence of 1 μM ICI 118551. Incubations were carried out during 10 min at 30°C and the reaction was stopped by adding 20 μl HCl 2.2 N and 10 nCi [^{3}H]-cyclic AMP (pH 7.4). The incubates were poured on to Dowex columns and eluted with 100 mM ammonium acetate buffer (pH 7.4). The radioactivity contained in the eluate was counted in liquid scintillation spectrophotometer. The results are expressed as pmol of formed cyclic AMP per mg of protein.

Data analysis

Results are presented as mean values ± s.e.mean. Statistical analysis was performed by the non parametric Mann–Whitney U test. A P value <0.05 was considered as significant.

Drugs and reagents

[^{125}I]-cyanopindolol (RAS: 2000 Ci mmol^{-1}) and [^{3}H]-cyclic AMP were purchased from Amersham (Amersham, England), [α^{32}P]-ATP from New England Nuclear (England). Bisoprolol was obtained from Merck Clevenot laboratories, ICI 118551 (erythro-DL-(7-methylindan-4-yloey)-3-isopropylaminobutan-2-ol) from ICI (Cheshire, U.K.), (−)-adrenaline from Sigma laboratories (Sigma France) ketanserin from Research Biochemical Inc (Ma, USA) and ritanserin from Janssen laboratories. All other drugs were of reagent grade.

Results

Blood pressure and catecholamine plasma levels

Cardiovascular parameters were measured in conscious dogs after a resting period of at least 60 min. As previously reported (Valet *et al.*, 1988; 1989), bilateral sinoaortic denervation induced a significant rise in blood pressure and heart rate after one month which persisted 18 months later (Table 1). Noradrenaline and adrenaline plasma levels were significantly higher one month after sinoaortic denervation than before surgery. In contrast, 18 months later, both plasma noradrenaline and adrenaline levels were in the normal range (Table 1).

Ratio heart weight/total body weight

In normotensive controls, R ratio (heart weight/total body weight) was 6.7 ± 0.1. In dogs studied 1 month after sinoaortic denervation, R was significantly greater than in controls (7.7 ± 0.1, $P < 0.05$). Eighteen months later, it remained significantly higher than in controls (7.8 ± 0.2, $P < 0.05$) but did not differ from the value observed after 1 month. LVT rose significantly in sinoaortic denervated dogs after a 1 (13.6 ± 1.3 mm, $P < 0.05$) or 18 (14.2 ± 0.9 mm, $P < 0.05$) months follow-up when compared with controls (9.3 ± 0.8 mm). Moreover, the weight of the dogs was not significantly modified one and eighteen months after the surgical procedure.

Cardiac β-adrenoceptors

Preliminary experiments were performed in order to check the selectivity of [^{125}I]-cyanopindolol binding on myocardial membranes. When saturation experiments were realized in standard conditions (i.e. in the presence of (−)-adrenaline for non-specific binding determination). Scatchard plots were hardly interpretable because of high non-specific binding values (around 75% at K_d value). This high non-specific binding can be explained by the fact that, first [^{125}I]-cyanopindolol is retained on the GF/C filters (this could be abolished by incubating the filters in presence of 1 mM polyethylene-imine) and secondly [^{125}I]-cyanopindolol binds to additional binding sites different from β-adrenoceptors. Competition experiments carried out with ritanserin and ketanserin in presence of 100 μM (−)-adrenaline indicated that the two drugs were able to inhibit [^{125}I]-cyanopindolol binding but with very low affinity, suggesting the existence of a non β-adrenoceptor specific binding. These data disagree with the pharmacological definition of 5-HT$_2$ receptors. Moreover, under these selective conditions ICI 118551 (but not bisoprolol) inhibited [^{125}I]-cyanopindolol binding (Figure 1). Thus, further experiments were performed in the presence of 10 μM ritanserin in all samples in order to avoid [^{125}I]-cyanopindolol interacting with this non β-adrenoceptor specific binding.

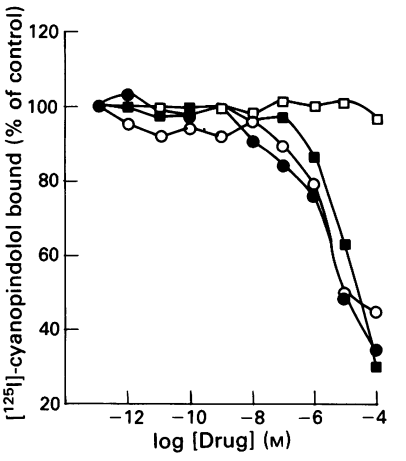

Figure 1 Inhibition of [^{125}I]-cyanopindolol binding in membranes from left ventricle of normotensive dogs by increasing concentrations of ritanserin (●), ketanserin (○), bisoprolol (□) and ICI 118551 (■). The experiments were performed in the presence of adrenaline (10 μM) in order to avoid any interaction of the radioligand with β-adrenoceptors. Each point is the mean of six separate experiments performed in duplicate.

In dogs studied 1 month after sinoaortic denervation, the number of β-adrenoceptors in the four measured cardiac cavities was significantly lower than that measured in controls. The decrease was around 50% except in the left ventricle where it reached 75%. In contrast, the number of cardiac β-adrenoceptors in dogs submitted to sinoaortic denervation for 18 months was similar to control values. No change in K_d values was observed in any of the groups (Table 2). Under these experimental conditions, the Hill number was always near unity indicating that [^{125}I]-cyanopindolol interacts with a homogeneous class of binding sites.

The respective percentage of β_1- and β_2-adrenoceptors was calculated in the right auricle and the left ventricle. In controls, β_1-adrenoceptors represent 75 ± 5% of the whole number of β-adrenoceptors in the right auricle and 82 ± 4% in the left ventricle. One month after sinoaortic denervation, the percentage of β_1-adrenoceptors was 33 ± 5% in the right auricle and 33 ± 6% in the left ventricle ($P < 0.05$ when compared with controls). After 18 months, the percentage was 55 ± 4% in the right auricle and 59 ± 3% in the left ventricle ($P < 0.05$ when compared with controls).

Adenylate cyclase activity

In the left ventricle of normotensive dogs, isoprenaline increased adenylate cyclase activity in a dose-dependent

Table 1 Systolic (SABP) and diastolic (DABP) arterial blood pressures, heart rate (HR) and plasma catecholamine levels (NA: noradrenaline, A: adrenaline) in normotensive controls (group 1) and sinoaortic denervated dogs studied 1 month (group 2) and 18 months (group 3) after sinoaortic denervation

	SABP (mmHg)	*DABP*	*HR* (b min^{-1})	*NA* (pg ml^{-1})	*A* (pg ml^{-1})
Group 1	148 ± 11	63 ± 5	85 ± 8	461 ± 54	85 ± 45
Group 2	202 ± 12*	101 ± 9*	130 ± 7*	861 ± 185*	191 ± 23*
Group 3	209 ± 11*	119 ± 5*	123 ± 5*	426 ± 132	110 ± 16

All measurements were obtained in conscious animals after a 60 min rest. Results are expressed as mean values ± s.e.mean; $n = 6$ in each group; *$P < 0.05$ when compared with group 1.

Table 2 Maximal number of β-adrenoceptors (B_{max}, fmol mg^{-1} protein) and dissociation constant at equilibrium (K_d, pM) in the four cardiac cavities in normotensive control (group 1) and sinoaortic denervated dogs studied 1 month (group 2) and 18 months (group 3) after sinoaortic denervation

	Group 1		*Group 2*		*Group 3*	
	B_{max}	K_d	B_{max}	K_d	B_{max}	K_d
Right auricle	29 ± 6	68 ± 5	13 ± 2*	40 ± 4	31 ± 3	55 ± 10
Left auricle	43 ± 3	61 ± 6	20 ± 4*	68 ± 8	42 ± 2	68 ± 5
Right ventricle	22 ± 3	61 ± 9	10 ± 2*	45 ± 10	21 ± 2	70 ± 6
Left ventricle	37 ± 11	72 ± 5	10 ± 3*	66 ± 8	37 ± 3	69 ± 4

The results are expressed as mean ± s.e.mean; $n = 6$ in each group; *$P < 0.05$ when compared with group 1.

manner (Figure 2a). The activity of adenylate cyclase following selective β_1-adrenoceptor activation was measured in the presence of ICI 118551 (1 μM), a selective β_2-adrenoceptor blocker. In the presence of ICI 118551, isoprenaline stimulated adenylate cyclase with a similar affinity (EC_{50} = 268 ± 36 nM) and efficiency as isoprenaline alone (EC_{50} = 195 ± 29 nM) (Figure 2b).

In dogs studied one month after sinoaortic denervation, basal activity of adenylate cyclase was significantly ($P < 0.05$) decreased (129 ± 12 pmol mg^{-1} protein) when compared with controls (178 ± 14 pmol mg^{-1} protein). Stimulation of adenylate cyclase activity by isoprenaline was significantly lower than in controls (Figure 2a), however, a slight increase in the EC_{50} value was observed (660 ± 79 nM). In the presence of 1 μM ICI 118551, isoprenaline failed to induce any activation of adenylate cyclase (Figure 2b).

In dogs studied 18 months after sinoaortic denervation, basal activity of adenylate cyclase was dramatically decreased (14 ± 2 vs 178 ± 14 pmol mg^{-1} protein in controls). However, isoprenaline alone or in the presence of ICI 118551 induced a similar increase of adenylate cyclase activity with no significant difference in the EC_{50} (248 ± 47 and 288 ± 52 nM respectively) values when compared to controls (Figure 3).

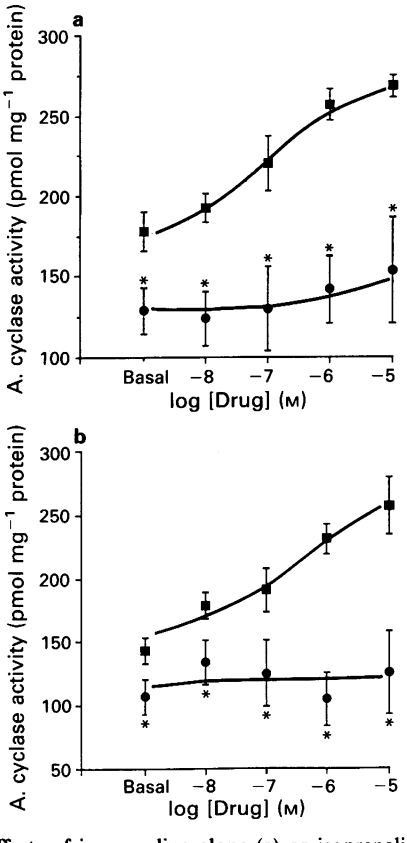

Figure 2 Effects of isoprenaline alone (a) or isoprenaline plus 1 μM ICI 118551 (b) on adenylate activity (in pmol mg^{-1} of protein) measured in membranes from the left ventricle of 6 normotensive controls (■) and 5 dogs studied 1 month after sinoaortic denervation (●). The results are expressed as mean with s.e.mean shown by vertical bars. *$P < 0.05$ using the Mann–Whitney U test when control animals were compared to sinoaortic denervated dogs.

Discussion

The present results demonstrate that (i) chronic sinoaortic denervation leads to an early development of left ventricular hypertrophy and (ii) total cardiac β-adrenoceptor number is negatively correlated with plasma catecholamine levels but apparently seems unrelated to the cardiac hypertrophy; (iii) the percentage of β_1-adrenoceptors dramatically decreases 1 month after sinoaortic denervation and does not return to normal values after 18 months and (iv) the basal values of adenylate cyclase activity in LV were significantly decreased after sinoaortic denervation. Isoprenaline alone or in the presence of a β_2-adrenoceptor antagonist (ICI 118551 1 μM) failed to stimulate adenylate cyclase activity 1 (but not 18) month after sinoaortic denervation.

The occurrence of left ventricular hypertrophy after sinoaortic denervation suggests that this experimental model is adequate for studying the visceral complications of arterial hypertension. In fact, recent data have shown that this experimental model is associated with renal vascular and glomerular lesions (Orfila *et al.*, 1990). Moreover, the early development of cardiac hypertrophy suggests that this phenomenon does not only depend on pressure overload. Other mechanisms may be involved, especially the rise in catecholamine plasma levels. In fact, *in vitro* data indicate that noradrenaline is able to induce protein synthesis in cultured rat myocytes (Simpson, 1983). In human essential arterial hypertension, there is a direct correlation between noradrenaline plasma levels and

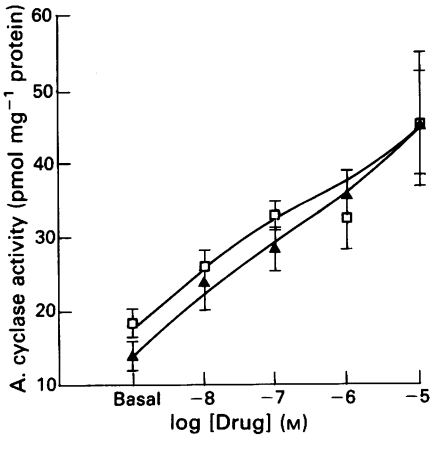

Figure 3 Effect of isoprenaline (□) or isoprenaline plus 1 μM ICI 118551 (▲) on adenylate activity (in pmol mg^{-1} of protein) measured in membranes from the left ventricle of five dogs studied 18 months after sinoaortic denervation. The results are expressed as mean with s.e.mean shown by vertical bars.

left ventricular mass (Corea *et al.*, 1983). In sinoaortic denervated dogs, cardiac hypertrophy can be initiated by the transient rise in catecholamine plasma levels observed in the early stage of hypertension. However, the role of arterial hypertension probably remains important, since, in this model, plasma catecholamines return to normal values within 2 months after surgical procedure (Valet *et al.*, 1988; 1989) whereas cardiac hypertrophy persists 18 months after sinoaortic denervation. At the end of the study, the dogs with left ventricular hypertrophy appeared fully compensated and none of them developed congestive heart failure.

Myocardial β-adrenoceptors were investigated by use of [^{125}I]-cyanopindolol, a non selective β-adrenoceptor antagonist. This ligand was used because of its high specific radioactivity which allows binding studies to be carried out on small myocardial fragments. Moreover, the β-adrenoceptor number identified with [^{3}H]-dihydroalprenolol is identical to that obtained with [^{125}I]-cyanopindolol (data not shown). Preliminary experiments indicated that [^{125}I]-cyanopindolol binds to β-adrenoceptors but also possesses affinity for additional binding sites. Competition experiments performed with 5-hydroxytryptamine receptor antagonists indicated that these additional binding sites probably correspond to non β-adrenoceptor specific binding sites. Thus, experiments were carried out in the presence of ritanserin (10 μM) in order to avoid any interaction of [^{125}I]-cyanopindolol with these additional sites.

Saturation experiments show that the number of myocardial β-adrenoceptors is decreased one month after sinoaortic denervation. In contrast, it returned to normal values after 18 months. These changes can be related to plasma catecholamine levels and not to myocardial hypertrophy. In fact, during the hyperadrenergic state (i.e. after 1 month), the density of β-adrenoceptors is decreased in the four cardiac cavities whereas only the left ventricle is subjected to pressure overload. Although we did not measure the number of β-adrenoceptors per ventricular myocardial cell, the present data suggest that the decreased β-adrenoceptor density is due to a down-regulation secondary to the rise in plasma catecholamines. In the same experimental model, Valet *et al.* (1989) reported a decrease in lymphocyte β-adrenoceptors in the early stage of hypertension followed by a return to normal values when plasma levels of catecholamines are normal. The lack of direct relationship between myocardial hypertrophy induced by pressure overload and the number of β-adrenoceptors is supported by the fact that B_{max} returned to normal values after 18 months in spite of unmodified left ventricular hypertrophy.

These data could explain the controversial results found in the literature concerning β-adrenoceptors and myocardial hypertrophy. In experimental models of arterial hypertension associated with increased sympathetic activity, β-adrenoceptor number was usually found to be decreased. This 'down-regulation' was reported in spontaneously hypertensive rats (Limas & Limas, 1978; Mukherjee *et al.*, 1980; Kuchii *et al.*, 1981; Kumano *et al.*, 1983; Yamada *et al.*, 1984), in rats with renal hypertension (Giachetti *et al.*, 1979; Woodcock & Johnston, 1980; Ayobe & Tarazi, 1983), and in the adrenaline-induced model of cardiac hypertrophy (Upsher & Khairallah, 1985). In contrast, after aortic banding in dogs (Vatner *et al.*, 1984) or in guinea-pigs (Karliner *et al.*, 1980), i.e. two experimental conditions where myocardial catecholamine stores are decreased (Fischer *et al.*, 1955), cardiac hypertrophy is associated with an increase of β-adrenoceptors. In rats with aortic banding, the results still remain controversial. β-Adrenoceptor number was reported to be either increased (Limas, 1979), decreased (Will-Shahab *et al.*, 1986; Chevalier *et al.*, 1989), or unchanged (Cervoni *et al.*, 1981). In man, no data are available in cardiac hypertrophy due to pressure overload. In hypertrophic cardiomyopathy, a disorder characterized by a non-dilated, hypertrophied left ventricle in the absence of any overt cause, the density of β-adrenoceptors in the right atrial tissue was found to be normal (Wagner *et al.*, 1989) but increased in the left ventricular endomyocardium (Takita *et al.*, 1990). Abnormal adrenergic function has been proposed as a cause of this cardiomyopathy.

In control normotensive dogs, β_1-adrenoceptors represent 75–80% of the total β-adrenoceptors. In the early stage of sinoaortic denervation a dramatic decrease in the number of β_1-adrenoceptors was observed with no change in the number of β_2-adrenoceptors. After an 18 month follow-up, the percentage of β_1-adrenoceptors increased when compared to values obtained 1 month after sinoaortic denervation but did not return to control values. Thus, the increase in catecholamine plasma levels after sinoaortic denervation induced a specific down-regulation of β_1-cardiac adrenoceptors. However, the fact that the β_1-adrenoceptor percentage did not return to basal values after 18 months despite the catecholamine normalization suggests that cardiac hypertrophy is also involved in the observed changes. Similar variations of myocardial β-adrenoceptor subtypes were previously reported after aortic banding in rats (Chevalier & Swynghedauw, 1990) and in spontaneously hypertensive rats (Michel *et al.*, 1987). In man at the final stage of cardiac heart failure (where plasma catecholamines are increased), myocardial β-adrenoceptor number and overall β_1-adrenoceptor subtypes were found to be significantly reduced (Bristow *et al.*, 1982; Fowler *et al.*, 1986). In other tissues (adipocytes and lymphocytes) from sinoaortic denervated dogs, down regulation preferentially concerns β_2-adrenoceptors (Valet *et al.*, 1988). These results indicate that the differential regulation of the subtypes of β-adrenoceptor is not only related to plasma catecholamine levels but also varies according to their location. In fat cells the relative proportions of β-adrenoceptor subtypes is very different from that in myocardial cells (respectively 67 and 18% of β_2-adrenoceptors).

The determination of adenylate cyclase activity first allows further insight into results obtained in the binding studies. In dogs submitted to sinoaortic denervation from 1 month, the stimulation of adenylate cyclase activity induced by either the whole β-adrenoceptor population (isoprenaline alone) or by β_1-adrenoceptors (isoprenaline plus ICI 118551) was significantly decreased. This phenomenon is no longer observed 18 months later. Taken together (Figure. 2), these data suggest that the stimulation of adenylate cyclase activity in myocardial cells involves mainly β_1-adrenoceptors. In fact, in the presence of isoprenaline plus ICI 118551, adenylate cyclase activity reached the same maximal values. Basal values of adenylate cyclase activity are lower than controls in 1 month sinoaortic denervated dogs and dramatically decreased 18 months after sinoaortic denervation. These results suggesting that adenylate cyclase activity falls in parallel with the development of left ventricular hypertrophy agree with the recent data of Chen *et al.* (1990) after aortic banding in dogs.

In conclusion, chronic sinoaortic denervation in dogs appears to be a convenient model for studying the regulation of β-adrenoceptors in the hypertrophied left ventricle. Homologous regulation by the increase in endogenous catecholamines during the early stage of sinoaortic denervation would seem to be the main factor for the down-regulation of cardiac β_1-adrenoceptors. Whilst left ventricular hypertrophy does not appear to regulate β-adrenoceptors directly, it could be involved in the alterations of transmembrane signalling (adenylate cyclase and/or G protein expression).

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Contributions of α_1-adrenoceptors, α_2-adrenoceptors and P_{2x}-purinoceptors to neurotransmission in several rabbit isolated blood vessels: role of neuronal uptake and autofeedback

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1 The roles of autofeedback and neuronal uptake in neurotransmission produced by electrical field stimulation in several rabbit isolated blood vessels were examined.

2 Blocking drugs were used to separate the possible purinergic and noradrenergic contributions to the end organ response: prazosin, antagonist at postjunctional α_1-adrenoceptors; rauwolscine and yohimbine, antagonists at pre- and postjunctional α_2-adrenoceptors; α,β-methylene ATP, desensitizing agent at postjunctional P_{2x}-purinoceptors. In addition to desensitizing postjunctional P_{2x}-purinoceptors, α,β-methylene ATP potentiated the noradrenergic component of the nerve-induced responses.

3 In the presence of an intact neuronal uptake mechanism, the vessels showed different contributions of purinergic (via P_{2x}-purinoceptors) and noradrenergic (via α_1-adrenoceptors and α_2-adrenoceptors) components to the end organ response to nerve stimulation: saphenous artery (approximately equal contributions from P_{2x}-purinoceptors and α_1-adrenoceptors), ileocolic artery (mainly P_{2x}-purinoceptors with a smaller contribution from α_1-adrenoceptors), plantaris vein (mainly α_1-adrenoceptors with a small contribution from α_2-adrenoceptors and P_{2x}-purinoceptors) and saphenous vein (α_1-adrenoceptors).

4 The presence of α_2-adrenoceptor-mediated autofeedback could be demonstrated for both purinergic and noradrenergic components of the nerve-induced responses in the artery preparations. In the veins, potentiation of nerve-induced responses by α_2-adrenoceptor antagonists could not be studied due to blockade of postjunctional α_2-adrenoceptor-mediated vasoconstriction.

5 Blockade of neuronal uptake with cocaine potentiated the noradrenergic component of the nerve-induced responses. Both α_1-adrenoceptor- and α_2-adrenoceptor-mediated components were potentiated, with a relatively greater potentiation of the α_2-adrenoceptor-mediated component. In the case of saphenous vein an α_2-adrenoceptor-mediated component which was previously absent was uncovered.

6 Blockade of neuronal uptake with cocaine had no effect or reduced the purinergic component of responses, the latter effect presumably due to enhanced α_2-adrenoceptor-mediated autofeedback.

7 In the presence of cocaine, nerve-induced responses in the saphenous vein were biphasic. Rauwolscine potentiated the first phase and inhibited the second phase thus demonstrating effects of pre- and postjunctional α_2-adrenoceptor-mediated activation in the same preparation.

8 In conclusion, neuronal uptake and autofeedback processes play important and complex interacting parts in determining the relative contributions of α_1- and α_2-adrenoceptors and P_{2x}-purinoceptors in the end organ response to neurotransmission in blood vessels.

Keywords: Blood vessels; co-transmission; neuronal uptake; autofeedback; α_1-adrenoceptors; α_2-adrenoceptors; P_{2x}-purinoceptors

Introduction

There is now strong evidence for cotransmission involving noradrenaline and ATP at sympathetic nerve terminals in vas deferens (Burnstock & Sneddon, 1985; Stjarne & Astrand, 1985) and blood vessels (see Burnstock, 1988). The present study was carried out to investigate the interaction between autofeedback and noradrenaline reuptake in determining the proportions of adrenergic and purinergic components of neurotransmission in several rabbit isolated blood vessels. The vessels studied included those where the purinergic component has previously been demonstrated, for example the ileocolic artery (von Kugelgen & Starke, 1985) and proximal saphenous artery (Burnstock & Warland, 1987), or where the α-adrenoceptor population is known to be heterogeneous, taking examples with different proportions of the two subtypes, α_1 and α_2, as demonstrated by agonist and antagonist potencies, i.e. saphenous vein (α_1 and α_2; Daly *et al.*, 1988a,b) and plantaris vein (mainly α_2; Daly *et al.*, 1988a).

Noradrenergic/purinergic cotransmission tends to have been investigated in systems where the noradrenergic component is mediated by α_1-adrenoceptors. Where postjunctional α_2-adrenoceptors are present, pharmacological isolation and analysis of the purinergic component is rendered complicated because blockade of α_2-adrenoceptors will reduce the noradrenergic component postjunctionally and remove autofeedback prejunctionally, thus overall exaggerating the purinergic component (Bulloch & Starke, 1990). This has been addressed by studying blood vessels with a heterogeneous α-adrenoceptor population over a wide range of stimulation frequencies and observing the interplay between α_2-adrenoceptor blockade and blockade of neuronal reuptake of noradrenaline.

Preliminary accounts of some of these results have previously been published (MacDonald *et al.*, 1989; McGrath *et al.*, 1990; Bulloch *et al.*, 1990; 1991).

Methods

Male New Zealand White rabbits (2–3 kg) were killed by stunning followed by exsanguination. The proximal saphenous artery, ileocolic artery, saphenous vein or plantaris vein were carefully removed with as little connective tissue as possible and placed in cold physiological salt solution (PSS). After careful dissection to remove the remaining connective

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tissue the vessels were divided into rings of 3–4 mm in length. Rings of blood vessels were then suspended between parallel platinum electrodes for stimulation and connected via cotton thread to Grass FTO3c transducers for isometric recording. Recordings were made on a Linseis L2065 chart recorder. After a period of equilibration (45–60 min), frequency-response curves (FRCs) were constructed using a Square One Instruments stimulator. Stimulation parameters used were 4–64 Hz, 1 s train duration, 0.1 ms pulse width, 35 volts. A low frequency long duration (4 Hz/10 s) train of pulses was also tested on the venous preparations. In experiments with the ileocolic artery preparations were also stimulated at 5 and 10 Hz for 20 s. In experiments using the veins, propranolol (1 μM) was present in the PSS.

Drugs and solutions

The composition of the PSS used for the saphenous artery and the veins was as follows: (mM) NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2 and glucose 11.1. Na_2EDTA 23 μM was included to prevent oxidative degradation of noradrenaline. The composition of the PSS used for the ileocolic artery was as follows (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 0.9, glucose 11, Na_2EDTA 0.03, ascorbic acid 0.3. The PSS were saturated with 95% O_2 5% CO_2 and maintained at 37°C.

The following drugs were used: prazosin HCl (Pfizer), rauwolscine HCl (Roth), α,β-methylene ATP (lithium salt; Sigma), cocaine HCl (Macarthys), propranolol HCl (Sigma), yohimbine HCl (Roth), CH-38083 (chinoin; 7,8-(methylenedioxi)-14-α-alloberbane HCl), corynanthine HCl (Sigma), UK-14,304 (5-bromo-6[2-imazolin-2-yl-amino] quinoxaline) bitartrate (Pfizer). All drugs were dissolved in distilled water.

Results

Proximal saphenous artery

Prazosin (1 μM) reduced responses at all frequencies (4–64 Hz) by around 50% leaving a sizeable prazosin-resistant component (Figure 1a). Desensitization with α,β-methylene ATP (α,β-MeATP) (3 μM) had a variable effect on the whole nerve responses. In some experiments α,β-MeATP reduced nerve responses, the reductions tending to be greater at the lower frequencies of stimulation (Figure 1b) while in other experiments it potentiated the nerve responses. In either case, responses were virtually abolished by subsequent addition of prazosin (Figure 1d). In experiments where prazosin was given before α,β-MeATP, subsequent desensitization with α,β-MeATP produced an almost complete inhibition of the prazosin-resistant component of the nerve-induced response (Figure 1c). Rauwolscine produced no inhibitory effect on either component of the response (see below).

Both the purinergic (prazosin-resistant) and noradrenergic (after α,β-MeATP desensitization) components of the nerve-induced response were inhibited by UK-14,304 and this inhibition was reversed by subsequent addition of rauwolscine (Figure 2). No contractile activity of UK-14,304 was observed at the concentrations used ($\leq 10^{-7}$ M). Rauwolscine itself potentiated both the purinergic and noradrenergic components of the nerve-induced response (Figure 3a,b).

Cocaine (3 μM) inhibited the nerve-mediated response at 4 Hz, had little overall effect at 8 and 16 Hz, and potentiated responses at 32 and 64 Hz (Figure 4a). The only significant effect of cocaine on the purinergic component of the response after prazosin was a significant reduction in responses at 4 Hz (Figure 4b). Responses at other frequencies were unaffected. Cocaine potentiated responses remaining after α,β-MeATP desensitization (noradrenergic component) (Figure 4c).

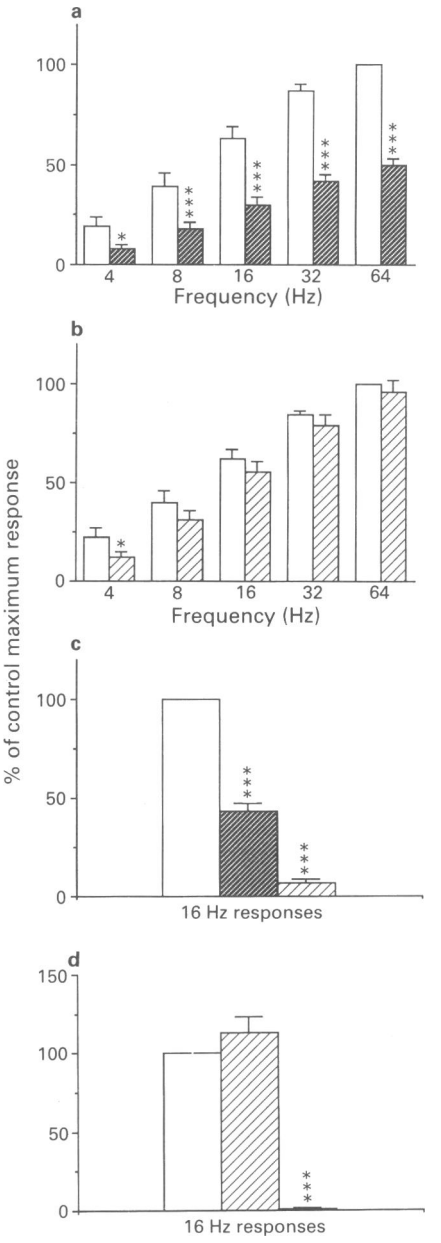

Figure 1 Effects of prazosin and α,β-methylene ATP (α,β-MeATP) on nerve induced responses in rabbit saphenous artery. (a) Effect of prazosin on responses at 4–64 Hz for 1 s. Open columns, control; dark hatched columns, prazosin (1 μM) ($n = 16$). (b) Effect of α,β-MeATP on responses at 4–64 Hz for 1 s. Open columns, control, hatched columns, α,β-MeATP (3 μM) ($n = 20$). (c) Effect of a combination of prazosin and α,β-MeATP on responses at 16 Hz. Open column, control; dark hatched column, prazosin (1 μM); hatched column, prazosin (1 μM) + α,β-MeATP (3 μM) ($n = 20$). (d) Effect of a combination of α,β-MeATP and prazosin on responses at 16 Hz. Open column, control; hatched column, a,β-MeATP (3 μM); dark hatched column, α,β-MeATP (3 μM) + prazosin (1 μM) ($n = 21$). In (a) and (b) asterisks indicate significant differences from controls. In (c) and (d) asterisks indicate significant difference from preceding column. P values: * < 0.05; ** < 0.01; *** < 0.001 (paired t test).

The potentiating effect of rauwolscine on either the purinergic or noradrenergic components of the response was not enhanced by the presence of cocaine (Figure 3c).

Ileocolic artery

Vasoconstrictor responses produced by electrically stimulating the isolated ileocolic artery rings for 1 second were frequency-dependent. Cocaine (10 μM) had little effect on the

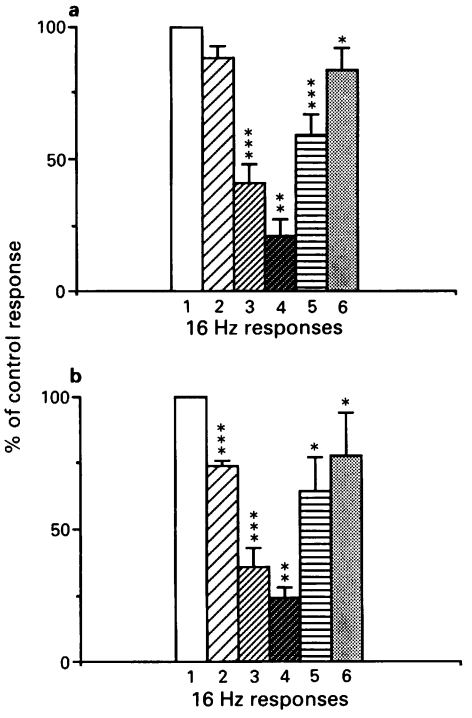

Figure 2 Effect of UK-14,304 on nerve-induced responses of rabbit saphenous artery at 16 Hz for 1 s. (a) Responses in the presence of prazosin (1 μM), i.e. purinergic component ($n = 6$). (b) Responses in the presence of α,β-methylene ATP (α,β-MeATP, 3 μM), i.e. noradrenergic responses ($n = 5$). In (a) and (b): column 1, control; column 2, UK-14, 304 (0.001 μM); column 3, UK-14,304 (0.01 μM); column 4, UK-14,304 (0.1 μM); column 5, UK-14,304 (0.1 μM) + rauwolscine (0.3 μM); column 6, UK-14,304 (0.1 μM) + rauwolscine (1.0 μM). Asterisks indicate significant difference from preceding column. P values: * < 0.05; ** < 0.01; *** < 0.001 (paired t test).

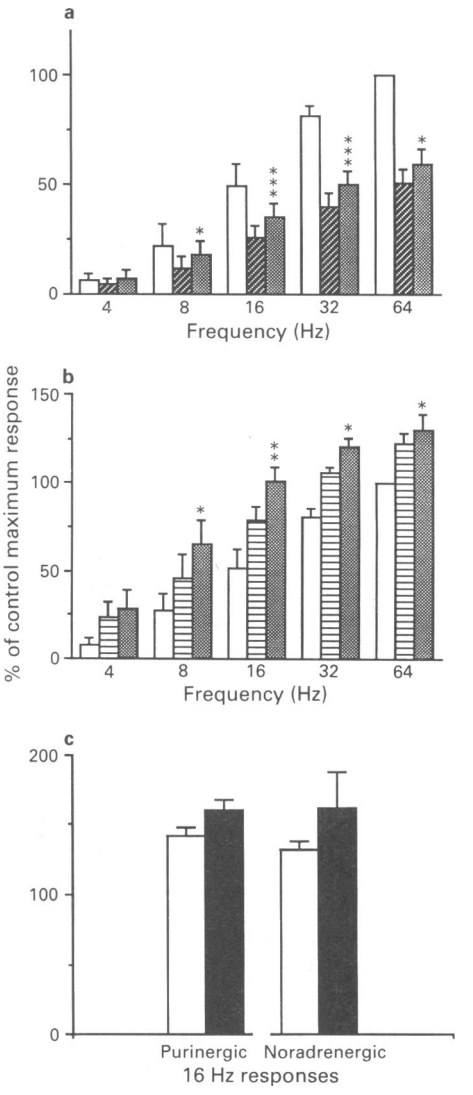

Figure 3 Effects of rauwolscine on nerve-induced responses of rabbit saphenous artery. (a) and (b) responses at 4–64 Hz for 1 s; (c) responses at 16 Hz for 1 s. (a) Open columns, control; hatched columns, prazosin (1 μM); stippled columns, prazosin (1 μM) + rauwolscine (0.3 μM) ($n = 5$). (b) Open columns, control; hatched columns α,β-methylene ATP (α,β-MeATP, 3 μM); stippled columns α,β-MeATP (3 μM) + rauwolscine (0.3 μM) ($n = 5$). (c) Columns on left are in presence of prazosin (1 μM), i.e. purinergic responses. Columns on right are in presence of α,β-MeATP (3 μM), i.e. noradrenergic responses. Open columns in absence of cocaine; solid columns in presence of cocaine (3 μM) ($n = 6$–8). Asterisks indicate significant difference from preceding column. P values * < 0.05; ** < 0.01; *** < 0.001 (paired t test).

electrically evoked responses at lower frequencies although a small potentiation of the response at 32 Hz was observed (Figure 5a). Subsequent administration of prazosin (0.3 μM) had no effect on responses at lower frequencies (4 and 8 Hz) but produced a significant reduction in the vasoconstrictor responses at higher frequencies (16 and 32 Hz) (Figure 5a). The responses remaining after prazosin were almost completely abolished by subsequent desensitization with α,β-MeATP (3 μM) (Figure 5a).

Yohimbine (0.3 μM) produced potentiation of the nerve-induced responses at 8, 16 and 32 Hz (Figure 5b). As above, subsequent administration of prazosin (0.3 μM) had no effect on responses at lower frequencies (4 and 8 Hz) but produced a significant reduction in the responses at 16 and 32 Hz (Figure 5b). Subsequent desensitization with α,β-MeATP (3 μM) almost completely abolished the remaining responses (Figure 5b).

Responses obtained by stimulation for 20 s at 5 and 10 Hz were biphasic in appearance (Bulloch & Starke, 1988; 1990). Cocaine (10 μM) had little effect on the first phase of the responses at either frequency but gave a clear potentiation of the second phase at both frequencies (Figure 6a). Prazosin (0.3 μM) antagonized these potentiated responses and also reduced the first phase of the 10 Hz response (Figure 6a). The biphasic responses remaining after prazosin were almost completely abolished by α,β-MeATP (3 μM) (Figure 6a).

Yohimbine (0.3 μM) produced potentiation of both phases of the response at 5 and 10 Hz (Figure 6b). Prazosin (0.3 μM) was effective in reducing both phases of the potentiated responses at 5 and 10 Hz (Figure 6b). Subsequent administration of α,β-MeATP after prazosin virtually abolished the responses (Figure 6b).

Plantaris vein

In the absence of cocaine, prazosin (0.1 μM) had little effect on responses at 4 and 8 Hz and reduced responses at the higher frequencies (Figure 7a). Desensitization with α,β-MeATP (3 μM) potentiated the response (not shown). Rauwolscine (1 μM) produced a small, but not significant, reduction of the whole nerve response (Figure 7c) and had no significant additional effect on the prazosin-resistant component (Figure 7a,e: analysis of variance, $P > 0.05$ at all frequencies). The combination of prazosin and rauwolscine therefore failed to abolish the responses. However, subsequent addition of α,β-MeATP after prazosin and rauwolscine almost completely abolished responses (Figure 7e).

Cocaine (10 μM) increased the overall height of responses and uncovered a small secondary component. The effect of prazosin was not significantly altered by cocaine (Figure 7a,b:

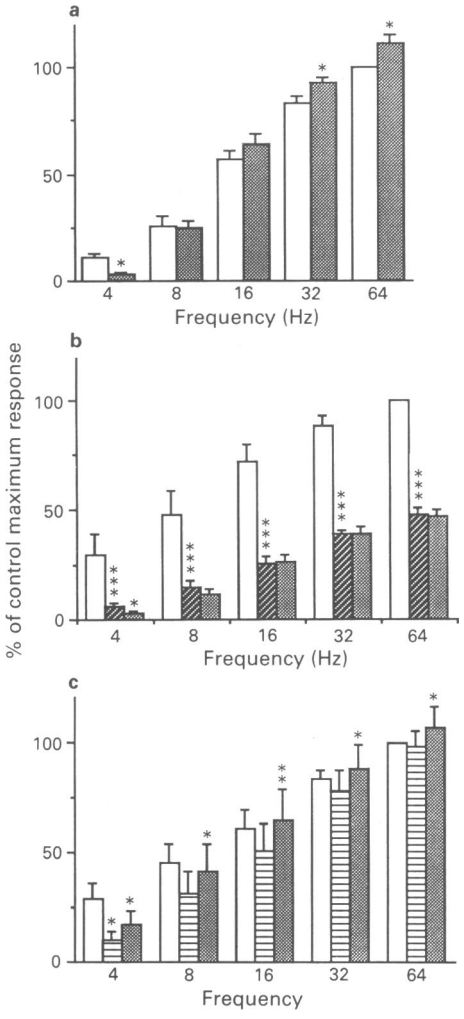

Figure 4 Effects of cocaine on nerve-induced responses of rabbit saphenous artery at 4–64 Hz for 1 s. (a) Open columns, control; stippled columns, cocaine (3 μM) ($n = 14$). (b) Open columns, control; dark hatched columns, prazosin (1 μM); stippled columns, prazosin (1 μM) + cocaine (3 μM) ($n = 6$). (c) Open columns, control; hatched columns α,β-methylene-ATP (α,β-MeATP 3 μM); stippled columns, α,β-MeATP + cocaine (3 μM) ($n = 7$). Asterisks indicate significant difference from preceding column. P values: * < 0.05; ** < 0.01; *** < 0.001 (paired t test).

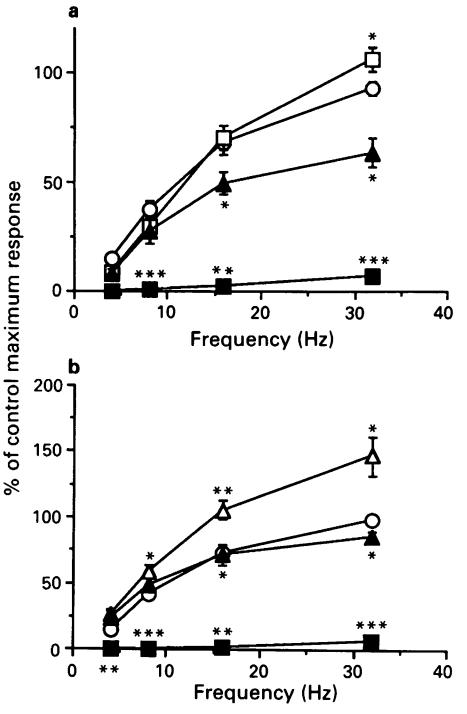

Figure 5 Effects of drugs on nerve-induced responses of rabbit ileocolic artery at 4–32 Hz for 1 s. (a) (○) Control; (□) cocaine (10 μM); (▲) cocaine (10 μM) + prazosin (0.3 μM); (■) cocaine (10 μM) + prazosin (0.3 μM) + α,β-methylene ATP (α,β-MeATP 3 μM) ($n = 5$). (b) (○) Control; (△) yohimbine (0.3 μM); (▲) yohimbine (0.3 μM) + prazosin (0.3 μM); (■) yohimbine (0.3 μM) + prazosin (0.3 μM) + α,β-MeATP (3 μM) ($n = 5$). Asterisks indicate significant difference from preceding treatment. P values: * < 0.05; ** < 0.01; *** < 0.001 (paired t test).

analysis of variance, $P > 0.05$ at all frequencies) but in the presence of cocaine, rauwolscine had a significant inhibitory effect at all frequencies (Figure 7d). A comparison (analysis of variance) of the effects of rauwolscine in the absence and presence of cocaine gave a significant difference ($P < 0.05$) at each frequency except at 4 Hz for 10 s. The effect of combined blockade with prazosin and rauwolscine however was not significantly altered in the presence of cocaine (Figure 7f: analysis of variance, $P > 0.05$ at all frequencies). There was no significant difference in the ability of α,β-MeATP to abolish responses in the presence of cocaine (Figure 7e,f: analysis of variance, $P > 0.05$ at all frequencies).

Lateral saphenous vein

In the absence of cocaine, prazosin (0.1 μM) produced a substantial reduction of responses (Figure 8a) and subsequent administration of rauwolscine (1 μM) had no further effect (Figure 8b).

Cocaine (10 μM) enhanced responses at all frequencies (e.g. Figure 9). In addition to an increase in peak height, at frequencies of 16 Hz and above a second phase emerged, appearing as a 'shoulder' on the response (Figure 9). Rauwolscine and CH-38083 (1 μM) potentiated the faster first phase of the response and at the same time inhibited the second phase (Figure 9 and Figure 10c,d). Prazosin and corynanthine (1 μM) exerted a non-selective inhibition of both phases of the responses (Figure 10a,b).

In the presence of cocaine the combination of prazosin and rauwolscine appeared to effect a greater inhibition of responses at all frequencies than in its absence (Figure 8b,c). However, the difference was statistically significant only at 8 and 16 Hz ($P < 0.05$, analysis of variance). The residual response was unaffected by subsequent desensitization with α,β-MeATP (results not shown).

Discussion

The blocking drugs were used to separate the possible purinergic and noradrenergic contributions and the adrenoceptor sub-types involved in the neurotransmission produced by electrical field stimulation. The blocking drugs were applied alone and in various combinations and sequences and the following premises were made concerning the selectivity of their blockade: prazosin (Cambridge *et al.*, 1977) and corynanthine (Weitzell *et al.*, 1979), competitive antagonists at postjunctional α_1-adrenoceptors; rauwolscine, yohimbine (Weitzell *et al.*, 1979) and CH-38083 (Vizi *et al.*, 1986), competitive antagonists at pre- and postjunctional α_2-adrenoceptors; α,β-methylene ATP, desensitizing agent at postjunctional P_{2x}-purinoceptors (Sneddon & Burnstock, 1984a,b). In addition, UK-14,304 was employed as a selective α_2-adrenoceptor agonist (Cambridge, 1981).

In using these blocking drugs several factors must be considered; for example, investigation of the presence of postjunctional α_2-adrenoceptor subtypes was complicated by the presence of prejunctional α_2-adrenoceptor-mediated

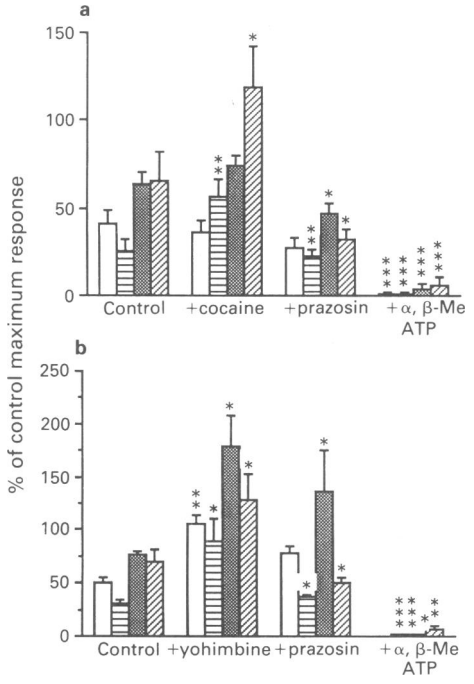

Figure 6 Effects of drugs on nerve-induced responses of rabbit ileocolic artery at 5 Hz and 10 Hz for 20 s. The first phase at 5 Hz (open columns) and 10 Hz (stippled columns) was taken as the peak constrictor height occurring 5–9 s after the onset of stimulation, and the second phase at 5 Hz (horizontal hatched columns) and 10 Hz (cross hatched columns) as the peak height between 18–23 s. (a) Controls, first set of responses; cocaine (10 μM), second set of responses; cocaine (10 μM) + prazosin (0.3 μM), third set of responses; cocaine (10 μM) + prazosin (0.3 μM) + α,β-methylene ATP (α,β-MeATP, 3 μM), fourth set of responses, (n = 5). (b) Controls, first set of responses; yohimbine (0.3 μM), second set of responses; yohimbine (0.3 μM) + prazosin (0.3 μM), third set of responses; yohimbine (0.3 μM) + prazosin (0.3 μM) + α,β-MeATP (3 μM), fourth set of responses (n = 5). Asterisks indicate significant difference of each phase from preceding treatment. P values: * < 0.05; ** < 0.01; *** < 0.001 (paired t test).

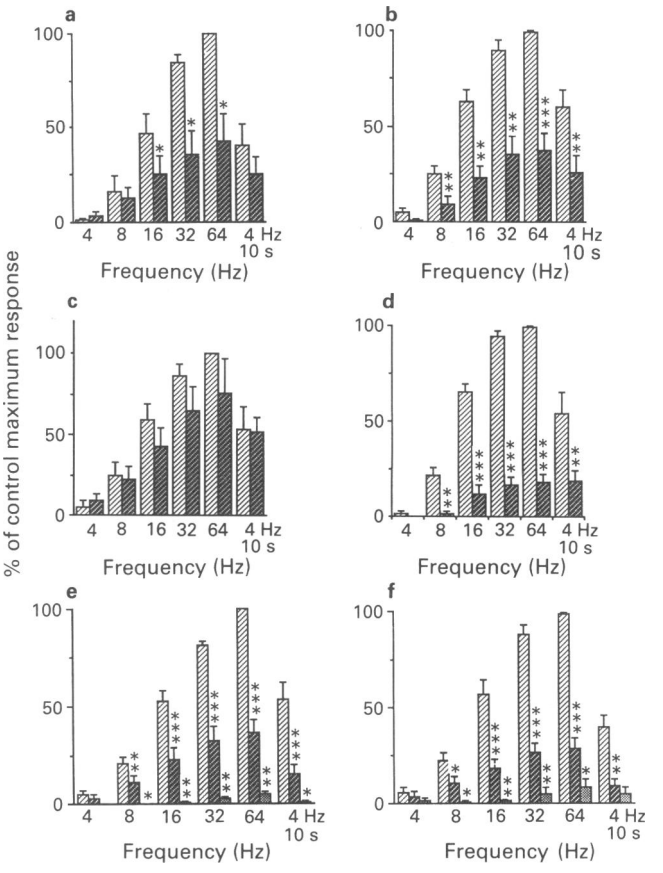

Figure 7 Effects of drugs on nerve-induced responses of rabbit plantaris vein at 4–64 Hz is for 1 s and 4 Hz for 10 s in absence, (a), (c) and (e), and presence, (b), (d) and (f), of cocaine (10 μM) (n = 5–6). (a) and (b) Controls, light hatched columns; prazosin (0.1 μM), dark hatched columns. (c) and (d) Controls, light hatched columns; rauwolscine (1 μM), dark hatched columns. (e) and (f) Controls, light hatched columns; prazosin (0.1 μM) + rauwolscine (1 μM), dark hatched columns; prazosin (0.1 μM) + rauwolscine (1 μM) + α,β-methylene ATP (3 μM), stippled columns. Asterisks indicate significant difference of each column from preceding treatment. P values: * < 0.05; ** < 0.01; *** < 0.001 (paired t test).

autofeedback whose blockade could enhance transmitter release and contractile responses. Another complicating factor was the potentiation of nerve-induced responses in saphenous artery and plantaris vein produced by α,β-MeATP when administered first. After α,β-MeATP-induced potentiation, nerve responses were blocked by subsequent adrenoceptor blockade. In addition, the potentiating effect was not seen after prazosin administration and therefore appears to be a potentiation of the noradrenergic component of the response. A potentiating effect on the noradrenergic component of the response by α,β-MeATP has also previously been observed by us in rat vas deferens *in situ* (Bulloch & McGrath, 1988) and in guinea-pig vas *in vitro* (Bulloch *et al.*, 1990) although in these cases the potentiating effect disappeared with time, leaving the P_{2x}-purinoceptor desensitizing effect unopposed. α,β-MeATP has also been shown to cause depolarization and potentiate noradrenergic nerve-induced contraction in rat tail artery (Neild & Kotecha, 1986). The potentiating effect of α,β-MeATP was mimicked by a similar degree of depolarization produced by KCl and was therefore attributed to depolarization. The mechanism of the potentiating effect in the present study may therefore be related to residual depolarization produced by α,β-MeATP, even after the muscle tension has returned to baseline. On the other hand a specific postjunctional synergism between exogenous noradrenaline and α,β-MeATP has also been reported (Ralevic & Burnstock, 1990) and such an effect cannot be ruled out. In any case, the results highlight difficulties encountered when using α,β-MeATP as an 'antagonist' of purinergic nerve-mediated contractions of smooth muscle.

Notwithstanding these difficulties, conclusions could be reached concerning the different contributions of purinergic and noradrenergic components to the end organ response to field stimulation of the intramural nerves in the different blood vessels studied. In the absence of any drugs to block neuronal uptake, the proximal saphenous artery gave approximately equal contributions from P_{2x}-purinoceptors and α_1-adrenoceptors; the response in the ileocolic artery was mediated mainly through P_{2x}-purinoceptors with a smaller contribution from α_1-adrenoceptors; in the plantaris vein there were contributions from α_1-adrenoceptors, α_2-adrenoceptors and P_{2x}-purinoceptors; in the saphenous vein the dominant response was mediated via α_1-adrenoceptors.

The presence of prejunctional α_2-adrenoceptors activated by stimulation-evoked release of noradrenaline could be shown by rauwolscine or yohimbine potentiation of nerve-induced responses although in the venous preparations this was complicated by the presence of postjunctional α_2-adrenoceptors. In saphenous artery, rauwolscine potentiated both the purinergic and noradrenergic component of the response showing that both components are influenced by α_2-adrenoceptor-mediated autofeedback, as shown previously in dog mesenteric (Muramatsu *et al.*, 1989) and rabbit ileocolic arteries (von Kugelgen & Starke, 1985; Bulloch & Starke, 1988; 1990) and as might be expected if cotransmission is taking place. The presence of prejunctional

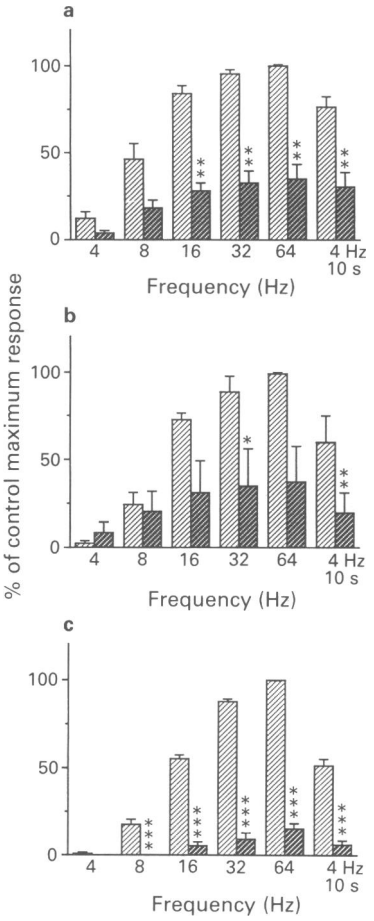

Figure 8 Effects of drugs on nerve-induced responses of rabbit saphenous vein at 4–64 Hz for 1 s and 4 Hz for 10 s. (a) Controls, light hatched columns; prazosin (0.1 μM), dark hatched columns ($n = 4$). (b) Controls, light hatched columns; prazosin (0.1 μM) + rauwolscine (1 μM), dark hatched columns ($n = 4$). (c) Cocaine (10 μM) present throughout. Controls, light hatched columns; prazosin (0.1 μM) + rauwolscine (1 μM), dark hatched columns ($n = 7$). Asterisks indicate significant difference of each column from preceding treatment. P values: * < 0.05; ** < 0.01; *** < 0.001 (paired t test).

α_2-adrenoceptors mediating inhibition of both noradrenaline and ATP release was confirmed in saphenous artery by the effects of UK-14,304, a selective α_2-adrenoceptor agonist (Cambridge, 1981). Administration of UK-14,304 reduced both the purinergic and noradrenergic components of the nerve-induced response and the reduction was antagonized by rauwolscine. Evidence has been produced that inhibition of α_2-adrenoceptor-mediated autofeedback may preferentially increase the release of noradrenaline, perhaps due to differences in the noradrenaline/ATP ratio in vesicles susceptible to autofeedback (Bulloch & Starke, 1990; Starke *et al.*, 1991). However, in the present study there was no evidence that this is the case and further experiments are required to clarify this.

The effects of rauwolscine on the venous preparations were complicated by the presence of postjunctional α_2-adrenoceptors. In the presence of an intact neuronal uptake mechanism, autofeedback could not be demonstrated. Rauwolscine either reduced (plantaris) or had little overall effect (saphenous), indicating that any potentiating effect due to removal of autofeedback was either absent or was masked by the blocking actions of rauwolscine at postjunctional α_2-adrenoceptors. Blockade of neuronal uptake with cocaine potentiated this postjunctional α_2-adrenoceptor component (see below) resulting in a larger inhibitory effect of rauwolscine on the plantaris vein. However, in the case of the saphenous vein, a biphasic response emerged after cocaine, the first phase of which was predominantly α_1-adrenoceptor-mediated and

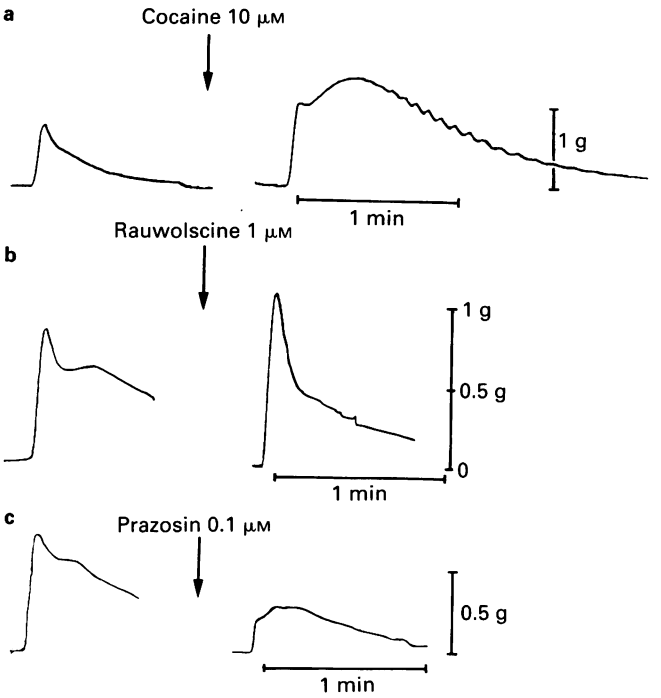

Figure 9 Representative tracings illustrating the effects of cocaine on nerve-induced response of rabbit saphenous vein. Responses were elicited by stimulation at 32 Hz for 1 s, 35 V. (a) Effect of cocaine (10 μM) on the time course of response. Responses in (b) and (c) are in the presence of cocaine; (b) effect of rauwolscine (1 μM); (c) effect of prazosin (0.1 μM).

potentiated by rauwolscine, and the second phase of which was α_1- and α_2-adrenoceptor-mediated and inhibited by rauwolscine. Thus in the saphenous vein, but not the plantaris, it was possible to demonstrate α_2-adrenoceptor-mediated autofeedback. Analysis of pre- and postjunctional α_2-adrenoceptor-mediated contributions to neurotransmission should be facilitated by the development of

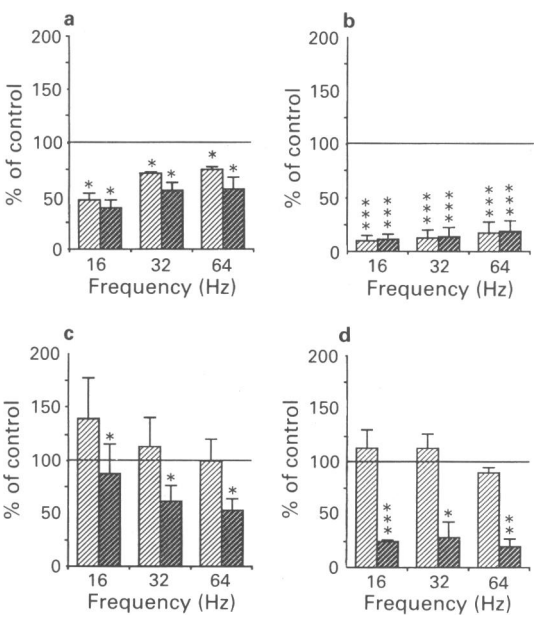

Figure 10 Effects of drugs on the first and second phases of the nerve-induced responses of saphenous vein obtained in the presence of cocaine. Light hatched columns, first phase; dark hatched columns, second phase. Each phase is expressed as a % of its own control value. (a) Corynanthine (1 μM) ($n = 3$); (b) prazosin (1 μM) ($n = 6$); (c) rauwolscine (1 μM) ($n = 6$); (d) CH 38083 (1 μM) ($n = 4$). Asterisks indicate significant difference of each phase from control value. P values: * < 0.05; ** < 0.01; *** < 0.001 (paired t test).

antagonists with selectivity for either site. Preliminary experiments with SKF 104078, an α_2-adrenoceptor antagonist with reputed selectivity for the postjunctional site (Ruffolo *et al.*, 1987), gave inhibition of both phases of the response in saphenous vein which may indicate a postjunctional α_2-adrenoceptor-mediated component of both phases of the response and a relative lack of activity of SKF 104078 at prejunctional α_2-adrenoceptors (unpublished data). Further investigations are required to confirm this.

Blockade of neuronal uptake with cocaine itself usually potentiated nerve-mediated responses due to potentiation of the noradrenergic component, although in vessels with a substantial purinergic component (saphenous and ileocolic arteries) at lower frequencies cocaine either had no effect or reduced the responses, the latter effect presumably due to enhanced autofeedback of the purinergic component by endogenous noradrenaline (Sneddon & Westfall, 1984; Muramatsu *et al.*, 1989). These effects were more clearly seen using the blockers to separate the different components: in saphenous and ileocolic arteries, cocaine potentiated the postjunctional α_1-adrenoceptor-mediated component; in the plantaris vein both the α_1- and α_2-adrenoceptor-mediated components of the response were potentiated, in particular the α_2-adrenoceptor-mediated component; in saphenous vein in addition to potentiation of the postjunctional α_1-adrenoceptor-mediated component, a postjunctional α_2-adrenoceptor component, previously absent, was uncovered. These findings might support the hypothesis that α_2-adrenoceptors are extrajunctional (Langer *et al.*, 1985), and thus accessible to noradrenaline only if it is allowed to diffuse away rather than be taken back up into the nerve terminals. An alternative explanation is that α_2-adrenoceptors are 'junctional' but, in the absence of other physiological factors which permit their expression e.g. angiotensin (Dunn *et al.*, 1989; 1991), need to be activated for longer than is possible with a pulse of released transmitter: thus the persistence of noradrenaline caused by uptake blockade allows their activation. Cocaine produced no potentiation of responses mediated by postjunctional P_{2x}-purinoceptors and in some case such responses were reduced, presumably due to enhanced prejunctional α_2-adrenoceptor-mediated feedback as discussed above.

The above analysis of the effects of cocaine on postjunctional components of the responses is, of course, complicated by the prejunctional effects of rauwolscine. Previous studies have shown that autofeedback can be more clearly demonstrated if neuronal uptake is blocked (Docherty & McGrath, 1979). A comparison was made in the present study between the potentiating effects of rauwolscine in the presence and absence of cocaine in a preparation uncomplicated by the presence of postjunctional α_2-adrenoceptors, proximal saphenous artery. No significant differences were seen in the effect of rauwolscine in the presence or absence of cocaine on either component of the response. Therefore in these experiments blockade of neuronal uptake with cocaine does not have a marked effect on the prejunctional effect of rauwolscine. On the other hand, the inhibition of purinergic responses by cocaine does suggest an enhanced autofeedback. It may be that feedback is enhanced by cocaine only at low frequencies (the inhibitory effects of cocaine in the saphenous artery were most marked at 4 Hz, while the comparison of rauwolscine with and without cocaine was carried out at 16 Hz). Further studies are indicated to resolve this.

In some instances cocaine altered the ability of the blocking drugs to affect the responses. Thus rauwolscine had a greater blocking effect in plantaris vein and responses in saphenous vein could be blocked by prazosin and rauwolscine more readily in the presence of cocaine. This may be due to more effective autofeedback occurring in the absence of neuronal uptake. The overall effects of neuronal uptake blockade on the ability of antagonists to block responses might depend on a complex interaction between enhanced autofeedback of noradrenergic and purinergic nerves and enhanced postjunctional α_1- and α_2-adrenoceptor stimulation.

To summarise, the effects of blockade of α_2-adrenoceptors and uptake$_1$ are shown in Table 1. Blockade of α_2-adrenoceptor autofeedback increases the contributions of α_1-adrenoceptors and P_{2x}-purinoceptors to the response due to increased release of noradrenaline and ATP. In preparations where postjunctional α_2-adrenoceptors are present this contribution is removed by α_2-adrenoceptor blockade, making demonstration of autofeedback difficult. Blockade of uptake$_1$ increases the persistence of noradrenaline, allowing greater activation of postjunctional α_1- and α_2-adrenoceptors. The increased activation of postjunctional α_2-adrenoceptors is particularly marked, perhaps because of the putative extrajunctional location of α_2-adrenoceptors or perhaps because the α_2-adrenoceptor requires a more prolonged activation than is permitted by neuronal uptake. The increased persistence of noradrenaline after uptake$_1$ blockade also reduces neurotransmitter release through the autofeedback process. For noradrenaline the reduction in release is outweighed by the reduction in neuronal uptake but the reduction in ATP release means purinergic transmission is reduced both in absolute terms and in relation to noradrenergic transmission. In conclusion, neuronal uptake and autofeedback processes play important and complex interacting parts in determining the relative contributions of α_1- and α_2-adrenoceptors and P_{2x}-purinoceptors in the end organ response to neurotransmission in blood vessels.

Table 1 Effect of α_2-adrenoceptor blockade and uptake$_1$ blockade on the contributions from postjunctional α_1-adrenoceptors, α_2-adrenoceptors and purinoceptors to nerve-induced contractile responses in rabbit isolated blood vessels

Pharmacological intervention	*Change in contribution to response*		
	α_1-Adrenoceptor	*α_2-Adrenoceptor*	*P_{2x}-purinoceptor*
Blockade of α_2-adrenoceptor	+	−	+
Blockade of uptake$_1$	+	++	−

Changes in contribution to the response indicated as follows: +, increased contribution to the response; ++, greater increase; −, decreased contribution.

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The effects of dietary treatment with essential fatty acids on sciatic nerve conduction and activity of the Na^+/K^+ pump in streptozotocin-diabetic rats

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1 This study examined the effects of dietary essential fatty acid supplementation (5% (w/w) evening primrose oil) upon sciatic motor nerve conduction velocity and $^{86}Rb^+$ pumping in sciatic nerve endoneurial preparations in rats with 4 to 5 weeks of streptozotocin-induced diabetes.

2 Control diabetic rats (dietary supplementation with 5% (w/w) hydrogenated coconut oil) exhibited a reduction in motor nerve conduction velocity (16%; $P < 0.05$) compared to similarly-fed non-diabetic controls, but there was no significant alteration in ouabain-sensitive $^{86}Rb^+$ pumping, a parameter reflecting activity of the Na^+/K^+ pump.

3 Treatment of diabetic rats with evening primrose oil prevented completely the development of the motor nerve conduction velocity deficit without affecting the severity of diabetes. Evening primrose oil treatment did not significantly affect motor nerve conduction velocity of non-diabetic animals.

4 Evening primrose oil treatment caused a significant reduction in activity of the Na^+/K^+ pump in sciatic nerves of diabetic animals (45%; $P < 0.05$).

5 These results suggest that the acute conduction velocity defect arising in streptozotocin-diabetic rats, and the actions of evening primrose oil upon this, are independent of any effect on activity of the Na^+/K^+ pump. Other putative mechanisms are discussed.

Keywords: ATPase; Na^+/K^+; diabetes mellitus; diabetic neuropathy; essential fatty acids; gamma-linolenic acid; evening primrose oil; nerve conduction; sciatic nerve; streptozotocin; rat

Introduction

Nerve conduction velocity deficits are often found in newly diagnosed type I diabetics, despite normal neurological examination and absence of symptoms of neuropathy (Gregersen, 1968), but this conduction deficit may reflect a derangement contributing to the development of overt polyneuropathy in some patients much later. Measurement of nerve conduction velocity may therefore be useful as a prognostic indicator of future clinical neuropathy (Dyck *et al.*, 1985).

Rats with acute experimental diabetes also show a reduced motor nerve conduction velocity (MNCV). This deficit can be corrected in both man and rats by tight control of glycaemia with insulin (Gregersen, 1968; Mayer & Tomlinson, 1983a) and other treatments such as aldose reductase inhibitors (Tomlinson *et al.*, 1984) which all prevent depletion of nerve free *myo*-inositol levels. This association led to the assumption that maintenance of normal conduction velocity in diabetes is limited by the nerve *myo*-inositol level. Experimental evidence has also linked *myo*-inositol depletion to a deficit in peripheral nerve Na^+/K^+-ATPase activity (Greene & Lattimer, 1983; 1984). Therefore hypotheses developed proposing that *myo*-inositol deficits caused impaired phosphoinositide turnover, thereby restricting a proposed activation of Na^+/K^+-ATPase by protein kinase C. It was further suggested that a reduction of neuronal Na^+/K^+-ATPase activity could cause conduction block in selected neurones, ultimately producing a reduction in nerve conduction velocity (Greene *et al.*, 1988).

Human and experimental diabetes are both associated with defective essential fatty acid metabolism (Eck *et al.*, 1979; Poisson, 1985). Studies in humans and rats, have shown that treatment with evening primrose oil, a preparation rich in essential fatty acids, can reverse and prevent the development of deficits in diabetic nerve function without affecting the severity of diabetes (Jamal *et al.*, 1986; Julu, 1988; Hondo *et al.*, 1987). A recent study by Tomlinson *et al.* (1989) demonstrated attenuation of a conduction velocity deficit by treatment with evening primrose oil, even though nerve *myo*-inositol levels were not normalised. Also, the diabetic state does not appear to alter protein kinase C activity (Simpson & Hawthorne, 1988; Gabbay *et al.*, 1990). These latter two pieces of evidence cast doubt upon hypotheses linking *myo*-inositol depletion to MNCV deficits via the Na^+/K^+-ATPase. The present study was, therefore, designed to investigate the effect of evening primrose oil treatment upon MNCV and activity of the Na^+/K^+ pump in sciatic nerves of rats with acute streptozotocin-induced diabetes.

Methods

Experimental organization

Forty male Wistar rats (220–240 g; 15–16 weeks of age; Charles River (U.K.) Ltd.) were assigned, at random, to four experimental groups. They were maintained under standard housing conditions with food and water available *ad libitum*. A two week period of equilibration accustomed the animals to the powdered diet (RM No. 1; Special Diet Services Ltd., Essex, U.K.; 65% food: 35% tap water, w/v) and established a 'normal-for-age' weight gain.

Rats were then fasted overnight before the induction of diabetes in two groups by a single intraperitoneal injection of streptozotocin (Sigma; 50 mg kg^{-1}, freshly dissolved in sterile 0.9% w/v saline). The remaining control groups received a single, intraperitoneal injection of sterile 0.9% w/v saline. Two days later, blood samples were obtained by tail prick from the streptozotocin-treated rats and morning (unfasted) blood glucose concentration measured by strip-operated reflectance photometry (Reflotest; Boehringer, Mannheim, Germany). Animals with a morning blood glucose concentration of less than 10 mmol l^{-1} were given a second 50 mg kg^{-1} intraperitoneal injection of streptozotocin.

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Immediately after confirmation of diabetes, one pair of control and diabetic groups were provided with a diet containing evening primrose oil ('Efamol', Scotia Pharmaceuticals, Guildford, Surrey) to comprise 5% of the dry weight (w/w). The other pair of groups were fed a diet containing 5% hydrogenated coconut oil to provide a control for the exaggerated fat intake associated with addition of evening primrose oil to the diet. The fatty acid composition of the two oils is shown in Table 1. These treatments were maintained for the 5 week protocol. Daily food and water consumption was monitored throughout the course of the study and it was estimated that each animal received approximately 1.9 g of oil per day. Body weight was recorded at least once a week.

Four weeks after the induction of diabetes the animals were anaesthetized with halothane (2–4% in O_2) for measurement of distal motor latency (see below). Three days later, when any effects of the anaesthetic would have worn off, the animals were killed by a sharp blow to the head. A blood sample was taken from each animal for the subsequent determination of plasma glucose concentration with the spectrophotometric glucose assay (GOD-PERID method, Boehringer, Mannheim, Germany). Both sciatic nerves were removed. Nerve length between the two cathodal stimulating points was measured *ex vivo* to enable calculation of MNCV from the distal motor latency measured previously. Endoneurial preparations were prepared from both nerves and $^{86}Rb^+$ uptake measured as described below.

Nerve conduction velocity

MNCV was calculated from distal motor latency and nerve length in the sciatic/interosseus system (Mayer & Tomlinson, 1983b). Rats were anaesthetized with halothane (4% in O_2 for induction and 2–2.5% for maintenance) and a small incision made in the left rear flank to allow a fine thermocouple to be placed adjacent to the mid-femoral portion of the sciatic nerve. Nerve temperature was monitored with a digital electronic thermometer (Comark Electronics, Rustington, Sussex) and maintained at 37°C with an infra-red lamp. Motor nerves were stimulated with fine percutaneous unipolar electrodes at the sciatic notch and the Achilles tendon. Electromyograms (EMG) were recorded via fine percutaneous unipolar electrodes with a digital oscilloscope (Gould, type 1421). Stored EMGs were plotted in a time-expanded format on a Bryans XY recorder for latency measurement. Measurement of nerve length between the two stimulation points *ex vivo* allowed MNCV to be calculated.

$^{86}Rb^+$ uptake

The method for measurement of the activity of the Na^+/K^+ pump, via ouabain-sensitive $^{86}Rb^+$ uptake, was based on that described by Simpson & Hawthorne (1988). Before excision, two ligatures (surgical silk) were applied to each sciatic nerve at its origin and before its division into terminal branches. The nerves were incubated for 7 min at 37°C in 5 mg ml^{-1} collagenase (Sigma) dissolved in incubation medium (Krebs-Henseleit bicarbonate buffered saline containing 0.5 mM *myo*-inositol, 5 mM glucose, 3% (w/v) bovine serum albumin, pH 7.4) gassed with 95% O_2/5% CO_2 in a shaking water bath (Techne S8-16). The nerves were washed 3 times in 2 ml prewarmed buffer minus bovine serum albumin. Under low magnification the loosened epineurium was gently peeled back in a proximo-distal direction with fine watchmakers forceps without stretching the nerve. The major fascicle was isolated and the perineurium fenestrated by creating slits along its length with the forceps. More colour coded ligatures were applied and the nerve bisected to yield two endoneurial preparations from each sciatic nerve. Endoneurial preparations were preincubated for 20 min at 37°C in 2 ml incubation medium containing 1% dimethylsulphoxide gassed with 95%O_2/5%CO_2. Half of the preparations were incubated with 2.0 mM ouabain (Sigma) and these were exposed to the ouabain-containing medium 10 min before addition of $^{86}Rb^+$. The endoneurial preparations were then transferred to identical incubation media but in the presence of 0.25 μCi ml^{-1} [^{86}Rb] Cl (Amersham International, Amersham, Bucks) and 0.7 μCi ml^{-1} [^{3}H]-sucrose (Amersham International) used as an extracellular marker. They were gassed and incubated at 37°C, with gentle shaking, for 30 min. Pilot studies showed $^{86}Rb^+$ uptake, under these conditions and in the presence and absence of ouabain (2 mM), to be linear for at least 40 min. Uptake of $^{86}Rb^+$ was terminated by washing the endoneurial preparations in ice-cold 0.1 M $MgCl_2$. Ligatures were cut off and each nerve section placed in 300 μl of 2 M NaOH for hydrolysis at 85°C for 60 min. Hydrolysates were neutralised by the addition of 600 μl 1 M HCl. Triplicate 20 μl aliquots were taken for protein determination by the Folin-Lowry method (Lowry *et al.*, 1951); duplicate 350 μl aliquots were taken for liquid scintillation counting between 8 and 110 nm and 110 and 212 nm in a 1211 'Minibeta' liquid scintillation counter. Active $^{86}Rb^+$ pumping was estimated by subtracting the proportion due to equilibration with extracellular space estimated as [^{3}H]-sucrose content as a proportion of the [^{3}H]-sucrose concentration in the bathing medium. Results were standardised for protein content and ouabain-sensitive $^{86}Rb^+$ uptake calculated by subtracting $^{86}Rb^+$ uptake in the presence of ouabain from $^{86}Rb^+$ uptake in the absence of the inhibitor.

Table 1 Lipid composition of evening primrose oil and coconut oil; figures are percentage by volume of each lipid

Chain length: double bonds	*Evening primrose oil*	*Coconut oil*
10:0	—	6.5
12:0	—	50.6
14:0	—	19.8
16:0	5.9	9.7
18:0	1.5	2.7
18:1 (oleic)	8.1	7.1
18:2 Ω6 (linoleic)	74.2	2.6
18:3 Ω6 (linolenic)	9.5	—

Analysis and data from Scotia Pharmaceuticals.

Statistical analysis

All data are presented as arithmetic mean ± s.d. Statistical analysis of treatment effects was carried out by one way analysis of variance (ANOVA). Where the F ratio gave $P < 0.05$, and where there was homogeneity of variance (Cochrans & Bartlett Box, $P > 0.05$), comparisons between individual group means were made by Duncan's Multiple Range Test at significance levels of $P = 0.05$ and $P = 0.01$.

Results

Animals

After primary blood glucose screening, one of the streptozotocin-treated rats in the coconut oil-supplemented group showed sub-threshold hyperglycaemia and was therefore given a second 50 mg kg^{-1} dose of streptozotocin which produced adequate hyperglycaemia. The remainder were sufficiently diabetic. Mean blood glucose concentration of diabetic animals was 17.99 ± 2.98 mmol l^{-1} ($n = 20$) while control animals had a mean concentration of 3.14 ± 0.63 mmol l^{-1} ($n = 20$). One diabetic animal died five days after induction of diabetes. Throughout the course of the experiment, the body weight of control rats increased by about 3–3.5 g per rat daily whereas, at the end of the protocol, the body weight of the diabetic rats was slightly lower than the initial value (Table 2). The mean final plasma glucose concentration of both diabetic groups was significantly higher than control values ($P < 0.01$;

Table 2 Body weight changes, final plasma glucose concentration and motor nerve conduction velocity (MNCV) of control rats and rats with streptozotocin-induced diabetes of 4–5 weeks duration

Group (n)	*Body weight* (g) *Initial*	*Final*	*Final plasma glucose concentration* ($mmol\,l^{-1}$)	*MNCV* ($m\,s^{-1}$)
Control (10) (CO supplement)	295.20 ± 24.06	395.85 ± 28.27[a]	8.66 ± 1.76[a]	45.64 ± 5.09[c]
Diabetic (9) (CO supplement)	292.00 ± 13.50	265.06 ± 20.06[b]	19.51 ± 9.26[b]	38.14 ± 7.23[a]
Control (10) (EPO treated)	274.65 ± 12.27	398.90 ± 26.53[a]	7.42 ± 1.07[a]	48.76 ± 7.48[b]
Diabetic (10) (EPO treated)	280.65 ± 12.36	279.95 ± 35.85[b]	25.04 ± 12.53[b]	51.29 ± 7.53[b]

CO = coconut oil; EPO = Evening primrose oil. Data expresed as arithmetic mean ± s.d. for the number of animals given in parentheses. Significance of differences was assessed by one-way analysis of variance. [a:b] $P < 0.01$; [a:c] $P < 0.05$.

Table 2). The water intake of diabetic animals was, on average, 8 times higher than that of control animals. Treatment with evening primrose oil had no effect on the general severity of diabetes since animal body weight, final plasma glucose concentration and water intake were not significantly altered (Table 2).

Motor nerve conduction velocity

Diabetic animals that received a coconut oil supplement showed a significant reduction in MNCV when compared to control animals 4 weeks after confirmation of diabetes ($P < 0.05$; Table 2). This deficit in conduction velocity was absent in the group of diabetic rats treated with evening primrose oil ($P < 0.01$) while the MNCV of non-diabetic rats treated with evening primrose oil was not significantly affected.

$^{86}Rb^+$ pumping

At 4 to 5 weeks after the induction of diabetes there was no significant difference between total $^{86}Rb^+$ uptake in nerves from both coconut oil-supplemented groups (Table 3). However, the total $^{86}Rb^+$ uptake in nerves from diabetic animals treated with evening primose oil was significantly lower than both coconut oil-supplemented groups ($P < 0.01$) and there was a trend towards a decrease when compared to evening primrose oil-treated controls, although this failed to reach significance by one way ANOVA. There was also a possible trend towards a decrease in the total $^{86}Rb^+$ uptake of evening primrose oil-treated controls when compared to both coconut oil-supplemented groups although, again, this failed to reach significance. These changes in total $^{86}Rb^+$ uptake appeared to be caused entirely by changes in ouabain-sensitive $^{86}Rb^+$ uptake, a parameter that reflects activity of the Na^+/K^+ pump, since there was no significant difference in ouabain-insensitive $^{86}Rb^+$ uptake between any groups. Ouabain-sensitive $^{86}Rb^+$ uptake ($^{86}Rb^+$ pumping) in nerves of both control groups and the coconut oil-supplemented diabetic animals was comparable. The ouabain-sensitive $^{86}Rb^+$ pumping of diabetic rats treated with evening primrose oil was, however significantly lower than both coconut oil-supplemented groups ($P < 0.05$).

Discussion

The treatment of rats with streptozotocin caused weight loss, polydipsia and hyperglycaemia; features characteristic of type I diabetes mellitus. There was, however, substantial variation in the final plasma glucose concentration of the diabetic animals. Animals with low plasma glucose concentrations at the end of the experiment were therefore excluded only if long term indicators of diabetes such as weight loss, increased water intake and an increase in urine volume were also diminished or absent.

Diabetic rats showed a deficit in MNCV after 4 weeks of streptozotocin-induced diabetes in accord with previous findings (Greene *et al.*, 1975; Mayer & Tomlinson, 1983b; Tomlinson *et al.*, 1984; 1989). It has been shown that this peripheral nerve defect can be reversed by fine control of glycaemia with insulin (Greene *et al.*, 1975) suggesting an aetiology linked to the diabetes generated by streptozotocin rather than to some other unrelated effect. Treatment with

Table 3 Uptake of $^{86}Rb^+$ in sciatic nerve endoneurial preparations from control rats and rats with streptozotocin-induced diabetes of 4–5 weeks duration

Group	*$^{86}Rb^+$ pumping* (c.p.m. $h^{-1}\,mg^{-1}$ protein) *Total*	*Ouabain-insensitive*	*Ouabain-sensitive*
Control (CO supplement)	21,667.2 ± 4507.8[a] (10)	8921.3 ± 1565.5 (9)	13,406.6 ± 4726.9[c] (9)
Diabetic (CO supplement)	22,010.7 ± 5965.3[a] (9)	7794.8 ± 1526.2 (9)	14,215.9 ± 5817.2[c] (9)
Control (EPO treated)	19,128.1 ± 4783.3 (10)	7098.1 ± 2589.2 (9)	12,076.0 ± 4672.7 (9)
Diabetic (EPO treated)	15,214.5 ± 3808.6[b] (9)	7300.3 ± 2094.1 (10)	7792.3 ± 2977.2[d] (9)

CO = coconut oil; EPO = Evening primrose oil. Data expressed as arithmetic mean ± 1 s.d. for the number of animals given in parentheses. Significance of differences was assessed by one-way ANOVA. [a:b] $P < 0.01$; [c:d] $P < 0.05$.

evening primrose oil prevented completely the development of this MNCV deficit in diabetic animals but had no effect on control values confirming previous findings (Julu, 1988; Tomlinson *et al.*, 1989).

The novel finding of this study was a complete dissociation of the MNCV deficit from any putative reduction in activity of the Na^+/K^+ pump. The activity of the Na^+/K^+ pump of untreated diabetic animals was not significantly different from control values despite a significant deficit in MNCV. In addition, treatment with evening primrose oil, which prevented the development of a MNCV deficit in diabetic animals was also associated with a significant reduction in activity of the Na^+/K^+ pump. These results are not consistent with the hypothesis that the acute MNCV defect arising in streptozotocin-diabetic rats is caused by impaired Na^+/K^+-ATPase activity (Greene *et al.*, 1988). Indeed, at first sight one should consider the prospect that reduced Na^+/K^+ pumping in the diabetic rats treated with evening primrose oil might explain the correction of the MNCV deficit associated with this treatment. Reduced Na^+/K^+ pumping might give a slight steady-state depolarization, possibly making the membrane more excitable and speeding nodal depolarization. However, the other effect of reduced Na^+/K^+ pumping would be to make the inward Na^+ gradient more shallow, which might have the opposite effect. In any case, increasing conduction velocity via reduced Na^+/K^+ pumping could not represent direct reversal of a diabetes-associated defect in the functioning of the Na^+/K^+-ATPase, because its activity was unaltered in the control diabetic preparations. Neither does there appear to be functional antagonism between diabetes and treatment via different mechanisms, because evening primrose oil did not inhibit the pump in the nerves of non-diabetic rats. Thus, there appears to be no clear relationship between the effect of evening primrose oil on the Na^+/K^+ pump and its effect on MNCV.

This is not the first demonstration of the independent nature of these two measurements. Other studies have also found a MNCV deficit to have developed without any apparent reduction in parameters of the Na^+/K^+ pump; although others have measured membrane Na^+/K^+-ATPase activity, rather than pumping of ions *per se* (Lambourne *et al.*, 1988). However, there is no contention that, at longer durations of diabetes, a deficit in Na^+/K^+-ATPase activity does develop; but under these conditions the Na^+/K^+-ATPase deficit is prevented by gangliosides (Calcutt *et al.*, 1988), whilst the MNCV deficit is not. Furthermore, the Na^+/K^+-ATPase deficit is resistant to aldose reductase inhibitors, when the MNCV deficit is prevented completely (Lambourne *et al.*, 1988). Lastly, administration of a galactose-supplemented diet to mice is associated with a deficit in MNCV which co-exists with increased Na^+/K^+-ATPase activity (Calcutt *et al.*, 1990).

If, as this study suggests, the diabetes-induced decrease in MNCV does not derive from reduced activity of the Na^+/K^+ pump, then another link must be found between diabetes and conduction velocity. Very little is known about the cause of defective MNCV in diabetes and there are no obvious putative mechanisms.

Anterograde axonal transport, the process that moves macromolecules and organelles manufactured in the neuronal cell body, to their sites of use in the periphery has been reported to be abnormal in diabetic rats (Schmidt *et al.*, 1975). Amino acid uptake and protein synthesis have also been shown to be reduced in the dorsal root ganglia of diabetic rats (Thomas *et al.*, 1984). A conduction deficit could arise because of a deficient delivery of voltage-sensitive Na^+ channels (Lombet *et al.*, 1985) which are vitally important for both depolarization and repolarization of the nerve membrane during the action potential. A large decrease in the number of voltage-sensitive Na^+ channels could, in theory, prolong the action potential and hence slow nerve conduction. Voltage-sensitive Na^+ channels are carried by fast anterograde axonal transport (Lombet *et al.*, 1985). Neurochemical insults arising as a consequence of diabetes may impair this transport (Meiri & McLean, 1982) or reduce cell body synthesis of the molecule as has been found for a similarly transported neuropeptide, substance P (Robinson *et al.*, 1987). Impaired Na^+ channel delivery could generate relatively brisk functional consequences, although this theory is purely speculative.

Evening primrose oil is rich in the essential fatty acids oleic, linoleic and gamma-linolenic acid (Manku, 1983). The biological activity of this treatment is probably not due solely to the presence of a single active constituent, but may depend on its total composition and triglyceride configuration (Myher *et al.*, 1977). Treatment with evening primrose oil probably enhances the synthesis of arachidonic acid by the conversion of linoleic acid through gamma-linolenic and dihomo-gamma-linolenic acids. This could increase the synthesis of inositol containing phospholipids in peripheral nerve (Simmons *et al.*, 1982) enhancing processes dependent on their second messenger function. The interaction of such effects with nerve conduction remains to be elucidated but the results of this study suggest that protection of the Na^+/K^+ pump activity is unlikely to be involved.

Another potential mechanism of action involves an increased prostaglandin E_1 production from gamma-linolenic acid (Horrobin *et al.*, 1984). This has been established in mesenteric vascular tissue (Watanabe *et al.*, 1987) although it remains to be demonstrated in peripheral nerve. Prostaglandin E_1 seems to have an important role in the maintenance of normal neuronal function. Blockade of prostaglandin E_1 synthesis is associated with a reduction in nerve action potential amplitude in the rat, and this abnormality could be corrected by prostaglandin E_1 administration (Horrobin *et al.*, 1977). Recent studies have shown that a structural analogue of prostaglandin E_1 can prevent the acute deficit in MNCV when administered to streptozotocin diabetic rats (Yasuda *et al.*, 1988; 1989). Prostaglandin E_1 and/or derived secondary mediators, might directly modulate the biochemical events increasing nerve conduction or might improve the endoneurial microcirculation by dilating arterioles and inhibiting platelet aggregation (Gorman, 1978) hence preventing the development of any MNCV deficit related to ischaemic endoneurial hypoxia (Tuck *et al.*, 1984; Dyck, 1989). An examination of the effect of drugs inhibiting prostaglandin biosynthesis on the capacity of evening primrose oil to prevent development of the nerve conduction deficit in diabetic rats will be of enormous value.

Polyunsaturated fatty acids are also key structural components of cell membranes and several important membrane-bound enzymes (Horrobin, 1988). Disturbances in essential fatty acid content and abnormality of their incorporation into myelin and axons have been shown in streptozotocin-diabetic rats (Spritz *et al.*, 1975; Lin *et al.*, 1985). It is possible that essential fatty acid dietary supplementation could restore normal neuronal membrane structure and function. One could speculate that an enhanced incorporation of essential fatty acids into the membrane lipids, as a result of their dietary supplementation, may act to inhibit the Na^+/K^+-ATPase of diabetic animals. Unpublished data from this laboratory have demonstrated that essential fatty acids cause a marked inhibition of activity of the Na^+/K^+ pump in crude sciatic nerve homogenates in accord with results from other *in vitro* systems (Ahmed & Thomas, 1971; Swann, 1984; Kelly *et al.*, 1986; Swarts *et al.*, 1990). Control rats appeared to be resistant, therefore diabetic rats may have a specific defect of their peripheral nerve making the Na^+/K^+-ATPase susceptible to the inhibitory effects of dietary essential fatty acids.

The results of this study are not consistent with the hypothesis that impaired Na^+/K^+-ATPase activity underlies the functional MNCV deficit in diabetes (Greene *et al.*, 1988). Correction of this deficit with evening primrose oil indicates that a deficiency of essential fatty acids and/or their derivatives may be connected with the cause of this defect, either directly or indirectly. Further work is however necessary to elucidate the intermediaries linking essential fatty acids with peripheral nerve conduction.

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Differential inhibitory effects of opioids on cigarette smoke, capsaicin and electrically-induced goblet cell secretion in guinea-pig trachea

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1 Goblet cell secretion in guinea-pig airways is under neural control. Opioids have previously been shown to inhibit neurogenic plasma exudation and bronchoconstriction in guinea-pig airways. We have now examined the effects of morphine and opioid peptides on tracheal goblet cell secretion induced by either electrical stimulation of the cervical vagus nerves, exogenous capsaicin, or acute inhalation of cigarette smoke. The degree of goblet cell secretion was determined by a morphometric method and expressed as a mucus score which is inversely related to mucus discharge.

2 Morphine, 1 mg kg^{-1}, completely blocked goblet cell secretion induced by electrical stimulation of the vagus nerves. Morphine also inhibited the response to cigarette smoke given either at a low dose (10 breaths of 1:10 diluted in air), which principally activates cholinergic nerves, or at a high dose (20 breaths of undiluted), which activates capsaicin-sensitive sensory nerves, by 100% and 73% respectively. In contrast, morphine had no significant inhibitory effect on capsaicin-induced goblet cell secretion. The inhibitory effect of morphine was reversed by naloxone.

3 Selective μ- or δ-opioid receptor agonists, [D-Ala^2, NMePhe^4, Glyol^5]enkephalin (DAMGO) or [D-Pen^2, D-Pen^5]enkephalin (DPDPE) respectively, caused a dose-related inhibition of low dose cigarette smoke-induced goblet cell discharge, with DPDPE more potent than DAMGO. A κ-receptor agonist, *trans*-3,4-dichloro-*N*-methyl-*N*-(2-(1-pyrollidinyl)cyclohexyl) benzeneacetamine (U-50,488H), had no inhibitory effect. DPDPE had no inhibitory effect on goblet cell secretion induced by exogenous methacholine.

4 DAMGO dose-dependently blocked the response to high dose cigarette smoke with a maximal inhibition of 95% at 2×10^{-7} mol kg^{-1}. Neither DPDPE nor U-50,488H had any significant inhibitory effect. The increase in goblet cell secretion induced by exogenous substance P was not affected by DAMGO.

5 We conclude that opioids inhibit neurally-mediated goblet cell secretion via actions at prejunctional δ- and μ-receptors on cholinergic nerves and at μ-receptors on sensory nerve endings, and that capsaicin activation of sensory nerves is via a different mechanism from that of electrical or cigarette smoke activation.

Keywords: Morphine; opioid; mucus secretion; goblet cell; cigarette smoke; capsaicin; μ- and δ-opioid receptor

Introduction

Morphine and opioid peptides inhibit neurally-mediated broncho-constriction (Frossard & Barnes, 1987; Bartho *et al.*, 1987; Belvisi *et al.*, 1988) and plasma exudation (Belvisi *et al.*, 1989) in guinea-pig airways, and mucus secretion in human bronchi (Rogers & Barnes, 1989) by blocking the release of tachykinins from capsaicin-sensitive sensory nerves. Opioids have also been shown to inhibit the cholinergic component of airway excitation in guinea-pigs (Johansson *et al.*, 1989; Belvisi *et al.*, 1990). Recently we have demonstrated that airway goblet cells are under neural control. Electrical stimulation of the cervical vagus nerves provokes a significant increase in goblet cell secretion partly via activation of cholinergic nerves and partly via capsaicin-sensitive sensory nerves (Tokuyama *et al.*, 1990). Acute injection of capsaicin, which activates sensory nerve endings to release tachykinins, was also demonstrated to cause goblet cell discharge (Kuo *et al.*, 1990a). Therefore, morphine and opioid peptides may have inhibitory effects on neurally-mediated airway goblet cell secretion.

The inhibitory effect of morphine or opioid peptides is inversely related to the frequency of stimulation used (Paton, 1957; Russell & Simons, 1985; Duckles & Budal, 1990). Because autonomic neurones generally discharge at relatively low frequencies *in vivo*, the inhibitory effect of opioids on neurally-mediated responses to more 'physiological' stimulation intensities might be different from those elicited by experimental stimulation. We recently demonstrated that acute inhalation of cigarette smoke, at concentrations giving plasma nicotine levels in the range reported for human smokers, stimulated neurally-mediated goblet cell discharge: 10 breaths of 1:10 diluted cigarette smoke (reported as low dose) activates predominantly parasympathetic cholinergic nerves (Kuo *et al.*, 1990b), whereas 20 breaths of undiluted cigarette smoke (reported as high dose) selectively activates capsaicin-sensitive sensory nerves (Kuo *et al.*, 1991). Thus, investigation of the modulatory effect of opioids on cigarette smoke-induced goblet cell secretion may provide an understanding of potentially important mechanisms under 'physiological' conditions of stimulation. The purpose of the present study was to examine the possible effect of opioids on goblet cell secretion in response to electrical stimulation of the vagus nerves, acute injection of capsaicin or acute inhalation of cigarette smoke in guinea-pig trachea *in vivo*, using morphine or the opioid agonists [D-Ala^2, NMePhe^4, Gly-ol^5]enkephalin (DAMGO), [D-Pen^2, D-Pen^5]enkephalin (DPDPE) or *trans*-3,4-dichloro-*N*-methyl-*N*-(2-(1-pyrollidinyl)cyclohexyl) benzeneacetamine (U-50,488H), which are selective at μ, δ or κ-opioid receptors respectively.

Methods

Male Dunkin-Hartley outbred guinea-pigs (256–450 g body wt, Charles River, Kent) anaesthetized with urethane (2 g kg^{-1}, i.p.), were laid supine on a heated blanket which maintained body temperature (rectal) at 37°C. The guinea-pigs

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were ventilated with a tracheal cannula and a constant-volume ventilator (Harvard Apparatus, MA, U.S.A.) at a tidal volume of 10 ml kg^{-1} and a frequency of 60 breaths min^{-1}. Pulmonary insufflation pressure, a measure of airway constriction, was determined via a side arm connected to the inspiratory limb of the ventilator tubing and connected to a differential pressure transducer (Franell Electronic Components Ltd., Leeds). To monitor the physiological condition of the animals and to establish drug activity, blood pressure and heart rate in the left carotid artery were recorded on a two-channel recorder (Devices, Ormed Ltd., Welwyn Garden City, U.K.) via an indwelling Portex cannula filled with heparin-saline (10 u ml^{-1}) linked to a pressure transducer (Bell and Howell, Basingstoke, Hants.).

Vagal nerve stimulation

Both cervical vagus nerves were carefully dissected free and sectioned (to avoid stimulating the central nervous system) at the level of the fifth tracheal cartilage ring. Their caudal ends were placed across bipolar platinum electrodes and were electrically stimulated (10 Hz, 5 ms, 5 V, for 3 min) by square-wave pulses (model S88 stimulator, Grass Instruments, Quincy, MA, U.S.A.). The effect of dissection and manipulation on goblet cell discharge was determined by repeating the above but without electrical stimulation (sham stimulation).

Capsaicin pretreatment

Aminophylline (25 mg kg^{-1}, i.p.) and terbutaline (0.1 mg kg^{-1}, s.c.) were injected 30 min before capsaicin administration to alleviate the bronchoconstriction. Guinea-pigs were anaesthetized with ketamine (50 mg kg^{-1}, i.m.) and xylazine (0.1 mg kg^{-1}, i.m.). Capsaicin (50 mg kg^{-1}, s.c.) was injected, and animals were studied 1 week later (Lundberg *et al.*, 1983). In these animals, the completeness of sensory denervation was determined by measuring bronchoconstriction in the presence of hexamethonium during experimentation. In vehicle pretreated animals, vagal stimulation increased airway pressure by $130 \pm 46\%$ ($n = 5$), whereas in capsaicin pretreated animals, the increase was only $2 \pm 2\%$ ($n = 5$), which indicates that capsaicin-pretreatment was completely effective.

Cigarette smoke exposure

Cigarettes were lit in a fume cupboard where a constant laminar flow prevented smoke accumulation. After the first quarter of the cigarette had burned, cigarette smoke was drawn through the cigarette into a 60 ml polypropylene syringe in less than 2 s. The cigarettes used were U.K. government category 'Middle Tar' (nicotine content 1.2 mg per cigarette; carbon monoxide content 11 mg per cigarette) which were commercially available and unfiltered. Two doses of cigarette smoke were used: 10 breaths diluted 1:10 in air (low dose) or 20 breaths of undiluted smoke (high dose). Diluted smoke was generated by expelling all but 6 ml smoke from the syringe and diluting to 60 ml in room air. Cigarette smoke was introduced into the animal's trachea and lungs via a 3-way tap just rostral to the endotracheal tube. Five or six ventilated breaths of room air were given after each tidal volume cigarette smoke. Control animals underwent the same procedure except air drawn through an unlit cigarette was used.

Protocol

To confirm that cholinergic nerves and capsaicin-sensitive sensory nerves were activated by electrical stimulation of the vagus nerves in the present batches of animals, hexamethonium, 5 mg kg^{-1}, was given 10 min before stimulation to establish that blocking ganglionic transmission to leave antidromic transmission had a significant but partial inhibitory effect on vagally-induced goblet cell secretion. The effectiveness of hexamethonium blockade was checked by determining its effect on nerve stimulation-induced changes in heart rate, blood pressure and airway pressure. Without hexamethonium, vagal stimulation caused mean heart rate to fall from 168 ± 8 ($n = 5$) to 82 ± 7 beats min^{-1}, decreased mean blood pressure by $55 \pm 5\%$ ($n = 5$) and increased airway pressure by $196 \pm 27\%$ ($n = 5$). Hexamethonium pretreatment significantly ($P < 0.05$) inhibited the reduction in heart rate (146 ± 7, $n = 5$, beats min^{-1} before stimulation; 134 ± 6 after stimulation), reduced the decrease in blood pressure to $6 \pm 4\%$ ($n = 5$) and the increase in airway pressure to $122 \pm 9\%$ ($n = 5$). The latter response was totally blocked by capsaicin pretreatment, increasing by $2 \pm 2\%$ ($n = 5$). This indicates that ganglionic blockade was virtually complete.

Morphine (1 mg kg^{-1}, i.v.) was administered 2 min before vagal stimulation, cigarette smoke exposure or exogenous capsaicin (1 $\mu g\,kg^{-1}$). The selective opioid receptor agonists, DAMGO (10^{-9}–2×10^{-7} mol kg^{-1}), DPDPE (10^{-9}–2×10^{-7} mol kg^{-1}) or U-50,488H (2×10^{-7} mol kg^{-1}), were studied in animals given cigarette smoke and were administered 2 min before exposure. The effect of the selective opioid receptor agonists on secretion induced by exogenous substance P (SP, 10^{-11} mol kg^{-1}, i.v.) or methacholine (10^{-13} mol kg^{-1}, i.v.) was also determined in two groups of animals. The dose of methacholine chosen gave an increase in goblet cell discharge which matched the response to low dose cigarette smoke. Naloxone (1 mg kg^{-1}) was injected 8 min before morphine in some separate studies to see whether reversal of the opioid effect was possible. Naloxone was also injected alone in other experiments to determine whether endogenous opioids had an inhibitory effect on goblet cell secretion in response to cigarette smoke exposure. The vehicle for these drugs (saline) was given at the same time points in control animals.

Tissue preparation

The methods used for tissue preparation and quantification of goblet cell discharge have been described in detail previously (Tokuyama *et al.*, 1990; Kuo *et al.*, 1990) and are outlined here. One minute after vagal nerve stimulation, or 15 min after cigarette smoke exposure or drug administration, the lungs were inflated by injecting 10% formal saline (approximately 10 ml) through the upper trachea until the lungs were fully expanded. The systemic circulation was perfused with 10% formal saline by incising the left ventricle, inserting a blunt-ended needle into the aorta, cross clamping the ventricles, and expelling blood via the incised right atrium at 100 mmHg pressure until the perfusate was clear. The trachea and lungs were removed, attached with thread to card to preserve their shape and orientation and fixed for at least 24 h in 10% formalin. Three micrometer thick sections of the trachea were cut in the coronal plane and stained with Alcian blue and periodic acid-Schiff (AB pH 2.5/PAS) in sequence to demonstrate the acidic and neutral intracellular glycoprotein of the secretory cells. Slides were coded to avoid observer bias during quantification and observed at a magnification of $\times 400$ with an Axioplan microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Plan-neofluar 40–0.75 objective lens. Sections with unequally sized cartilage in the two walls of the trachea or apparent thickening of the epithelium indicated oblique sectioning and were discarded before analysis.

Quantification of goblet cell discharge

The intracellular mucin of the epithelial secretory cells appeared as purple coloured oval discs which were graded according to size: Grade 1, the vertical distance of the stained area was within 1/3 of the epithelial layer, measured from basement membrane to cell apices. Grade 2: the vertical distance of the stained area exceeded 1/3 of the epithelial layer. Stained areas were graded in 20 consecutive high power fields

(HPF) along both sides of the lower airways (a total of 40 HPF), starting at the carina and moving cranially. A mucus score (MS) was calculated for each animal as $n_1 + 2n_2$, where n_1 and n_2 were the number of cells in each grade respectively. Thus, MS was inversely related to degree of goblet cell discharge: the lower the score, the greater the degree of discharge and *vice versa*. Stained areas which were agranular or had ill-defined boundaries were not included in the counts.

Drugs

Drugs and chemicals were obtained from the following sources: SP, capsaicin, Tween 80, urethane, methacholine chloride, and hexamethonium chloride (Sigma Chemical Co., Poole, Dorset); atropine sulphate BP (Phoenix Pharmaceuticals Ltd., Oxford); naloxone hydrochloride (DuPont U.K., Hertfordshire); morphine sulphate (May and Baker, Dagenham, Essex); DAMGO, DPDPE (Bachem Feincheikalien AG, Bubendorf, Switzerland); U-50,488 (The Upjohn Company, Kalamazoo, U.S.A.); heparin injection BP (CP Pharmaceuticals Ltd., Wrexham, Wales); aminophylline (Antigen, Roscrea, Ireland); terbutaline (Astra Pharmaceuticals, Kings Langley, U.K.); xylazine (Bayer, Bury St Edmunds, U.K.). Capsaicin for pretreatment was prepared at a concentration of $50\,mg\,kg^{-1}$ in 10% ethanol and 10% Tween 80 vol/vol in saline. Capsaicin for acute injection was dissolved in absolute ethanol at a concentration of $1\,g\,ml^{-1}$, then diluted to $1\,mg\,ml^{-1}$ in saline before experimentation. All other drug solutions were freshly prepared on each day of experimentation in saline or vehicle.

Statistical analysis

Data did not approximate a Gaussian distribution and the probability of differences between groups was initially assessed by Kruskal-Wallace analysis followed by the Mann-Whitney U-test (two-tailed) to determine the significance of differences in MS between groups. Data in Results are mean ± s.e.mean. The null hypothesis was rejected at $P < 0.05$.

Results

Effect of morphine on goblet cell secretion induced by electrical stimulation of the vagus nerves

Bilateral cervical vagus nerve stimulation produced a significant ($P < 0.01$) decrease of 45% in MS (indicative of secretion) compared with sham stimulated controls (Figure 1). Pretreatment with hexamethonium ($5\,mg\,kg^{-1}$) partially and significantly inhibited the response to vagal stimulation (Figure 1). The hexamethonium-resistant part of the response was significantly ($P < 0.02$) different both from hexamethonium with sham stimulation and from vagal stimulation alone (Figure 1). Capsaisin pretreatment significantly blocked the hexamethonium-resistant part of the response compared with vehicle pretreated controls (Figure 1). Morphine, $1\,mg\,kg^{-1}$, completely ($P < 0.01$) inhibited the response to vagal stimulation (Figure 1). The inhibitory effect of morphine was significantly ($P < 0.05$) reversed by naloxone ($1\,mg\,kg^{-1}$) (Figure 1).

Effect of morphine on responses to cigarette smoke-induced goblet cell secretion

Acute inhalation of either low dose of cigarette smoke or high dose cigarette smoke caused a significant ($P < 0.01$) reduction in MS of 55% and 44% respectively compared with corresponding sham air controls (Figure 2). Morphine significantly ($P < 0.01$) inhibited the response to either low or high dose cigarette smoke by 100% and 73% respectively (Figure 2).

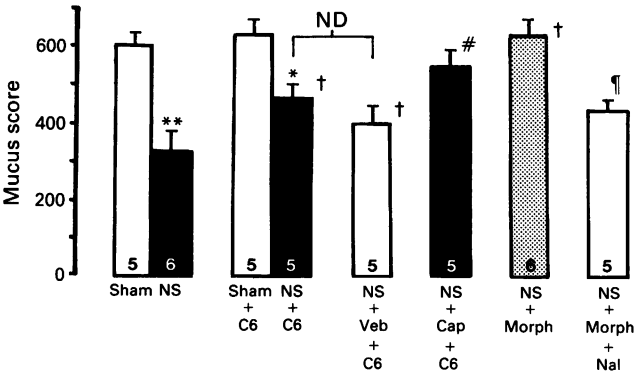

Figure 1 Effects of hexamethonium (C6, $5\,mg\,kg^{-1}$, i.v.), alone or after pretreatment with capsaicin (Cap.) or its vehicle (Veh.), as well as morphine (Morph., $1\,mg\,kg^{-1}$ i.v.) in the presence or absence of naloxone (Nal, $1\,mg\,kg^{-1}$, i.v.) on electrical stimulation of the vagus nerves (NS)-induced decrease in mucus score in guinea-pig trachea. Data are mean mucus score (bar = s.e.mean) for numbers of animals indicated in the histograms. * $P < 0.05$, ** $P < 0.01$ compared with corresponding sham stimulation (Sham). † $P < 0.05$ compared with NS alone; # $P < 0.05$ compared with vehicle control; ¶ $P < 0.05$ compared with NS + Morph. ND: no significant difference.

Naloxone reversed both inhibitory effects of morphine (Figure 2). Naloxone alone given 10 min before either dose of cigarette smoke exposure failed to influence the responses without naloxone (Figure 2).

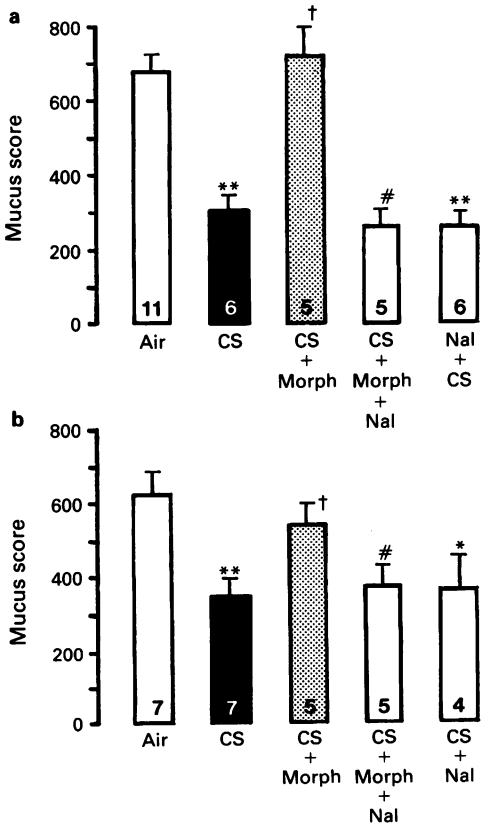

Figure 2 Effect of morphine (Morph, $1\,mg\,kg^{-1}$, i.v.) on (a) low dose or (b) high dose cigarette smoke (CS)-induced decrease in mucus score in guinea-pig trachea in presence or absence of naloxone (Nal, $1\,mg\,kg^{-1}$, i.v.). Data are mean mucus scores (bars = s.e.mean) for numbers of animals indicated in the histograms. ** $P < 0.01$, * $P < 0.05$ compared with air control (Air); † $P < 0.05$ compared with CS alone; # $P < 0.05$ compared with morphine-treated group.

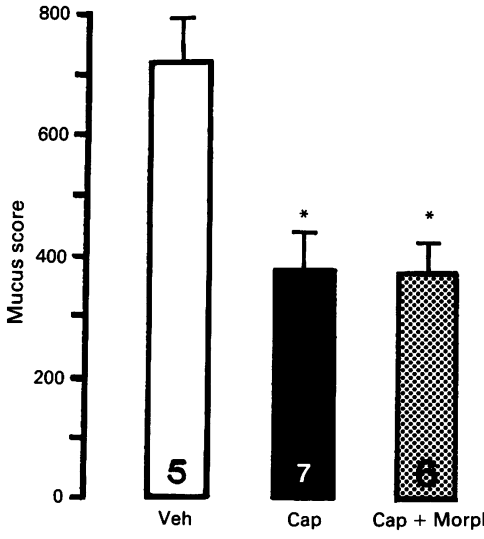

Figure 3 Effect of morphine (Morph, 1 mg kg^{-1}, i.v.) on capsaicin (Cap, 1 μg kg^{-1})-induced goblet cell secretion in guinea-pig trachea. Data are mean mucus scores (bars = s.e.mean) for numbers of animals indicated in the histograms. * $P < 0.05$ compared with vehicle control (Veh).

Effect of morphine on exogenous capsaicin-induced goblet cell secretion

Acute injection of capsaicin (1 μg kg^{-1}, i.v.) caused a significant ($P < 0.01$) decrease in MS of 48% compared with vehicle controls (Figure 3). Morphine did not alter the response to capsaicin (Figure 3).

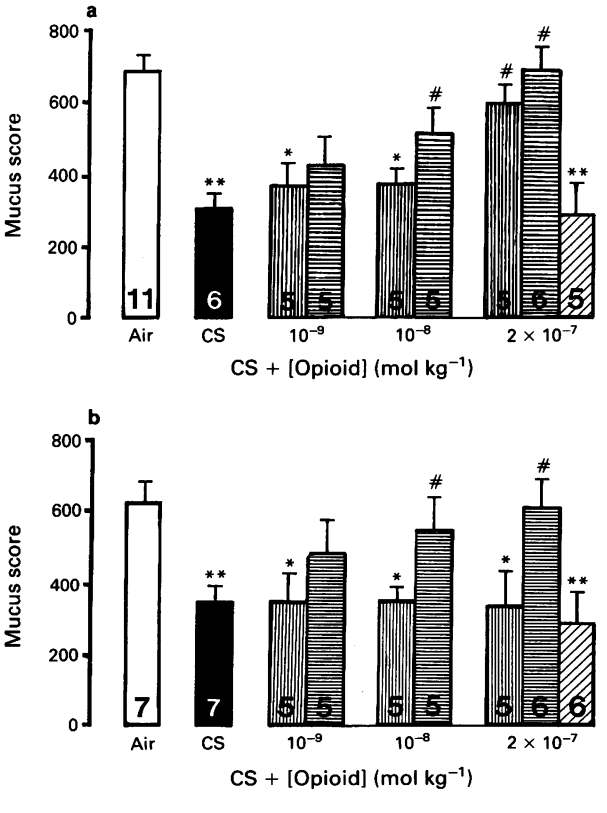

Figure 4 Effect of DPDPE (▥), DAMGO (▤) or U-50,488H (▨) on goblet cell secretion induced by (a) low dose or (b) high dose cigarette smoke (CS) in guinea-pig trachea. Data are mean mucus scores (bars = s.e.mean) for numbers of animals indicated in the histograms. ** $P < 0.01$, * $P < 0.05$ compared with air control (Air); # $P < 0.05$ compared with CS alone.

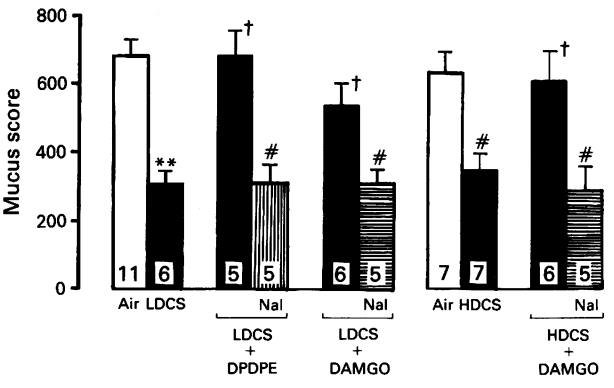

Figure 5 Effect of naloxone (Nal, 1 mg kg^{-1}) on the inhibitory effects of DPDPE or DAMGO (2 × 10^{-7} mol kg^{-1} respectively) on goblet cell secretion induced by low dose cigarette smoke (LDCS), or the effect of DAMGO on the response to high dose cigarette smoke (HDCS). Data are mean mucus scores (bars = s.e.mean) for numbers of animals indicated in the histograms. ** $P < 0.01$ compared with corresponding air controls (Air). † $P < 0.05$ compared with corresponding cigarette smoke alone groups. # $P < 0.05$ compared with corresponding DPDPE or DAMGO-treated groups.

Morphine and naloxone alone had no significant effect on mucus score in vehicle controls (635 ± 71, $n = 5$; 542 ± 30, $n = 6$, respectively).

Effect of selective opioid receptor agonists on responses to cigarette smoke

The δ-opioid receptor agonist, DPDPE (10^{-9}–2 × 10^{-7} mol kg^{-1}, $n = 5$–6) or the μ-selective agonist DAMGO (10^{-9}–2 × 10^{-7} mol kg^{-1}, $n = 5$–6) produced dose-related inhibitions of the secretory response to low dose cigarette smoke (Figure 4a) with maximal inhibitions of 100% and 75% at 2 × 10^{-7} mol kg^{-1} of each agonist respectively. The κ-selective agonist, U-50,488H, had no significant inhibitory effect on the response (Figure 4a). DAMGO also caused a dose-dependent inhibition of the response to high dose cigarette smoke with a maximal inhibition of 95% at 2 × 10^{-7} mol kg^{-1} (Figure 4b). In contrast, DPDPE had no significant effect on the response to high dose cigarette smoke (Figure 4b) neither did U-50,488H have any significant effect (Figure 4b). The inhibitory effects of DAMGO or DPDPE on the responses to either dose of cigarette smoke were completely reversed by naloxone (Figure 5).

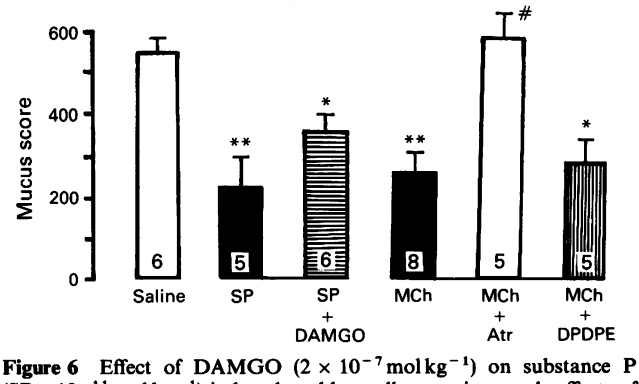

Figure 6 Effect of DAMGO (2 × 10^{-7} mol kg^{-1}) on substance P (SP; 10^{-11} mol kg^{-1})-induced goblet cell secretion and effect of DPDPE (2 × 10^{-7} mol kg^{-1}) or atropine (Atr, 0.1 mg kg^{-1}) on methacholine (MCh, 10^{-13} mol kg^{-1})-induced goblet cell discharge in guinea-pig trachea. Data are mean mucus scores (bars = s.e.mean) for numbers of animals indicated in the histograms. ** $P < 0.01$, * $P < 0.05$ compared with saline control (saline); # $P < 0.05$ compared with MCh alone.

Effect of opioids on the response to exogenous methacholine and substance P

Exogenous methacholine (10^{-13} mol kg^{-1}) caused a similar degree of goblet cell discharge as low dose cigarette smoke in decreasing MS by 51% compared with saline controls. The effect of methacholine was inhibited completely by atropine (0.1 mg kg^{-1}, MS = 585 ± 56, $n = 5$, $P < 0.01$). Substance P (10^{-11} mol kg^{-1}) also caused a significant ($P < 0.05$) reduction in MS of 59% compared with saline controls. The response to methacholine was not significantly affected by DPDPE 2×10^{-7} mol kg^{-1} (Figure 6) neither was the response to SP inhibited by DAMGO 2×10^{-7} mol kg^{-1} (Figure 6).

Discussion

We have demonstrated in the present study that morphine inhibits the secretory responses of tracheal goblet cells to vagal stimulation and acute inhalation of cigarette smoke in guinea-pigs. The inhibitory effects of morphine were significantly reversed by naloxone suggesting that the inhibitory effects were mediated via opioid receptors. Our present study showed that vagal stimulation-induced goblet cell secretion was partly inhibited by the ganglionic blocker hexamethonium with the remaining hexamethonium-resistant part of the response inhibited by capsaicin pretreatment, which suggests that capsaicin-sensitive sensory nerves as well as cholinergic or adrenergic nerves were involved in the secretory response. These results are consistent with our previous study which showed that electrical stimulation of the cervical vagus nerves provokes airway goblet cell discharge by activating cholinergic and capsaicin-sensitive sensory nerves (Tokuyama *et al.*, 1990). Therefore, morphine might have inhibitory effect on both components.

The failure of morphine to block the acute effect of capsaicin on goblet cell discharge suggests that the mechanism underlying capsaicin-induced activation of sensory nerves might be different from that of vagal stimulation or acute inhalation of cigarette smoke. Acute administration of capsaicin induces release of tachykinins including substance P from sensory nerve endings via a Ca^{2+}-dependent but tetrodotoxin-resistant mechanism, suggesting that capsaicin has a direct action on nerve endings (Szolcsanyi, 1983; Maggi *et al.*, 1987). It has been reported that capsaicin-induced release of tachykinins (Maggi *et al.*, 1988) or depolarization of sensory neurones (Rang *et al.*, 1987) is resistant to ω-conotoxin, which blocks neuronal depolarization-coupled transmitter secretion (Augustine *et al.*, 1987; Miller, 1987), which suggests that capsaicin-induced release of tachykinins is different from that induced by antidromic stimulation of nerves and independent of action potential propagation. Morphine and opioid peptides inhibit nerve cell discharge by postsynaptic inhibition of cell firing, or a presynaptic reduction in the release of neurotransmitters by blocking the propagation of action potentials and by reducing the entry of calcium (North & Williams, 1983). Morphine and opioid peptides might, therefore, be expected to have no inhibitory effect on the direct effect of capsaicin on sensory nerves. This suggestion is consistent with the inability of morphine to inhibit capsaicin-induced release of CGRP from guinea-pig isolated heart (Franco-Cereceda *et al.*, 1989) and the resistance of capsaicin-induced bronchoconstriction in the guinea-pig to [D-Met^2, Pro^5]enkephalin (Bartho *et al.*, 1987).

The inhibitory effect of morphine on both low and high dose cigarette smoke-induced goblet cell discharge indicated that morphine might inhibit both cholinergic and capsaicin-sensitive mechanisms. That is consistent with a previous report in which morphine was shown to inhibit both cholinergic and non-cholinergic excitatory constrictor responses in guinea-pig airways (Johansson *et al.*, 1989). With low dose cigarette smoke, both the δ-selective opioid receptor agonist, DPDPE and the μ-opioid agonist, DAMGO inhibited cigarette smoke-induced goblet cell secretion. DPDPE was more potent than DAMGO which suggests a predominance of δ-opioid receptors. The inhibitory effects of DPDPE and DAMGO were completely reversed by naloxone, confirming that these effects were mediated via opioid receptors. The site of action of the inhibitory opioid receptors in the airways is not conclusively determined in this study. Because DPDPE did not significantly affect methacholine-induced goblet cell secretion, a presynaptic site of action seems likely. Low dose cigarette smoke principally activates parasympathetic ganglia to activate cholinergic systems (Kuo *et al.*, 1990b). Enkephalins have been reported to inhibit presynaptically cholinergic neurotransmission at autonomic ganglia (Konishi *et al.*, 1979). Morphine and enkephalins also inhibit the nicotinic receptor-mediated release of catecholamines in the adrenal medulla (Kumakura *et al.*, 1980). Therefore, it is possible that opioids inhibit goblet cell discharge at the level of the ganglion. However, it is also possible that opioids block neuroeffector transmission (Cowie *et al.*, 1968; Oka, 1980).

Only DAMGO significantly inhibited the response to high dose cigarette smoke, which selectively stimulates capsaicin-sensitive sensory nerves (Kuo *et al.*, 1991), and was reversed by naloxone indicating that inhibition was via an action on μ-opioid receptors. μ-Opioid receptors have been shown to be present on a capsaicin-sensitive population of sensory nerves (Laduron, 1984) which contain neuropeptides including the tachykinins substance P and neurokinin A as well as calcitonin gene-related peptide. Opioid agonists have been demonstrated to inhibit stimulus-evoked release of substance P from the rat trigeminal nucleus *in vitro* (Jessell & Iversen, 1977). Substance P has been shown to be the most potent of a number of neuropeptides, including those mentioned above, in causing goblet cell discharge (Kuo *et al.*, 1990a). Therefore, μ-opioid receptor inhibition of high dose cigarette smoke-induced goblet cell secretion is probably mediated via inhibition of the release of substance P. DAMGO had no significant effect on SP-induced goblet cell secretion which further suggests that inhibition is at a presynaptic site. Neither the δ-opioid receptor agonist, DPDPE, nor the κ-opioid receptor agonist, U-50,488, had significant inhibitory effect on high dose cigarette smoke-induced goblet cell secretion. Pretreatment with naloxone did not significantly affect the response to either high or low dose cigarette smoke, indicating that the role of endogenous opioids in this response is minimal.

Our present results extend the spectrum of inhibitory effects of morphine and opioid peptides on neurally-mediated airway responses. Opioid inhibition of cigarette smoke-induced goblet cell secretion may have interesting clinical implications. In rodent airways, cigarette smoke triggers capsaicin-sensitive nerves to induce microvascular leakage (Lundberg *et al.*, 1983) and mucus secretion from goblet cells (Kuo *et al.*, 1991). In addition, cigarette smoke inhalation has been shown to stimulate cholinergic nerves to induce secretion from submucosal glands (Peatfield *et al.*, 1986) and goblet cells (Kuo *et al.*, 1990b). Similar neurally-mediated responses may contribute to the hypersecretory state of cigarette smokers and patients with chronic bronchitis, which is a cigarette smoke-related condition. The hyperfunction of goblet cells in the distal airways may adversely affect small airway function in smokers (Lumsden *et al.*, 1984). Since the response of goblet cells can be effectively blocked by opioids acting on prejunctional receptors on cholinergic or sensory nerves, opioids may be useful in blocking airway hypersecretory states in bronchial diseases associated with cigarette smoking. The mucus hypersecretion of asthma may also be susceptible to this treatment, since activation of cholinergic nerves and axon reflexes by inflammatory mediators have been suggested as important in the pathophysiology of asthma (Barnes, 1986).

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The electrical and mechanical responses of the rabbit saphenous artery to nerve stimulation and drugs

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1 Electrical and mechanical responses to field stimulation (1–64 Hz, 0.5 ms supramaximal voltage) were recorded simultaneously in the rabbit saphenous artery. The electrical response consisted entirely of excitatory junction potentials (e.j.ps) which were abolished by α, β methylene ATP (α,β MeATP, 10^{-6} M) and by tetrodotoxin (TTX, 10^{-6} M) but were unaffected by the α_1-adrenoceptor antagonist, prazosin (10^{-6} M). No additional electrical response was evoked by field stimulation, even in the presence of normetanephrine (NMN) and desmethylimipramine (DMI, each 10^{-6} M), which block neuronal and extraneuronal uptake of noradrenaline (NA) respectively. Action potentials to field stimulation were produced only in the presence of tetraethylammonium (10^{-3} M) which also enhanced the contraction.

2 Contractions to field stimulation were reduced (by some 50%) by prazosin (10^{-6} M) and abolished by the additional presence of α,β MeATP (10^{-6} M), which blocks purinoceptors by desensitization, suggesting the involvement of both NA and an ATP-like substance in the contractile response.

3 Idazoxan (10^{-6} M) which blocks prejunctional α_2-adrenoceptors, significantly increased the amplitude of both e.j.ps and the contraction to field stimulation (10 pulses, 1–4 Hz, 0.5 ms, supramaximal voltage).

4 NA (10^{-2} M by pressure ejection) did not alter membrane potential even in the presence of NMN and DMI (each 10^{-6} M). ATP (10^{-2} M by pressure ejection) produced a concentration-dependent, α,β MeATP-sensitive depolarization.

5 In tissues desensitized by constant infusion of α,β MeATP (10^{-6} M) contractions to NA (10^{-7}–3×10^{-5} M), histamine ($10^{-7} - 3 \times 10^{-5}$ M) and KCl (1–1.6×10^{-2} M) were unaffected.

6 Following restoration of the membrane potential, after an initial depolarization, α,β MeATP (4×10^{-6} M) did not change the amplitude of electrotonic hyperpolarizing current pulses significantly but abolished evoked e.j.ps. The rates of recovery of evoked e.j.ps and the depolarization to ATP (10^{-2} M by pressure ejection) following desensitization to α,β MeATP (10^{-6} M) were comparable. These results suggest that the effects of α,β MeATP are mediated selectively via receptors (purinoceptors).

7 Suramin (10^{-3} M) abolished e.j.ps and the prazosin (10^{-6} M), insenstive component of the contractile response and antagonized contractions to α,β MeATP (10^{-7}–10^{-5} M), ATP (10^{-5}–10^{-3} M), histamine (10^{-7}–3×10^{-5} M) and 5-hydroxytryptamine (10^{-7}–10^{-5} M) but those to NA (10^{-7}–10^{-5} M) and KCl (1–1.6×10^{-2} M) were unaffected. Suramin is insufficiently selective, under these conditions, as a purinoceptor antagonist.

Keywords: Noradrenaline; adenosine 5′-triphosphate; nerve stimulation; α,β methylene ATP; suramin; rabbit saphenous artery; vascular smooth muscle

Introduction

In many vascular smooth muscles, stimulation of sympathetic nerves releases more than one excitatory substance, noradrenaline (NA) and adenosine 5′-triphosphate (ATP) or a closely related substance, the phenomenon of co-transmission (see Burnstock, 1976; Campbell, 1987; Bartfai *et al.*, 1988). The contribution of individual transmitters in situations of co-transmission varies. In rabbit ear artery for example, the main electrical response to nerve stimulation, excitatory junction potentials (e.j.ps), are non-adrenergic, followed by a late, small, slow depolarization which is adrenergic and accounts for the contraction (Allcorn *et al.*, 1985). On the other hand, in rabbit mesenteric artery (Lim *et al.*, 1986), the evoked e.j.ps fuse and summate to give action potentials and are unaffected by α_1-adrenoceptor antagonists but abolished by α,β methylene ATP (α,β MeATP) which blocks purinoceptors by desensitization. The accompanying contractions are reduced by α-adrenoceptor antagonists and abolished by the additional presence of α,β MeATP, indicating the involvement of both transmitters in the mechanical response.

Clearly, (i) responses to sympathetic nerve stimulation vary with the blood vessel being investigated and must be examined individually and (ii) electrical responses are neither a constant nor a significant feature of the response to neuronally-released NA though this transmitter is in most cases involved in the contractile response. This raises the question as to the basis of the contractile response to NA where little or no electrical change is observed.

The present investigation addressed the questions raised in these observations. In this paper, the relationship between the electrical and mechanical responses to sympathetic nerve stimulation was examined in the rabbit saphenous artery, a tissue in which co-transmission has already been proposed (Burnstock & Warland, 1987). The contribution of the individual co-transmitters was analysed pharmacologically and the suitability of α,β MeATP as an antagonist assessed. The results of these experiments indicated that sympathetic nerve stimulation produced membrane potential changes, mediated exclusively by the non-adrenergic transmitter, while the contraction involved both excitatory substances. There remained the question of the basis for the NA-mediated component of the contraction. A biochemical basis for this was sought by measuring the effect of NA and α,β MeATP on membrane phospholipids. Preliminary results of both these investigations have been communicated (Muir & Nally, 1989; Nally & Muir, 1989; Nally *et al.*, 1990; 1991).

Methods

Male New Zealand White rabbits (1.5–2.5 kg) were killed either by stunning or by an overdose of CO_2, followed by

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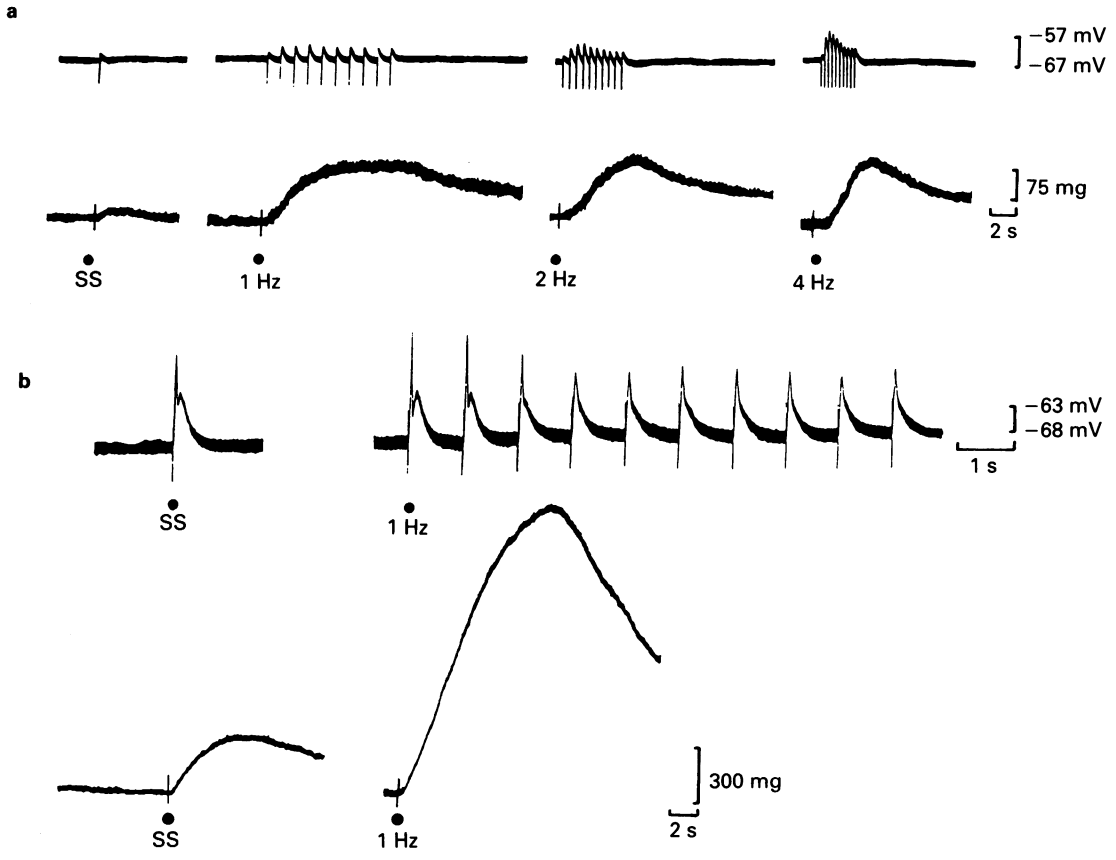

Figure 1 The intracellularly-recorded electrical (upper trace in each case) and mechanical responses to field stimulation (●, supramaximal voltage, 0.5 ms, single stimulus SS and 10 pulses at 1, 2 and 4 Hz) in the same cell of the rabbit saphenous artery, in (a) the absence of and (b) in the presence of tetraethylammonium (TEA, 10^{-3} M). Action potentials were superimposed on e.j.ps in the presence of TEA and the contractions greatly enhanced.

exsanguination. Sections of saphenous arteries (4 cm long), were dissected out from 2 cm above to 1 cm below the knee, (Holman & Surprenant, 1980) and transferred to a Petri dish containing Krebs solution, where connective tissue and adhering fat were removed.

Contractile responses were measured from ring segments (0.5–1 cm long) of arteries mounted in organ baths (10 ml) under tension (1 g) by inserting two Pt wires into the lumen, one anchored, the other attached to a force displacement transducer (Grass FT03T). Field stimulation (supramaximal voltage, 0.5 ms, 1–64 Hz) of intramural nerves was carried out via two Ag/AgCl electrodes arranged parallel to the artery. In those experiments where 6-hydroxydopamine (6-OHDA 5×10^{-4} M) was used, the drug was added to the bath 2 h before the start of experiments (Wadsworth, 1973). The absence of any contraction to field stimulation indicated the success of this treatment.

For simultaneous intracellular electrical and mechanical recording, lengths of artery (2 cm) were mounted in a horizontal perspex bath (2 ml). One end of the artery was cut into a spiral and attached to a force displacement transducer (Grass FT03T). The other end was passed through Ag/AgCl ring electrodes for field stimulation (supramaximal voltage, 0.5 ms, 1–32 Hz) of intramural nerves and pinned on to a Sylgard base. Intracellularly-recorded signals, obtained with conventional glass microlectrodes (40–80 mΩ) filled with 3 M KCl were passed to a high impedance amplifier (Neurolog), displayed on a storage oscilloscope and u.v. recorder and stored on a frequency-modulated (Racal 4D) tape recorder.

Changes in conductance and resistance were measured by the technique of Abe & Tomita (1968). A length of artery (2 cm) was placed in a horizontal organ bath, which was divided into two compartments, one for stimulating the tissue, the other for recording responses, by one of two Ag plate electrodes (1 cm × 1 cm, 50 μm thickness, 1 cm apart), through which a small hole had been drilled (0.2 mm) to allow the passage of the tissue. The surface of the Ag plate exposed to the recording chamber was insulated by coating with epoxy resin. The electrodes were connected to a constant current device. One end of the tissue was passed through Ag/AgCl ring electrodes for stimulation of intramural nerves. The relative intensity of the stimulating current was obtained from the voltage gradient of the solution in the stimulating chamber, as recorded by two Ag electrodes, 2 mm apart in the wall of the organ bath. Changes in membrane potentials were recorded as before and both chambers were separately irrigated with Krebs solution. All experiments were done at 37 ± 0.5°C, pH 7.4, in Krebs solution of the following composition (mM): NaCl 118.4, $NaHCO_3$ 25, NaH_2PO_4 1.1, KCl 4.7, $MgCl_2$ 1.3, $CaCl_2$ 2.7 and glucose 11. Where the ionic composition of the Krebs was altered, isotonicity was maintained by substitution with appropriate ions. $[Na^+]_o$ was reduced by replacing NaCl with choline chloride. $[K^+]_o$ was reduced by replacing KCl with equivalent amounts of NaCl and increased using an equivalent reduction in NaCl. $[Cl^-]_o$-free solutions were made by replacing NaCl with sodium benzenesulphonate, KCl with K_2SO_4, $MgCl_2$ with $MgSO_4$ and $CaCl_2$ with $CaSO_4$. Tissues were bubbled with 95% O_2, 5% CO_2 and allowed to equilibrate for 1 h before starting recordings. Antagonists were added 15 min prior to investigation. Drugs were added, either as a bolus injection to the bathing fluid, or by hydrostatic pressure ejection with a Picospritzer (General Valve Corp., N.J. U.S.A.).

Drugs

The following drugs were used: α,β methylene adenosine 5'-triphosphate (α,β MeATP, Sigma), adenosine 5'-triphosphate (ATP, Sigma), atropine sulphate (Sigma), desmethylimipramine hydrochloride (DMI, Ciba-Geigy), diltiazem hydro-

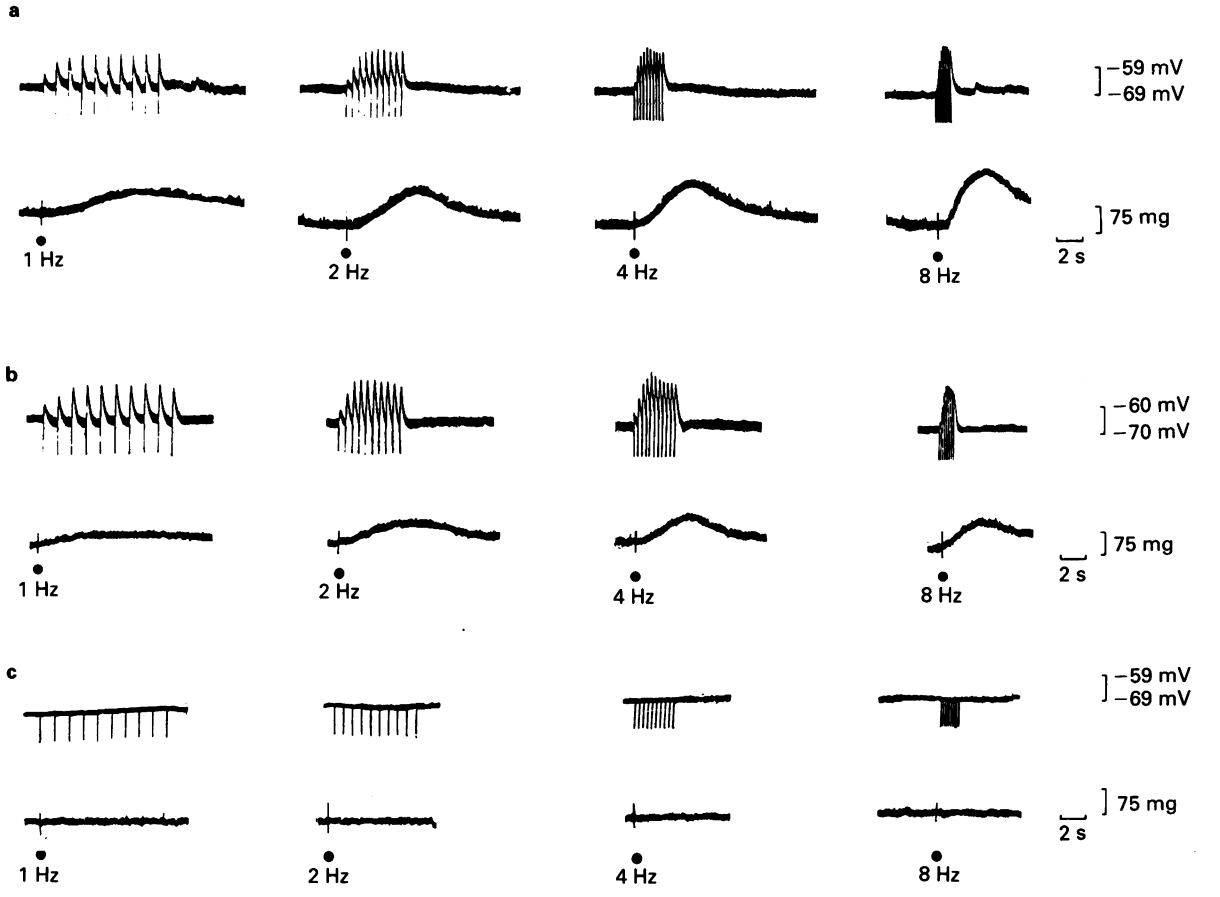

Figure 2 The effects of prazosin (10^{-6} M) alone (b) and together with α,β methylene ATP (α,β MeATP, 10^{-6} M, c) on the intracellularly-recorded electrical (upper trace) and mechanical responses to field stimulation (●, supramaximal voltage, 0.5 ms, 10 pulses at 1, 2, 4 and 8 Hz) in the rabbit saphenous artery compared with controls (a). E.j.ps were unaffected by prazosin, while the contraction was reduced by some 50%. Prazosin and α,β MeATP together abolished both the electrical response and the contraction to field stimulation. Recordings made from 2 different cells.

chloride (Sigma), guanethidine monosulphate (Ciba-Geigy), histamine diphosphate (Sigma), 5-hydroxytryptamine hydrochloride (5-HT, Sigma), 6-hydroxydopamine hydrobromide (6-OHDA, Sigma), idazoxan, 2-(2-(1,4, benzodioxanyl)) 2-imidazoline hydrochloride (Reckitt & Colman), (−)-noradrenaline bitartrate (NA, Koch-Light), (±)-normetanephrine hydrochloride (NMN, Sigma), potassium chloride (KCl, Koch-Light), (±)-prazosin hydrochloride (a gift from Pfizer), (±)-propranolol hydrochloride (Sigma), suramin, 8, 8′ [carbonyl*bis* [imino-3, 1-phenylene-carbonylimino (4-methyl-3, 1-phenylene) carbonylamino]] bu-1,3,5-napthalene-trisulphonic acid hexasodium (a gift from ICI), tetraethylammonium bromide (TEA, Sigma), tetrodotoxin (TTX, Sigma).

Drug concentrations in the text refer to the salt, with the exception of TTX which is expressed as the base.

Statistical analysis

Results are expressed as mean ± s.e.mean. Statistical significance between data samples was tested by an unpaired Student's *t* test, or by analysis of regression. A probability level of $P < 0.05$ was considered statistically significant. ***indicates $P < 0.001$ and **$P < 0.05$. The number of observations exceeded 3 in each case.

Results

The mean resting membrane potential was -69 ± 0.3 mV, $n = 257$. Each tissue exhibited individual, spontaneous e.j.ps (2–6 mV) which occurred at random intervals unaccompanied by contractions.

The response to field stimulation

Field stimulation of intramural nerves, (supramaximal voltage, 0.5 ms) evoked e.j.ps and contractions. Single stimuli evoked e.j.ps which could reach 14 mV (mean value, 4.2 ± 0.3 mV, $n = 153$, range 0.5–14 mV). These were rarely accompanied by contractions. Trains of stimuli (1–64 Hz) produced e.j.ps (3–30 mV) which facilitated (at or above 0.5 Hz) and summated (at or above 2 Hz), accompanied by contractions. The size of the e.j.ps and the contractions were graded with frequency (optimum 4–8 Hz). The electrical component of the response consisted solely of e.j.ps; there was no additional late slow membrane depolarisation, as observed in the rabbit ear artery (Allcorn *et al.*, 1985). Action potentials were not observed in response to field stimulation, unless in the presence of TEA (10^{-3} M) which blocks certain K channels (Ito *et al.*, 1970; Figure 1). In the presence of this drug, the mean amplitude of the e.j.ps was increased some 300% (to 10–25 mV) ($P < 0.005$) and spikes of up to 40 mV were observed, superimposed on e.j.ps of 12 mV or more, in response to both single pulses and trains of (10) stimuli. There was no apparent change in the frequency of the spontaneous e.j.ps. The action potentials produced in the presence of TEA were abolished by the Ca^{2+} channel blocker, diltiazem (10^{-5} M).

Responses to field stimulation were neuronally-mediated, being abolished by TTX (10^{-6} M), or guanethidine (10^{-6} M).

Alteration of external ionic environment

Removal of $[K^+]_o$ (control 4.7 mM), produced no significant change in membrane potential ($n = 9$), but significantly attenuated ($P < 0.001$) the amplitude of evoked e.j.ps. An

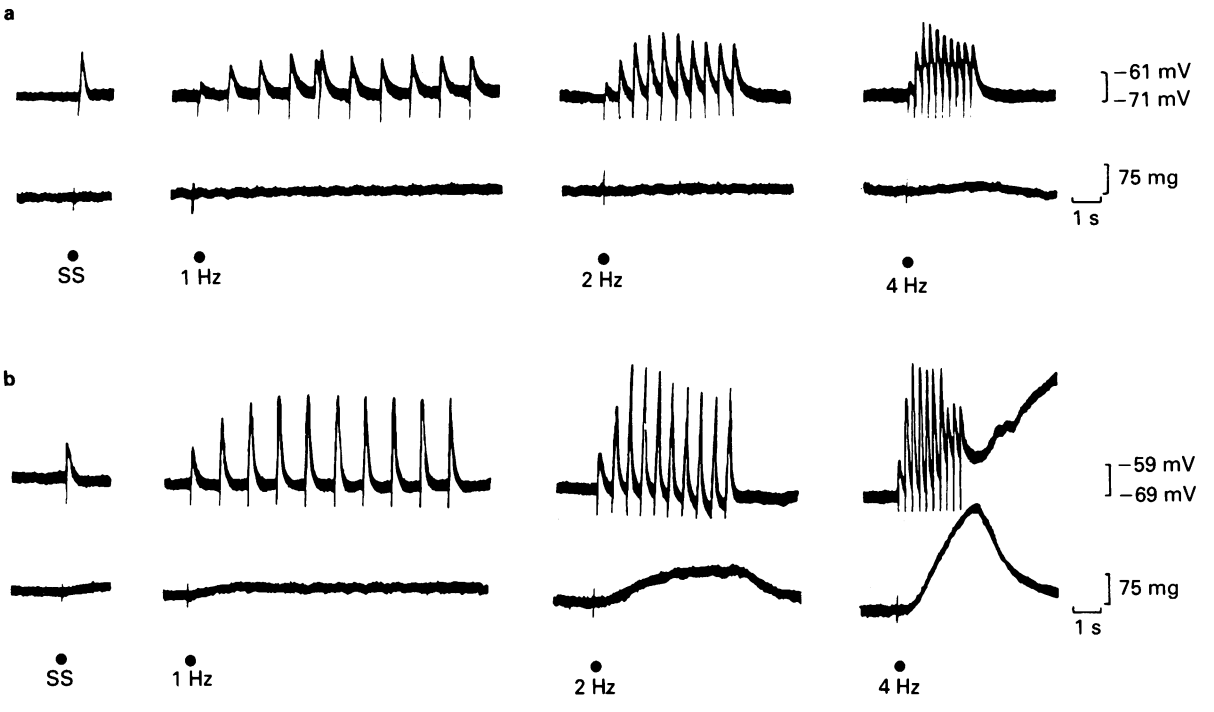

Figure 3 The effect of idazoxan (10^{-6} M, b) on the electrical (upper trace) and mechanical responses to field stimulation (●, supramaximal voltage, 0.5 ms, single stimulus SS and 10 pulses at 1, 2 and 4 Hz) from the same cell in the rabbit saphenous artery; (a) Control responses. Both the e.j.ps and the contraction were enhanced by idazoxan. Impalement was lost following the final response.

increase in $[K^+]_o$ to 9.4 mM, depolarized the membrane ($P < 0.001$) to -59 ± 0.55 mV ($n = 62$) and ($P < 0.005$) reduced the amplitude of evoked e.j.ps.

A reduction in $[Na^+]_o$ from 144.5 mM (control) to 108.3 mM, significantly ($P < 0.001$) depolarized the membrane to -58 ± 1.1 mV ($n = 18$) and greatly reduced the amplitude of subsequently-evoked e.j.ps. A reduction in $[Na^+]_o$ to 72.2 mM further depolarized the membrane potential to -54 ± 2.0 mV, ($n = 9$) and abolished evoked e.j.ps. Removal of $[Cl^-]_o$, significantly depolarized ($P < 0.05$) the membrane to

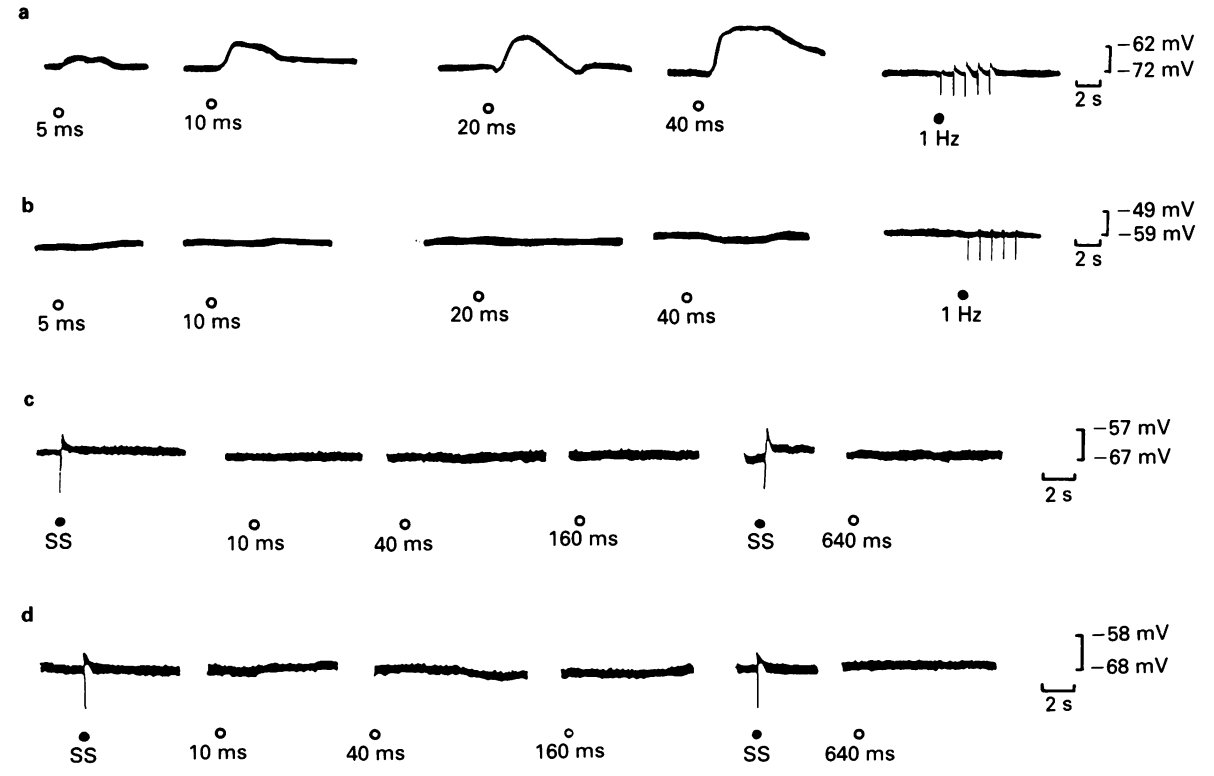

Figure 4 The intracellularly-recorded electrical response from two different cells (a) and (c) in the rabbit saphenous artery to field stimulation (●, supramaximal voltage, 0.5 ms, single stimulus, SS, and 5 pulses at 1 Hz) and to pressure ejection of ATP (○, 10^{-2} M, a and b) or noradrenaline (NA ○, 10^{-2} M, c and d) for the durations (ms) indicated. α,β Methylene ATP (α,β MeATP, 10^{-6} M) and normetanephrine (NMN, 10^{-6} M) together with desmethylimipramine (DMI, 10^{-6} M) were present respectively in (b) and (d) and absent in the corresponding controls (a) and (c). Both the evoked e.j.ps and the depolarization to ATP were abolished by α,β MeATP. NA failed to alter membrane potential even in the presence of NMN and DMI.

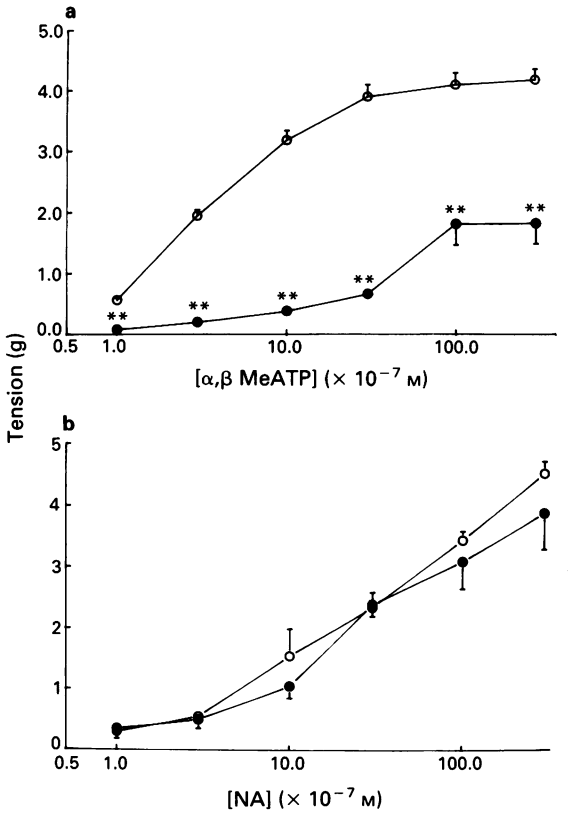

Figure 5 The effect, compared with controls (○), in the rabbit saphenous artery, of a desensitizing infusion of α,β methylene ATP (α,β MeATP, ●, 10^{-6} M) on the contractions to (a), α,β MeATP (10^{-7}–3×10^{-5} M) and (b) noradrenaline (NA, 10^{-7}–3×10^{-5} M). Infusion of α,β MeATP antagonized the response to individual doses of α,β MeATP, but not that to NA.

-58 ± 2.0 mV ($n = 15$) and subsequently reduced, or abolished evoked e.j.ps.

The effects of antagonists and uptake blockers on the response to nerve stimulation and drugs

Evoked e.j.ps were unaffected by the α_1-adrenoceptor antagonist, prazosin (10^{-6} M), abolished by α,β MeATP (10^{-6} M) and were accordingly presumably mediated via P_{2x}-purinoceptors. The amplitude of the contraction to field stimulation was approximately halved by either prazosin (10^{-6} M), or by α,β MeATP (10^{-6} M) alone and abolished by a combination of these drugs (Figure 2). Clearly two substances are released following stimulation of the sympathetic nerves; NA and ATP (or a closely related substance). Only the non-adrenergic transmitter, however, appears to be involved in the electrical response. Attempts to maximize the effect of any nerve-released NA by either prolonged periods of stimulation (up to 1 min) over a range of frequencies (1–64 Hz) or by addition to the perfusate of normetanephrine (NMN, 10^{-6}) and desmethylimipramine (DMI, 10^{-6} M), which respectively block neuronal and extraneuronal uptake of the transmitter, failed to uncover an electrical noradrenergic response to nerve stimulation, although the mechanical response was enhanced. Idazoxan (10^{-6} M) which blocks prejunctional α_2-adrenoceptors significantly ($P < 0.05$) increased both the mean amplitude of evoked e.j.ps in response to trains of pulses (10 pulses, 1–4 Hz) and the mean mechanical response ($P < 0.001$) (Figure 3). Approximately 50% of the increased mechanical response was blocked by prazosin, while α,β MeATP abolished both the electrical and the residual mechanical responses.

If NA and ATP are indeed co-transmitters in the rabbit saphenous artery (see Burnstock & Warland, 1987), their exogenous application should mimic the response to nerve stimulation.

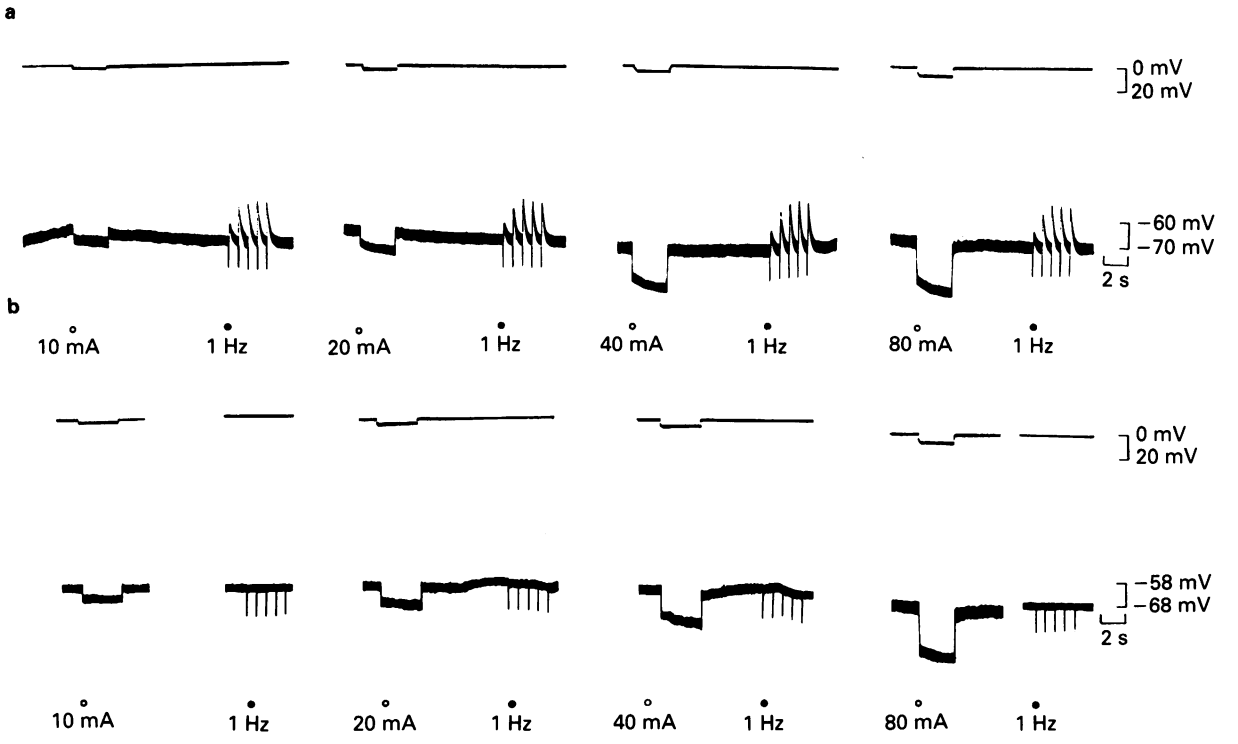

Figure 6 The membrane responses in the rabbit saphenous artery, to hyperpolarizing current pulses (○, 10–80 mA) and to field stimulation (●, supramaximal voltage, 0.5 ms, 5 pulses at 1 Hz) in the absence (a) and the presence (b) of α,β methylene (α,β MeATP, 4×10^{-6} M), using the technique of Abe & Tomita (1968). Changes in resistance, as measured by changes in membrane voltage, were not significantly affected by α,β MeATP, while e.j.ps were abolished, suggesting the absence of any significant non-selective membrane effect of the compound. Recordings were made from the same cell.

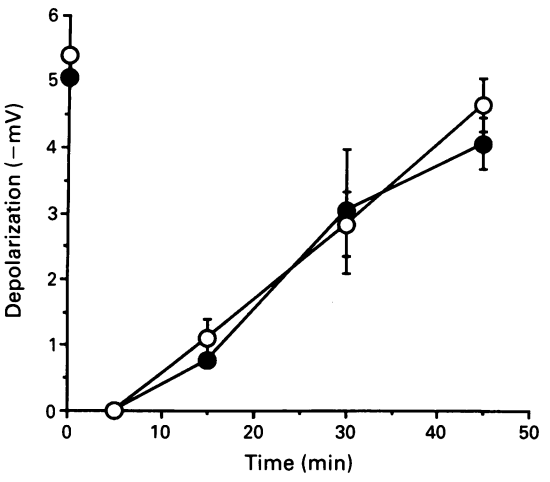

Figure 7 The time taken for recovery of intracellularly-recorded electrical responses to field stimulation (●, e.j.ps, 5 pulses at 1 Hz, 0.5 ms, supramaximal votlage) and to ATP (○, 10^{-2} M, 20 ms by pressure ejection), on washout following a desensitizing infusion of α,β methylene ATP (α,β MeATP, 10^{-6} M). Both the evoked e.j.ps and the depolarization to ATP were abolished by α,β MeATP. The times taken for recovery of the responses to nerve stimulation and to ATP, on washout of α,β MeATP, were similar ($n = 9$). Values for control e.j.ps (●) and ATP depolarization (○) are indicated on the ordinate scale.

ATP (10^{-2} M) added by pressure ejection by Picospritzer (40 p.s.i., 5–40 ms) produced a dose-dependent, α,β MeATP-sensitive depolarization of up to 15 mV (Figure 4). In contrast, NA (10^{-2} M) added by Picospritzer (40 p.s.i. 10–640 ms) failed to change the membrane potential, even in the presence of NMN and DMI (each 10^{-6} M, Figure 4).

Because of its importance in evaluating co-transmission, the suitability of α,β MeATP as a P_{2x}-purinoceptor antagonist was examined. Three series of experiments were carried out. In the first, the effects of prolonged exposure to the drug on the contractile sensitivity of a number of agonists was examined. α,β MeATP (10^{-6} M), was continuously infused until contractions either to bolus injections of α,β MeATP (10^{-7}–10^{-5} M, Figure 5a) or to ATP (10^{-5}–10^{-3} M) were reduced or abolished. This was taken as an indication of the successful desentization of P_{2x}-purinoceptors (Figure 5). In contrast, contractions to NA (10^{-7}–3×10^{-5} M, Figure 5b) and histamine, (10^{-7}–3×10^{-5} M, not shown) were each unaffected by a desensitizing infusion of α,β MeATP (10^{-6} M).

The depolarizing agent KCl, which evokes contraction by opening voltage-dependent calcium channnels, was used to indicate if α,β MeATP infusion inhibited contraction by a receptor-independent mechanism. To avoid prejunctional release of transmitters by KCl, tissues were pretreated *in vitro* with 6-OHDA (5×10^{-4} M, 2 h) to destroy adrenergic nerves (Wadsworth, 1973). After this treatment, contractions evoked by KCl (1–1.6×10^{-2} M) were unaffected by infusion of α,β MeATP (10^{-6} M).

In a second series of experiments, the effect of α,β MeATP infusion (4×10^{-6} M) on displacement of membrane potential was measured (Abe & Tomita, 1968), to determine the effect of the compound on membrane properties. α,β MeATP initially depolarized (mean value, -53 ± 1.02 mV, $n = 28$) the membrane potential which returned to control levels after 3–5 min. Recordings were taken when the membrane potential values had been restored. Figure 6 shows the effects of α,β MeATP on changes in membrane resistance produced by hyperpolar-

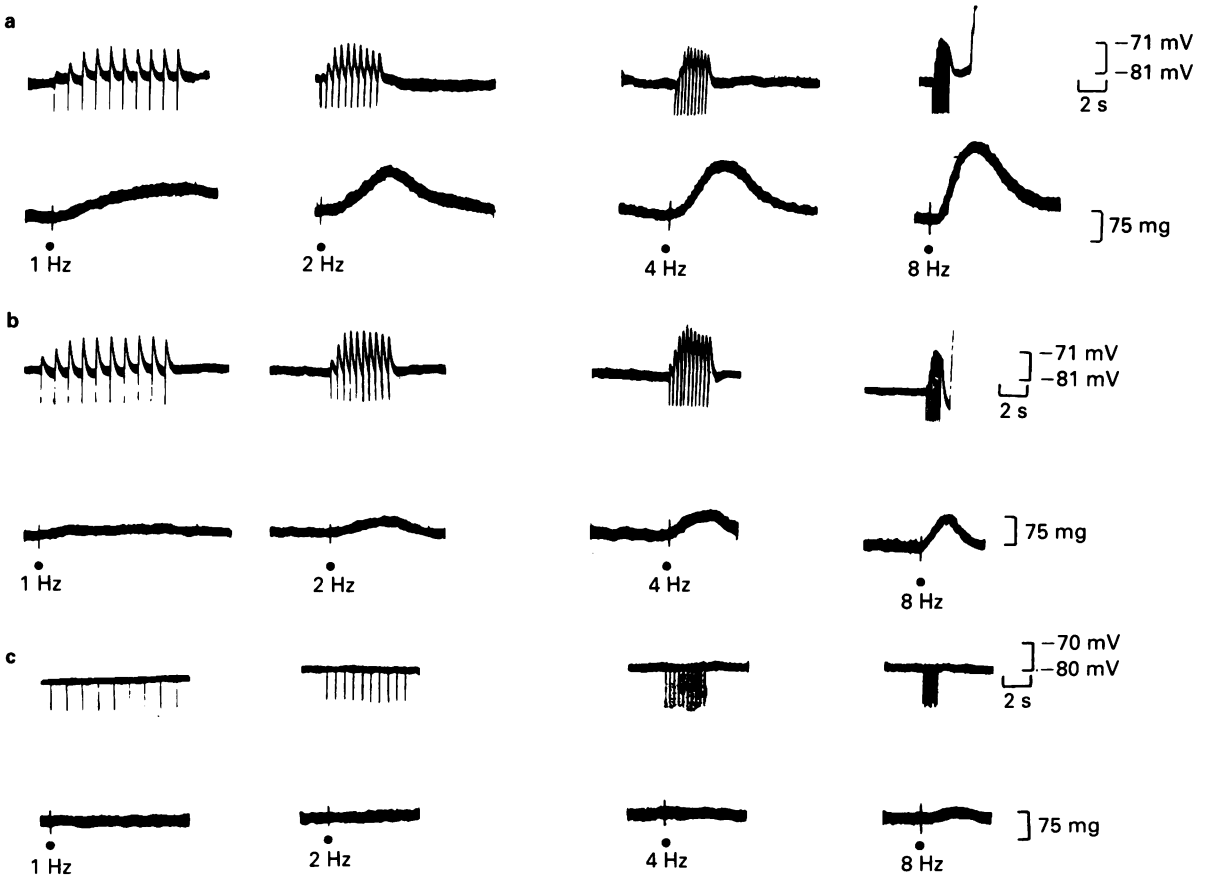

Figure 8 The effect of prazosin alone (10^{-7} M, b) and in combination with suramin (10^{-3} M, c), on the intracellularly-recorded electrical (upper trace) and mechanical responses to field stimulation (●, 10 pulses at 1, 2, 4 and 8 Hz, 0.5 ms, supramaximal voltage). E.j.ps were unaffected by prazosin, but the contraction was reduced by some 50% compared with controls (a). Suramin and prazosin together abolished both the electrical and mechanical responses to field stimulation in the rabbit saphenous artery. Recordings made from 3 different cells (a, b, c) in the same preparation.

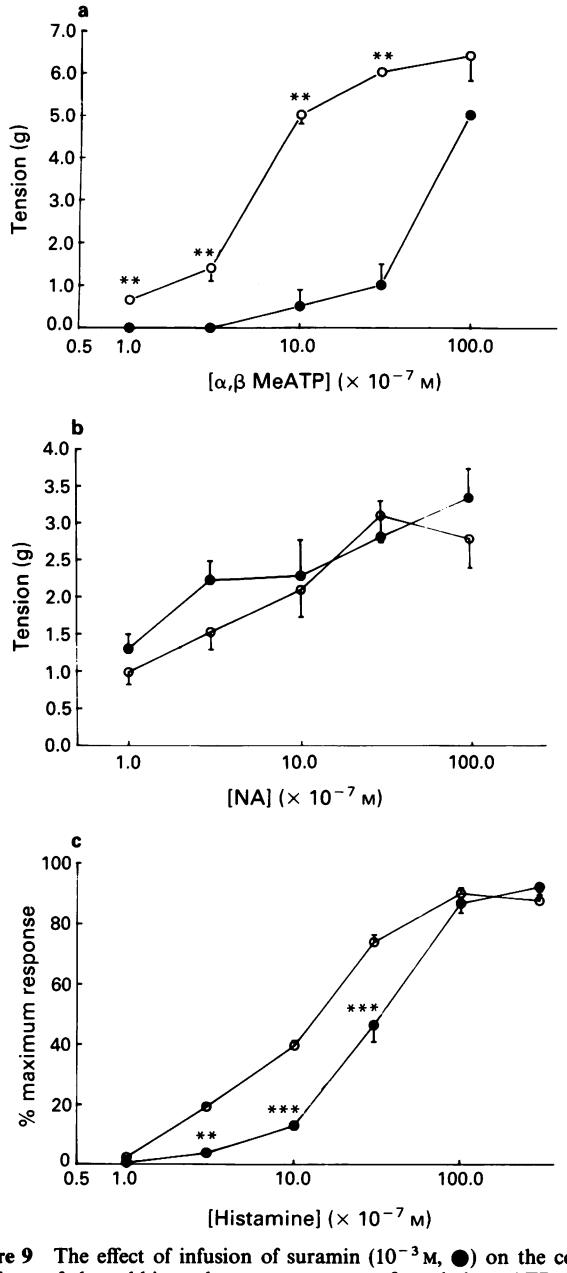

Figure 9 The effect of infusion of suramin (10^{-3} M, ●) on the contractions of the rabbit saphenous artery to α,β methylene ATP (α,β MeATP, 10^{-7}–10^{-5} M, a), noradrenaline (NA, 10^{-7}–10^{-5} M, b) and histamine (10^{-7}–3×10^{-5} M c), compared with respective controls (○). Suramin antagonized the responses to α,β MeATP and to histamine, but not to NA.

izing current (1–200 mA). α,β MeATP (4×10^{-6} M) did not significantly change the amplitude of the evoked electrotonic potential. On the other hand, evoked e.j.ps (5 pulses at 1 Hz) were abolished by the infusion.

In a third series of experiments, intracellular electrical recording was used to compare the rates of recovery of the membrane depolarization produced by ATP (10^{-2} M), added by pressure ejection, with those of evoked e.j.ps during washout of α,β MeATP, following desensitization of purinoceptors. The time taken for recovery of the two responses (Figure 7) was comparable, suggesting a similar effect of α,β MeATP on both neuronally released non-adrenergic transmitter and exogenously added ATP. Together these results indicate that α,β MeATP did not produce any non-selective membrane effects and was acting via P_{2x}-purinoceptors.

Suramin (10^{-3} M) which may also antagonize purinoceptors (Dunn & Blakeley, 1988) produced neither a contraction nor an alteration in resting membrane potential, but abolished both evoked e.j.ps and those contractions to field stimulation (0.5 ms, 8 Hz, supramaximal voltage) remaining after pretreatment with prazosin (10^{-6} M, Figure 8). Suramin (10^{-3} M) also antagonized contractions to α,β MeATP (10^{-7}–10^{-5} M, Figure 9a) and ATP (10^{-5}–10^{-3} M), but those to NA (10^{-7}–10^{-5} M, Figure 9b) and KCl (1–1.6×10^{-2} M), the latter after pretreatment with 6-OHDA (5×10^{-4} M) were each unaffected. Suramin, however, antagonized the contractions to histamine (10^{-7}–3×10^{-5} M, Figure 9c) and 5-HT (10^{-7}–10^{-5} M).

Discussion

The mean resting membrane potential of the rabbit saphenous artery (−69 mV) was similar to that of the guinea-pig mesenteric artery (−70 mV, Kuriyama & Suzuki, 1981) and somewhat greater than that in arteries of rabbit ear (−63 mV, Droogmans *et al.*, 1977), or in the internal layers of sheep carotid arteries (−59.5 mV Mekata & Keatinge, 1975). Lower measurements in these tissues may have included those from damaged cells (Holman & Suprenant, 1980; Suprenant, 1980) or from different regions of the arterial smooth muscle. The electrical response of cells in this region, at least to adrenaline in the sheep carotid artery (Mekata & Keatinge, 1975) differed from those of the inner non-innervated layer. The cells impaled in the present experiments were most likely to lie in the outer innervated layer. There was no evidence of there being more than one population of cells in this study.

Field stimulation, of the rabbit saphenous artery, it was confirmed, released two excitatory substances (Burnstock & Warland, 1987), each of which contributed to the contraction. Similar results were found in the rabbit mesenteric artery by Kugelgen & Starke (1985). Both ATP and NA also mediate contraction in the rabbit jejunal artery, the extent of individual transmitter involvement being affected by train length (Cunnane & Evans, 1990). In response to short trains of stimuli (10 pulses), similar to those used in the present investigation, contractions at 2 Hz and 10 Hz, were largely purinergic and only with longer (100) pulse trains was a substantial noradrenergic contractile component revealed. In the rabbit saphenous artery, the contraction to 10 pulses at 1, 2, 4 Hz was inhibited by prazosin and abolished by the additional presence of α,β MeATP. These gradations in the contribution of individual transmitters to contraction reflect not only tissue differences *per se* (see Introduction) but the anatomical position of the arteries chosen for study (see for example Ramme *et al.*, 1987). The ability of idazoxan to increase both the amplitude of the e.j.ps and the contractions in this artery, implies that there is an α_2-adrenoceptor-mediated control of the release of both putative transmitters from the same nerve endings. The primary electrical event arising from field stimulation was an e.j.p. and, from exogenously added adenine nucleotides, a membrane depolarization. No change in membrane potential accompanied either the release of NA by sympathetic nerve stimulation, under conditions calculated to maximize its effect, or the application of large quantities (up to 3×10^{-4} M) of the catecholamine itself. NA it seems, contracts the saphenous artery by a voltage-independent mechanism (cf. Sneddon & Burnstock, 1984; Sneddon & Westfall, 1984; Bolton & Large, 1986).

A second electrical effect, an action potential, was obtained to nerve stimulation in the presence of TEA, which blocks certain K^+ channels (Ito *et al.*, 1970). Under these circumstances, the voltage-dependent channels responsible for initiation of action potentials were activated at a threshold potential of some −57 mV. The lack of action potentials in the absence of TEA is reminiscent of transmission in the rat anococcygeus (Creed *et al.*, 1975) and bovine retractor penis (Byrne & Muir, 1984) muscles. In these tissues, sympathetic field stimulation evoked only e.j.ps accompanied by contractions. Action potentials were initiated in the presence of TEA.

In the rabbit saphenous artery, the action potentials and the accompanying contractions were inhibited by diltiazem,

which inhibits voltage-dependent Ca^{2+} channels (Beattie *et al.*, 1986). The upstroke of the action potential may therefore be carried by calcium current. The unmasking of the action potential by TEA suggests that the repolarization may also utilise a Ca^{2+}-activated gK^+ which is blocked by the drug. In contrast, the e.j.ps were unaffected by diltiazem (not shown). Thus, in the rabbit saphenous artery, contraction is normally effected by e.j.ps, action potentials can be demonstrated but are observed only when the truncating K^+ conductance is blocked as with TEA. The ionic mechanisms underlying the development of e.j.ps were only briefly addressed in this study. While the depolarization produced by increasing $[K^+]_o$ to 9.4 mM was expected, the absence of any significant change in membrane potential when the tissue was exposed to K^+-free Krebs solution was not. A similar mechanism in the guinea-pig taenia coli, may exist (Casteels, 1970); thus although K^+-free Krebs solution increased K^+ permeability, and decreased intracellular K^+ concentration, an accompanying increase in intracellular Na^+ ions may have offset any membrane hyperpolarization. This would also alter the ionic gradient of Na^+ and account for the decrease in e.j.p. amplitude observed. The decline in evoked e.j.p. amplitude following reduction or removal of $[Na^+]_o$ or $[K^+]_o$, together with the evidence from the use of TEA, suggests that each of these ions may contribute to the evoked response in this artery. The ability of Cl^- withdrawal to reduce or abolish e.j.ps may be due to its effect on Na^+ conductance and the Na^+/K^+ ATPase pump. Cl^- conductance may contribute only about 4% of the total membrane conductance, at least in the vas deferens smooth muscle (Aickin & Brading, 1983).

To date much of the evidence for co-transmission has relied on the activity of α,β MeATP. By desensitizing P_{2x}-purinoceptors, use of this compound enables the role of the two proposed transmitters NA and ATP in the neuronal response to be demonstrated and studied (Burnstock & Warland, 1987). Clearly the drug could have other effects and while there is no evidence that it modifies transmitter release in rabbit ear artery (Allcorn *et al.*, 1985) the possibility of its having non-specific effects as observed by Byrne & Large, (1986) and Kotecha & Neild, (1986) in other arteries and by Komori & Ohashi, (1988) in the chicken rectum, remained. In the rabbit saphenous artery there was no evidence of α,β MeATP having a non-specific effect either on receptors other than purinoceptors or on non-receptor voltage-mediated events. Maintained exposure to α,β MeATP did not reduce the postjunctional response of the saphenous artery to histamine, 5-HT, NA or KCl. The times taken for the responses to nerve stimulation (e.j.ps) and to exogenously-added ATP to recover on wash out following desensitization by α,β MeATP, were comparable, suggesting that α,β MeATP exerts a similar effect on both neuronally-released and exogenously-added ATP i.e. via purinoceptors. α,β MeATP did not alter the amplitude of applied electrotonic hyperpolarizing potentials at a time when e.j.ps were abolished, implying a lack of any non-selective membrane effect of the compound. α,β MeATP is a selective and useful tool in the study of co-transmission in the rabbit saphenous artery. In contrast, though suramin has some affinity for purinoceptors, large concentrations (10^{-3} M) were required. This may reflect the ability of the drug to inhibit ectonucleotidases responsible for the breakdown of ATP (Hourani & Chown, 1989). Suramin inhibited the effects of histamine and 5-HT perhaps as a result of the ability to bind a protein (Wills & Wormall, 1950) including those of receptor sites. While the responses to NA and KCl were unaffected by the drug, suramin seems insufficiently selective to be useful in the study of co-transmission in this tissue.

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Evidence for multiple endothelin receptors in the guinea-pig pulmonary artery and trachea

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1 The responses of the three peptides, endothelin 1 (ET-1), endothelin 2 (ET-2) and endothelin 3 (ET-3) were analysed on isolated circular segments of pulmonary arteries and trachea from the guinea-pig.

2 In the pulmonary artery, the vasomotor responses to the endothelins, expressed as the maximum contraction (E_{max}%), had the order ET-1 > ET-2 > ET-3 while the order of potency (pD_2) was ET-1 = ET-2 > ET-3. ET-1 and ET-2 caused cross-desensitization, but did not affect the responses to ET-3. ET-3 did not cause cross-desensitization to ET-1 or ET-2 although it induced homologous desensitization. Finally, the effects of ET-1 and ET-2 were additive to those of ET-3. The additive effect of ET-3 to those of ET-1 or ET-2 was more difficult to demonstrate, given the profound contraction produced by ET-1 and to a lesser extent by ET-2.

3 In the trachea, the rank order of potency, additivity and desensitization were different from the pulmonary artery. Basically, all three peptides were equipotent but less potent than ET-1 in the artery. There was no evidence for additivity and only a slight tendency to tachyphylaxis was seen.

4 The guinea-pig pulmonary artery appears to be endowed with one receptor type which is sensitive to ET-1/ET-2 and with another receptor type which responds preferentially to ET-3. In the trachea, neither of these receptors appears to be present since all three peptides apparently act on a homogeneous population of receptors with characteristics different from those of the two arterial receptors. This suggests a third non-isopeptide selective type of endothelin receptor.

Keywords: Endothelin; *in vitro* pharmacology; pulmonary blood vessels; tachyphylaxis; trachea; endothelin receptors

Introduction

Three endothelin genes with vasoactive products have been described in the human genome (Inoue *et al.*, 1989). The products expressed by these genes are distinct from each other but display a considerable homology. The 'classical' endothelin, endothelin 1 (ET-1), is the product originally isolated from porcine aortic endothelial cells. ET-3 differs from ET-1 in 6 out of 21 residues (Yanagisawa *et al.*, 1988a,b). ET-2 bears a close resemblance to ET-1, with Leu^6 and Met^7 substituted for Trp^6 and Leu^7, respectively (Inoue *et al.*, 1989). The three endothelins are potent vasoconstrictors as originally shown in porcine isolated aorta and coronary arteries (Inoue *et al.*, 1989). Different pharmacological profiles have led to the suggestion that there are endothelin receptor subtypes (Inoue *et al.*, 1989; Yanagisawa & Masaki, 1989). This assumption has gained further support from both ligand binding studies (Watanabe *et al.*, 1989; Masuda *et al.*, 1989) as well as recent work with cloned receptors (Arai *et al.*, 1990; Sakurai *et al.*, 1990). In the guinea-pig pulmonary vascular bed a remarkable degree of tachyphylaxis was noted for ET-1 (Cardell *et al.*, 1990). This is in agreement with findings in guinea-pig femoral artery (Wiklund *et al.*, 1988) and porcine aorta (Ishikawa *et al.*, 1988). In the present study we have examined endothelin-induced responses in the pulmonary artery and trachea of guinea-pig in an attempt to differentiate between different types of endothelin receptors. A preliminary account of some of this work was presented at the IUPHAR symposium on Chemistry and Biology of Endothelin (Cardell *et al.*, 1991).

Methods

Young male guinea-pigs (200–300 g) were killed by a blow on the neck. The lungs, including the heart and trachea, were quickly removed and immersed in a cold (+4°C) buffer solution (for composition, see below). Lobar arteries (second to third branches) and a distal portion of the trachea were dissected free of surrounding tissue. The vessels and the trachea were used in the experiments either immediately or, occasionally, following overnight storage in a cold buffer solution. Circular segments were mounted on two L-shaped metal prongs. One prong was connected to a force displacement transducer attached to a Grass polygraph for continuous registration of isometric tension and the other to a displacement device. The mounted segments were immersed in small (2.5 ml) temperature-controlled (37°C) tissue baths containing the buffer solution. The solution was equilibrated with 5% CO_2 in O_2, giving a pH of 7.4.

Initially, a tension of 1–2 mN was applied to the arterial segments and 2–3 mN was applied in the tracheal segments. The segments were subsequently allowed to stabilize at this level of tension for 90 min. The contractile ability of each segment was then examined by exposure to a potassium rich (60 mM) buffer solution (for composition, see below). Only when two reproducible contractions could be elicited was the individual segment used in further studies. The integrity of the vascular endothelium was assessed at the end of most experiments by obtaining a dilator response to acetylcholine (10^{-6} M) (Furchgott, 1984). The presence of tracheal epithelium was confirmed by staining with a 5% silver nitrate solution followed by light microscopy (Abrol *et al.*, 1984). There were no differences in the response to the endothelins when concentration-response curves obtained by cumulative application were compared to those obtained by a single dose procedure (Cardell *et al.*, 1990).

The log concentration-response relationship was approximated by linear regression analysis of the data within the 5% to 95% response interval. The pD_2 value (i.e. the negative logarithm of the concentration eliciting half the maximum response) and E_{max}% (the maximal contraction elicited by an agonist expressed as a percentage of the contraction induced by 60 mM K^+) was calculated for each experiment.

The strong tachyphylaxis caused by the endothelins was used as the basis of a series of desensitization tests (Miasiro & Paiva, 1990). Both the homologous and the cross-desensitization patterns for the three peptides were investigated. In the homologous desensitization tests the segments

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were first contracted by ET-1, ET-2 or ET-3. After repeated washes during a 90 min recovery period the initially contracted segment had returned to its 'resting' state (baseline). A single dose of the peptide was then reapplied to the segment. The cross desensitization test was performed according to the same principle, but at the point of reapplication a different endothelin was administered.

Solutions and drugs

The following solutions were used: (a) standard buffer solution (mM): NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2, glucose 11. (b) 60 mM K^+ buffer solution: as above, but substituting equimolar amounts of NaCl with KCl. Analytical-grade chemicals and twice-distilled water were used for preparing all solutions.

The following drugs were used: acetylcholine chloride (Sigma, U.S.A.), endothelin 1, endothelin 2, endothelin 3 (Peninsula, U.S.A.) 5-hydroxytryptamine creatine sulphate (Sigma, U.S.A.), histamine dihydrochloride (Sigma, U.S.A.), and prostaglandin $F_{2\alpha}$ (Astra, Sweden).

Endothelins were dissolved in and further diluted in saline containing bovine serum albumin (1%) and used in the experiments within 30 min to avoid possible degradation. The concentrations of the agents are expressed as the final molar concentration in the bath.

Statistics

Data were tested by analysis of variance (ANOVA) followed by Scheffe's method in order to test specific differences between groups (Wallenstein *et al.*, 1980).

Results

Contractile responses

ET-1, ET-2 and ET-3 elicited strong concentration-dependent contractions of the pulmonary lobar artery (Figure 1a) and the trachea (Figure 1b). The responses were slow in onset and long lasting.

In the pulmonary artery, ET-1 induced the greatest maximum constriction of the three peptides. The potencies of ET-1 and ET-2 were similar, while ET-3 was less active as compared to the other two peptides (Figure 1a and Table 1). Notably, ET-3 did not produce a contraction in vessels from approximately every sixth animal tested. These experiments were not included in the calculations. In the segments that did not respond to ET-3, histamine elicited strong reproducible contractions which could be reversed by acetylcholine (not illustrated).

Table 1 Guinea-pig pulmonary artery and trachea: maximum response (E_{max}%) to endothelin, expressed as a percentage of the contraction induced by 60 mM potassium, and sensitivity (pD_2) expressed as the negative logarithm of the concentration eliciting half maximum response

Pulmonary artery	n	E_{max}%	pD_2
ET-1	14	168 ± 21	8.65 ± 0.18
ET-2	9	121 ± 19 **	8.67 ± 0.16 NS
ET-3	9	100 ± 19 * **	7.79 ± 0.16 ** **
ANOVA		**	**
Trachea	n	E_{max}%	pD_2
ET-1	8	74 ± 13	7.81 ± 0.26
ET-2	5	75 ± 15	8.03 ± 0.39
ET-3	7	67 ± 15	7.94 ± 0.16
ANOVA		NS	NS

ET-1, endothelin 1; ET-2, endothelin 2; ET-3, endothelin 3.
The values represent the mean ± standard deviation.
Analysis of variance (ANOVA) was used followed by Scheffe's method in order to test specific differences between groups. * $P < 0.05$; ** $P < 0.01$; NS = no significant difference.

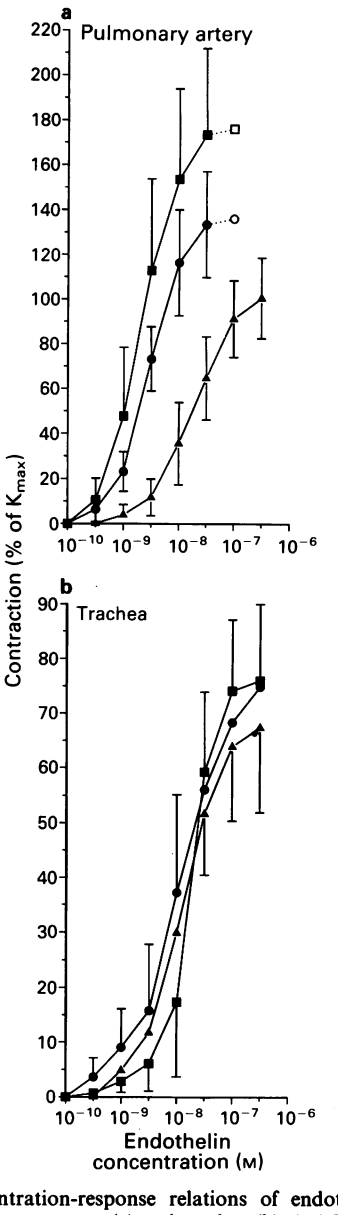


Figure 1 Concentration-response relations of endothelins (ET) in guinea-pig pulmonary artery (a) and trachea (b): (■) ET-1; (●) ET-2 and (▲) ET-3. Responses are expressed as a percentage of potassium (60 mM)-induced contraction and each point is the mean with s.d. shown by vertical bars. Pulmonary artery $n = 9$–14. Trachea: $n = 5$–8. In some arterial experiments a concentration of 10^{-7} M of ET-1 (□) and ET-2 (○) was also tested (172 ± 28%, $n = 7$ and 124 ± 13%, $n = 4$, respectively). Since there was no significant difference compared to 3×10^{-8} M for these peptides, the concentration 3×10^{-8} M was used for calculation of E_{max}%.

In the trachea, the maximum contractile responses to ET-1, ET-2 and ET-3 were smaller ($P < 0.01$) than the corresponding responses in the pulmonary arteries. The potencies of ET-1 and ET-2 were significantly less in the trachea than in the pulmonary artery. In contrast to the findings in the pulmonary arteries, there was no significant difference in the maximum contractile response or in potency between the peptides (Table 1).

Additivity experiments

In arterial segments maximally constricted by ET-3, application of ET-1 or ET-2 induced an additional contraction (Figure 2a). In segments maximally contracted by ET-1 or

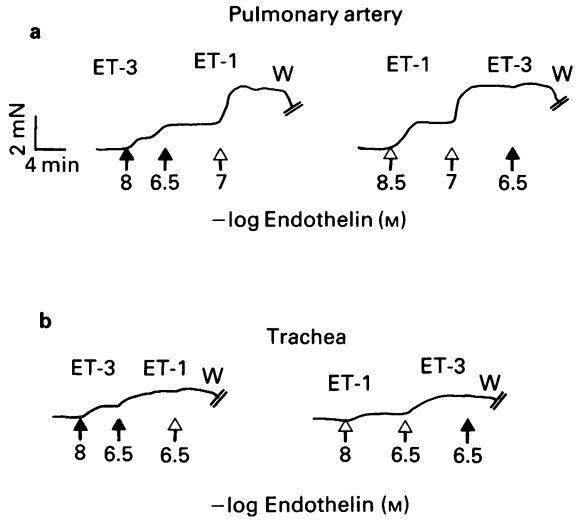


Figure 2 Typical examples of endothelin (ET)-induced contractions of guinea-pig pulmonary artery (a) and trachea (b). Cumulative application of ET-3 followed by ET-1 and cumulative application of ET-1 followed by ET-3, respectively. This type of experimentation was repeated four times in the trachea and six times in the pulmonary artery. At each arrow ET-1 (open) or ET-3 (filled) was added to the buffer solution. W, wash.

ET-2, only a small additive contraction was induced by ET-3. This contraction was more obvious in vessels precontracted by ET-2 than ET-1. None of the peptides induced an additional constriction of tracheal segments already maximally contracted by one of the other two peptides (Figure 2b).

The time course for the development of contraction of pulmonary arteries and trachea was equally long lasting and did not differ appreciably among the three peptides. However, the tracheal segments reached their maximum contraction somewhat more slowly than the vessel segments (Figure 2).

Homologous desensitization

After 2 h of repeated washing, arteries initially contracted by ET-1, ET-2 or ET-3 had returned to their 'resting' state (baseline). When ET-1 or ET-2 was reapplied, no or occasionally a small transient contraction (<7%) was seen (Figure 3a,b). Tachyphylaxis could also be demonstrated with ET-3; reapplication induced only about 40% of the initially induced contraction (Figure 3c). In the trachea, the tachyphylaxis seen after reapplication of the same peptide was rather weak for all three peptides. Furthermore, there were no major differences in the tachyphylaxis patterns for the three peptides. They all reached about 70% of the initial contraction (Figure 3d–f).

Cross desensitization

Vessel segments, initially contracted by ET-1, returned to their 'resting' contractile state after 2 h of repeated washes. When ET-2 was added to these segments, no or at most a weak transient contraction (<10%) was seen (Figure 4a). The same pattern was seen when ET-1 was given to segments previously exposed to ET-2 (Figure 4b). There was no apparent tachyphylaxis when applying ET-1/ET-2 after ET-3 (Figure 4c,d) or when applying ET-3 after ET-1/ET-2 (Figure 4e,f). Preparations that were tachyphylactic to ET-1/ET-2 still responded not only to ET-3 but also to histamine (10^{-6} M), 5-hydroxytryptamine (10^{-6} M), KCl (60 mM) and prostaglandin $F_{2\alpha}$ (10^{-6} M) (not illustrated).

In the trachea, the end tachyphylaxis seen after cross desensitization was identical for all three peptides and did not differ from the tachyphylaxis seen after homologous desensitization (Figure 4g–l).

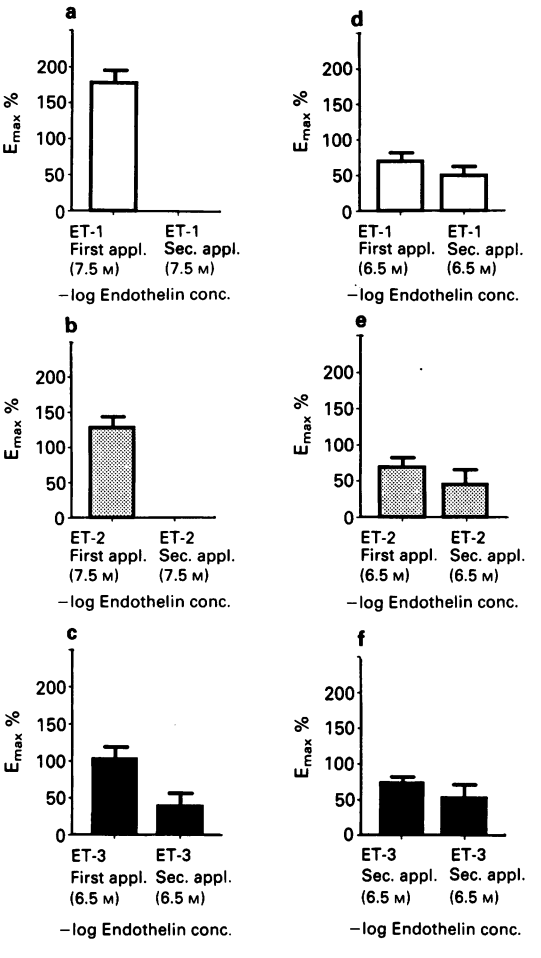
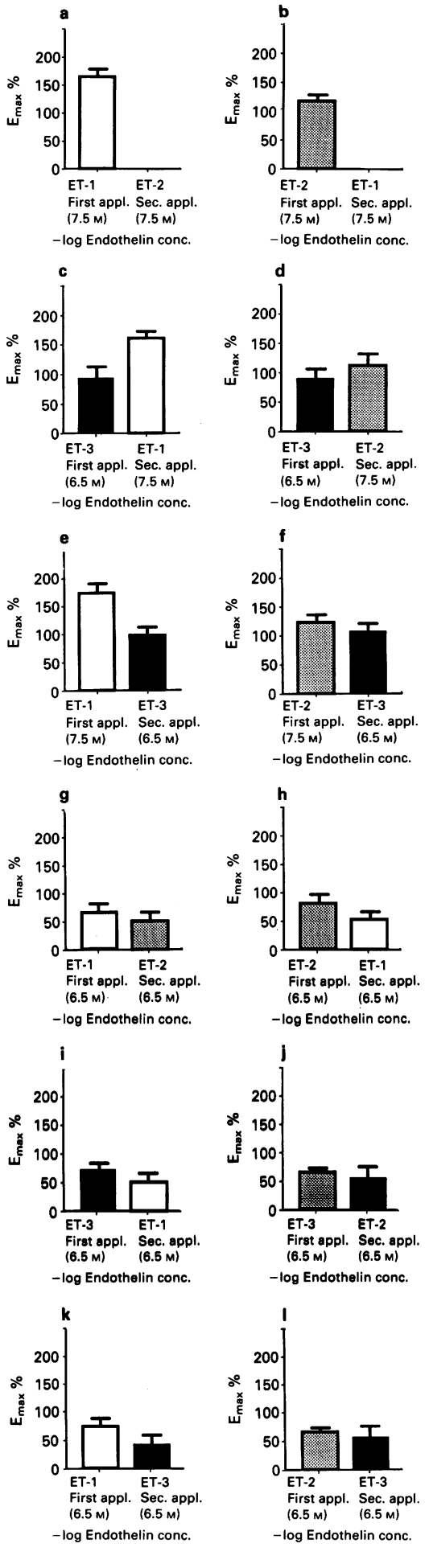


Figure 3 Homologous desensitization of endothelin 1 (ET-1), ET-2 and ET-3. Quantitative data for guinea-pig pulmonary arterial (a–c) and tracheal (d–f) segments initially contracted by ET-1, ET-2 or ET-3. After 2 h of repeated washings the segments had returned to their baseline. (a–b) When ET-1 or ET-2 was reapplied no persistent contraction was induced. (c) When ET-3 was reapplied a small contraction was seen. (d–f) After reapplication of the same peptide, ET-1, ET-2 or ET-3 to the tracheal segments a second contraction, slightly smaller than the first was seen. The responses are expressed as a percentage of potassium (60 mM)-induced contraction and the columns represent the mean with s.d. shown by vertical bars. n = 3–4 for each experiment.

Discussion

The present study demonstrates that ET-1, ET-2 and ET-3 induce a slowly developing, long lasting contraction of guinea-pig isolated tracheal and pulmonary arterial segments. Repeated washes for up to 2 h are required before the muscle tension returns to the control level (Ohlstein *et al.*, 1989). This may reflect slow dissociation of the endothelins from their receptors and/or long lasting intracellular effects (Gu *et al.*, 1988; Borges *et al.*, 1989). The vasoconstrictor activity in terms of maximum tension showed the order ET-1 > ET-2 > ET-3 and the rank order of potency was ET-1 = ET-2 > ET-3. This profile is in agreement with findings in rat pulmonary artery (Nakajima *et al.*, 1989) and porcine coronary artery (Inoue *et al.*, 1989). In the tracheal segments the order of maximum contractile activity was ET-1 = ET-2 = ET-3 and the order of potency was the same.

In vivo, a bolus injection of ET-1, ET-2 or ET-3 produces an initial depressor response, followed by an increase of the systemic blood pressure (Wright & Fozard, 1988; de Nucci *et al.*, 1988; Le Monnier de Gouville *et al.*, 1990). Although the initial relaxation is characteristic of the systemic response, such a response is seldom seen *in vitro* (Folta *et al.*, 1989). The tachyphylaxis pattern seen *in vitro* is also different from that



found under *in vivo* conditions since only the vasodepressor effects of the endothelins display tachyphylaxis *in vivo* (Le Monnier de Gouville *et al.*, 1990). Furthermore, regional and species-related differences in the endothelin responses are well known phenomena (Minkes & Kadowitz, 1989; Faraci, 1989; Lippton *et al.*, 1988). Endothelin is believed to exert its biological activity through interaction with specific receptors on the target cells (Miyazaki *et al.*, 1989; Watanabe *et al.*, 1989). The pharmacological profiles of the three structurally distinct endothelins suggest the existence of different receptor types (Inoue *et al.*, 1989) and two receptors for endothelin have been proposed (Randall *et al.*, 1989; Warner *et al.*, 1989; Fu *et al.*, 1989). In the present study, the three endothelins were used in an attempt to illustrate the existence of different types of endothelin receptors in the guinea-pig lung.

Treatment of pulmonary arteries with ET-1 led to desensitization towards ET-1. The desensitization seemed to be of the homologous type, since previous treatment with ET-1 did not alter the expected responses of either potassium or histamine. The same homologous desensitization pattern was seen for ET-2 and ET-3. The strong cross-tachyphylaxis reaction between ET-1 and ET-2 in the pulmonary artery implies that they act at the same functional receptor site or sites. However, it must be realized that tachyphylaxis does not have to involve receptor inactivation or desensitization, post receptor events may also desensitize. ET-3, on the other hand, produced tachyphylaxis upon reapplication, but there was no evidence of cross-tachyphylaxis towards ET-1 or ET-2.

ET-3 has been shown to be more easily dissociated from the relevant endothelin receptor than ET-1, probably due to a more polar nature of the N-terminal part of the molecule (Yanagisawa *et al.*, 1988a). This alone cannot explain the difference in the tachyphylaxis pattern. A more likely explanation is that ET-1 and ET-2 act on the same functional site while ET-3 acts on another site. In recent ligand binding studies on chicken cardiac membranes and rat lung membranes, two distinct types of endothelin binding sites have been found (Watanabe *et al.*, 1989; Masuda *et al.*, 1989). One site has a high affinity for ET-1 and ET-2 whereas the other site interacts preferentially with ET-3.

In the additivity experiments carried out in the present study the arterial segments which had reached the E_{max}-level for ET-3, could be further contracted by ET-1/ET-2. In the reverse situation, where ET-1 or ET-2 were used to maximally contract the arteries, ET-3 induced a small additive contraction. The latter situation was more difficult to demonstrate, given the profound contraction produced by ET-1 and to a lesser extent, ET-2. These findings give further support to the view that different receptors are involved in the vasoconstrictor responses to the endothelins.

In the trachea the pharmacological profile of the three endothelins differed from that in the pulmonary artery in that there was no difference between them in potency or intrinsic activity. Furthermore, ET-1, ET-2 or ET-3 did not further contract segments precontracted by either of the other two peptides. This is partly in agreement with the conclusions of Maggi *et al.* (1989) who postulated an endothelin receptor in the guinea-pig bronchi different from that in the guinea-pig aorta.

Figure 4 Cross desensitization of endothelin 1 (ET-1), ET-2 and ET-3. Quantitative data for guinea-pig pulmonary arterial (a–f) and tracheal (g–l) segments initially contracted by ET-1, ET-2 or ET-3. After repeated washing and a 2 h recovery period another peptide was applied in the same dose. (a–b) Strong cross tachyphylaxis reaction between ET-1 and ET-2. (c–d) No sign of tachyphylactic interference between ET-3 and ET-1/ET-2. (e–f) Application of ET-3 after contraction induced by ET-1 or ET-2 resulted in a normal contraction slightly smaller than control. (g–l) Only a slight tendency of tachyphylaxis is seen between the three peptides in the tracheal segment. The responses are expressed as a percentage of potassium (60 mM)-induced contraction and the columns represent the mean with s.d. shown by vertical bars. $n = 3$–4 for each experiment.

Recently, two separate endothelin receptors were cloned. Through ligand binding studies one was found to correspond to an ET-1 selective receptor type (Arai *et al.*, 1990), whereas the other was a non-isopeptide-selective type designated ET_B (Sakurai *et al.*, 1990). The ET_B receptor mRNA was not found to be present in vascular smooth muscle cells. This receptor could very well correspond to the tracheal endothelin receptor described in this paper.

According to Sakurai *et al.* (1990) the ET-1/ET-2 selective receptor found in the vascular smooth muscle cells should be referred to as an ET_A receptor. It is therefore tempting to propose that the contractile vascular endothelin receptor population in the pulmonary artery is dominated by the ET-1/ET-2 receptor site (most likely an ET_A-receptor) while the postulated ET-3 receptor site is of minor significance. In the trachea a third non-isopeptide-selective type of endothelin receptor is found, probably being of the ET_B-type.

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Characterization of postsynaptic α-adrenoceptors in the arteries supplying the oviduct

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1 *In vitro* experiments were designed to characterize postjunctional α-adrenoceptor subtypes in ring segments (1 mm length; outer diameter 300–500 μm) from arteries supplying the oviduct of the heifer.

2 Noradrenaline, adrenaline and phenylephrine evoked concentration-dependent contractile responses. The pD_2 values were 5.67, 5.89 and 5.93, respectively. Medetomidine, clonidine and B-HT 920 (2-amino-6-allyl-5,6,7,8-tetra-hydro-4H-(thiazo)-4,5-d-azepine) were ineffective.

3 The α-adrenoceptor selective antagonists, prazosin (1 nM–0.1 μM) and rauwolscine (0.1–10 μM) competitively antagonized the response to noradrenaline. The pA_2 values were 9.38 and 6.83, respectively.

4 The dissociation constant (K_D) for noradrenaline calculated by use of the irreversible antagonist, dibenamine, was 3.95 (2.09–5.81) μM. The occupancy-response relationship was non-linear. Half-maximal response to noradrenaline was obtained with 22% receptor occupancy while maximal response required 100% occupancy.

5 B-HT 920 evoked a biphasic contractile concentration-dependent response in preparations incubated in a physiological solution containing 20 mM K^+, 0.1 μM prazosin and 1 μM propranolol. Rauwolscine 0.1 μM significantly ($P < 0.01$) blocked the first component of the B-HT 920 concentration-response curve with an apparent pA_2 value of 8.52 (7.86–9.18).

6 These results strongly suggest that α-adrenoceptors in oviductal arteries are mainly of the α_1 subtype, although a possible role for α_2-adrenoceptors cannot be excluded.

Keywords: α-Adrenoceptors; vascular smooth muscle; oviductal arteries

Introduction

The oviduct and arteries which supply it (i.e. oviductal arteries) are richly innervated by sympathetic nerves (Brunding *et al.*, 1969; Black, 1974). It is well established that the sympathetic nervous system is involved in the control of the contractile activity of the oviduct (Howe & Black, 1973; Rodriguez-Martinez, 1984; Samuelson & Sjöstrand, 1986; Isla *et al.*, 1989). This control is also related to the hormonal status through the oestrous cycle, which could influence the catecholamine concentration as well as the receptor predominance (Samuelson & Sjöstrand, 1986; Juorio *et al.*, 1989).

The role of the sympathetic nervous system in non-contractile processes that take place in the oviduct, such as oviduct fluid formation, is yet to be elucidated (Forman *et al.*, 1986). It has been suggested that the sympathetic nervous system could be involved in regulating this process through a control of the blood supply to the oviduct, so that its agents would be expected to inhibit oviduct fluid formation (Leese, 1988).

Limited information exists on the effects of noradrenergic agents on the arteries supplying the oviduct. It is known that noradrenaline evokes a concentration-dependent contractile response in human isolated oviductal arteries (Forman *et al.*, 1985); however, the α-adrenoceptor subtypes involved in this response, have not yet been determined.

Selective receptor agonists and antagonists have shown to be useful tools in the pharmacological characterization of receptors (Kenakin, 1987). Irreversible antagonists such as dibenamine, partially inactivate the α-adrenoceptor population so that the fraction of receptor occupied at each concentration of agonist can be calculated (Furchgott & Bursztyn, 1967).

Since the importance of the sympathetic nervous system in the oviduct vascular bed is still rather unknown, the present investigation was undertaken to characterize the α-adrenoceptor subtypes involved in the contractile activity of isolated oviductal arteries. The dissociation constant and the receptor reserve for noradrenaline-induced responses were also determined. Oviductal arteries from immature heifers, in which an influence of the sex hormones can be ruled out, were used in this study.

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Methods

Vascular preparations

Genital tracts from immature heifers with ovaries macroscopically quiescent were collected daily from the slaughterhouse, placed in ice-cold physiological salt solution (PSS) (composition in mM: NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2, glucose 11 and EDTA 0.01 at pH 7.4) and transported to the laboratory.

The oviduct and a small portion of the tip of the uterine horn were separated from the rest of the genital tract and fixed in a Petri dish filled with ice cold PSS. The uterine branch of the ovarian artery was identified and segments of its secondary branches (300–500 μm outer diameter) supplying the oviductal isthmus were carefully dissected free from the mesosalpinx and surrounding tissue with the aid of micro-scissors and a stereomicroscope (Nikon SMZ 2B). Ring preparations of approximately 1 mm in length were obtained and transferred to 5 ml organ baths containing PSS at 37°C bubbled with 95% O_2 and 5% CO_2. The preparations were gently slid onto parallel stainless steel legs (75 μm diameter) of two L-shaped steel hooks (Högestätt *et al.*, 1983). One of the hooks was attached to a displacement unit allowing the fine adjustment of tension and the other was connected to a force-displacement transducer (GRASS FT O3C). Isometric wall tension was recorded on a GRASS model 7B polygraph. Preparations were allowed to equilibrate for about 1 h in PSS. During this period the organ baths were washed with fresh (37°C) PSS every 15 min and the passive tension was set at

1.75 mN mm^{-1} in length by stepwise stretching of the rings. This tension was obtained in previous experiments in which the level of passive tension was correlated to the maximum response elicited by K-PSS (PSS with 119 mM KCl substituted for NaCl). In brief, arterial ring segments ($n = 7$) were exposed every 30 min to K-PSS and the passive tension (resulting tension after washout in PSS) was increased stepwise until a maximal contraction to K-PSS was obtained in all vessel segments.

Experimental procedure

After the equilibration period, rings were contracted two or three times with K-PSS at 30 min intervals. Potassium depolarization served as a control of reproducibility of the contractions and as an internal standard for each vessel ring (Fallgren & Edvinsson, 1986).

The integrity of the endothelium was tested by applying acetylcholine (ACh) (1 μM) to vessels constricted with increasing concentrations of noradrenaline until the level of tension was approximately 50–60% of the response induced by K-PSS. Preparations with intact endothelium relaxed well (80–100% of the noradrenaline response) in the presence of ACh, whereas in the absence of endothelium, ACh had either no effect or even contracted rings.

Contractile concentration-response curves to the different α-adrenoceptor agonists were obtained by adding increasing concentrations of the agonists to the bath in half log unit steps, when the previous concentration had produced its equilibrium response, or after 5 min if no response was obtained (Van Rossum, 1963).

In the experiments where the effects of cocaine, propranolol, prazosin and rauwolscine were investigated, the preparations were incubated for 45 min with the drug before a second concentration-response curve to the agonist was obtained. In order to determine the dissociation constant (K_D) to noradrenaline, the irreversible α-blocker, dibenamine, was used. After determination of the concentration-response curve to noradrenaline, rings were exposed to dibenamine (50 nM) for 10 min and then rinsed at 5 min intervals for the next 60 min. After this period, reproducible concentration-response curves to noradrenaline were obtained which were depressed and shifted to the right with respect to the control. In all the experiments, at least one vessel ring that received no antagonist was run in parallel with the experimental rings. These control experiments showed no change in either the maximum effect or sensitivity after five applications of noradrenaline. In experiments involving α-adrenoceptor antagonists, tissues were pretreated with iproniazid (0.36 mM) for 45 min at the beginning of the experiment to block monoamine oxidase (MAO) and the bath solution contained propranolol (1 μM) to block β-adrenoceptors, corticosterone (10 μM) and cocaine (10 μM) to inhibit the extraneuronal and neuronal uptake mechanisms and tropolone (10 μM) to inhibit catechol-*O*-methyltransferase (COMT).

In order to investigate the α_2-adrenoceptor-mediated contractile activity in pre-depolarized arteries the following experiments were performed. Vascular rings were incubated for 45 min in a K-PSS (20 mM K^+) containing propranolol (1 μM) and prazosin (0.1 μM) to block β- and α_1-adrenoceptors, respectively. After this treatment, concentration-response curves to B-HT 920 (2-amino-6-allyl-5,6,7,8-tetra-hydro-4H-(thiazo)-4,5-d-azepine) in the absence and in the presence of 0.1 μM rauwolscine were established.

Calculations and statistics

The contractile response induced by each concentration of agonist was expressed in mN of tension developed above basal levels and used in the construction of the concentration-response curves. The concentration of each agonist eliciting 50% of its own maximum response (EC_{50}) was determined graphically for each curve by linear interpolation. The relative potencies of the agonists were calculated by the method of the 2 and 2 dose assay (Tallarida & Murray, 1987). The pA_2 values for prazosin and rauwolscine were determined from a Schild plot (Arunlakshana & Schild, 1959) using noradrenaline as the agonist. The concentration ratio (CR) produced by the antagonist (i.e., the ratio of concentrations of noradrenaline giving an equal response in the presence and in the absence of the competitive antagonist: always measured as the EC_{50} value) was determined for various concentrations of antagonist. The apparent pA_2 of rauwolscine in pre-depolarized preparations was determined with only one concentration of the antagonist and B-HT 920 as the agonist. K_B was calculated using the equation (Furchgott, 1972):

$$K_B = \frac{[\text{antagonist}]}{(\text{CR}-1)}$$

where CR is the concentration-ratio (i.e., the ratio of concentrations of B-HT 920 giving 50% of the control maximum response in the presence and in the absence of rauwolscine, 0.1 μM). The control maximum response was established at 10 μM B-HT 920.

The noradrenaline dissociation constant (K_D) was calculated according to the procedure described by Furchgott & Bursztyn (1967); after partial receptor inactivation by an irreversible antagonist, equieffective concentrations of noradrenaline before ([A]) and after ([A']) treatment were compared using the equation (Furchgott, 1966):

$$\frac{1}{[A]} = \frac{1}{q[A']} + \frac{1-q}{qK_D}$$

where K_D is the functional equilibrium dissociation constant for the agonist and q is the fraction of the active receptors remaining after partial irreversible blockade. A plot of 1/[A] against 1/[A'] was constructed. The slope of the regression line and y-intercept were used to calculate K_D from the equation:

$$K_D = \frac{(\text{Slope} - 1)}{\text{Intercept}}$$

The fraction of receptors occupied (RA/RT) in control tissues at each concentration of agonist [A] was calculated from the following equation (Furchgott & Bursztyn, 1967):

$$\frac{[RA]}{[RT]} = \frac{[A]}{K_D + [A]}$$

The control response for noradrenaline was then replotted as a function of the fractional receptor occupation and appropriate curves were constructed. Estimates of the receptor reserve were made from K_D/ED_{50} (Ruffolo, 1982; Kenakin, 1987).

Results are expressed as means ± s.e.mean or as mean values with 95% confidence intervals. One-way analysis of variance (ANOVA) was used when more than two groups were analysed. Statistical differences between two means were determined by Student's t test for paired or unpaired observations where appropriate. A value of $P < 0.05$ was considered to be statistically significant. The least squares method was used for calculating linear regressions. For the Schild plot, differences between the slope and unity were tested with Student's t test, under a null hypothesis (slope = 1). n denotes the number of animals from which vessels were taken.

Drugs

The following drugs were used: (±)-noradrenaline HCl and (−)-adrenaline (Serva, Germany); B-HT 920 (2-amino-6-allyl-5,6,7,8-tetra-hydro-4H-(thiazo)-4,5-d-azepine) (Boehringer Ingelheim, Spain); prazosin HCl (Pfizer, U.S.A.); rauwolscine HCl (Carl Roth, Germany); dibenamine HCl (Smith Kline & French, U.K.); medetomidine HCl (Farmos Group, Finland);

phenylephrine HCl, clonidine HCl, propranolol HCl, cocaine HCl, corticosterone, tropolone and iproniazid (Sigma, U.S.A.). Prazosin was dissolved in warm water (50°C) at pH 4–5 by constant agitation. Dibenamine and corticosterone were prepared in 99% ethanol and adrenaline in 0.25 N HCl and further diluted in water containing ascorbic acid (1 mM). Previous experiments showed that the solvents used had no effect on preparations. Stock solutions of drugs were stored at −20°C and fresh dilutions were made daily except for dibenamine which was prepared immediately before use for each experiment. All drugs were added directly to the bath in volumes of 5–50 μl and the concentrations given are the calculated final concentration in the bath solution.

Results

The α-adrenoceptor agonists adrenaline, noradrenaline and phenylephrine induced concentration-dependent contractions in the arteries supplying the oviduct of the heifer (Figure 1), whereas clonidine, B-HT 920 and medetomidine were ineffective in all vessels tested. The maximum contractile effect to these agonists as well as the sensitivity, expressed as pD_2, were not significantly different. The order of potency, was noradrenaline = phenylephrine = adrenaline (Table 1).

The maximum contractile response (E_{max}) and the EC_{50} (expressed as pD_2) to noradrenaline (E_{max} = 7.40 ± 1.52 mN, pD_2 = 5.87 ± 0.18, n = 5) were unaffected by the addition to the bath of 1 μM cocaine (E_{max} = 7.30 ± 1.46 mN, pD_2 = 5.63 ± 0.27, n = 5) or 10 μM cocaine (E_{max} = 7.30 ± 0.06 mN, pD_2 = 5.61 ± 0.03, n = 5). On the other hand, the concentration-response curves to noradrenaline (E_{max} = 6.50 ± 0.74 mN, pD_2 = 5.84 ± 0.18, n = 5) and adrenaline (E_{max} = 5.95 ± 0.34 mN, pD_2 = 5.91 ± 0.07, n = 7) were not significantly affected by 1 μM propranolol (E_{max} = 6.80 ± 2.20 mN, pD_2 = 5.88 ± 0.24, n = 5 for noradrenaline and E_{max} = 5.91 ± 0.37 mN, pD_2 = 5.88 ± 0.11, n = 7 for adrenaline).

The selective α_1-adrenoceptor antagonist, prazosin (1–100 nM), produced parallel shifts to the right in the noradrenaline concentration-response curve (Figure 2a). A significant antagonism was observed even at concentrations as low as 1 nM. The Schild plot constructed for prazosin against noradrenaline yielded a straight line with a slope not significantly different from unity and an intercept with the abscissa (pA_2) of 9.57 (Figure 2b). Concentration-response curves to noradrenaline were unaffected by 0.1 μM rauwolscine, whereas, increasing the concentration to 1–10 μM rauwolscine, significantly ($P < 0.001$) shifted the concentration-response curves to noradrenaline to the right (Figure 3a). The Schild plot demonstrating the effects of rauwolscine appeared also to fit in well with a single straight regression line close to unity and gave a pA_2 value of 6.83 (Figure 3b). Although some reduction in the maximal response to noradrenaline was seen at the high prazosin and rauwolscine concentrations, the antagonism can be considered competitive, since the concentration-response curves before and after the antagonist were parallel and construction of a Schild plot gave straight lines with slopes not significantly different from unity.

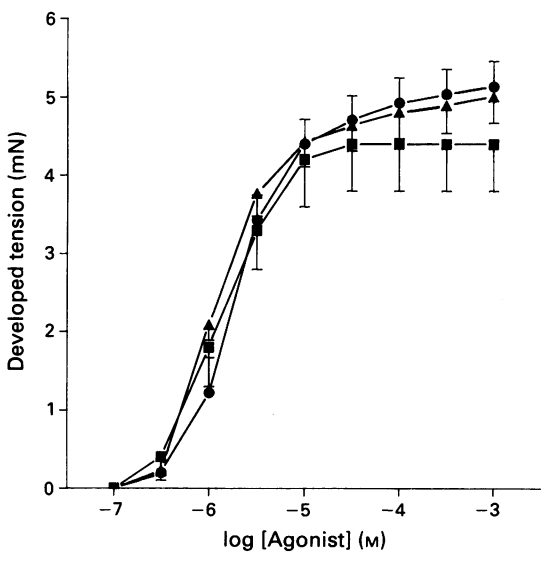

Figure 1 Concentration-response curves in heifer oviductal arteries to noradrenaline (●, n = 12), adrenaline (▲, n = 13) and phenylephrine (■, n = 8). Each point represents mean and vertical lines show s.e.mean.

Table 1 Comparative properties of adrenoceptor agonists on the heifer oviductal arteries

Agonist	n	E_{max} (mN)	pD_2	*Relative potency*
Noradrenaline	12	5.14 ± 0.32	5.67 ± 0.05	1
Adrenaline	13	5.01 ± 0.34	5.89 ± 0.05	0.71
Phenylephrine	8	4.40 ± 0.60	5.93 ± 0.08	0.87

E_{max} values reflect the maximum contractile effects, expressed as the maximal developed tension.
Relative potency obtained by the 2 and 2 dose assay method (Tallarida & Murray, 1987).
pD_2 = −log EC_{50}. Data shown are means ± s.e.mean. n = number of animals.

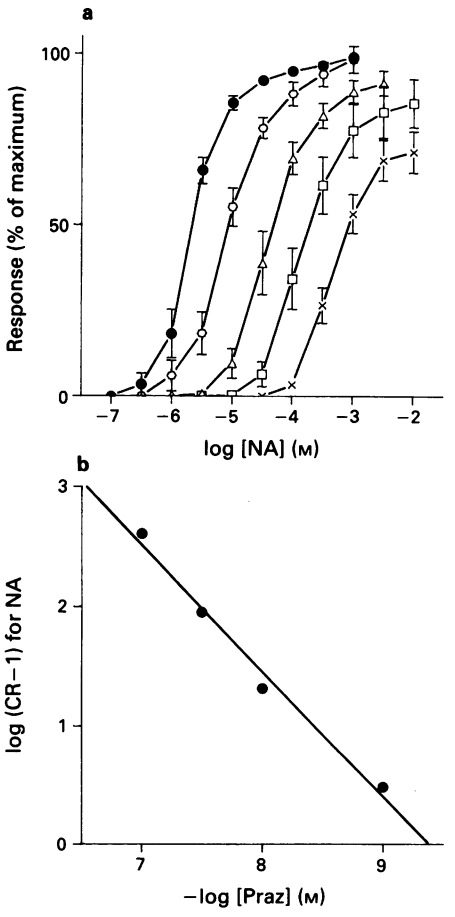

Figure 2 (a) Concentration-response curves for noradrenaline (NA) in the absence (●) and presence of various concentrations of prazosin (Praz): (○) 1 nM, (△) 10 nM, (□) 30 nM, (×) 100 nM. Each point represents the mean and vertical lines show s.e.mean (n = 11). (b) Schild plot of log [CR-1] against −log [antagonist] for Praz-NA antagonism on isolated ring segments of oviductal arteries from heifers. The intercept on the abscissa scale gives the pA_2 value. $y = -1.06x + 9.95$; $r = 0.99$; $pA_2 = 9.38$.

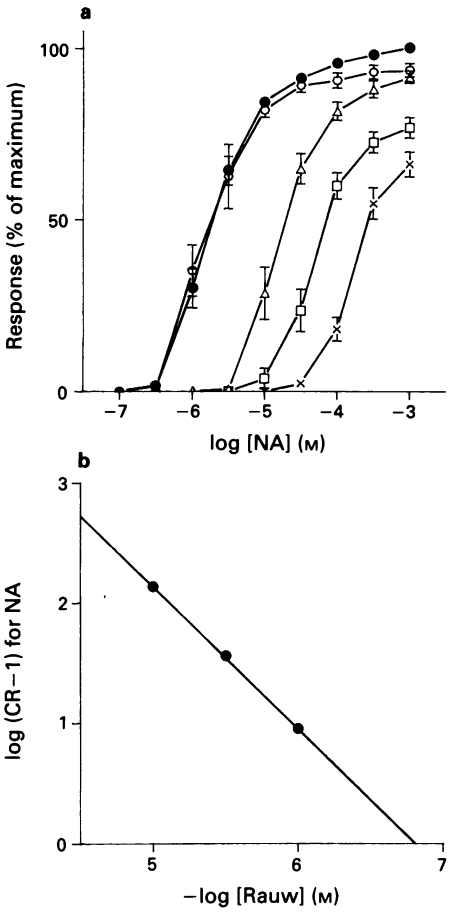

Figure 3 (a) Concentration-response curves for noradrenaline (NA) in the absence (●) and presence of various concentrations of rauwolscine (Rauw): (○) 0.1 μM, (△) 1 μM, (□) 3 μM, (×) 10 μM. Each point represents the mean and vertical lines show s.e.mean ($n = 12$). (b) Schild plot of log [CR-1] against −log [antagonist] for Rauw-NA antagonism on isolated ring segments of oviductal arteries from heifers. The intercept on the abscissa scale gives the pA_2 value. $y = -17x + 8.01$; $r = 0.99$; $pA_2 = 6.83$.

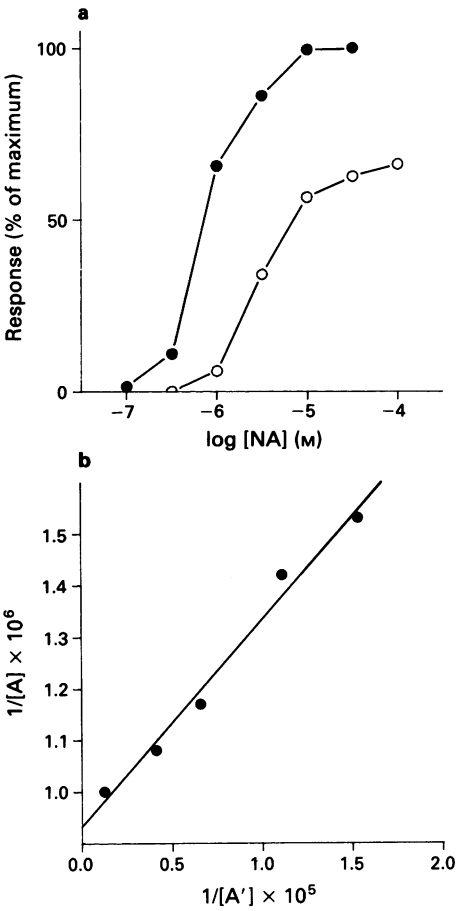

Figure 4 Typical experiment to determine the dissociation constant (K_D) for noradrenaline (NA) in the oviductal arteries of the heifer. (a) NA concentration-response curves before (●) and after (○) exposure of the preparation to dibenamine (50 nM for 10 min). (b) Plot of reciprocals of equiactive concentrations before (1/[A]) and after (1/[A']) treatment with dibenamine from which K_D was calculated as described under 'Methods'. $y = 4.04x + 933962.6$; $K_D = 3.26\,\mu$M.

The dissociation constant (K_D) for noradrenaline was determined by the method of partial alkylation of the α-adrenoceptor population with the irreversible antagonist, dibenamine. An example of these experiments is presented in Figure 4. The mean K_D value was calculated to be 3.95 (2.09–5.81) μM ($n = 11$). The concentration-response relationship for noradrenaline (Figure 5a) was replotted as a function of the receptor occupancy using the calculated K_D mean value (see Methods) and is shown in Figure 5b. A non-linear stimulus-response relationship was obtained, since the maximal response to noradrenaline needed 100% receptor occupancy, whereas the half maximal response was obtained with only 22% of the available receptors. The receptor reserve expressed as K_D/EC_{50} was 2.86.

In preparations pre-depolarized with K-PSS (20 mM K^+) and in the presence of propranolol (1 μM) and prazosin (0.1 μM), B-HT 920 evoked a biphasic concentration-response relationship with an initial small contractile component observed at concentrations up to 10 μM and a second component showing a greater contractile response at higher concentrations. Rauwolscine (0.1 μM) significantly ($P < 0.01$) blocked the first contractile component with an apparent pA_2 (−log K_B) value of 8.52 (9.18 − 7.86) ($n = 9$), whereas the second was unaffected (Figure 6).

Discussion

The results obtained in the present investigations suggest that responses to α-adrenoceptor agonists are mediated predominantly by α_1-adrenoceptors, although the presence of α_2-adrenoceptors cannot be excluded.

The contractile responses obtained with the endogenous agonists noradrenaline and adrenaline, which exhibit mixed α_1- and α_2-adrenoceptor activity (Timmermans & Van Zwieten, 1981), indicate the presence of α-adrenoceptors in the oviductal arteries of the heifer. The lack of effect of propranolol, a β-adrenoceptor antagonist, on the response to noradrenaline and adrenaline suggest that β-adrenoceptor activation is not involved in their effects.

Cocaine, which inhibits neuronal catechol uptake mechanisms and thus, increases the agonist concentration in the neuroeffector junction, had no effect on the noradrenaline-induced contraction, suggesting that no neuronal uptake occurs in the oviductal arteries of the heifer. This is in agreement with the results obtained in uterine arteries from the guinea-pig (Fallgren & Edvinsson, 1986) and ewe (Isla & Dyer, 1990).

Phenylephrine, a selective α_1-adrenoceptor agonist (Starke & Docherty, 1982) seems to behave as a full agonist since its maximum contractile effect was not significantly different from that obtained with the endogenous agonists adrenaline and noradrenaline. Phenylephrine was shown to be equipotent with noradrenaline and adrenaline suggesting the predominance of α_1-adrenoceptor activation by these agonists. Furthermore, the fact that the selective α_2-adrenoceptor agonists B-HT 920 (Timmermans & Van Zwieten, 1982) and medetomidine (Scheinin *et al.*, 1987) failed to contract oviductal arteries argues in favour of the α-adrenoceptors being of the α_1-subtype.

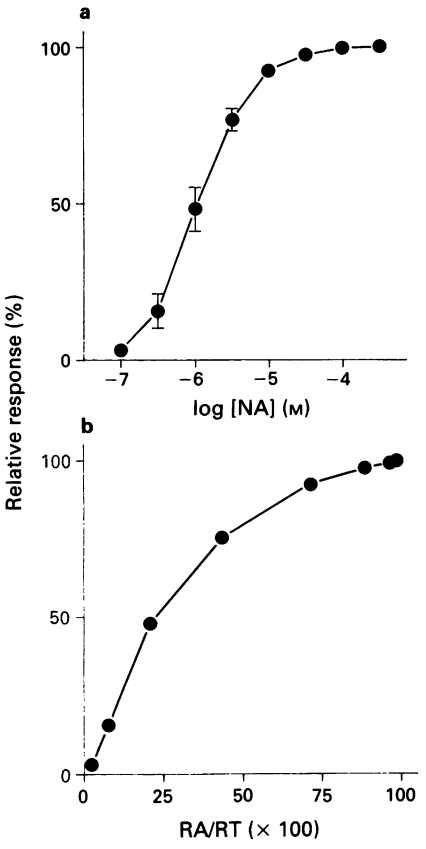

Figure 5 Relationship of the relative response to the log concentration and the fraction of receptors occupied in the noradrenaline (NA) stimulation. (a) Concentration-response curve for the contractile effects of NA in heifer oviductal arteries. Responses are expressed as a percentage of the maximum contractile response elicited by NA. Each point represents the mean and vertical lines show s.e.mean ($n = 15$). (b) Replot of data from (a) showing the relative response as a function of receptors occupied by NA. The fraction of receptors occupied was calculated employing the average K_D value for NA (3.95 μM).

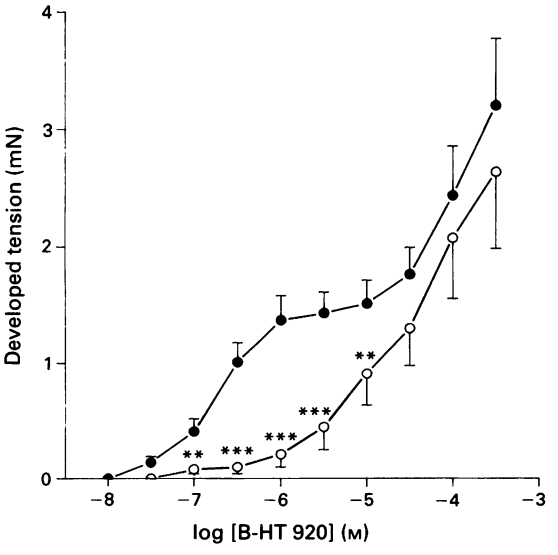

Figure 6 Concentration-response curve for B-HT 920 in the absence (●) and presence of 0.1 μM rauwolscine (○). The bath solution contained 20 mM K^+, propranolol (1 μM) and prazosin (0.1 μM). Tissues were exposed to rauwolscine for 45 min. Each point represents the mean and vertical lines show s.e.mean ($n = 9$). $^{**}P < 0.01$; $^{***}P < 0.001$.

It is generally held that antagonists are better tools than agonists for classifying receptors. This is mainly due to differences in efficacies between agonists, which may seriously hamper the interpretation of the results (Starke, 1981). Prazosin and rauwolscine are generally believed to be among the most selective antagonists for α_1- and α_2-adrenoceptors, respectively (Starke, 1981). The pA_2 value for an antagonist in blocking the response to an agonist should be an accurate indication of its affinity for binding to the receptor site if appropriate precautions are taken and certain criteria fulfilled (Furchgott, 1972; Ruffolo, 1982; Kenakin, 1984; 1987). In the present study, the experiments with prazosin and rauwolscine were carried out in the presence of blockers of neuronal and extraneuronal noradrenaline uptake, β-adrenoceptors, MAO and COMT. Furthermore, in order to maintain the functional integrity of the vessels, care was taken not to remove the endothelium.

The slope of the Schild plot for prazosin in this study was not different from unity indicating that the antagonism is caused in a competitive manner and therefore that the obtained pA_2 value can be assimilated as the $-\log K_B$ value (Arunlakshana & Schild, 1959). According to Furchgott (1972), the K_B value for a specific antagonist acting on the same type of receptor in different preparations should be the same. The pA_2 value of prazosin obtained in the present study (9.38) indicates a high affinity for this α_1-adrenoceptor and is within the range reported for α_1-adrenoceptor blockade (Agrawal *et al.*, 1984; Skärby & Larsson, 1987). However, the Schild plot for rauwolscine gave a slope which was not significantly different from unity and a pA_2 value of 6.83. According to Andersson *et al.* (1984), concentrations of rauwolscine around 10 nM selectively interact with α_2-adrenoceptors; however, concentrations higher than 0.3 μM have been reported to be non-selective for α_2-adrenoceptors, interacting in addition with α_1-adrenoceptors (Skärby & Larsson, 1987). Representative pA_2 values for the interaction of rauwolscine with α_1-adrenoceptors have been shown to be in the range 5.1 to 5.9 (Andersson *et al.*, 1984; Högestätt & Andersson, 1984). Although the rauwolscine pA_2 value in oviductal arteries was higher than this range, the high prazosin/rauwolscine affinity ratio (> 500) and the high rauwolscine concentration needed to obtain a significant inhibition of contraction indicates that rauwolscine is acting non-selectively on α_1-adrenoceptors in oviductal arteries of the heifer.

The obtained K_D value for noradrenaline is in the range reported for K_D in other vascular beds in which a predominance of α_1-adrenoceptors exists (Oriowo *et al.*, 1989; Isla & Dyer, 1990).

Partial receptor alkylation of α-adrenoceptors with dibenamine revealed a non-linear relationship between contraction and percentage receptor occupancy for noradrenaline. Noradrenaline evoked a half maximal contractile response by occupying only 22% of the α-adrenoceptors but maximal response required 100% occupancy. As originally defined, the receptor reserve is the fraction of the total receptor pool not required for a maximal tissue response. From our results, it can be seen that virtually all the receptors are required for maximal response (no receptor reserve as originally defined). The quantification of the receptor reserve by the ratio K_D/EC_{50} better expresses the efficiency of coupling (Ruffolo, 1982; Kenakin, 1987). In oviductal arteries, this ratio was higher than unity suggesting that noradrenaline behaves as a full agonist and that a receptor reserve for the half maximal response exists. However, this ratio is low compared to other vascular beds in which a receptor reserve exists. Thus, in the rabbit aorta, K_D was 21 fold higher than the EC_{50} value (Ruffolo, 1982). This may indicate that in the oviductal arteries of the heifer the coupling between stimulus and response is inefficient.

The results discussed here correlate well with the suggestion of an exclusive population of α-adrenoceptors of the α_1-subtype in oviductal arteries of the heifer. However, it has been noticed that agents causing an elevation of intracellular

calcium concentration such as KCl (Harker et al., 1990), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Furuta, 1988), arginine vasopressin (Templeton et al., 1989), Bay K 8644 (Sulpizio & Hieble, 1987) and endothelin-1 (MacLean & McGrath, 1990) are able to 'unmask' or increase the expression of α_2-adrenoceptor activity. In this sense, contractions evoked by α_2-adrenoceptor stimulants are shown to rely more heavily on the influx of extracellular calcium than those caused by α_1-adrenoceptor activation (Cavero et al., 1983; Van Zwieten & Timmermans, 1987). It is most likely that this influx occurs through voltage-dependent calcium channels (Cavero et al., 1983) and would therefore be sensitive to any alteration in membrane potential. In oviductal arteries partially depolarized with KCl in the presence of prazosin, the α_2-adrenoceptor agonist B-HT 920 evoked a biphasic contractile concentration-dependent curve. This pattern of contractile behaviour has been previously shown with noradrenaline and could reflect the action of receptors other than α-adrenoceptors (Bevan, 1981). From our results, we can speculate that the slight initial contractile component to B-HT 920 could be related to the expression of α_2-adrenoceptor activity, whereas the more marked second component could reflect non-α-adrenoceptor activation or a nonspecific action of this compound. Thus, rauwolscine had a significant blocking effect on the first component with a pA_2 value (8.52) consistent with an interaction with α_2-adrenoceptors (Andersson et al., 1984; Skärby & Larsson, 1987), while the second component was not affected. The physiological significance of this α_2-population is not clear. Ford et al. (1989) have suggested that in uterine arteries, α_2-adrenoceptors could be involved in maintaining the vascular tone rather than in evoking contraction. However, further studies are necessary to ascertain the functional role of this α_2-adrenoceptor population.

In conclusion, we suggest that the α-adrenoceptors involved in the contractile activity of the oviductal arteries are predominantly of the α_1-subtype, without excluding a possible role for α_2-adrenoceptors. A receptor reserve for α-adrenoceptors exists in this tissue although a less efficacious stimulus-response coupling seems to operate compared to other noradrenergically innervated vascular smooth muscles.

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Increase in vascular permeability produced in rat airways by PAF: potentiation by adrenalectomy

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1 The effect of bilateral adrenalectomy on the sensitivity of blood vessels in rat airways to mediators that increase vascular permeability was examined.

2 An increase in vascular permeability was induced by intravenous platelet activating factor (PAF, 50, 100, 500, 1000 ng kg^{-1}) and measured by quantifying the extravasation of Evans blue dye.

3 PAF consistently increased the amount of Evans blue extravasation in the larynx, trachea, main bronchi and intrapulmonary airways in sham-operated rats.

4 The magnitude of this extravasation was significantly greater in the larynx ($P < 0.05$), trachea ($P < 0.05$) and main bronchi ($P < 0.05$) of the adrenalectomized rats than it was in these tissues of the sham-operated rats.

5 When adrenalectomized rats were given subcutaneous dexamethasone (0.2 mg kg^{-1} 4 h before PAF) the amount of plasma extravasation produced by PAF was decreased to the level of the sham-operated rats.

6 We conclude that adrenalectomy potentiates the increase in airway vascular permeability induced by PAF in rats and that this effect may be due to the depletion of endogenous corticosteroids.

Keywords: Glucocorticosteroids; airway oedema; anti-inflammatory agents; inflammation; blood vessels; permeability; asthma; PAF.

Introduction

Adrenalectomy influences the effect on vascular permeability of different stimuli in different organs or tissues. In rats, adrenalectomy potentiates the vascular permeability induced by histamine and 5-hydroxytryptamine in the skin (Leme & Wilhelm, 1975), the pulmonary vascular permeability induced by hypoxia (Stelzner *et al.*, 1988), and the gastric vascular permeability induced by ethanol (Nishiwaki *et al.*, 1989). Interestingly in all these models, the potentiating effect of adrenalectomy was abolished by exogenous corticosteroids, suggesting that endogenous corticosteroids can diminish the response of blood vessels to certain mediators that increase vascular permeability.

In the airways, exogenously administered corticosteroids are potent inhibitors of mediator-induced increase in vascular permeability (Boschetto *et al.*, 1991), whereas it is unknown to what extent endogenously secreted corticosteroids contribute to the natural control of the airway permeability response.

In the present study we investigated whether bilateral adrenalectomy enhances the amount of plasma extravasation induced by platelet activating factor (PAF) in rat airways, and whether exogenous corticosteroid replacement prevents the increase of PAF response in adrenalectomized rats. We used rats because it has been demonstrated that corticosteroids are markedly more potent in this species than, for example, in guinea-pigs (Hirshman & Downes, 1985). We have used PAF because it is highly potent in inducing airway vascular permeability (Bussolino *et al.*, 1987; Evans *et al.*, 1987; O'Donnell & Barnett, 1987; Dillon & Duran, 1988).

Methods

A total of 96 male Sprague-Dawley rats were used, weighing 200–350 g; they were purchased from Morini Farm (Reggio Emilia, Italy).

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Protocol

We first examined the increase of airway vascular permeability induced by increasing doses of PAF in adrenalectomized and sham-operated rats, and we observed that adrenalectomy potentiates the response to 500 and 1000 ng kg^{-1} PAF. Then, we examined the effect of 1000 ng kg^{-1} PAF in adrenalectomized and sham-operated rats after pretreatment with subcutaneous dexamethasone.

Eighty-four rats were anaesthetized with ether and then bilaterally adrenalectomized or sham-operated (42 rats for each group). These animals were studied between the 7th and 8th day after surgery, based on the observation that adrenalectomy, as previously shown by others (Akana *et al.*, 1985), is associated with depletion of corticosterone at that time.

Rats were, then, premedicated with diazepam and anaesthetized with droperidol and fentanyl citrate. In 60 experiments (30 adrenalectomized and 30 sham-operated rats) we first injected Evans blue dye intravenously (i.v.), as a tracer, to assess vascular permeability and 1 min later increasing doses of PAF or of its vehicle. Five minutes after PAF or its vehicle, we opened the chest and perfused the rats through the heart with 0.9% NaCl. Thereafter, we processed larynx, trachea, main bronchi and intrapulmonary airways to determine the amount of plasma extravasation. In the other 36 experiments (12 normal, 12 sham-operated and 12 adrenalectomized rats) animals were treated with dexamethasone or its vehicle 4 h before PAF injection.

Bilateral adrenalectomy

Eighty-four rats were used throughout the experiments. In each individual experiment, age-matched controls were employed. Bilateral adrenalectomy was carried out under ether anaesthesia. Through small bilateral retroperitoneal incisions, the adrenals were identified at the superior pole of each kidney, removed, and the skin sutured with 3–0 silk. Sham operations were performed in the same manner except that the adrenal glands were left undisturbed. The adrenalectomized rats were given 0.9% NaCl solution for drinking and normal laboratory food. Control animals were given tap water instead of NaCl solution (Caesar *et al.*, 1970). Adrenalectomies

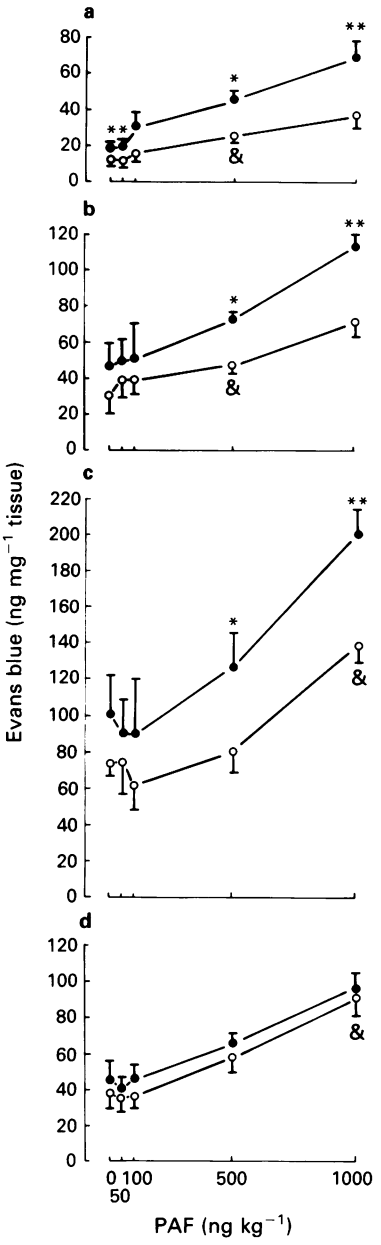

Figure 1 Effects of platelet-activating factor (PAF) and its vehicle, 0.35% bovine serum albumin (BSA) saline (1 ml kg^{-1}), on the amount of extravasation of Evans blue dye in (a) the larynx, (b) trachea, (c) main bronchi and (d) intrapulmonary airways in adrenalectomized (●) and sham-operated (○) rats. Values shown are means of six animals per group; s.e.mean shown by vertical bars. Minimum dose of PAF causing significant ($P < 0.01$) increase in vascular permeability compared with BSA-saline in sham-operated rats is shown (&). * $P < 0.05$; ** $P < 0.01$ compared with sham-operated rats which received the same dose of PAF.

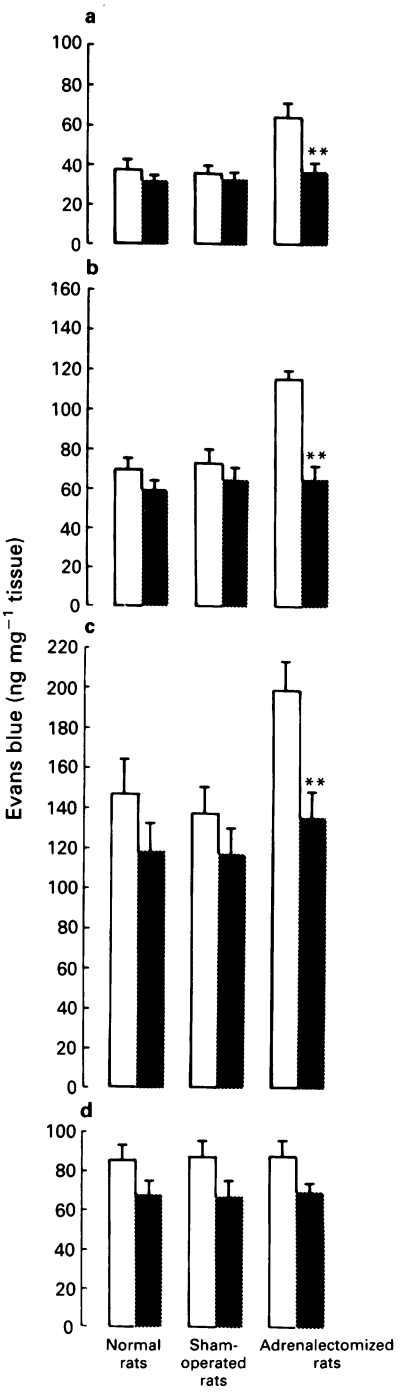

Figure 2 Effects of dexamethasone (0.2 mg kg^{-1}, 4 h before PAF; solid columns) and its vehicle (open columns) on vascular permeability induced by 1000 ng kg^{-1} PAF in normal, sham-operated, and adrenalectomized rats in (a) the larynx, (b) trachea, (c) main bronchi and (d) intrapulmonary airways. Dexamethasone did not significantly inhibit PAF-induced vascular permeability in normal and sham-operated rats. Values shown are means of six animals per group; s.e.mean shown by vertical bars. * $P < 0.05$; ** $P < 0.01$ compared with corresponding group not treated with dexamethasone.

were confirmed at autopsy and by plasma corticosterone determinations. Plasma corticosterone determinations were done in 15 adrenalectomized and in 15 sham-operated rats.

Corticosteroid treatment

Dexamethasone 0.2 mg kg^{-1} in 0.9% NaCl or 0.9% NaCl alone were administered subcutaneously 4 h before PAF in adrenalectomized, sham-operated and normal rats. We previously studied the effect of different doses and different times of administration of dexamethasone on PAF-induced vascular permeability in normal rats. We chose the above combination of dose and time of administration of dexamethasone because it reduces PAF-induced vascular permeability without having a significant inhibitory effect (Figure 2) in normal and sham-operated rats. In fact dexamethasone 2 mg kg^{-1} administered 4 h before PAF did not reduce the Evans blue extravasation in all the tissues studied (larynx 27 ± 2, trachea 70 ± 5, main bronchi 123 ± 6, intrapulmonary airways 75 ± 4 ng Evans blue mg^{-1} tissue), whereas dexamethasone at a dose of 0.2 mg kg^{-1} given 24 h and 4 h before PAF significantly inhibited plasma extravasation in all tissues (Boschetto *et al.*, 1991).

Measurement of plasma exudation

Rats were premedicated with diazepam (3 mg kg^{-1}) by intraperitoneal injection and anaesthetized with 0.4 ml of Leptofen

(containing 1 mg droperidol and 0.032 mg fentanyl citrate) by intramuscular injection. Vascular permeability was quantified by measuring the extravasation of Evans blue (EB) dye by a method described previously (Boschetto *et al.*, 1989) which correlates well with extravasation of radiolabelled albumin (Rogers *et al.*, 1989). EB dye (30 mg ml^{-1} in 0.9% NaCl, filtered through a 0.22 μm Millipore filter), 30 mg kg^{-1}, was injected into the internal jugular vein followed 1 min later by injection of PAF or its vehicle. PAF was kept as stock solutions of 10 mg ml^{-1} in ethanol at −80°C. Solutions of 100 ng ml^{-1} and 1000 ng ml^{-1} in 0.35% (weight/vol) bovine serum albumin in saline were freshly prepared on each experimental day. The vehicle for PAF (1 ml kg^{-1} 0.35% BSA saline) or an equal volume of PAF solution, in doses of 50, 100, 500, and 1000 ng kg^{-1}, were injected into the internal jugular vein 5 min before perfusion with saline. Then later the chest was opened and the rats were perfused with 100 ml of 0.9% NaCl solution (pH 5.5) from a cannula inserted through the left ventricle into the ascending aorta. Approximately 10 s elapsed from the time the left ventricle was incised to insert the cannula and the beginning of the perfusion. Blood and 0.9% NaCl solution drained from the right atrium through another incision; 0.9% NaCl solution was at a temperature of 21°C and was perfused at a pressure of 100 mmHg.

The larynx, trachea, main bronchi, and lungs were removed. The main bronchi were separated from the trachea, and the remaining intrapulmonary airways gently stripped of parenchyma with a razor blade. Wet weights of all tissues were recorded. EB dye was extracted from the tissues by incubation at 37°C for 16 h in 2 ml formamide in stoppered tubes. The concentration of EB dye was determined by light absorbance at 620 nm wavelength (Lambda 5 spectrophotometer; Perkin-Elmer Corp., Northwalk, CT, U.S.A.) and by interpolation on a standard curve of EB in the range 0.5–10 ng ml^{-1} in formamide. EB dye content of each sample was expressed as ng mg^{-1} wet weight tissue.

Plasma corticosterone determination

Blood samples were obtained from jugular veins in adrenalectomized and sham-operated rats 1 week after surgery. Plasma was separated by centrifugation. Plasma samples were then frozen at −20°C for later analysis. Plasma corticosterone levels were measured by a radioimmunoassay. All the assays were performed according to the method of the manufacturer (Eurogenetix, Milan, Italy), using a gamma-counter (Mod. Cristall Packard). By this method, normal values of corticosterone are 50–400 ng ml^{-1}. All samples were analysed in duplicate and results were averaged.

Drugs and chemicals

Drugs and chemicals were obtained from the following sources: Evans blue dye, formamide, bovine serum albumin, acetic acid, 3,4 dihydroxbenzylamine and alumina: Sigma Chemicals Ltd, St Louis, MO, U.S.A.; diazepam: Roche Pharmaceuticals, Basel, Switzerland; Leptofen: Farmitalia Carlo Erba S.p.a., Milano, Italy; dexamethasone: Merck Sharp & Dohme B.V., Haarlem, Holland; PAF: Bachem AG, Bubendorf, Switzerland; diethyl ether: E Merck, Darmstadt, Germany. Drug dilutions and perfusions were made with 0.9% sodium chloride for intravenous infusion: Bieffe Biochimici S.p.a., Firenze, Italy. Drugs were diluted to give injection volume of 1 ml kg^{-1}.

Statistical analysis

Average values are expressed as the mean ± standard error of the mean (s.e.mean). Data for the concentration of EB dye extractable from tissues do not approximate a Gaussian (normal) distribution but show positive skewness and the Mann-Whitney U test (Siegel, 1956) was used to test the null hypothesis. Bonferroni's correction (Wallenstein *et al.*, 1980) was used when appropriate to allow for multiple comparisons. Differences having P values less than 0.05 were considered statistically significant.

Results

Effect of PAF on leakage

Injection of PAF induced immediate tachypnoea in all animals, which subsided after 1 min. PAF consistently increased extravasation of EB in sham-operated rats in all tissues examined. The minimum tested dose of PAF required to cause a significant increase in vascular permeability compared with the 0.35% BSA saline control was 500 ng kg^{-1} for larynx and trachea; 1000 ng kg^{-1} for main bronchi and intrapulmonary airways (Figure 1).

The effect of bilateral adrenalectomy on PAF-induced leakage

The effects of PAF in adrenalectomized rats were compared with those in sham-operated rats which received the same dose of PAF. Bilateral adrenalectomy did not cause observable changes in the health and behaviour of animals. Adrenalectomy augmented the PAF-induced increase in Evans blue extravasation in the larynx, trachea, main bronchi but not in the intrapulmonary airways. Adrenalectomy significantly increased the baseline (0 ng kg^{-1} PAF) amount of Evans blue extravasation in the larynx ($P < 0.05$). At 500 ng kg^{-1} of PAF, the increase was significant also in the trachea ($P < 0.05$) and main bronchi ($P < 0.05$); the difference was larger at a dose of 1000 ng kg^{-1} of PAF (Figure 1). Adrenalectomy failed to produce an enhancement of the PAF response in intrapulmonary airways (Figure 1). The potentiation of the amount of plasma extravasation induced by PAF in adrenalectomized rats was not due to differential effects of adrenalectomy on the weight of the tissues studied, as the weights were 115 ± 4 mg and 110 ± 5 mg ($P > 0.05$) for larynx, 57 ± 3 mg and 55 ± 3 mg ($P > 0.05$) for trachea and 23 ± 2 mg and 26 ± 3 mg ($P > 0.05$) for main bronchi respectively in adrenalectomized and sham-operated rats.

Effect of adrenalectomy on plasma corticosterone

Adrenalectomy markedly decreased plasma corticosterone. At 1 week after surgery mean (±s.e.) plasma corticosterone was 2.5 ± 1.2 ng ml^{-1} and 380 ± 35 ng ml^{-1} ($P < 0.01$) in adrenalectomized and sham-operated rats respectively.

Effect of dexamethasone in adrenalectomized rats

As illustrated in Figure 2, dexamethasone (0.2 mg kg^{-1}, 4 h before PAF) significantly reduced the amount of plasma extravasation induced by PAF in the larynx, trachea and main bronchi of adrenalectomized rats but this effect was absent in the control rats.

Discussion

We have demonstrated that, in rats, adrenalectomy potentiated the PAF-induced increase in vascular permeability in the extrapulmonary airways but not in the intrapulmonary

airways. Our study extends previous observations in other organs and shows that adrenalectomy also potentiates PAF-induced vascular permeability in the airways. This potentiation was accompanied by more than a 99% reduction in the plasma concentration of corticosterone. The effect of adrenalectomy was reversed by pretreating rats with dexamethasone, suggesting a major role of endogenous steroids in the regulation of microvascular permeability.

The effect of inflammatory mediators on airway vascular permeability is regionally dependent (Evans *et al.*, 1989), and indeed we observed that a significant amount of plasma extravasation was induced by a lower dose of PAF ($500\,ng\,kg^{-1}$) in the larynx and trachea compared with smaller airways. We also observed that adrenalectomy potentiates PAF-induced increase in vascular permeability in the larynx, trachea, main bronchi, but not in the intrapulmonary airways. Thus, we suggest that not only the response to PAF but also the regulatory effect of corticosteroids may be regional.

As we expressed all the data as ng Evans blue mg^{-1} of tissue, an increase in extravasation would be inferred from an increase in the amount of dye or a decrease in the weight of the tissue. The potentiation of PAF-induced increase in vascular permeability in corticosteroid-depleted rats was not due to differential effects of adrenalectomy on the weight of tissues studied because the weight of tissues was not significantly different in the groups of rats examined.

In the present study the Evans blue extravasation induced by PAF in the larynx, trachea, main bronchi and intrapulmonary airways of sham-operated rats was lower than that observed in control, non-operated rats examined in our previous study carried out in a different laboratory (Boschetto *et al.*, 1991). Sham-operation might have influenced the dose-response curve through unknown mechanisms. On the other hand, we also examined control non-operated rats in the present study (Figure 2), and found no difference in the response to 1000 ng PAF in non-operated vs sham-operated rats. Thus we do not have a clear explanation for the differences between this and the previous study.

Before adrenalectomy and sham-operation, animals were anaesthetized with ether for 3–4 min and ether is an irritant that is known to increase vascular permeability in the airways (Lundberg & Saria, 1983). The oedema induced by ether has been shown immediately after 5 min of inhalation. We, therefore, suspect that ether exposure did not contribute to the sensitivity of airway vasculature 7 days after the short anaesthesia.

The potentiating effect of adrenalectomy could have been influenced by airway infections, as it is known that such infections make the airways of rats abnormally sensitive to certain mediators which increase vascular permeability (McDonald, 1988). Although we believe that this is a possibility, we do not have the facilities to test whether the rats acquired such infections.

Bilateral adrenalectomy may also decrease the production of endogenous adrenaline, and adrenaline decreases the sensitivity of blood vessels to mediators that increase vascular permeability (Beets & Paul, 1980; Boschetto *et al.*, 1989). Thus, a role for adrenaline in the observed phenomena remains to be investigated. However, the observation that adrenalectomy produced an almost complete depletion of circulating corticosterone and that corticosteroid repletion completely prevented the potentiation of vascular permeability induced by PAF, suggest a prominent role of endogenous corticosteroids. In addition, corticosteroids regulate both the biosynthesis (Wurtman, 1966) and the urinary excretion of adrenaline (Parvez *et al.*, 1974), and thus it would be difficult to separate the relative effect of corticosteroids and adrenaline.

Corticosteroids are potent inhibitors of inflammatory oedema and this probably represents a major component of their therapeutic activity in inflammatory diseases. Corticosteroids, indeed, inhibit skin reactions, caused by vascular permeability increasing factors, including IgE antibody-mediated homologous passive cutaneous anaphylaxis (Inagaki *et al.*, 1988). In rat tracheobronchial mucosa, corticosteroids failed to reduce local oedema induced by cigarette smoke and by instillation of hydrochloric acid and gastric juice (Lundberg *et al.*, 1983; Martling & Lundberg, 1988). However, dexamethasone blocked the potentiation of the capsaicin-induced increase in vascular permeability in the tracheas of rats with respiratory tract infections (Tu Huang *et al.*, 1989). These apparent differences in the effectiveness of corticosteroids, may relate to differences in the dose of the corticosteroid or in the time interval from when the corticosteroid is injected to when the inflammation is induced. In fact dexamethasone and budesonide have been shown to reduce significantly the bradykinin- and histamine-induced increase in permeability of hamster cheek pouch venules in a time-dependent manner (Svensjo & Roempke, 1985). Corticosteroids have also been demonstrated to inhibit the increase in vascular permeability caused by PAF in the hamster cheek pouch, probably acting by a direct effect on vascular endothelial cells (Bjork *et al.*, 1985).

The mechanisms by which corticosteroids act on plasma extravasation is unknown. There are several potential target sites through which corticosteroids can influence oedema formation. Three of the most likely sites of action are mediator generation, endothelium, and leukocyte-endothelial interactions (Williams & Yarwood, 1990). Thus corticosteroids can inhibit vascular permeability by suppressing the generation of permeability-increasing mediators. There is also evidence that corticosteroids can inhibit the response of endothelial cells to permeability-increasing mediators *in vivo*.

In conclusion, we have shown that adrenalectomy potentiates the increase of airway vascular permeability induced by PAF in rats and that this effect may be due to the depletion of endogenous corticosteroids. These results suggest that endogenous corticosteroids may play a role in the physiological regulation of airway microvascular permeability.

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An ubiquitous modulating function of rabbit tracheal epithelium: degradation of tachykinins

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1 To examine the role of epithelium in the responsiveness of tracheal smooth muscle in rabbit, we measured the contractile responses to acetylcholine (ACh), KCl, 5-hydroxytryptamine (5-HT), histamine, substance P (SP), neurokinin A (NKA), and electrical field stimulation (EFS) in intact and epithelium-denuded preparations.

2 Removal of epithelium did not alter the contractile response to any agonist examined, except SP.

3 Removal of epithelium enhances the contractile response to SP. In the presence of phosphoramidon, the contractile response to SP was not significantly different in either group. The results suggest that the effect of epithelium is largely due to degradation of SP by enzymes in the epithelium.

4 Arachidonic acid metabolites did not seem to be related to the responses induced by contractile agonists or EFS.

5 In the presence of SP, the contractile responses and [^{3}H]-choline outflow evoked by EFS were dose-dependently enhanced. Contractile responses to EFS and [^{3}H]-choline outflow evoked by EFS were enhanced by SP significantly more than by NKA. Both were abolished by atropine or tetrodotoxin.

6 These results suggest that rabbit tracheal epithelium may modulate SP-induced contractions, both direct and indirect, by inactivation of SP. This phenomenon is widespread in mammals. The rabbit may be a useful model to examine airway cholinergic functions.

Keywords: Airway epithelium; tachykinins; enkephalinase; epithelium-derived relaxing factor (EpDRF)

Introduction

The airway epithelium is thought to have defensive mechanisms such as clearance by mucociliary action, a diffusion barrier, and a contraction or coughing reflex initiated through irritant receptors. Airway smooth muscle responses induced by several agonists are modulated by epithelial cells (Cuss & Barnes, 1987). Studies have shown that mechanical removal of airway epithelium potentiated responses to acetylcholine (ACh), 5-hydroxytryptamine (5-HT) and histamine in a number of animal species including the guinea-pig, canine, bovine, rat and in man (Fedan *et al.*, 1988). It is well known that relaxation of vascular smooth muscle induced by ACh requires the presence of the endothelium (Furchgott & Zawadski, 1980), but inhibition of contraction of airway smooth muscle by epithelium is small by comparison and no precise inhibitory mechanism is known.

Tschirhart *et al.* (1987) described inhibition of airway epithelium that was partially mediated by cyclo-oxygenase products. It is generally recognized that airway epithelial cells can synthesize prostaglandins from unsaturated fatty acids along the cyclo-oxygenase pathway of arachidonic acid (AA) metabolism (Fedan *et al.*, 1988). The capacity to synthesize prostaglandins is different in different species, and from region to region of the tracheobronchial tree (Butler *et al.*, 1987). Cyclo-oxygenase products are known to be responsible for spontaneous contraction of guinea-pig tracheal smooth muscle (Yamane & Kobayashi, 1990). Endogenous prostaglandins modulate histamine-induced contractions (Shore *et al.*, 1985) and cholinergic neurotransmission in canine tracheal smooth muscle (Walters *et al.*, 1984), but little is known about these phenomena in the rabbit. There are several reports that substance P (SP) may be involved in airway smooth muscle function and contractile responses induced by SP are thought to be via direct effects on smooth muscle and indirect effects through cholinergic nerves (Tanaka & Grunstein, 1984; 1986; Sekizawa *et al.*, 1987). In rabbit tracheal smooth muscle, previous studies did not evaluate the effects of epithelium on response to tachykinins. SP is degraded by epithelial neutral endopeptidase (NEP; EC 3.4.24.11), which is present in airway smooth muscle, submucosa, and epithelium (Dusser *et al.*, 1989). Therefore, epithelium may modulate the effect of SP on airway smooth muscle contraction and facilitate ACh release from cholinergic nerve terminals.

The present study was designed to determine whether or not epithelium modulates the responsiveness of rabbit tracheal smooth muscle to several agonists or to electrical field stimulation (EFS) *in vitro*, and especially to examine the possibility of epithelial modulation of ACh-release evoked by EFS in the presence or absence of tachykinins.

Methods

Tissue and preparations

Japanese white rabbits (age 16–20 weeks, weight 2.8–3.2 kg) were anaesthetized with pentobarbitone sodium (25 mg kg^{-1}, i.v.) and exsanguinated. The trachea was quickly removed and immersed in ice-cold Krebs-Henseleit (K-H) solution (composition (mM): NaCl 118, KCl 4.5, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 11, $MgSO_4$ 1.5 and KH_2PO_4 1.2), gassed with 95% O_2 and 5% CO_2. Connective tissue and adipose tissue were carefully removed, and the trachea was cut along the cartilaginous longitudinal axis. The middle portion of the trachea was used to make strips, each containing two adjacent cartilage bands. Epithelium was removed from alternate strips with fine forceps. These strips were tied at each end with silk threads, one connected to a holder and the other to a force displacement transducer (Nihon Kohden SB 1-T) for continuously measuring isometric tension and recording on a pen recorder (Sanei Recti-Horiz 8K). Strips of trachea were placed between two rectangular platinum electrodes (10 mm × 5 mm) for EFS. These strips were initally loaded with 1 g tension and equilibrated for 60 min at 37°C during which time they were washed in K-H solution every 10 min. EFS, generated by an electronic stimulator (Nihon Kohden SEN-3201), was applied trans-axially through these electrodes. Study of responses to applied tension indicated that the optimum resting load was unchanged after epithelial removal.

Effects of epithelium removal and endogenous arachidonic acid products on concentration-response curves of several agonists

Concentration-response curves of ACh, KCl, 5-HT and histamine were constructed from responses to cumulative addition of these agents in both preparation groups, in the presence or absence of 10^{-7} M atropine. To study the effect of endogenous AA metabolites, 10^{-5} M indomethacin was added 15 min before, and retained during, concentration-response determination.

Concentration-response curves to substance P and neurokinin A and effect of neutral endopeptidase inhibitor

To study whether the epithelium modifies contractile responses to SP and neurokinin A (NKA), cumulative concentration-response curves were constructed using SP and NKA in concentrations ranging from 10^{-10} to 10^{-6} M in the presence or absence of 10^{-7} M atropine. The reason for using atropine was that contractions induced by SP and NKA were thought to occur via both direct effects on smooth muscle and indirect effects on cholinergic nerves. To study whether NEP inhibitor affected contractile responses to SP or NKA, 3×10^{-9} and 10^{-6} M SP, and the same concentrations of NKA were used to induce contraction in the presence or absence of 10^{-5} M phosphoramidon. These measurements were made in the presence of 10^{-7} M atropine, 10^{-6} M phentolamine, 10^{-6} M propranolol and 10^{-6} M guanethidine together.

Contractile responses to electrical field stimulation and augmented effects of tachykinin

Contractile responses to EFS were constructed by applying 10 V, 2 ms, 5–20 Hz for 45 s to both the intact and denuded preparations. To study how SP or NKA augmented the contractile responses to EFS, each preparation was electrically stimulated 8 times; twice in control and twice in the presence of either SP or NKA at both 5 and 20 Hz. The intervals between stimulation were 3 min. Different, but similar preparations were used for SP and NKA. The mean of the two responses in each trial was taken as the value for that trial. When SP or NKA was used, 10 min was allowed for equilibration before testing.

[^{3}H]-choline release

The release of [^{3}H]-choline was determined according to the method described by Kilbinger *et al.* (1982). The strips of rabbit trachea were incubated for 60 min at 37°C in 5 ml of K-H solution containing 1 μCi ml^{-1} of [^{3}H]-choline (specific activity, 80 Ci mmol^{-1}). During incubation, the preparations were stimulated with 0.2 Hz, 1 ms square wave pulses. The strips were then washed in K-H solution containing 10 μM hemicholinium-3 for 60 min. The strips were stimulated for two 3 min periods with 10 V, 2 ms square wave pulses at 20 Hz. The perfusate was collected every 5 min and counted for radioactivity in a liquid scintillation spectrometer (Packard, model, TRI-CARB A3255). EFS of a strip started 15 min (S1) and 45 min (S2) after the end of the washout period. The drugs to be tested were added to the perfusate from 5 min before the initiation until 2 min after the termination of EFS(S2). In these experiments, we used tachykinin doses that did not induce smooth muscle contraction (SP 10^{-10}–10^{-9} M, NKA 10^{-10} M).

Drugs

The following drugs were used: acetylcholine chloride, atropine sulphate, (±)-propranolol hydrochloride, hexamethonium chloride, 5-hydroxytryptamine, tetrodotoxin, physostigmine (Sigma Chemical Co., St Louis, Mo, U.S.A.), substance P, neurokinin A, phosphoramidon, spantide, calcitonin gene-related peptide (CGRP) (Peptide Institute Inc., Osaka, Japan), guanethidine sulphate (TCI, Tokyo), phentolamine mesylate (Ciba-Geigy), [^{3}H]-choline (NEN product, Boston, Mass, U.S.A.), nordihydroguaiaretic acid (NDGA), 5,8,11,14-eicosatetraynoic acid (EYTA) (Paesel GmbH & Co., Frankfurt, Germany), hemicholinium-3 hydrate (Aldrich Chemical Co., Milwaukee, Wis, U.S.A.).

Statistical analysis

The results are expressed as means ± s.e. Statistical evaluation of the data was by Student's t test for paired or unpaired observations. At $P < 0.05$, means were considered to be significantly different.

Results

The histology of a tracheal cross section showed that airway epithelium could be removed by gentle separation with fine forceps. The epithelium removal had no effect on basal tone. There was no noticeable qualitative or quantitative difference in the relation between applied load and developed tension for the intact and denuded preparations.

Contractile response of tracheal smooth muscle to acetylcholine, KCl, 5-hydroxytryptamine and histamine

The concentration-effect curves to ACh in epithelium-intact and epithelium-denuded trachea are shown in Figure 1. Maximal responses and EC_{50} values in intact preparations were not different from those for denuded preparations in the presence or absence of physostigmine. The contractile response of 3×10^{-4} M ACh was abolished by 10^{-7} M atropine. The dose-response curves of tracheal strips with and without epithelium responding to increasing concentrations of KCl are shown in Figure 2a and b. In the presence or absence of atropine, maximal responses of intact preparations were not different from those in epithelium-denuded preparations. In the presence of 10^{-7} M atropine, contractions induced by 20 mM and 30 mM KCl were significantly depressed in both preparation groups and the values of EC_{50} were significantly larger than in the absence of atropine ($P < 0.001$), but maximal responses were the same in both groups in the pre-

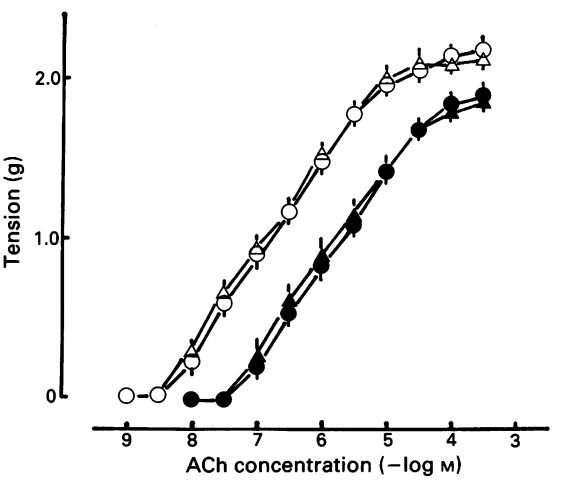

Figure 1 Effect of epithelium removal on concentration-response curves to acetylcholine in rabbit tracheal smooth muscle strips in the presence or absence of 10^{-6} M physostigmine. Points are mean values from 8 observations and vertical bars represent s.e. (●) Intact preparations in the absence of physostigmine; (▲) epithelium-denuded preparations in the absence of physostigmine; (○) intact preparations in the presence of 10^{-6} M physostigmine; (△) epithelium-denuded preparations in the presence of 10^{-6} M physostigmine.

Table 1 Effect of epithelium removal and indomethacin on contractions produced by acetylcholine (ACh), KCl and 5-hydroxytryptamine (5-HT) in tracheal strips from rabbit

		No indomethacin			*With indomethacin*	
Agonist	n	*+Epithelium*	*−Epithelium*	n	*+Epithelium*	*−Epithelium*
			EC_{50} (−log M)			
ACh	8	5.83 ± 0.03	5.82 ± 0.04	8	5.82 ± 0.03	5.83 ± 0.04
KCl	8	1.58 ± 0.02	1.59 ± 0.03	8	1.58 ± 0.02	1.57 ± 0.03
5-HT	8	6.13 ± 0.03	6.15 ± 0.05	8	6.13 ± 0.03	6.15 ± 0.05
			Maximum contraction (g)			
ACh	8	1.98 ± 0.08	1.94 ± 0.05	8	1.96 ± 0.07	1.94 ± 0.04
KCl	8	1.13 ± 0.04	1.14 ± 0.05	8	1.13 ± 0.05	1.14 ± 0.05
5-HT	8	1.04 ± 0.06	1.03 ± 0.07	8	1.06 ± 0.08	1.01 ± 0.08

Results are expressed as the means ± s.e.mean.

sence of 10^{-7} M atropine. In the absence or presence of atropine, the concentration-response curves of both preparation groups to 5-HT were not significantly different (Figure 2c and d), but in the presence of 10^{-7} M atropine, contractions induced by 3×10^{-7} M 5-HT were significantly depressed in both preparation groups, although maximal responses were different in the two groups. Histamine did not induce contraction in either group. Effects of indomethacin on contractile responses of these agonists are shown in Table 1. Indomethacin did not affect EC_{50} values or maximal responses of these agonists. Neither NDGA nor ETYA affected the curves of response to change in concentration of ACh, 5-HT, or KCl for either group (data not shown).

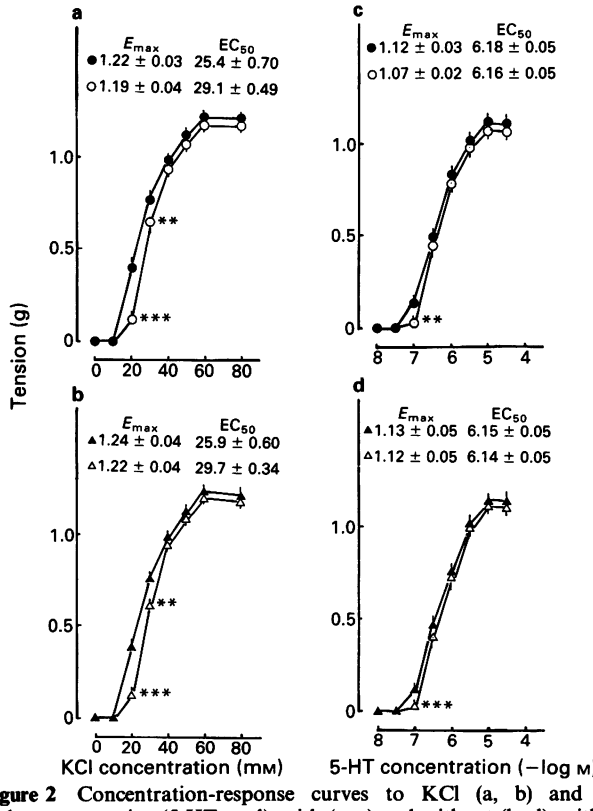

Figure 2 Concentration-response curves to KCl (a, b) and 5-hydroxytryptamine (5-HT, c, d), with (a, c) and without (b, d) epithelium, in the presence or absence of atropine. Points are mean values from 8 observations and vertical bars represent s.e. Differences between contractile response in the absence of atropine and those in the presence of 10^{-7} M atropine were analyzed. $^{**}P < 0.01$; $^{***}P < 0.001$. Removal of epithelium did not affect the values of EC_{50} or E_{max}, in the presence or absence of atropine. Atropine significantly depressed the EC_{50} values for KCl in both preparation groups ($P < 0.001$) and did not affect the EC_{50} values for 5-HT in both preparation groups. (●) Intact preparations in the absence of atropine; (▲) epithelium-denuded preparations in the absence of atropine; (○) intact preparations in the presence of 10^{-7} M atropine; (△) epithelium-denuded preparations in the presence of 10^{-7} M atropine.

Contractile response of tracheal smooth muscle to substance P, neurokinin A and calcitonin gene-related peptide

The concentration-response curves to SP in both groups are shown in Figure 3a. Atropine depressed contractile responses induced by 3×10^{-9} M SP in both groups. The concentration-response curves to NKA by tracheal strips with and without epithelium are shown in Figure 3b. Atropine had no effect on the NKA concentration-response curves. In the presence of phosphoramidon, removal of the epithelium did not change the contractile responses to 3×10^{-9} or 10^{-6} M SP (Figure 4). CGRP did not induce contraction in either group. CGRP did not affect SP-induced potentiation of EFS (Figure 7c,d).

Contractile response of tracheal smooth muscle to electrical field stimulation and potentiation of these effects by tachykinins

Contractile responses induced by 5, 10 or 20 Hz EFS are shown in Figure 5. Contractile responses induced by EFS in intact preparations were not significantly different from those

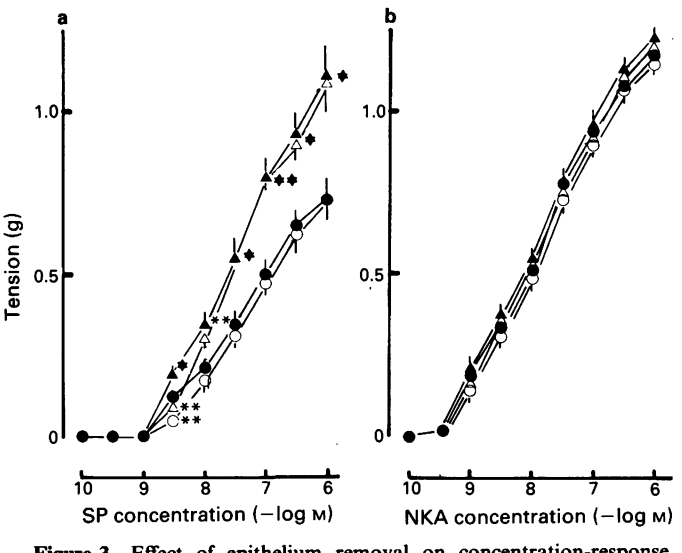

Figure 3 Effect of epithelium removal on concentration-response curves to substance P (SP, a) and neurokinin A (NKA, b) in the presence or absence of atropine. Points are mean values from 8 observations and vertical bars represent s.e. Difference between contractile responses in intact preparations (●, ○) and those in denuded preparations (▲, △) were analyzed: $^{*}P < 0.05$; $^{**}P < 0.01$. Difference between contractile response in the absence of atropine and contractile response in the presence of 10^{-7} M atropine were analyzed: $^{**}P < 0.01$. EC_{50} values for SP or NKA were the same with or without epithelium and in the presence or absence of atropine. EC_{50} for SP (●: 7.51 ± 0.01; ○: 7.51 ± 0.01; ▲: 7.50 ± 0.01; △: 7.50 ± 0.01). EC_{50} for NKA (●: 7.74 ± 0.01; ○: 7.75 ± 0.01; ▲: 7.75 ± 0.01; △: 7.75 ± 0.01). Symbols are the same as in Figure 2 legend.

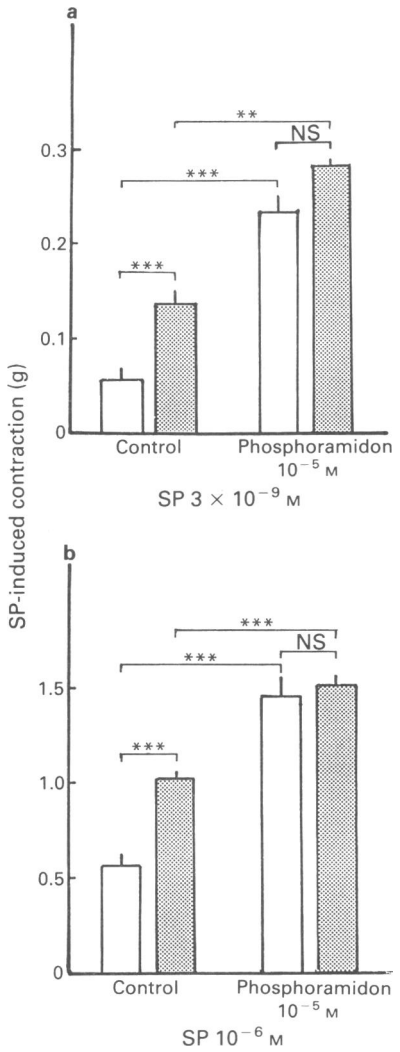

Figure 4 Effect of phosphoramidon on contraction induced by (a) 3×10^{-9} and (b) 10^{-6} M substance P (SP). Columns are mean values from 8 observations and vertical bars represent s.e. Open columns are intact preparations; stippled columns are epithelium-denuded preparations. Difference between contractile responses in intact preparations and those in epithelium denuded preparations were analyzed. Significant difference between groups: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; NS, not significant.

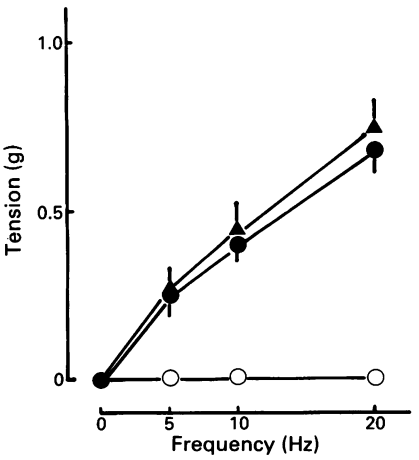

Figure 5 Contractile response to electrical field stimulation of rabbit tracheal smooth muscle in intact and epithelium-denuded preparations. Points are mean values from 8 observations and vertical bars represent s.e. (●) Intact preparations in the absence of atropine or tetrodotoxin (TTX); (▲) epithelium-denuded preparations in the absence of atropine or TTX; (○) in the presence of atropine or TTX.

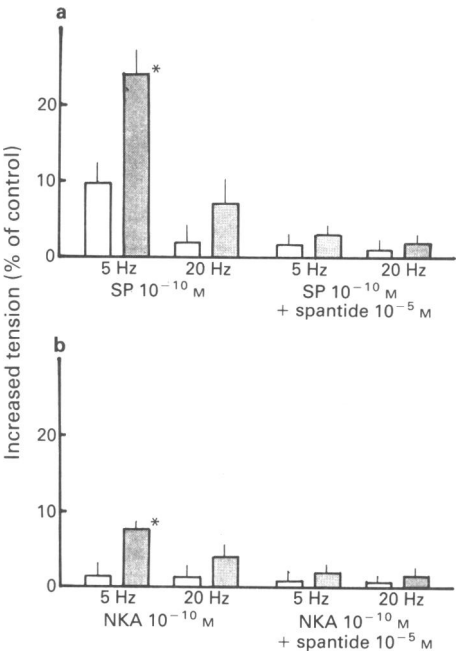

Figure 6 Effects of (a) 10^{-10} M substance P (SP) and (b) 10^{-10} M neurokinin A (NKA) on contractile response to electrical field stimulation at 5 or 20 Hz. Columns are mean values from 8 observations and vertical bar represents s.e. Open columns are intact preparations; stippled columns are epithelium-denuded preparations. Difference between contractile response of each frequency in intact preparations and those in epithelium denuded preparations: $*P < 0.05$.

in denuded preparations. The contractile responses induced by EFS were abolished by 10^{-7} M atropine or 1 μg ml^{-1} TTX. SP and NKA enhanced the contractile responses to EFS.

Contractile responses to EFS were enhanced significantly more by SP than by NKA. The percentage increase in tension at 5 Hz was significantly greater than that at 20 Hz (Figure 6).

Electrical field stimulation-evoked [^{3}H]-choline outflow

In the absence of tachykinins, the ratio of S2/S1 for [^{3}H]-choline outflow was 0.97 ± 0.02 in intact preparations and 0.98 ± 0.02 in denuded preparations (Figure 8). EFS of both groups of preparations induced an increase in [^{3}H]-choline outflow, together with enhancement of contractile responses (Figure 7a). In the presence of SP, the ^{3}H outflow evoked by EFS in denuded preparations was significantly larger than in intact preparations. In the presence of 10^{-5} M phosphoramidon, the effects of 10^{-10} M SP on [^{3}H]-choline release were enhanced in both groups of preparations. The [^{3}H]-choline outflow evoked by EFS was not affected by 10^{-10} M NKA. Significant increase in [^{3}H]-choline outflow evoked by EFS was caused by 10^{-9} M SP in both groups of preparations. It also caused the S2/S1 ratio in denuded preparations to be significantly larger than that in intact preparations. The increase in [^{3}H]-choline outflow evoked by EFS was abolished by 1 μg ml^{-1} TTX or by 10 min pretreatment with calcium-free medium containing 0.1 mM EGTA.

Discussion

In the present study, removal of epithelium did not change the responsiveness of tracheal smooth muscle to ACh, 5-HT, KCl or NKA in partial agreement with results reported by Raeburn *et al.* (1986). However, there have been several reports that removal of the epithelial layer increased the responsiveness of *in vitro* preparations of airway smooth muscle (Barnes *et al.*, 1985; Flavahan *et al.*, 1985). It is unlikely that removal of the epithelium changed the mechani-

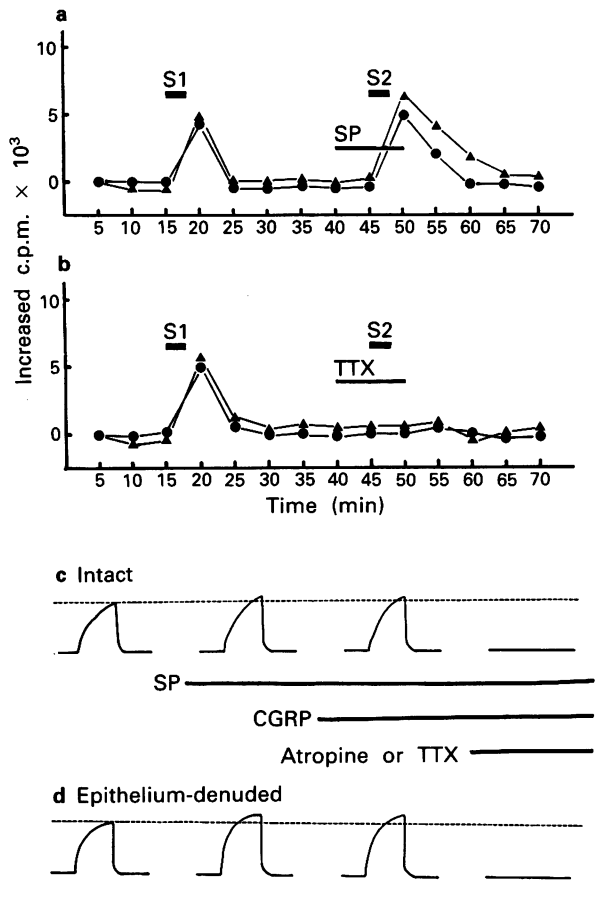

Figure 7 Effect of substance P (SP, a) and tetrodotoxin (TTX, b) on electrical field stimulation (EFS)-evoked [^{3}H]-choline outflow in rabbit tracheal smooth muscle. The perfusate was collected in 5 min fractions (5 ml) and their radioactivity was measured. EFS was performed 15 min (S1) and 45 min (S2) after the end of washout period. SP and TTX were applied 5 min before the initiation of EFS (S2) until 2 min after the end. Effects of SP, calcitonin gene-related peptide (CGRP), tetrodotoxin, or atropine on EFS-induced contractile responses in intact preparation (c). Effects of SP, CGRP, tetrodotoxin, or atropine on EFS-induced contractile responses in epithelium-denuded preparation are shown in (d). (●) Intact preparation; (▲) epithelium-denuded preparation.

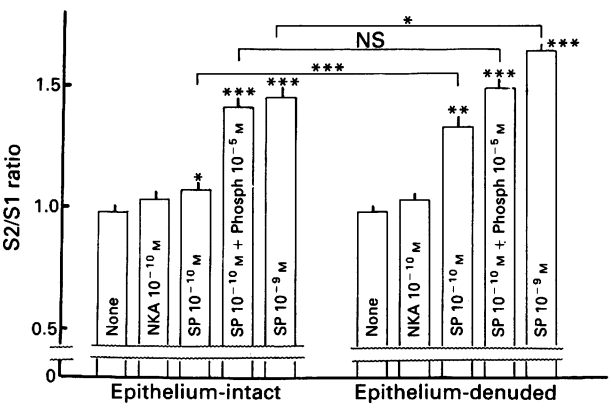

Figure 8 Effects of substance P (SP), neurokinin A (NKA) and phosphoramidon (Phosph) on the ratio of [^{3}H]-choline outflow (S2/S1) evoked by electrical field stimulation. Drugs were given 5 min before S2 and lasted until 2 min after end of S2. Asterisks on the top of the columns indicate significant difference between the S2/S1 ratio with agonist ($n = 6$) and without agonist ('None' $n = 9$) within each preparation group. Asterisks on the horizontal lines combining the columns indicate a significant difference in the S2/S1 ratio between epithelium-intact and epithelium-denuded preparations in the presence of same agonists. Significant difference between groups: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; NS, not significant.

cal properties of tracheal smooth muscle in view of the lack of effect on the dose-response curves to ACh, KCl and 5-HT. Small *et al.* (1990) showed that epithelium could modulate smooth muscle activity by both release of epithelium-derived relaxing factors (EpDRF), and by acting as a barrier to drug diffusion in guinea-pig trachea. We cannot evaluate the barrier function because most of the muscle surface in both of our preparations was exposed to the test solution. The epithelium may inactivate some chemical mediators by acting as a metabolic sink. The epithelium of rabbit trachea does not appear to represent a significant source of cholinesterase due to the lack of effect of epithelium removal on the ACh concentration-effect curve. Furthermore, although physostigmine potentiated the response to ACh in epithelium-intact preparation, similar potentiation was evident in epithelium-denuded preparations. KCl- and 5-HT-induced contractions may be partially mediated by a muscarinic mechanism. KCl may induce ACh release from the intramural nerve plexus by depolarization. Sheller *et al.* (1982) showed that 5-HT facilitated ACh release in canine lungs. In rabbit trachea, 5-HT may induce ACh release from the intramural nerve plexus. Removal of epithelium did not affect ACh release induced by KCl or 5-HT. Rabbit tracheal epithelium may be inactive in the cyclo-oxygenase and lipoxygenase pathways because indomethacin, NDGA or ETYA did not change the contractile responses to KCl, ACh, 5-HT or EFS. In culture, rabbit tracheal epithelial cells did not spontaneously produce prostaglandins, but were easily stimulated to produce them as well as hydroxyheptadecatrienoic acid (HHT) and hydroxyeicosatetraenoic acid (HETE) (Xu *et al.*, 1986). In rabbits, airway injury can easily disturb production of AA metabolites, regulation of electrolyte transport and mucus secretion and these may affect airway hyperreactivity (Xu *et al.*, 1986).

SP and structurally related peptides belong to the family of tachykinins. These peptides have a common C-terminal sequence and exhibit similar biological actions (Devillier *et al.*, 1988). SP and neurokinin A (NKA) are coded by the same precursor gene (Nakanishi, 1987). Noxious stimulation induces an inflammatory response known as neurogenic inflammation which is characterized by release of tachykinins, smooth muscle contraction, increased vascular permeability, gland secretion, and facilitation of ACh release (Barnes, 1986). Responses to tachykinins are normally modulated by enzymes. NEP and peptidyl dipeptidase A (angiotensin converting enzyme, ACE, EC3,4,15,1.) are thought to be involved in degradation of tachykinins (Skidgel *et al.*, 1984). An immunohistochemical study showed the presence of NEP in airway smooth muscle, submucosa and epithelial cells (Sekizawa *et al.*, 1987). The presence of ACE was demonstrated on the luminal surface of vascular endothelium (Johnson *et al.*, 1985). Matas *et al.* (1983) showed that NEP was the principal enzyme for hydrolyzing SP. Our results demonstrate that removal of the epithelium increased the contractile response to SP, which may be due to loss of enzymatic degrading sites or loss of EpDRF. Figure 4 suggests that NEP outside the epithelium (in smooth muscle, submucosal gland, and vagus nerve) can also modulate contractile responses to SP. It is unlikely that EpDRF, whether produced along the cyclo-oxygenase pathway or not, significantly affected either preparation. Figure 3b suggests that NKA resists enzymatic degradation, or does not induce the release of EpDRF. It is probable that epithelial NEP degraded SP more rapidly than it degraded NKA. Hopper *et al.* (1985) showed that the hydrolysis of neurokinins could be attributed to either NEP or to a bestatin-sensitive aminopeptidase, or to both. Devillier *et al.* (1988) showed that NKA and neurokinin B might be resistant to NEP. Atropine shifted the concentration-response curves to SP to the right in both preparation groups. Contraction induced by SP was partially mediated by a muscarinic mechanism. In the presence or absence of tachykinins, contractile responses to EFS were completely abolished by atropine and TTX in both preparation groups. Our results show that a cholinergic mechanism is responsible for contractile responses

and tachykinin potentiation of EFS effects. Neither hexamethonium nor indomethacin affected potentiation by SP or NKA (data not shown). Spantide abolished tachykinin potentiation of EFS effects. The action site of tachykinins is likely to be on a postganglionic tachykinin receptor, and modulation of cholinergic neurotransmission by tachykinins is not due to cyclo-oxygenase products of AA metabolism. More ACh was released at 20 Hz than at 5 Hz, so the tachykinin-induced potentiating effect might be masked by a large amount of ACh. SP dose-dependently facilitated [^{3}H]-choline outflow evoked by EFS (Figure 8). The potentiating effect on [^{3}H]-choline outflow evoked by EFS in denuded preparations was larger than that in intact preparations. In the presence of phosphoramidon, [^{3}H]-choline outflow evoked by EFS was potentiated in both groups of preparations. These results suggested that epithelial NEP, and extra-epithelial NEP may both modulate SP facilitation of [^{3}H]-choline outflow evoked by EFS by breaking down the SP. The potentiation of the contractile response and [^{3}H]-choline outflow induced by SP was significantly greater than that induced by NKA. Atropine did not shift the NKA concentration-effect curves. These results suggest that tachykinin receptors on rabbit tracheal intramural nerves may be NK_1 receptors. In the study of [^{3}H]-choline outflow, the potentiating effect of 10^{-10} M SP in denuded preparations was similar to that of 10^{-9} M SP in intact preparations. Epithelial damage would lead to loss of NEP so that released tachykinin would have a greater effect. CGRP and SP are co-localized in both the central and peripheral nervous systems, including peripheral sensory nerves (Lundberg *et al.*, 1985). In rabbit trachea CGRP had no effect alone and did not affect the contraction induced by EFS in the presence of SP. Wiesenfeld-Hallin *et al.* (1984) reported that effects of SP were potentiated by CGRP at doses below threshold in the rat spinal cord. This difference in results could be explained by the absence of CGRP receptors in the rabbit trachea, or by rapid and complete degradation of CGRP by NEP in the rabbit trachea, or both.

In summary, contractile responses to EFS were mainly mediated by cholinergic functions, and SP facilitated cholinergic neurotransmission in the rabbit trachea. The epithelium may modulate directly and indirectly SP-induced contractions because of the presence of NEP. In rabbit trachea, no AA metabolites are concerned with contractile responses to EFS and drug-induced contractions. Thus, the presence of NEP in rabbit trachea can modulate cholinergic neurotransmission, and this function is ubiquitous in several species. The rabbit may be a useful animal for examination of airway cholinergic functions.

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Characterization of the P_1-purinoceptors mediating contraction of the rat colon muscularis mucosae

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1 Previous studies had shown that adenosine and adenine nucleotides including adenosine 5'-triphosphate (ATP) caused contraction of the rat colon muscularis mucosae via P_1 and P_{2Y}-purinoceptors respectively, and that the stable ATP analogue adenylyl 5'-(β, γ-methylene)diphosphonate (AMPPCP) had an unexpected direct action on the P_1-purinoceptors. The P_1-purinoceptors have now therefore been further characterized by use of the adenosine analogues 5'-N-ethylcarboxamidoadenosine (NECA) and N^6-cyclopropyladenosine (CPA) and the antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), which is selective for the A_1 subtype. The P_2-purinoceptor antagonist suramin was also used, to investigate the selectivity of the P_2 agonists.

2 The order of potency of P_1 agonists for contraction was CPA > NECA > AMPPCP $\geq$ adenosine, and DPCPX (1 nM) caused greater than two fold shifts to the right of the log concentration-response curves for each of these agonists, although the shifts were not always parallel and Schild analysis of the inhibition of the effect of adenosine resulted in a plot with a slope greater than unity. These results indicate that the P_1-purinoceptor mediating contraction is of the A_1 subtype, as has been found in other tissues in which adenosine causes contraction.

3 The P_2-purinoceptor antagonist suramin (300 μM) had no effect on the responses to adenosine or to AMPPCP, but abolished contractions induced by the related stable ATP analogue adenosine 5'-(α,β-methylene)triphosphonate (AMPCPP). Contractions induced by ATP, which were not affected by DPCPX (10 nM) alone, were only partially inhibited by suramin (300 μM), revealing an A_1 component to its action which could be blocked by DPCPX (10 nM).

4 In conclusion, these results show that the rat colon muscularis mucosae possesses contractile A_1 receptors in addition to the previously characterized P_{2Y} receptors, and confirms our finding that the stable ATP analogue, AMPPCP, has an unexpected direct action on these A_1 receptors.

Keywords: Purinoceptors; adenosine; ATP; suramin; rat colon muscularis mucosae

Introduction

The pharmacological effects of extracellular adenosine and adenine nucleotides are mediated by specific receptors known as purinoceptors, of which two main classes are recognised, P_1-purinoceptors which respond to adenosine and P_2-purinoceptors which respond to adenosine 5'-triphosphate (ATP) and adenosine 5'-diphosphate (ADP) (Burnstock, 1978). The methylxanthines such as theophylline and its analogues are competitive antagonists at P_1-purinoceptors and inhibit the effects of adenosine but not of ATP (Burnstock, 1978), while until recently no reversible competitive antagonists existed for the P_2-purinoceptor (Fedan & Lamport, 1990). However the trypanocidal drug, suramin, has now been demonstrated to be a competitive antagonist of the actions of ATP on a number of smooth muscle preparations, although it appears to equilibrate with the receptors very slowly (Dunn & Blakely, 1988; Den Hertog *et al.*, 1989a,b; Hoyle *et al.*, 1990; Leff *et al.*, 1990; Von Kugelgen *et al.*, 1990). A complicating factor in the study of purinoceptor pharmacology is that as well as acting on P_2-purinoceptors, ATP (and some of its analogues) is rapidly and sequentially dephosphorylated by ectonucleotidases present on the surface of cells, ultimately to adenosine which has its own effects on P_1-purinoceptors and is also removed by uptake into cells and by deamination to the inactive inosine (Pearson & Slakey, 1990).

P_1-purinoceptors have been subdivided into A_1 and A_2, on the basis of different structure-activity relationships for agonists and antagonists and, in general, different second messenger coupling systems, with A_1 receptors causing inhibition of adenylate cyclase and A_2 receptors causing stimulation, although this is not a criterion for receptor subdivision and other coupling systems probably exist. On A_1 receptors N^6-substituted adenosine analogues such as N^6-cyclopentyladenosine (CPA) are more potent than 5'-substituted analogues such as 5'-N-ethylcarboxamidoadenosine (NECA), whereas on A_2 receptors the potency order is reversed. Radioligand binding studies with A_1 and A_2 selective ligands have confirmed the existence of these two subtypes, and selective antagonists have been developed (for reviews see Bruns, 1990a,b; Jacobson, 1990; Daly, 1990). However, characterization of adenosine receptor subtypes in isolated smooth muscle preparations has not always been clear-cut, as agonist potency orders have not always fallen into one of the two patterns expected, and indeed this has led to the suggestion of a third major subclass, A_3 (Ribeiro & Sebastiao, 1986), although this has not gained wide acceptance. Another problem has been that some of the antagonists which have been reported to be A_1-selective in other tissues fail to show selectivity in functional studies in smooth muscle, although recently 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) has been shown to act as a selective, competitive A_1 antagonist in guinea-pig and rat tissues (Collis *et al.*, 1987; 1989; Collis, 1990).

P_2-purinoceptors on smooth muscle have also been subdivided, the major division being into P_{2X} and P_{2Y}, mediating contraction or relaxation of smooth muscle respectively (Burnstock & Kennedy, 1985; Gordon, 1986; Burnstock, 1990). This receptor classification is largely based on different structure-activity relationships for ATP analogues (Cusack & Hourani, 1990), as well as on the different responses elicited. On P_{2X}-purinoceptors stable analogues of ATP, such as adenylyl 5'-(β,γ-methylene)diphosphonate (AMPPCP) and adenosine 5'-(α,β-methylene)triphosphonate (AMPCPP) are more potent than ATP which is equipotent with its 2-substituted analogues such as 2-methylthioadenosine 5'-

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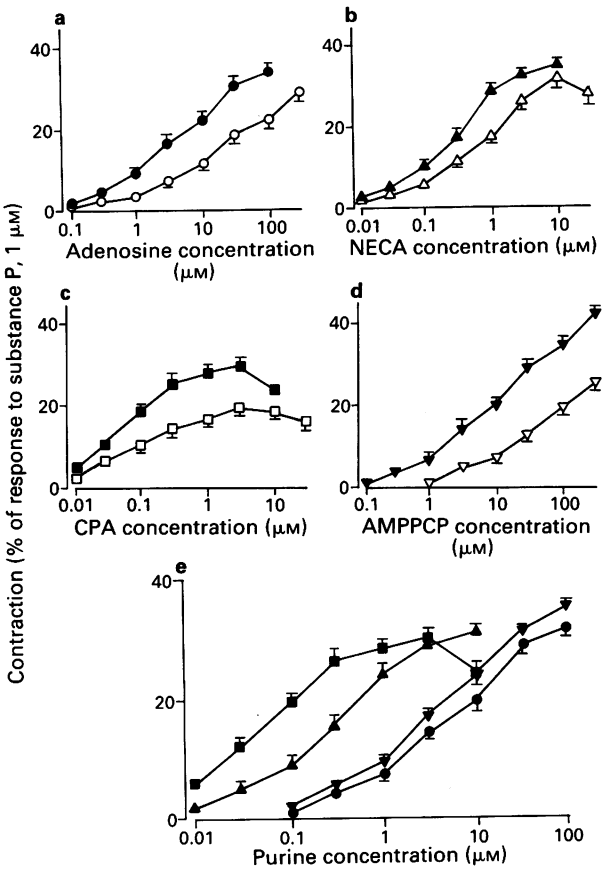

Figure 1 Contractions of the rat colon muscularis mucosae to (a) adenosine (●, ○), (b) 5'-N-ethylcarboxamidoadenosine (NECA, ▲, △), (c) N^6-cyclopropyladenosine (CPA, ■, □) or (d) adenylyl 5'-(β, γ-methylene)diphosphonate (AMPPCP ▼, ▽) alone (closed symbols) or in the presence of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 1 nM) (open symbols). Each point is the mean of at least 4 determinations, and the vertical bars show the s.e.mean. Control curves for each agonist alone are also shown in (e), for comparison. Each point here is the mean of at least 12 determinations, and the vertical bars show s.e.mean.

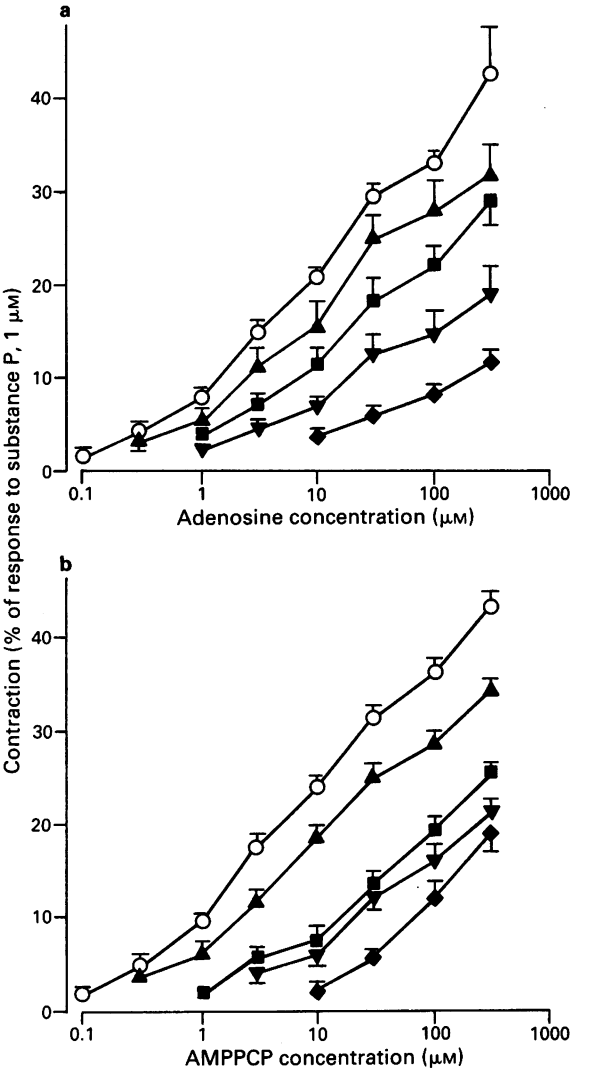

Figure 2 Contractions of the rat colon muscularis mucosae to (a) adenosine or (b) AMPPCP alone (○) or in the presence of 0.3 nM (▲), 1 nM (■), 3 nM (▼) or 10 nM (◆) DPCPX. Each point is the mean of at least 6 determinations, and the vertical bars show s.e.mean. For abbreviations, see legend to Figure 1.

triphosphate (2-MeSATP), whereas on P_{2Y}-purinoceptors 2-MeSATP is more potent than ATP which is more potent than AMPPCP and AMPCPP (Burnstock & Kennedy, 1985). These structure-activity relationships may be complicated in some tissues by the rapid breakdown by ectonucleotidases of ATP and its 2-substituted analogues to adenosine or its 2-substituted analogues, which may affect the observed potency of these nucleotides (Welford *et al.*, 1986; 1987). The P_2-purinoceptor antagonist, suramin, does not distinguish between the two proposed subtypes, but has a pA_2 value of about 5 on both P_{2X}- and P_{2Y}-purinoceptors (Dunn & Blakely, 1988; Den Hertog *et al.*, 1989a,b; Hoyle *et al.*, 1990; Leff *et al.*, 1990; Von Kugelgen *et al.*, 1990).

We have recently shown that in the rat colon muscularis mucosae preparation both P_1- and P_2-purinoceptors exist and mediate contraction, and that the P_2-purinoceptor present is, unexpectedly, of the P_{2Y} subtype. Furthermore, in this preparation AMPPCP, but not ATP, MeSATP or AMPCPP, acts via the P_1 rather than the P_2-purinoceptors, as it is inhibited by the selective P_1-purinoceptor antagonist, 8-sulphophenyltheophylline (8-SPT), and this P_1 effect is direct as the breakdown of AMPPCP in this tissue is far slower than that of ATP which itself is unaffected by 8-SPT (Bailey & Hourani, 1990). Because of these unexpected findings, we decided to investigate further the purinoceptors in this tissue by using the adenosine analogues NECA and CPA, as well as the A_1-selective antagonist DPCPX, to determine whether A_1 or A_2 receptors were present, and by using the P_2 antagonist suramin to confirm that AMPPCP was acting on P_1- and not P_2-purinoceptors in this tissue.

Methods

Pharmacological studies

Male Wistar rats (150–200 g) were killed by cervical dislocation and the distal colon removed and placed in warm (32°C) Tyrode buffer (ionic composition (mM): Na^+ 149.1, K^+ 2.8, Ca^{2+} 1.8, Mg^{2+} 2.1, Cl^- 147.5, $H_2PO_4^-$ 0.3, HCO_3^- 11.9 and glucose 5.6) pregassed with 95% O_2 : 5% CO_2. The dissection of the rat colon muscularis mucosae was carried out as described by Bailey & Jordan (1984), with minor modifications. Briefly, a glass pipette, external diameter 5 mm, was placed inside the colon and the outer layer of longitudinal muscle was removed by gentle rubbing with wet cotton wool, leaving a thick walled tube, the muscularis mucosae. This was suspended in a 3 ml organ bath at 32°C in gassed Tyrode solution (containing atropine 1 μM), and contractions were recorded isometrically under a resting tension of 1 g with a Grass FT03 strain gauge and displayed on a Grass 79C polygraph and expressed as a percentage of the contraction induced by substance P (1 μM). The tissue was allowed to equilibrate for 3 h before control concentration-response

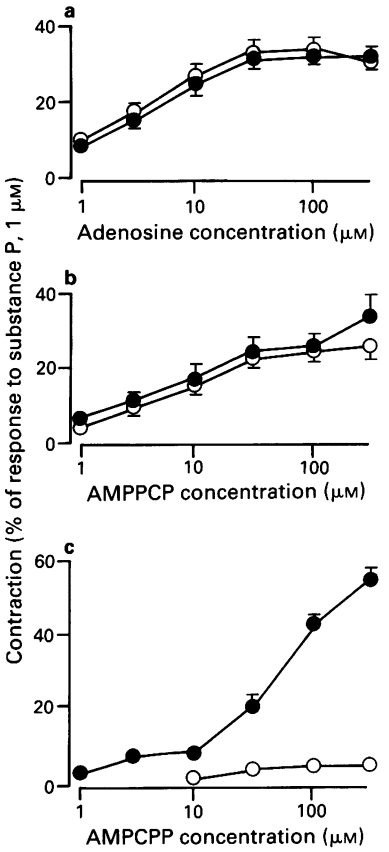

Figure 3 Contractions of the rat colon muscularis mucosae induced by (a) adenosine, (b) AMPPCP or (c) AMPCPP alone (●) or in the presence of suramin (300 μM) (○). Each point is the mean of at least 4 determinations, and the vertical bars show the s.e.mean. For abbreviations, see legend to Figure 1.

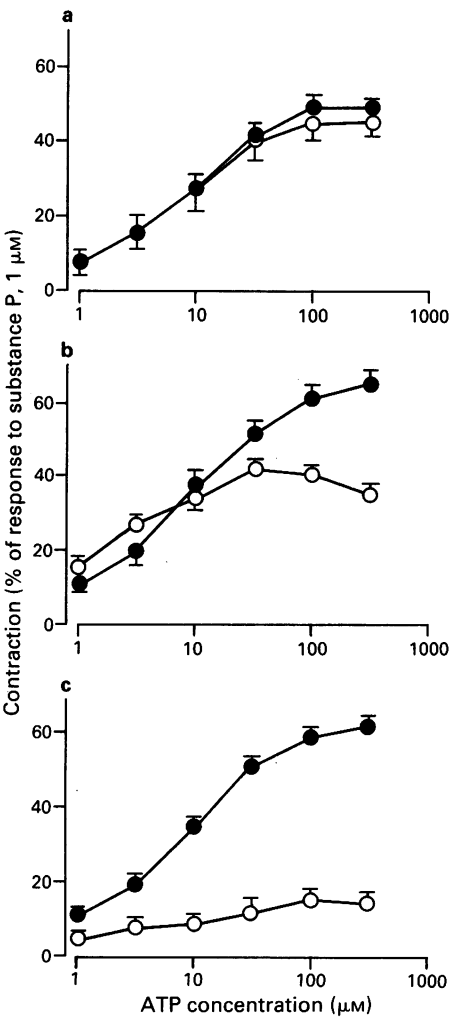

Figure 4 Contractions of the rat colon muscularis mucosae induced by adenosine 5'-triphosphate alone (●) or in the presence of the following antagonists (○): (a) DPCPX (10 nM); (b) suramin (300 μM); (c) DPCPX (10 nM) plus suramin (300 μM). Each point is the mean of at least 4 determinations, and the vertical bars show the s.e.mean. For abbreviations, see legend to Figure 1.

curves to purinoceptor agonists were determined, followed by incubation with an antagonist for 40 min before the concentration-responses curves were repeated in the presence of the antagonist. Recovery of responses to the agonists was established following washout of the antagonist for up to 40 min; 12–20 min were allowed between doses of agonist and the purinoceptor agonists were left in contact with the tissue for 45–90s.

Materials

ATP, AMPPCP, AMPCPP, adenosine and SP were obtained from Sigma UK Ltd, DPCPX, CPA and NECA from Research Biochemicals, and suramin from Bayer, UK. CPA (10 mM) was dissolved in 20% aqueous ethanol and DPCPX (1 mM) was dissolved in 2% aqueous dimethylsulphoxide (DMSO) containing 6 mM NaOH. After dilution corresponding to the final bath concentration of the substances used, these solvents had no effect on the responses of the tissues.

Results

CPA, NECA, adenosine and AMPPCP each contracted the rat colon muscularis mucosae, and the order of potency was CPA > NECA > AMPPCP ≥ adenosine, although maximal responses to adenosine and to AMPPCP could not be achieved due to their rather low potency. Responses to each of these agonists were inhibited by DPCPX (1 nM), although the maximal contractions were depressed by the antagonist (Figure 1). Increasing concentrations of DPCPX (0.3–10 nM) caused increasing inhibition of the responses to adenosine but also depressed the slope of the log concentration-response curve (Figure 2a), and because of the non-parallel nature of the shifts Schild analysis was unsatisfactory and resulted in a plot with a slope greater than unity (results not shown). DPCPX (0.3–10 nM) caused similar shifts in the log concentration-response curve to AMPPCP, but did not depress the slope of the curves to the same extent (Figure 2b). Contractions to adenosine and to AMPPCP were unaffected by suramin (300 μM), whereas those to AMPCPP were almost abolished (Figure 3). Contractions to ATP were unaffected by DPCPX (10 nM), partially inhibited by suramin (300 μM) and almost abolished by a combination of DPCPX (10 nM) and suramin (300 μM) (Figure 4). Whereas the contraction to ATP

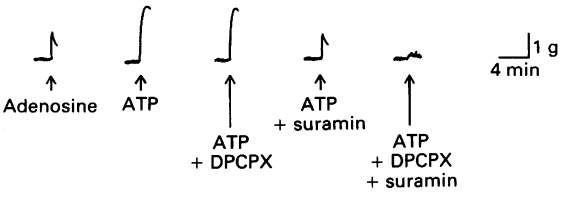

Figure 5 Representative traces showing contractions induced by adenosine (100 μM) or by ATP (100 μM) alone or in the presence of DPCPX (10 nM), suramin (300 μM) or DPCPX (10 nM) plus suramin (300 μM). The arrows indicate the point of addition of adenosine or ATP. For abbreviations, see legend to Figure 1.

alone or in the presence of DPCPX (10 nM) was rather slow and sustained, in the presence of suramin (300 μM) this was converted into a faster, quickly reversible contraction similar to that seen with adenosine (see Figure 5 for representative traces).

Discussion

These results show that in the rat colon muscularis mucosae there is a potency order for adenosine agonists of CPA > NECA and that an A_1-selective concentration of DPCPX (1 nM) inhibits the effects of adenosine agonists, suggesting that the P_1-purinoceptor mediating contraction of this tissue is of the A_1 subtype (Bruns, 1990b; Collis, 1990; Daly, 1990). Adenosine itself was rather less potent than NECA, although its potency may have been reduced by uptake or deamination, and was roughly equipotent with the stable ATP analogue, AMPPCP. In general the postsynaptic inhibitory effects of adenosine in smooth muscle are mediated by A_2 receptors while the presynaptic inhibition of transmitter release is mediated by A_1 receptors, although these presynaptic receptors have also been proposed to be of the putative A_3 subclass (Ribeiro & Sebastiao, 1986; White, 1988; Kennedy, 1990; Olsson & Pearson, 1990; Stone, 1991). In those few tissues in which adenosine has been shown to cause contraction, such as the renal vasculature (Kenakin & Pike, 1987) and the guinea-pig myometrium (Smith *et al.*, 1988), this also appears to be via A_1 receptors, although the contractions induced by adenosine in the rat anococcygeus have been reported to be insensitive to methylxanthines and may therefore not be mediated by P_1-purinoceptors (Stone, 1983). It has also been reported that the guinea-pig aorta and trachea each possess both A_1 and A_2 receptors which mediate contraction and relaxation respectively, although for adenosine the A_2-mediated relaxation is dominant (Farmer *et al.*, 1988; Stoggall & Shaw, 1990). The existence in the rat colon muscularis mucosae of contractile P_1-purinoceptors of the A_1 subtype is therefore consistent with this general pattern.

Although the dissociation constant for DPCPX in the rat colon muscularis mucosae is clearly in the nanomolar range as 1 nM caused greater than a two fold shift in the log concentration-response curves for all the adenosine agonists, an accurate dissociation constant could not be obtained because the shift was not always parallel and Schild analysis resulted in lines with slopes greater than unity. Although this could indicate that DPCPX was not acting in a purely competitive manner in this tissue, we have observed the same problem with 8-SPT in this tissue (unpublished observations) and in the rat duodenum although not in the guinea-pig taenia caeci (Hourani *et al.*, 1991), while Schild plots have been successfully obtained to both antagonists in rat atria and in guinea-pig aorta, atria and trachea (Collis *et al.*, 1989; Collis, 1990). The reason for the differences in Schild slope between the different tissues is not known, but Leung *et al.* (1990) have suggested a model whereby competitive antagonists may appear to be noncompetitive. This model is based on their finding that unoccupied A_1 adenosine receptors appear to be tightly bound to G-proteins, and suggests that antagonists bind preferentially to the free receptors whereas agonists bind preferentially to the receptors coupled to the G-proteins, resulting in apparently noncompetitive binding kinetics (Leung *et al.*, 1990). Another possible explanation for the Schild plot having a slope greater than unity is that DPCPX was not at equilibrium with the receptors, but this is unlikely because preliminary experiments with 1 nM DPCPX using a longer incubation time (2 h) did not result in increased inhibition. A further possibility is that there is also a small population of A_2 receptors present on this tissue opposing the A_1 effects of adenosine and resulting in apparently non-surmountable antagonism by DPCPX, but although this possibility cannot be discounted no relaxation to adenosine or its analogues was revealed in the presence of the antagonist.

That the P_2-purinoceptor antagonist, suramin, inhibited the effects of ATP and of AMPCPP but not of adenosine or AMPPCP confirms the selectivity of suramin for P_2-purinoceptors and our previous conclusion (Bailey & Hourani, 1990) that AMPPCP unexpectedly acts on P_1- and not P_2-purinoceptors in this tissue. That DPCPX, like 8-SPT (Bailey & Hourani, 1990), dose-dependently inhibited the effects of AMPPCP in a similar fashion to those of adenosine also confirms that the major action of AMPPCP is via the A_1 receptors. Blockade of P_1 receptors with 8-SPT did reveal a P_2 component to the action of very high concentrations ($\geq$100 μM) of AMPPCP (Bailey & Hourani, 1990), and this might explain why suramin slightly inhibited the effect of 300 μM AMPPCP (Figure 3b), and why DPCPX did not depress the slopes of the log concentration-response curves to AMPPCP to quite the same extent as those of adenosine (Figure 2). We have also observed this P_1 effect of AMPPCP in the rat duodenum and, to a lesser extent, in the guinea-pig taenia caeci, and as in all these tissues AMPPCP (unlike ATP) is highly resistant to degradation these effects are likely to be direct and not via its potential breakdown product adenosine (Bailey & Hourani, 1990; Hourani *et al.*, 1991).

Although DPCPX, like 8-SPT, did not inhibit the effects of ATP on the rat colon muscularis mucosae even at 10 nM, the results reported here show that suramin, which abolished the effects of the P_2 agonist AMPCPP, only partially inhibited the effects of ATP, and revealed a P_1 component of its action. In this tissue responses to P_2 agonists are slower than responses to P_1 agonists (see Bailey & Hourani, 1990 for representative traces), and in the presence of suramin the response to ATP changed in nature, becoming faster and quickly reversing, like that to adenosine. This residual response was blocked by DPCPX, indicating that it was indeed mediated by the A_1 receptors in this tissue, and a combination of suramin and DPCPX abolished responses to ATP. It is not clear from these results whether this P_1 component of the effect of ATP is direct or is mediated by its rapid breakdown to adenosine.

In conclusion, the results presented here and those previously published (Bailey & Hourani, 1990) show that in the rat colon muscularis mucosae contractile responses to purines are mediated by A_1 and P_{2Y} receptors, which can be selectively blocked by DPCPX and suramin respectively. Although adenosine and its analogues act only on the A_1 receptors and AMPCPP acts only on P_{2Y} receptors, the stable ATP analogue AMPPCP acts directly via the A_1 receptors and ATP itself also has an A_1 component to its action which can be revealed by suramin.

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Adenosine receptors in rat basophilic leukaemia cells: transductional mechanisms and effects on 5-hydroxytryptamine release

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1 The presence of adenosine receptors linked to adenylate cyclase activity and their functional role in calcium-evoked 5-hydroxytryptamine (5-HT) release was investigated in rat basophilic leukaemia (RBL) cells, a widely used model for studying the molecular mechanisms responsible for stimulus-secretion coupling.

2 In [^{3}H]-5-HT-loaded cells triggered to release by the calcium ionophore A23187, a biphasic modulation of 5-HT secretion was induced by adenosine analogues, with inhibition of stimulated release at nM and potentiation at μM concentrations, suggesting the presence of adenosine receptor subtypes mediating opposite effects on calcium-dependent release. This was also confirmed by results obtained with other agents interfering with adenosine pharmacology, such as adenosine deaminase and the non-selective A_1/A_2 antagonist 8-phenyl-theophylline.

3 Similar biphasic dose-response curves were obtained with a variety of adenosine analogues on basal adenylate cyclase activity in RBL cells, with inhibition and stimulation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) production at nM and μM concentrations, respectively. The rank order of potency of adenosine analogues for inhibition and stimulation of adenylate cyclase activity and the involvement of G-proteins in modulation of cyclic AMP levels suggested the presence of cyclase-linked A_1 high-affinity and A_2-like low-affinity adenosine receptor subtypes. However, the atypical antagonism profile displayed by adenosine receptor xanthine antagonists on cyclase stimulation suggested that the A_2-like receptor expressed by RBL cells might represent a novel cyclase-coupled A_2 receptor subtype.

4 Micromolar concentrations of adenosine analogues could also increase inositol phospholipid hydrolysis and inositol tris-phosphate formation in both unstimulated cells and in cells triggered to release by the calcium ionophore. The stimulation was constant, small and additive to that exerted by the calcium ionophore.

5 It is concluded that RBL cells express both A_1 and A_2-like adenosine receptors which exert opposite effects on 5-HT release and intracellular cyclic AMP levels. However, besides modulation of cyclic AMP levels, additional transduction pathways, such as modulation of phospholipase C activity, may contribute to the release response evoked by adenosine analogues in this cell-line.

Keywords: Adenosine receptors; rat basophilic leukaemia cells; cyclic AMP levels; inositol-tris-phosphate production; 5-hydroxytryptamine release

Introduction

Adenosine and adenine nucleotides have been implicated in a variety of functions in different organs and systems. The effects of adenosine are thought to be mediated by activation of A_1 and A_2 receptor subtypes (Burnstock, 1989). Of particular importance among the many effects of adenosine is the modulation of calcium-dependent neurotransmitter release (Stone & Bartrup, 1989). Inhibition of release of a variety of 'classical' neurotransmitters (e.g. catecholamines, acetylcholine, 5-hydroxytryptamine (5-HT), excitatory amino acids and γ-aminobutyric acid (GABA) to a lesser extent) has been shown to be mediated by A_1 receptors located on presynaptic nerve terminals (Fredholm & Dunwiddie, 1988), whereas the A_2 receptor subtype seems to induce opposite effects (Spignoli *et al.*, 1984). A_1 high-affinity adenosine receptors are linked to inhibition of membrane adenylate cyclase activity, whereas activation of A_2 low-affinity receptors leads to stimulation of this enzyme and consequently to increases in intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels (Londos & Wolff, 1977; Van Calker *et al.*, 1979). More recently, additional transduction mechanisms, such as modulation of inositol-phosphates production and of calcium and potassium currents have also been implicated in the effects of adenosine on target cells, particularly those mediated by the A_1 receptor subtype (Cooper *et al.*, 1989; Scott & Dolphin, 1989).

Adenosine receptors have been described by several authors in serosal and parenchymal mast cells as well as in blood basophils where they modulate mediator release, triggered by immunological and non-immunological stimuli. The precise type of modulation exerted by adenosine receptors in these cells is still controversial. There are several possible sources of variability in these results, such as the different origin of the mast cells used (Church *et al.*, 1983; Marone *et al.*, 1989; Marquardt & Waker, 1990), the fact that adenosine is released from activated mast cells (Marquardt *et al.*, 1984) and can therefore interfere with pharmacological studies if adenosine deaminase (ADA) is not used to remove it, and the possible co-existence of different classes of adenosine receptors with different or even opposite functions.

We have characterized adenosine receptor-mediated regulation of secretion in a mastocyte tumour cell line, rat basophilic leukaemic (RBL) cells. These cells have their physiological counterpart in mucosal mast cells and therefore represent a suitable *in vitro* model to study degranulation (Barsumian *et al.*, 1981). Release of histamine, 5-HT, arachidonate metabolites and other allergic mediators can be induced by antigen in RBL cells sensitized by specific IgE, or by causing influx of external calcium with the calcium ionophore A23187, which

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bypasses some of the early events in the transduction process and directly triggers the final response. Ali et al. (1990) have found that in RBL cells, adenosine analogues potentiate antigen-induced 5-HT release by increasing phosphoinositide hydrolysis and influx of calcium. The rank order of potency of adenosine analogues (N-ethyl-carboxamido-adenosine > adenosine > R-phenyl-isopropyl-adenosine) was consistent with the activation of adenosine A_2 receptors. However, since these effects were relatively insensitive to classical adenosine receptor antagonists and apparently not related to modulation of intracellular cyclic AMP concentrations, these authors suggested the involvement of a novel type of adenosine receptor.

We show here that A_1- and A_2-like receptors regulating adenylate cyclase activity are both expressed by RBL cells, and that these modulate calcium/ionophore-induced release of 5-HT, with different functional effects depending on the receptor subtype. The role of cyclic AMP in the release response in RBL cells is discussed.

Methods

Cell culture

RBL-2H3 cells were grown in 75 cm^2 tissue culture flasks in Eagle's minimum essential medium, supplemented with 16% foetal calf serum.

Adherent cells were sub-cultured weekly after detachment in a buffer containing (mM): NaCl 125, EDTA 1.5, glucose 5.6 and HEPES 10, at pH 7.2.

Release of 5-hydroxytryptamine

The release of 5-HT was evaluated according to the method of De Matteis et al. (1991). Briefly, cells were detached and resuspended in complete growth medium containing [^{3}H]-5-HT (1 μCi ml^{-1}) and plated at a density of 3.7×10^5 cells/250 μl/well. Twenty-four hours later, cells were washed 3 times with Tyrode solution (composition mM: NaCl 125, KCl 5, glucose 5.6, HEPES 10, pH 7.2) to remove unincorporated radioactivity. Release of [^{3}H]-5-HT was then stimulated with 1 μM A23187 in the absence (control release) or presence of adenosine agonists and antagonists. Stock solutions of 10 mM A23187 in dimethyl sulphoxide (DMSO) were used. In control experiments, DMSO diluted 1:1000 did not affect 5-HT release. Release was terminated after 30 min by adding ice-cold release buffer supplemented with 5 mM EDTA. Supernatants were centrifuged to remove detached cells and aliquots counted by liquid scintillation to determine released [^{3}H]-5-HT. Adherent cells were lysed with 0.2% Triton X 100 and counted to determine residual incorporated radioactivity. Results are expressed as percentage release with respect to total incorporated radioactivity.

Adenylate cyclase activity

Adenosine-dependent adenylate cyclase was determined at 37°C as previously described (Abbracchio et al., 1989), with an established assay which is a minor modification of the method of Salomon et al. (1974).

Briefly, cells were detached from dishes, centrifuged for 10 min at 1100 g and stored at −80°C until day of assay (no longer than 1 month). On day of use, cells were rapidly thawed at 37°C, centrifuged in phosphate buffer twice at 1100 r.p.m. for 10 min, homogenized in 50 mM Tris-maleate pH 7.2 containing 2 mM dithiothreitol (12 strokes in a Teflon/glass potter) and aliquots (approximately 10–15 μg protein/tube) incubated in absence (basal activity) or presence of graded concentrations of the adenosine receptor analogues, N-ethyl-carboxamido-adenosine (NECA), cyclo-pentyl-adenosine (CPA), R-phenyl-isopropyl-adenosine (R-PIA) or 2-chloro-adenosine (2-Cl-ADO).

The reaction was started from addition of the cell homogenate to tubes containing, in a final volume of 100 μl, 1 μCi of [α^{32}P]-ATP (final concentration: 50 μM), 50 μM EGTA, 1 mM $MgCl_2$, 1 mM cyclic AMP, 50 mM Tris-maleate (pH 7.5), 20 μM GTP, 0.2 mM papaverine, 20,000 d.p.m. of [^{3}H]-cyclic AMP to determine chromatography recovery, an ATP-regenerating system consisting of 6.3 mM creatine phosphate and 60 μg/sample of creatine-phosphokinase, and 2 iu of adenosine-deaminase (ADA) ml^{-1} of cocktail. As described in more detail in Results, to evaluate better the inhibitory effects of adenosine on adenylate cyclase activity, 1 mM $MnCl_2$ was also added to increase basal enzyme activity (Jakobs & Watanabe, 1985). Incubation was continued for 40 min at 37°C and stopped by addition of 100 μl of a 'stop' solution containing 2% sodium dodecyl sulphate, 40 mM ATP and 1.3 mM cyclic AMP and by subsequently boiling samples for 4 min.

Cyclic AMP was separated by double chromatography on Dowex (AG $50X_4$ 200–400 mesh, H^+ form) and alumina columns (Salomon et al., 1974). After loading samples onto Dowex columns, cyclic AMP was eluted with distilled water onto alumina columns and finally eluted into scintillation vials with 0.1 M imidazole buffer, pH 7.5. Recoveries of cyclic AMP varied between 70 and 80%.

Results are expressed as percentage inhibition or stimulation of basal enzyme activity.

Protein content was determined according to the method of Bradford (1976).

Measurement of [^{3}H]-inositol phospholipids hydrolysis

Cells were detached, resuspended in complete growth medium and plated at a density of 1.5×10^6 cells ml^{-1} in 12-well plates, two days before the release experiment. The day before, complete growth medium was replaced with Medium 199 containing either [^{3}H]-inositol (5 μCi ml^{-1}) or [^{3}H]-5-HT (1 μCi ml^{-1}). The release experiment was conducted in parallel for inositol 1,4,5-tris-phosphate (IP_3) and 5-HT under identical conditions in the solutions described above with the addition of 10 mM LiCl. In plates containing [^{3}H]-inositol, the reaction was stopped by the addition of 1 ml cold methanol. Cells were then scraped and wells were rinsed once with a solution of methanol:water 1:3 (0.8 ml). Chloroform (1.25 ml) was then added. Samples (1 ml) of the aqueous phase were loaded on Dowex formate columns and the separation of the products of inositol lipids hydrolysis was carried out as described by Berridge et al. (1983).

Materials

Minimal essential medium with Eagle's salts and foetal calf serum were purchased from GIBCO. A23187, R-PIA, NECA, theophylline (Theo), 8-phenyl-theophylline (8-Ph-Theo), creatine phosphate and creatine-phosphokinase were from Sigma. ADA was obtained from Boehringer-Mannheim; 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 1,3-dipropyl-8-(2-amino-4-chlorophenyl)xanthine (PACPX) and CPA were from RBI. The Dowex resins for cyclic AMP and IP_3 chromatography were from Biorad. [^{3}H]-5-HT, [^{3}H]-inositol and [α^{32}P]-ATP were obtained from Dupont-NEN (Florence, Italy). All other chemicals were reagent grade and were obtained from Merck AG.

Results

5-Hydroxytryptamine release studies

Exposure of RBL cells to the calcium ionophore A23187 (1 μM) significantly stimulated 5-HT release. Basal and A23187-stimulated release were respectively 4% and 35% of the total 5-HT cellular content. Figure 1 shows that both ADA and the A_1/A_2 blocking agent 8-Ph-Theo markedly reduced the effect of A23187 (compare columns b and g with

a), indicating that a significant degree of occupation of stimulatory adenosine receptors occurs even in the absence of added adenosine. The inhibition exerted by these agents was specifically linked to the blockade of adenosine activity on purinoceptors since it was completely reversed by the exogenous addition of adenosine analogues such as CPA or NECA at μM concentrations in the case of ADA (Figure 1c,e), and partially reversed by the same agonists in the case of 8-Ph-Theo (Figure 1h,i). The stimulatory effects of either CPA or NECA were only partially sensitive to the xanthine antagonist (Figure 1d,f). In order to obtain a clear pharmacological profile of adenosine receptors, therefore, we found it necessary to eliminate endogenous adenosine from the assay buffer by adding ADA both in the release studies and in the adenylate cyclase and IP_3 studies. The addition of CPA in the presence of ADA inhibited A23187-stimulated release at nM concentrations and potentiated it at μM concentrations (Figure 2). Basal release was not modified. The maximal inhibiting ($40 \pm 4\%$) and potentiating activity ($170 \pm 5\%$) were observed respectively at 1 nM and 100 μM CPA. Both inhibitory and stimulatory effects of CPA on 5-HT release were only partially (50%) antagonized by the A_1/A_2 blocking agent 8-Ph-Theo (3 μM) (data not shown).

Similar results were obtained with other adenosine receptor analogues such as **R**-PIA, the only difference being their reduced ability to inhibit calcium-evoked release at nM concentrations (data not shown).

Adenylate cyclase studies

Since the data concerning 5-HT release suggested the presence of both A_1 and A_2 receptors in RBL cells, which exerted opposite effects on 5-HT release, we optimized our adenylate cyclase assay conditions, in order to be in a position to detect both inhibitory and stimulatory effects of adenosine analogues on cyclic AMP production. Whereas hormonal stimulation of adenylate cyclase was demonstrable, the detection of inhibition of cyclic AMP production by adenosine analogues required elevation of basal adenylate cyclase activity, as reported in other studies (Fredholm *et al.*, 1986). Since this cell line does not show a classical stimulatory response to forskolin (Jakobs & Watanabe, 1985), we investigated the effects

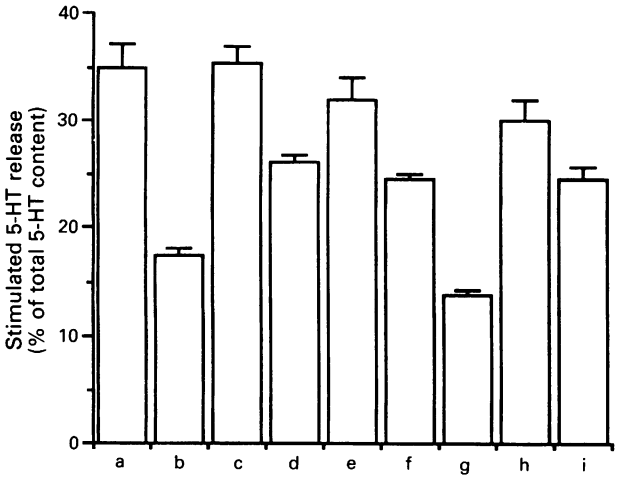

Figure 1 Effects of adenosine deaminase (ADA) and 8-phenyl-theophylline (8-Ph-Theo) on 5-hydroxytryptamine (5-HT) release induced by 1 μM A23187 in rat basophilic leukaemia cells. In cells prelabelled with [^{3}H]-5-HT, release was stimulated with the calcium ionophore in the absence (control release) or presence of either ADA (2 iu ml^{-1}) or 8-Ph-Theo (3 μM) alone or in combination with adenosine analogues (10 μM) as indicated: (a) Control; (b) ADA; (c) ADA + cyclopentyl-adenosine (CPA); (d) ADA + CPA + 8-Ph-Theo; (e) ADA + N-ethyl-carboxamide-adenosine (NECA); (f) ADA + NECA + 8-Ph-Theo; (g) 8-Ph-Theo; (h) 8-Ph-Theo + CPA; (i) 8-Ph-Theo + NECA. Each column represents the mean value from 5 independent experiments run in triplicate; s.e. shown by vertical bars.

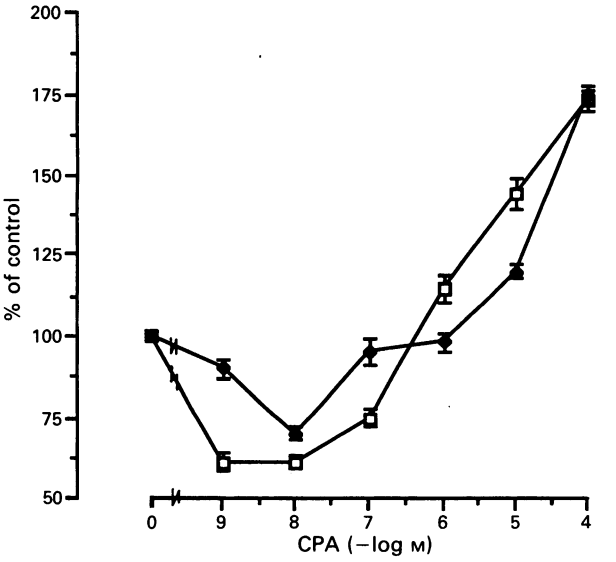

Figure 2 Effects of cyclo-pentyl-adenosine (CPA) on A23187-induced 5-hydroxytryptamine (5-HT) release and on adenylate cyclase activity in rat basophilic leukaemia cells. 5-HT release (□) and adenylate cyclase activity (◆) were determined as described in Methods. Results are expressed as percentage of control activities (i.e., A23187-induced 5-HT release for release experiments, and basal adenylate cyclase activity for cyclic AMP determinations). Basal unstimulated release (in absence of calcium ionophore) accounted for $4 \pm 1\%$ of the total 5-HT cellular content, and was not influenced by CPA at any of the indicated concentrations. Each point in figure represents the mean value (±s.e.) obtained from 6 independent experiments run in triplicate.

of another cyclase-stimulatory agent, namely manganese (Bockaert *et al.*, 1984). As shown in Figure 3, Mn^{2+} increased basal adenylate cyclase activity in homogenates of RBL cells in a dose-dependent manner. Since elevated Mn^{2+} concentrations (e.g. 2 mM; Figure 3) can also mask the response of adenylate cyclase to inhibitory hormones (Bockaert *et al.*, 1984), an intermediate concentration (1 mM) was chosen.

Under such conditions, the dose-response curve to a typical adenosine receptor agonist such as CPA (Figure 2) was biphasic, with inhibition of cyclic AMP generation in the nM range and stimulation at μM concentrations. The discrepancy between CPA dose-response curves for 5-HT release and for cyclic AMP production (Figure 2) is likely to be due to the

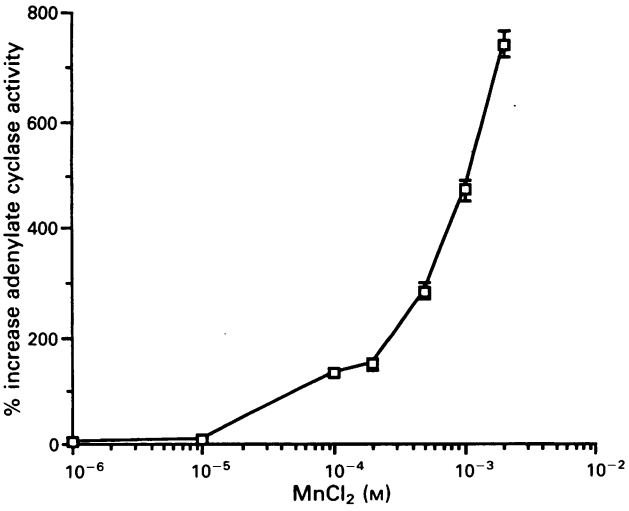

Figure 3 Effect of manganese chloride on membrane adenylate cyclase activity in rat basophil leukaemia cells. Adenylate cyclase activity was measured in absence (basal activity) or presence of increasing concentrations of $MnCl_2$. Each point represents the mean from 3 independent experiments run in triplicate; s.e. shown by vertical bars.

different experimental conditions under which the two assays were performed, since 5-HT release was measured in intact cells and cyclase activity in broken cell homogenates. In addition, this discrepancy might be due to a marked sensitivity of the secretory response to even slight changes of intracellular cyclic AMP concentrations. Alternatively, cyclic AMP-independent effects of CPA on release cannot be excluded.

Similar results on adenylate cyclase activity were obtained with other adenosine receptor analogues, such as NECA, R-PIA and 2-Cl-ADO (Table 1). The rank order of potency of analogues for cyclase inhibition (CPA > R-PIA = 2-Cl-ADO > NECA) and activation (NECA > R-PIA = 2-Cl-ADO > CPA) (Table 1 and Figure 2) is consistent with the classification of A_1 and A_2 receptors (Fredholm, 1986; Williams, 1987). In separate experiments in which either the inhibitory (nM range) or the stimulatory (μM and mM range) effects of CPA and NECA on adenylate cyclase were studied in more detail, inhibition was found to be more marked with CPA (mean maximal inhibition: $-25 \pm 3\%$ with respect to basal cyclase activity, mean of 6 determinations; IC_{50}: 5 ± 0.8 nM) and less evident with NECA (mean maximal inhibition: -9%); conversely, cyclic AMP increases were higher with NECA (mean maximal stimulation, $+102 \pm 7\%$ over basal enzyme activity, mean of 6 determinations; EC_{50}: $0.5 \pm 0.02\,\mu$M), than with CPA (mean maximal stimulation: $+72\%$ over basal activity; EC_{50}: $25 \pm 4\,\mu$M).

Since the phospholipase CPLC-coupled adenosine receptors described by Ali et al. (1990) in this cell line were relatively insensitive to classical adenosine receptor xanthine antagonists, we performed studies to verify whether the effects of adenosine analogues on cyclic AMP production could be antagonized by xanthine derivatives. To do so, we constructed dose-response curves with various adenosine receptor xanthine antagonists on both adenylate cyclase inhibition induced by nM CPA and adenylate cyclase stimulation induced by μM NECA concentrations.

As shown in Figure 4, the two selective A_1 receptor antagonists PACPX and DPCPX could counteract adenylate cyclase inhibition induced by 10^{-8} M CPA in a dose-dependent manner. In agreement with previously reported data (Bruns et al., 1987), DPCPX seemed more potent than PACPX and could completely antagonize CPA-induced adenylate cyclase inhibition at a concentration (50 nM) which is known virtually to abolish A_1-receptor binding (Lee & Reddington, 1986). On the other hand, antagonism of adenosine-dependent cyclase stimulation by Theo and 8-Ph-Theo did not fulfil 'classic' A_2 receptor criteria (Figure 5). In fact, although antagonism of cyclic AMP production was dose-dependent and 8-Ph-Theo seemed more potent than Theo (consistent with results of Bruns et al., 1980), no antagonism was observed up to 10^{-5} M and a complete block of cyclase stimulation was obtained

Table 1 Effects of several adenosine receptor agonists on membrane adenylate cyclase in rat basophilic leukaemia (RBL) cells

Agonist concentration (M)	*Adenylate cyclase activity* (% of basal)		
	NECA	*R-PIA*	*2-Cl-ADO*
10^{-9}	92 ± 3	87 ± 3	89 ± 4
10^{-8}	88 ± 4	79 ± 4	81 ± 3
10^{-7}	135 ± 5	102 ± 5	104 ± 5
10^{-6}	172 ± 6	120 ± 3	122 ± 2
10^{-5}	202 ± 8	158 ± 4	163 ± 3
10^{-4}	207 ± 6	172 ± 5	171 ± 7

Adenylate cyclase activity was assayed in homogenates of RBL cells in absence (basal activity) or presence of graded concentration of either N-ethyl-carboxamido-adenosine (NECA), R-phenyl-isopropyl-adenosine (R-PIA) or 2-chloro-adenosine (2-Cl-ADO).
Results are expressed as % of basal enzyme activity set as 100%, and represent the mean ± s.e. of 3 experiments run in triplicate.

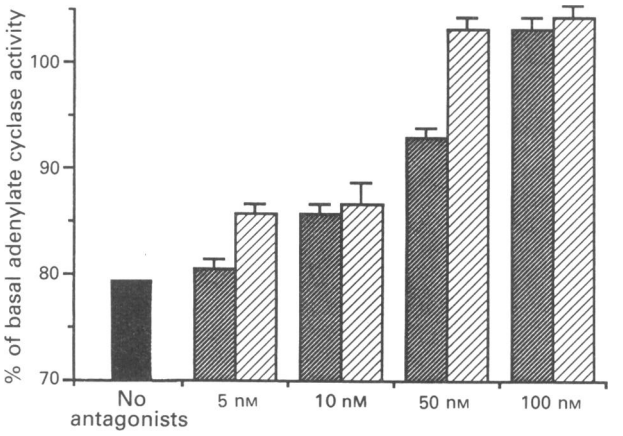

Figure 4 Effects of the two A_1 receptor antagonists, 1,3-dipropyl-8-(2-amino-4-chlorophenyl) xanthine (PACPX) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) on cyclo-pentyl-adenosine (CPA)-induced inhibition of membrane adenylate cyclase activity in rat basophilic leukaemia cells. Adenylate cyclase activity was determined in cell homogenates in presence of either 10^{-8} M CPA alone (solid column) or in presence of both 10^{-8} M CPA and either PACPX (left hand column of each pair) or DPCPX (right-hand column of each pair) at the indicated concentrations. Results are expressed as % of basal adenylate cyclase activity. Each value represents the mean of triplicate determinations; s.e. indicated by vertical bars. Similar results were obtained in 3 independent experiments.

only at mM concentrations, in contrast to what has been described for other adenosine-regulated cyclase systems (Fredholm et al., 1982; Psychoyos et al., 1982). Moreover, the 20–30 fold higher affinity of 8-Ph-Theo over Theo, usually observed for other A_2 receptor systems (Bruns et al., 1980; Daly, 1985) was not evident. Similar data were obtained with these two adenosine receptor antagonists on adenylate cyclase stimulation evoked by either R-PIA or CPA (data not shown).

These results together indicate that RBL cells express both A_1 and A_2-like membrane adenosine receptor subtypes linked

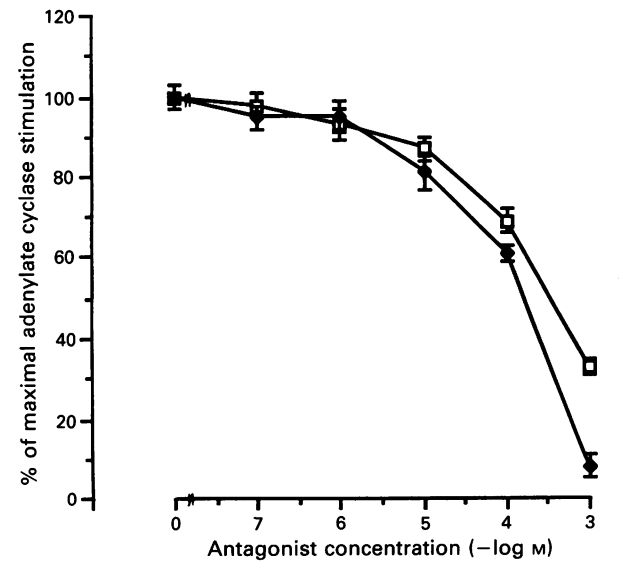

Figure 5 Antagonism by theophylline (Theo) (□) and 8-phenyl-theophylline (8-Ph-Theo) (◆) of N-ethyl-carboxamido-adenosine (NECA) stimulation of membrane adenylate cyclase activity in rat basophilic leukaemia cells. Adenylate cyclase activity was determined in cell homogenates in the presence of either 10^{-4} M NECA alone (value of maximal cyclase stimulation set at 100%) or in the presence of both 10^{-4} M NECA and either Theo or 8-Ph-Theo at the indicated concentrations. Points represent the mean of triplicate determinations; s.e. shown by vertical bars. Similar results were obtained in 3 independent experiments and with other adenosine receptor agonists such as cyclopentyl-adenosine and R-phenyl-isopropyl-adenosine used at maximal concentrations.

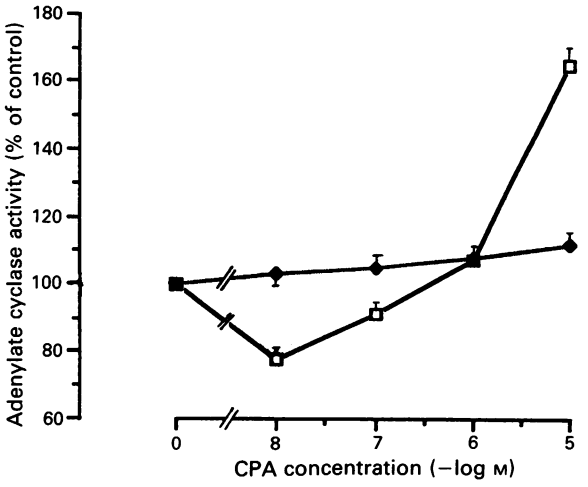

Figure 6 Abolition by guanosine 5'-O-(2-thiodiphosphate) (GDP-β-S) of A_1 and A_2 receptor-mediated modulation of adenylate cyclase activity in rat basophilic leukaemia cells. Cyclo-pentyl-adenosine (CPA) dose-response curve on adenylate cyclase activity was performed in the absence (control) (□), or presence of 1 mM GDP-β-S (◆). Each point represents the mean value obtained from 3 independent experiments run in triplicate; s.e. shown by vertical bars.

to inhibition and stimulation of adenylate cyclase activity, respectively.

Modulation of cyclic AMP levels by adenosine receptors is mediated by guanosine triphosphate (GTP)-binding proteins (G-proteins) in many cell types. The observation that the G-protein inhibitor guanosine 5'-O-(2-thiodiphosphate) (GDP-β-S) completely abolished both A_1 receptor-induced inhibition and A_2 receptor-induced stimulation of adenylate cyclase (Figure 6) indicates that this is the case also in RBL cells. Conversely, the non-hydrolyzable GTP analogue guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S) stimulated adenylate cyclase activity in a dose-dependent manner (mean stimulation at 1 and 10 μM: +480 ± 25% and +1320 ± 54% with respect to basal activity).

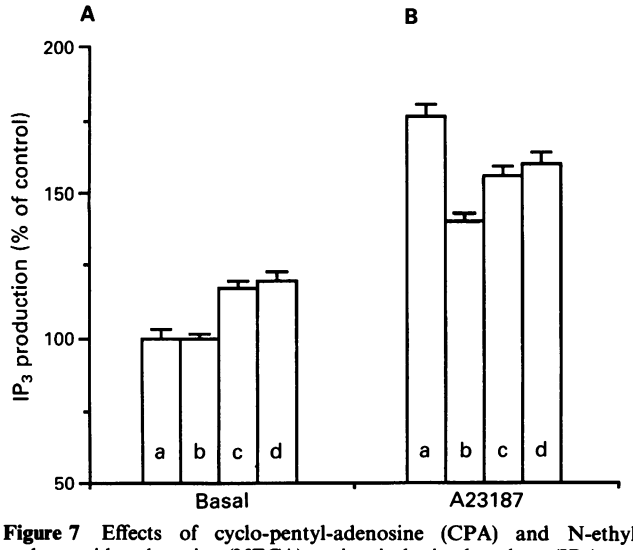

Figure 7 Effects of cyclo-pentyl-adenosine (CPA) and N-ethyl-carboxamido-adenosine (NECA) on inositol tris-phosphate (IP_3) production in rat basophilic leukaemia cells. IP_3 formation was determined in the absence (basal condition in A) or presence of 1 μM A23187 (B) as described in Methods, in the absence (a) or presence of either 2 iu ml^{-1} adenosine deaminase alone (b) or in combination with 10 μM CPA (c) or NECA (d), as indicated. Phosphoinositide hydrolysis was stopped after a 30 min exposure to the calcium ionophore. Results represent the mean of triplicate determinations; s.e. shown by vertical bars. Similar results were obtained in 4 independent experiments.
Panel A: c, d: $P < 0.05$ with respect to b.
Panel B: b: $P < 0.02$ with respect to a; c, d: $P < 0.05$ with respect to b.

Inositol tris-phosphate (IP_3) studies

Formation of IP_3 by membrane phosphoinositides was measured in parallel with 5-HT release in intact cells under identical experimental conditions. A significant stimulation of IP_3 production by A23187 was observed after 30 min of exposure (176 ± 5% over basal value, Figure 7). The ionophore effect was smaller (130 ± 7%) after 15 min and undetectable after 2 min of exposure to the calcium ionophore. ADA inhibited IP_3 production induced by A23187 at 30 min (Figure 7), and had no effect at either shorter stimulation times (15 or 2 min) or on basal activity. By contrast, the purinoceptor agonists CPA and NECA, tested over the concentration-range used for cyclase and 5-HT release studies, had no inhibitory activity, but at μM concentrations exerted a slight stimulatory effect (+20% over control value) both on basal and on calcium ionophore-evoked PLC activity. Results shown in Figure 7 refer to a 30 min exposure to the calcium ionophore; however, the effect of adenosine analogues was independent of the duration of the exposure and was detected also after 2 and 15 min. Finally, CPA and NECA stimulation of IP_3 production was insensitive to 8-Ph-Theo (data not shown).

Discussion

This study aimed to verify the presence of membrane adenosine receptors able to modulate adenylate cyclase activity in the tumour mast cell line RBL, and also to investigate the possible functional role of these receptors in controlling 5-HT release. It was crucial for these studies to optimize assay conditions in order to be able to investigate both stimulatory and inhibitory effects of adenosine analogues on basal cyclic AMP production. Elevation of basal adenylate cyclase activity was achieved by including in the assay 1 mM $MnCl_2$. In contrast to our results, Jakobs & Watanabe (1985) could not detect any significant increase of basal cyclase activity by Mn^{2+} in RBL cells. This discrepancy might be due to the different assay conditions used. We assayed cyclase activity in homogenates of RBL cells, whereas Jakobs & Watanabe (1985) disrupted cells by freezing in liquid nitrogen and then prepared a membrane fraction. It might well be that, as has been reported for other adenylate cyclase systems (Minneman *et al.*, 1979), homogenates contain some endogenous factor(s) that modulate adenylate cyclase sensitivity and that are lost upon preparation of membranes. Certainly, the Mn^{2+}-induced increase of basal cyclase activity detected by us in RBL cells is consistent with results obtained for other cyclase systems (Bockaert *et al.*, 1984). A second step that we had to take was to eliminate endogenous adenosine by including ADA (2 iu ml^{-1}) in the assay buffers.

Under such conditions, two typical adenosine analogues, CPA (a selective and potent activator of A_1 inhibitory receptors) and NECA (a potent activator of A_2 stimulatory receptors; Williams, 1987), showed biphasic dose-response curves on adenylate cyclase activity, although inhibition of cyclic AMP formation was far more evident with CPA and stimulation greater with NECA (Figure 2 and Table 1). The calculated IC_{50} and EC_{50} values for inhibition and stimulation of cyclic AMP formation were consistent with data obtained on other adenylate cyclase systems (Dunwiddie & Fredholm, 1984; Fredholm *et al.*, 1986) and with what was expected from the known relative potencies of these two adenosine receptor analogues (Williams *et al.*, 1986; Williams, 1987); similar effects on adenylate cyclase were demonstrated with all the other adenosine analogues tested (Table 1). Whereas cyclase inhibition was counteracted by specific A_1 receptor antagonists, cyclase stimulation did not respond to adenosine receptor xanthine antagonists as expected for 'classic' A_2 receptors.

Taken together, these results suggest that, in common with other cell types, RBL cells express both A_1-high affinity and A_2-like lower affinity receptors mediating opposite effects on

membrane adenylate cyclase activity. However, the atypical potency profile displayed by 8-Ph-Theo and Theo on cyclase stimulation by adenosine analogues suggests that the pharmacological properties of A_2-like receptors in RBL cells might differ from those of 'classic' A_2 receptors. The mechanisms by which adenosine receptors modulate cyclase activity involve a G-protein, as indicated by the results obtained with the guanine-nucleotides in the present paper (Gilman, 1987).

The effects of adenosine analogues on 5-HT release appeared to occur in parallel with the modulation of cyclic AMP synthesis. CPA exerts a biphasic effect (inhibitory at low and stimulatory at high concentrations) on calcium-dependent secretion at the same concentrations that are effective on cyclase activity. This indicates that activation of cyclase-linked A_1 and A_2 receptors mediated inhibition and potentiation of release, respectively. Of the two adenosine receptor subtypes expressed by these cells, the A_2 receptor function (potentiation of release) appears to be more prominent than the A_1 function (inhibition of release).

These cyclase-coupled purinoceptors seem to co-exist with PLC-coupled receptors on RBL cell membrane, since both CPA and NECA, in the presence of ADA, stimulated IP_3 production at μM concentrations, an effect that was insensitive to 8-Ph-Theo. The effect of purinoceptors on PLC activity is only stimulatory, since adenosine agonists never exerted inhibition of either basal or calcium ionophore-induced IP_3 production. Stimulation of PLC activity by adenosine analogues, and its insensitivity to 8-Ph-Theo have been previously reported by Ali *et al.* (1990). These authors have discounted a role for cyclic AMP and attributed the release-potentiating effects of the adenosine analogues only to a simulation of phosphoinositide breakdown and calcium influx. Moreover, since the pharmacology of this response did not match that of known adenosine receptors (A_1, A_2), they proposed the involvement of a novel adenosine receptor subtype. These conclusions appear to be in contrast with our findings, that release in RBL cells is also modulated by cyclase-linked A_1 and A_2 receptors. An explanation for the above discrepancies can be found at least in part in the different experimental conditions used in the two laboratories. We find that in order to observe a typical A_1/A_2 receptor profile in the regulation of 5-HT release, it is necessary to eliminate endogenous adenosine from the assay buffers. Release of adenosine from mast cells has been reported to occur in response to antigen, calcium ionophore and compound 48/80 (Marquardt *et al.*, 1984). If ADA is not added to the assay buffers, A_1 and A_2 receptors are likely to be substantially occupied by the endogenous ligand, as indicated by the significant reduction of A23187-induced release caused by the non-specific A_1/A_2 receptor blocker 8-Ph-Theo or by the addition of ADA itself (Figure 1). The inhibitory effect of ADA on A23187-induced IP_3 production (Figure 7) is also in line with the above conclusions. Moreover, the effect of ADA on IP_3 formation became evident only after 30 min of exposure to the calcium ionophore, when presumably sufficient adenosine has been released. Since Ali *et al.* (1990) did not use ADA in their experiments, the involvement of A_1 and A_2 cyclase-linked receptors would have been difficult to demonstrate. Another important difference between the two sets of data might relate to the methods used to measure cyclic AMP formation, since the RIA assay used by Ali *et al.* gives very low signals in the absence of PDE inhibitors; such inhibitors however cannot be used in their cyclic AMP assay because they interact with adenosine receptors.

The role of cyclic AMP in regulating secretion is far from defined. Calcium-evoked secretion has been shown to be potentiated by increases in cyclic AMP levels in some secretory systems (Jones *et al.*, 1986; Guild *et al.*, 1988), probably through modulation of the functional state of some components of the exocytotic apparatus. Conversely, in GH3 cells inhibition of cyclic AMP formation subsequent to activation of A_1 receptors is associated with a reduction of prolactin release (Delahunty *et al.*, 1988). In mast cells and RBL cells, in particular, the role of cyclic AMP and of other second messengers in 5-HT release is, at present, still unclear. The available results are rather controversial, with some groups reporting a biphasic effect of permeant analogues of cyclic AMP on 5-HT release (McCloskey, 1988), and others reporting inhibition (Narashiman *et al.*, 1988) or no effect (Ali *et al.*, 1990). It is likely that some of the discrepancies might arise from differences in the experimental conditions used by various authors, as discussed in the Introduction. We found a good correlation between the degree of cyclase activation by adenosine receptors and the extent of 5-HT release, indicating a modulatory role for cyclic AMP in the secretory process in RBL cells. However, adenosine stimulatory effects on release and on adenylate cyclase activity were only partially sensitive to xanthines, suggesting that A_2 receptors mediating both effects in RBL cells are somehow different from 'classic' A_2 receptors and might represent a novel cyclase-coupled A_2 receptor subtype. Moreover, an additional transduction pathway contributing to the release response is recruited by A_2-like receptors, as suggested by Ali *et al.* (1990) and also by our demonstration of the effects of adenosine and adenosine analogues on IP_3 formation. Whether A_2-like receptors modulating PLC activity are indeed the same receptors responsible for elevation of cyclic AMP levels still remains to be established.

In conclusion, our results demonstrate that RBL cells express both A_1 and A_2-like cyclase-coupled and PLC-coupled purinoceptors and suggest that they may both be involved in the modulation of calcium-evoked secretion. In particular, the inhibitory phase of this modulation is likely to be sustained by A_1 receptors, whereas the stimulatory phase may involve both A_2-like cyclase-coupled and PLC-linked receptors. Preliminary results from our laboratories suggest that a cross-talk may exist between the two transduction pathways activated by adenosine in these cells. Stimulation of protein-kinase C by phorbol esters potentiates both basal and adenosine-induced cyclase activity, whereas down-regulation of protein kinase C is associated with a strong inhibition of purinoceptor cyclase activation.

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Effects of systemic non-steroidal anti-inflammatory drugs on nociception during tail ischaemia and on reperfusion hyperalgesia in rats

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1 We have investigated the effects of five non-steroidal anti-inflammatory drugs (NSAIDs) on nociception during ischaemia and on reperfusion hyperalgesia in rats.

2 We induced tail ischaemia in conscious rats by applying a tourniquet at the base of the tail until the rats exhibited co-ordinated escape behaviour when we released the tourniquet.

3 We assessed hyperalgesia by measuring the tail flick latency following tail immersion in water at 49°C, before applying and immediately after releasing the tourniquet, and then at 30 min intervals for 2 h.

4 Intraperitoneal injection of NSAIDs prior to applying the tourniquet had no effect on the co-ordinated escape behaviour during ischaemia, nor on tail flick latency in the absence of prior ischaemia. However all the drugs attenuated reperfusion hyperalgesia in a log dose-dependent manner. Doses required to abolish hyperalgesia, were indomethacin 5 mg kg^{-1}, diclofenac sodium 42 mg kg^{-1}, ibuprofen 54 mg kg^{-1}, dipyrone 168 mg kg^{-1} and paracetamol 170 mg kg^{-1}.

5 We conclude that the mechanisms underlying nociception during ischaemia are not the same as those underlying reperfusion hyperalgesia. Moreover our procedure provides a rapid and more humane method for measuring the antinociceptive potency of NSAIDs.

Keywords: Reperfusion hyperalgesia; ischaemia; tail flick test; prostaglandin; non-steroidal anti-inflammatory drugs

Introduction

Inflamed or damaged tissue, including previously ischaemic tissue, becomes more sensitive to subsequent noxious or previously innocuous stimulation. This hyperalgesia could result from changes in excitability of central nervous system neurones (Woolf, 1983; Wall & Woolf, 1984) or from the local release of metabolites, such as prostaglandins, which have the potential to sensitize nociceptors (Ferreira & Vane, 1974; Sicuteri *et al.*, 1974; Juan, 1978; Lynn, 1987). Prostaglandins also are believed to be released during ischaemia and to increase activity in afferent nociceptive pathways (Staszewska-Barczak *et al.*, 1976; Sachetti *et al.*, 1980; Stebbins *et al.*, 1985; Longhurst & Dittman, 1987; Pal *et al.*, 1989). Thus the non-steroidal anti-inflammatory drugs, which all inhibit prostaglandin synthesis, might be expected to attenuate hyperalgesia and to be antinociceptive during ischaemia.

We have shown that lysine acetylsalicylate, a prostaglandin synthesis inhibitor, did indeed abolish the hyperalgesia to a noxious thermal stimulus during reperfusion of the rat's tail following ischaemia, but, contrary to expectations, did not affect the latency to co-ordinated escape behaviour following application of a tourniquet to the tail (Gelgor *et al.*, 1986b). Our observations indicate either that prostaglandins did not contribute to nociception during ischaemia itself, or that the particular drug and dose we used did not inhibit their synthesis.

It is important to resolve what role prostaglandin synthesis plays in nociception during ischaemia and in reperfusion, and other types of hyperalgesia. Non-steroidal anti-inflammatory drugs have potential clinical use as analgesics during ischaemic events. Also, several assays developed to assess the efficacy of non-steroidal anti-inflammatory drugs depend on their action during hyperalgesia (Randall & Selitto, 1957; Weichman, 1989).

We have examined the effect of a range of non-steroidal anti-inflammatory drugs of varying therapeutic potency on nociception during ischaemia induced by applying a tourniquet to the tail of the rat, and on the resultant hyperalgesia following reperfusion of the tail. Our results imply that prostaglandins or related substances do indeed contribute to nociception during hyperalgesia but not during ischaemia. They also provide the basis for a new assay of the antinociceptive potency of non-steroidal anti-inflammatory drugs which is free of the ethical problems which beset many existing assays, in which experimental animals are exposed to inescapable, and often chronic, noxious stimuli.

Some of the results have been reported at the Second International Pain Symposium, Jerusalem, and the Physiological Society of Southern Africa (Gelgor *et al.*, 1990).

Methods

Animals

Male Sprague-Dawley rats weighing 250–300 g were used. The animals were housed in groups of five per cage at an ambient temperature of 21–23°C on a 12 h dark 12 h light cycle, and were allowed free access to standard rat chow and tap water. Different groups of 10 rats were used for each of the different drugs.

Ischaemia

Ischaemia was induced by applying an inflatable cuff to the base of the rat's tail, as previously described (Gelgor *et al.*, 1986a,b). The cuff was connected to a sphygmomanometer, and was inflated to a pressure of 200 mmHg, well above systolic pressure of the rat. The moment the rat exhibited an escape response the cuff was deflated. The time between application of the tourniquet and the escape response, that is the escape latency, is a measure of the noxious effect of the ischaemia. If the rat had not responded within 30 min, the cuff was deflated, to avoid excessive tissue damage.

Application of a similar tourniquet to the arms of the authors and other human subjects caused an intense, aching, poorly localized pain. Following removal of the tourniquet

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there was hyperaemia, temporary paraesthesia, and then resolution of all pain within a few minutes. There were no sequelae.

Tail flick test

Nociception before ischaemia and during reperfusion was tested by the modified tail flick test which we have employed previously (Gelgor *et al.*, 1986a,b). The rat's tail was submerged in a water bath controlled at 49°C, and tail flick latency measured on a stop watch, as the time from submergence to the first coordinated motor response, indicated by a flicking of the tail. A mean of three measurements 1 min apart was recorded as the latency. To avoid thermally induced tissue damage, animals which failed to respond within 20 s had their tails removed from the water.

Experimental procedure

The rats were placed in clear perspex restrainers which allowed free movement of the tail and slightly restricted movement of the rest of the body. The rats were placed in these restrainers for 2–3 h per day on two consecutive days before any experimentation to allow them to habituate to these conditions. On experimental days the animals were placed in the restrainers for 15 min before any testing. At least 48 h were allowed between successive measurements on individual animals. Experiments were carried out between 09 h 00 min and 13 h 00 min at an ambient temperature of 24°C. Tail flick latencies were measured before any treatment, immediately after cuff deflation, and then at 0.5 h intervals for 2 h. Control experiments were performed by placing an uninflated (sham) cuff on the tail for 12 min; this time is equal to the mean escape latency measured in previous experiments in our laboratory.

Because tail flick latency varies with tail temperature, in a pilot experiment, we measured tail skin temperature, using copper-constantan thermocouples taped to the dorsal surface of the tail 30 mm from the base, before, during and after ischaemia. Temperatures were stored on a data logger (MCS 120; MC Systems). Temperatures were recorded for 30 min before any testing and at 10 min intervals throughout the experiment. Specific temperatures were recorded during ischaemia and immediately before tail flick latencies were measured.

All agents as well as their vehicles were administered in 0.5 ml boluses intraperitoneally 30 min before application of tourniquet (test groups) or sham tourniquet (control groups). The vehicle was normal saline except for ibuprofen where it was polyethylene glycol, and paracetamol for which we used corn oil. The drugs and dosages used were: indomethacin (Merck, Sharp and Dohme Research Laboratories) 1, 2, 5 mg kg^{-1}; diclofenac sodium (Ciba Geigy) 5, 20, 50 mg kg^{-1}; ibuprofen (Lennon) 10, 30, 100 mg kg^{-1}; paracetamol = 4-acetamidophenol (Lennon) 10, 50, 200 mg kg^{-1}; dipyrone (Hoechst AG) 50, 100, 200 mg kg^{-1}.

Student's t test with Bonferroni correction for multiple comparisons and a one-way analysis of variance were used for data analysis.

The experimental procedures were approved by the Animal Ethics Committee of the University of the Witwatersrand (Certificate number 89/23/5) and complied with the recommendations of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmerman, 1980).

Results

Tail temperature decreased significantly during ischaemia ($P < 0.001$, $n = 10$, paired t test). Following release of the tourniquet, tail temperature recovered to values which did not differ significantly from those before the cuff was applied ($P > 0.05$, $n = 10$, paired t test). Figure 1 represents change in tail flick latency and in tail temperature following release of the tourniquet. Also included in the figure are the changes in tail flick latency predicted from the changes in temperature, using the coefficient of $-0.25\,s\,°C^{-1}$ increase in temperature derived in previous work (Milne & Gamble, 1989; Han & Ren, 1991). We found no significant change in tail skin temperature, from that prevailing before application of the tourniquet, whereas there was a significant reduction in tail flick latency ($P < 0.01$, $n = 10$, paired t test with Bonferroni correction) at all three measurement times following release of the tourniquet. This hyperalgesia therefore was unrelated to changes in tail temperature.

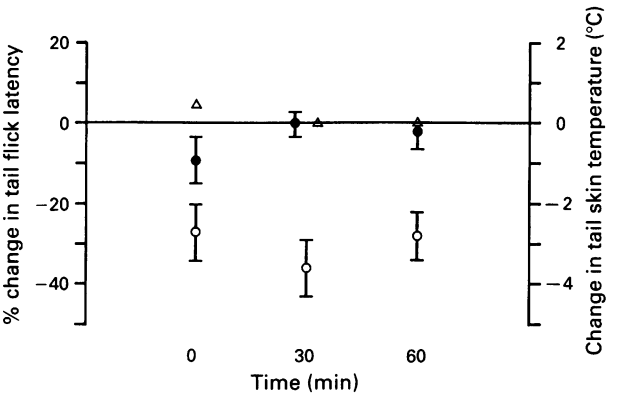

Figure 1 Percentage change in tail flick latency, (mean, with s.e. shown by vertical bars, $n = 10$) (left y-axis) and change in tail temperature (mean ± s.e., $n = 10$) (right y-axis) from values evident before application of a tourniquet, measured during tail reperfusion following removal of the tourniquet. Temperature (●) did not change significantly ($P > 0.05$, paired t test.) The tail flick latency (○) decreased significantly at all time intervals ($0.05 > P > 0.001$, paired t test with Bonferroni correction for repeated measures.) Tail flick latency (△) predicted from the tail temperature, by use of the coefficient $-0.25\,s\,°C^{-1}$ (Milne & Gamble, 1989; Han & Ren, 1991).

The latency to coordinated escape behaviour following the induction of ischaemia in the absence of any of the drugs was 13.8 ± 0.7 min (mean ± s.e., $n = 50$). There was no significant difference in escape latency between different groups of rats ($P > 0.05$, $n = 10$, unpaired t test). None of the five different drugs had any significant effect on the escape latency to ischaemia at any dosage ($P > 0.05$, $n = 10$, unpaired t test, see Table 1). Also none of the drugs tested had any effect on the tail flick latency in the absence of ischaemia ($P > 0.05$, $n = 10$, unpaired t test).

Following reperfusion of the tail after a period of ischaemia, there was a significant hyperalgesia, as indicated by a reduction in tail flick latency, which was greatest immediately

Table 1 Effect of non-steroidal anti-inflammatory drugs (NSAIDs) on ischaemia and tail flick latency in the absence of ischaemia

Agents administered		*Escape latency during ischaemia* (min)	*Tail flick latency* (s)
Sterile water		14.8 ± 2.3	7.1 ± 0.4
Polyethylene glycol		13.1 ± 2.2	7.1 ± 0.3
Corn oil		15.6 ± 2.2	7.9 ± 0.3
Indomethacin	5 mg kg^{-1}	15.4 ± 2.7	7.0 ± 0.3
Diclofenac sodium	50 mg kg^{-1}	15.6 ± 2.1	7.6 ± 0.4
Ibuprofen	100 mg kg^{-1}	12.0 ± 2.3	7.3 ± 0.2
Paracetamol	200 mg kg^{-1}	15.1 ± 2.0	7.9 ± 0.3
Dipyrone	200 mg kg^{-1}	13.5 ± 1.9	7.1 ± 0.4

Escape latencies during ischaemia (mean ± s.e.) and tail flick latencies in the absence of ischaemia (mean ± s.e.), following intraperitoneal administration of vehicles and of the highest doses of each of the NSAIDs. The drugs did not affect escape latency or tail flick latency.

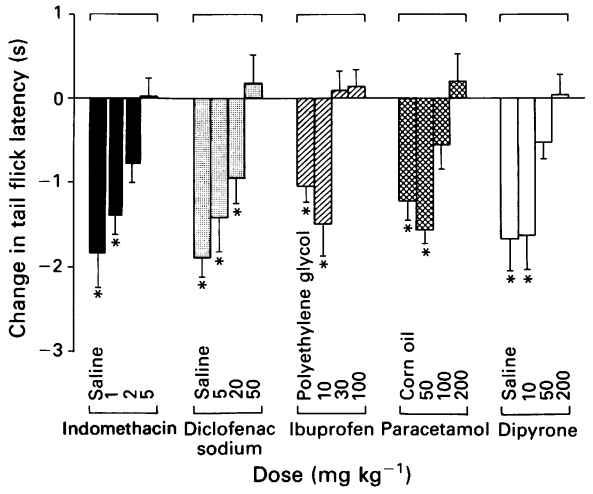

Figure 2 Change in tail flick latency (mean with s.e. shown by vertical bars) measured immediately after relief of ischaemia, following treatment with vehicles and agents. The asterisks represent those groups in which treatment resulted in a significant decrease in tail flick latency from pre-treatment values ($P < 0.01$, $n = 10$, paired t test), that is groups in which there was a significant hyperalgesia. All agents abolished this hyperalgesia, at sufficient dose. All doses are mg kg^{-1}.

following removal of the tourniquet, and persisted for 60 min, after administration of the vehicles ($0.05 > P > 0.001$, $n = 10$, unpaired t test, with Bonferroni correction for repeated measures). Figure 2 represents the change in tail flick latency from that evident before application of the tourniquet, measured immediately after release of the tourniquet, for five different groups of rats, each treated with either the vehicle or one of the drugs. There were no significant differences between the values measured during treatment with the lowest dose of each of the drugs tested and during treatment with the corresponding vehicle ($P > 0.05$, $n = 10$, paired t test). However, the change in tail flick latency for both the vehicle and the lowest dose of each drug was significantly different from zero ($0.02 > P > 0.001$, $n = 10$, t test) indicating the presence of significant reperfusion hyperalgesia in each case. Administration of the highest dose of each of the drugs resulted in the tail flick latency during reperfusion being no different to that prevailing before ischaemia ($P > 0.05$, $n = 10$, paired t test). Therefore all the drugs abolished reperfusion hyperalgesia.

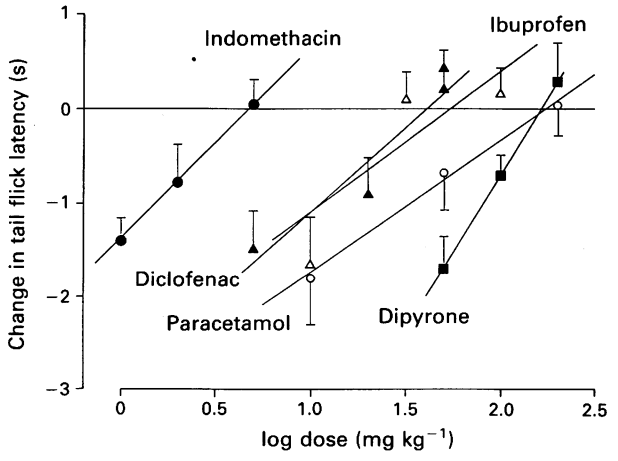

Figure 3 Change in tail flick latency (mean with s.e. shown by vertical bars) measured immediately after relief of ischaemia, plotted against log dose of the five agents. Reperfusion hyperalgesia was attenuated in a dose-dependent manner. Doses required to abolish hyperalgesia, calculated from regression analysis, were: indomethacin 5 mg kg^{-1}, diclofenac sodium 42 mg kg^{-1}, ibuprofen 54 mg kg^{-1}, dipyrone 168 mg kg^{-1} and paracetamol 170 mg kg^{-1}.

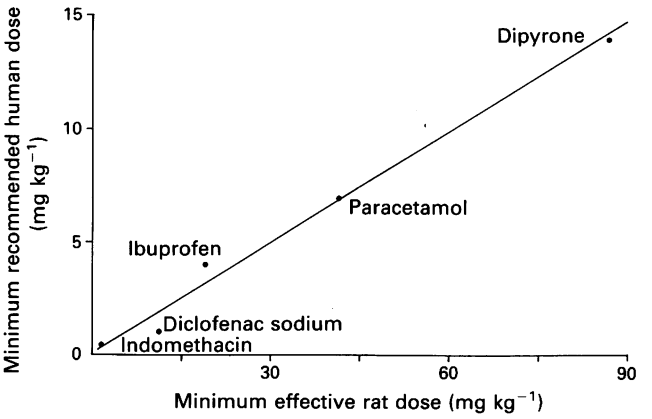

Figure 4 Minimum effective rat dose, calculated, from regression analysis, as the dose of agent which induces a change in tail flick latency following ischaemia equal to the change in tail flick latency induced by administration of the vehicle, plotted against minimum recommended human dose, taken from standard therapeutic references for physicians. Regression analysis gave $y = 0.056 + 0.164x$, $r = 0.993$, $P < 0.001$.

However, none of the drugs, at the doses we used, induced analgesia; tail flick latency never increased significantly.

Figure 3 shows change in tail flick latency, measured immediately after release of the tourniquet, plotted against the log dose of each drug. Reperfusion hyperalgesia was attenuated in a dose-dependent manner. Dosages of each drug required to abolish hyperalgesia, calculated from the intercepts of the regression line with the x-axis, were: indomethacin 5 mg kg^{-1}, diclofenac sodium 42 mg kg^{-1}, ibuprofen 54 mg kg^{-1}, dipyrone 168 mg kg^{-1} and paracetamol 170 mg kg^{-1}.

Another measure of the relative potency of the drugs could be derived by calculating, from the regression lines, the doses at which the change in tail flick latency was equal to that produced by the vehicle. These doses could be considered the minimum effective doses, since any higher dose would attenuate the hyperalgesia. Figure 4 shows the correlation between these minimum effective doses and the minimum recommended human therapeutic dose for the same drugs. The minimum human therapeutic doses were taken from the manufacturer's information recorded in a compendium of such information for physicians (MIMS Desk Reference Vol. 22 1986/87), assuming a 75 kg patient. There was a very high degree of correlation between the experimental measure of potency and the human dose.

Discussion

Previous work indicates that tail flick latency is negatively correlated with tail skin temperature (Berge *et al.*, 1988; Tjolsen *et al.*, 1989) and that some factors which alter tail flick latency, usually interpreted as affecting nociception, may really just alter tail skin temperature (Tjolsen *et al.*, 1988; Lund *et al.*, 1989). The induction of ischaemia in the rat's tail reduces blood flow and consequently tail skin temperature at the ambient temperature we used. However, following release of the tourniquet, tail temperature returned to pre-ischaemic values whilst tail flick latency remained significantly decreased. We have therefore established that the hyperalgesia observed on reperfusion of the previously ischaemic tail is not the result of a change in tail skin temperature.

Non-steroidal anti-inflammatory drugs attenuated the hyperalgesia evident on reperfusion of the rat tail following a period of ischaemia. We were able to rank the potency of a range of non-steroidal anti-inflammatory drugs by their ability to attenuate reperfusion hyperalgesia. Rank order of potency was: indomethacin > diclofenac sodium > ibuprofen > paracetamol > dipyrone. The behavioural response to the ischaemic stimulus itself was not altered

following administration of the drugs. This observation leads us to suggest that the mechanisms underlying nociception during ischaemia and reperfusion hyperalgesia are not the same, because doses of drugs which abolished hyperalgesia had no observable effect on nociception during ischaemia. Similarly, the same doses of the drugs did not appear to affect nociception during application of noxious heat to the tail. In experiments conducted in the absence of ischaemia, the tail flick latency was not altered by the administration of any of the non-steroidal anti-inflammatory drugs. It has been reported previously that diclofenac is not anti-nociceptive in the tail flick or hot-plate tests (Bjorkmann *et al.*, 1990). In our experiments, all five of the non-steroidal anti-inflammatory drugs exhibited an anti-algesic effect during reperfusion of the previously ischaemic tail, but did not exhibit any antinociceptive effects.

Ischaemia or hypoxia is believed to induce the release of prostaglandins and bradykinin which together stimulate afferent nerve endings (Staszewska-Barczak *et al.*, 1976; Stebbins *et al.*, 1985; Pal *et al.*, 1989). Bradykinin is present in inflammatory exudates before prostaglandins (Juan, 1978; 1981; Terenius, 1981; Neugebauer *et al.*, 1989), and stimulates the release of prostaglandins, which, in turn, may sensitize the nociceptor to the algogenic action of other mediators released during tissue damage. In studies of cardiac ischaemic pain, the NSAIDs, aspirin and indomethacin, reduced bradykinin-induced excitation of afferent nerve endings (Vogt *et al.*, 1979; Sachetti *et al.*, 1980). Arachidonic acid, a precursor of prostaglandins, accumulates in flow-deprived cardiac tissue and its concentration increases significantly in the first hour following reperfusion of the heart (Van der Vusse *et al.*, 1989). Prostaglandin concentrations measured in the brain after 15 min global cerebral ischaemia were at their highest levels in the 15–60 min reperfusion period, when compared with pre-ischaemic levels (Stevens & Yaksh, 1988). The hyperalgesia we observed following reperfusion of the tail was greatest immediately after release of the tourniquet and lasted for 60 min (Gelgor *et al.*, 1986b).

Arachidonic acid accumulation in ischaemic conditions can elevate the rate of prostaglandin synthesis only if the local oxygen concentration is sufficiently high (Lands, 1979). We believe that prostaglandin precursors accumulate during ischaemia, and are metabolised to prostaglandins, or other eicosanoids, when oxygen becomes available during reperfusion. NSAIDs, which inhibit the cyclo-oxygenase enzyme, consequently will have no effect during ischaemia itself, but will attenuate prostaglandin synthesis during reperfusion. We also conclude that prostaglandins do not play a role in the tail flick response.

Hyperalgesic assays currently used to assess the efficacy of non-steroidal anti-inflammatory drugs involve the administration of chemical irritants such as brewers' yeast, trypsin, formalin, kaolin, carrageenan, acetic acid or *Mycobacterium butyricum* in the adjuvant arthritis model (Randall & Selitto, 1957; Vinegar *et al.*, 1976; 1990; Menasse *et al.*, 1978; Ferreira *et al.*, 1978; Maier *et al.*, 1979; Van Kolfschoten *et al.*, 1983; Okuyama & Aihara, 1984; Shibata *et al.*, 1989). In many cases the hyperalgesia takes hours to develop and no simple intervention can terminate the noxious stimulus. The procedure of reperfusion of the rat tail following a period of ischaemia does not involve the administration of a chemical irritant to induce hyperalgesia, but rather relies on the endogenous release of humoral mediators. In the absence of a deliberately applied stimulus, like noxious heat, the animal is in no distress and the duration of the hyperalgesia is relatively short. Although the doses of NSAIDs required to abolish reperfusion hyperalgesia in rats are considerably higher than the therapeutic doses used in man, they were highly significantly, and linearly, correlated with minimum recommended human dose, so our procedure provides the basis for an assay which may be used to rank the potency of new non-steroidal anti-inflammatory drugs. Rank order of potency of the drugs we tested correlates with potency ranking in other studies which have employed the carrageenan paw oedema test, Randall-Selitto test, acetic acid writhing test and the adjuvant arthritis model (Vinegar *et al.*, 1976; Van Kolfschoten *et al.*, 1983; Okuyama & Aihara, 1984; Tolman *et al.*, 1984; Vane & Botting, 1987; Weichman, 1989).

Indomethacin is a potent inhibitor of prostaglandin synthesis in man and in animal anti-inflammatory assays (Ferreira & Vane, 1974; Robinson *et al.*, 1978), and, of the drugs we tested in our procedure, indomethacin proved to be the most potent. Also, the dosage range of efficacy of indomethacin in our study (1.5–5 mg kg^{-1}) correlates well with ED_{50} dosages reported in the carrageenan paw oedema assay, which were in the range of 1.3–6.5 mg kg^{-1} (Flower *et al.*, 1972; Vinegar *et al.*, 1976; Van Kolfschoten *et al.*, 1983; Vane & Botting, 1987). Diclofenac sodium, in some tests, has been found to be equipotent to indomethacin (Menasse *et al.*, 1978; Maier *et al.*, 1979; Skoutakis *et al.*, 1988; Small, 1989), but was less potent in our hands. Different non-steroidal anti-inflammatory drugs inhibit prostaglandin synthesis to varying degrees in different tissues, and hence have different potential therapeutic effects (Flower & Vane, 1972). Paracetamol and dipyrone are more potent in inhibiting prostaglandin synthesis in brain tissue and do not possess anti-inflammatory properties (Flower *et al.*, 1972; Ferreira *et al.*, 1978; Vane, 1983; Carlson *et al.*, 1986). High doses, 168 and 170 mg kg^{-1} respectively, were required to abolish reperfusion hyperalgesia.

In conclusion, we have shown that five NSAIDs attenuate reperfusion hyperalgesia at doses at which they do not influence the responses to a noxious ischaemic or noxious thermal stimulus. Our observations are consistent with a role for prostaglandins in nociception during hyperalgesia, but not during noxious ischaemia or thermal stimulation. We can interpret our results in terms of a peripheral action of prostaglandins, but they do not exclude a central action (Ferreira *et al.*, 1978; Jurna & Brune, 1990). Finally, we believe we have indicated a way to test potency of NSAIDs which has far fewer ethical problems than other existing procedures.

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Cytoprotection by iloprost against paracetamol-induced toxicity in hamster isolated hepatocytes

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1 The ability of iloprost (ZK36374) to protect hamster isolated hepatocytes from the toxic effects of paracetamol and its reactive metabolite N-acetyl-*p*-benzoquinoneimine (NABQI) was investigated. The cytoprotection provided by iloprost was compared with that of N-acetyl-L-cysteine.

2 Treatment of hepatocytes with either NABQI (0.4 mM) or paracetamol (2 mM) alone resulted in a considerable loss of cell viability, as assessed by trypan blue exclusion or leakage of lactate dehydrogenase, accompanied by an increase in the percentage of viable cells that were blebbed. N-acetyl-L-cysteine (1.25 mM) pretreatment diminished the loss of cell viability and the percentage of blebbed cells resulting from exposure to NABQI or paracetamol, whereas iloprost (10^{-16} M to 10^{-10} M) pretreatment reduced only the loss of cell viability, not the percentage of viable cells exhibiting blebbing. Pretreatment with N-acetyl-L-cysteine significantly attenuated the depletion by paracetamol of glutathione and decreased the covalent binding of [^{14}C]-paracetamol to cellular proteins, whereas iloprost was without any such effects.

3 The effects of iloprost and N-acetyl-L-cysteine were also investigated by use of a model of paracetamol toxicity in which it is possible to study the biochemical events leading to cell injury separate from the generation of toxic metabolites. Hamster hepatocytes were incubated with paracetamol (4 mM) for 90 min at 37°C during which metabolism of paracetamol occurs with minimal loss of cell viability. Following washing of cells, to remove paracetamol and its metabolites, there was a progressive loss of viability and increase in the percentage of cells exhibiting blebbing when incubated in buffer alone. Addition of either N-acetyl-L-cysteine (1.25 mM) or iloprost (10^{-14} M to 10^{-8} M), following washing, significantly reduced the expected loss of cell viability. Iloprost at concentrations outside this range was without effect.

4 Paracetamol toxicity to isolated hepatocytes could be prevented or delayed by treatment with either N-acetyl-L-cysteine or iloprost, but whereas the former prevented or even reversed plasma membrane blebbing with a resultant reduction in the percentage of viable cells that were blebbed, the prostanoid appeared only to delay the progression from plasma membrane blebbing to loss of viability. Hence, the percentage of viable cells that were ultimately blebbed following exposure to paracetamol was not significantly reduced by addition of iloprost.

5 Aspirin or ibuprofen exacerbated the loss of viability induced by prior incubation with paracetamol. Thus, there may be a role for endogenous prostaglandins in protecting hepatocytes from paracetamol toxicity.

6 Iloprost is cytoprotective without any effect upon toxin metabolism or detoxication. The mechanism of action of iloprost probably does not involve induction of prostaglandin synthesis or activation of the previously-characterized prostacyclin receptor.

Keywords: Cytoprotection; iloprost; hepatocytes; paracetamol; prostaglandins; toxicity

Introduction

The administration of exogenous prostaglandins to rats can prevent necrosis of the gastric mucosa following its exposure to a variety of noxious stimuli (Robert *et al.*, 1968), including mineral acids, alkali, boiling water, ethanol, corticosteroids, nonsteroidal anti-inflammatory compounds and bile acids (Robert, 1976; 1979; Lancaster & Robert, 1978; Robert *et al.*, 1979). This property of prostaglandins has been termed cytoprotection (Chaudhury & Jacobson, 1978), although more properly it should be described as organoprotection (Szabo & Szelenyi, 1987). It is now known that the cytoprotective effects of prostaglandins extend to tissues other than the stomach, including the heart (Lefer *et al.*, 1978; Ferrari *et al.*, 1989), pancreas (Manabe & Steer, 1980), kidney (Papanicolaou *et al.*, 1975; Ruwart *et al.*, 1981) and liver (Stachura *et al.*, 1980). It is likely that in many of these tissues cytoprotection is by a different, or additional mechanism, from that which operates in the stomach.

A number of compounds can cause hepatotoxicity, involving a variety of different mechanisms (Boobis *et al.*, 1989). Prostaglandins have been reported to prevent the hepatic damage caused by many such agents, including: aflatoxin B_1 (Rush *et al.*, 1989), bromobenzene (Funck-Brentano *et al.*, 1984; Bursch & Schulte-Hermann, 1987), carbon tetrachloride (Ruwart *et al.*, 1981; Stachura *et al.*, 1981; Ujhelyi *et al.*, 1984; Guarner *et al.*, 1985; Bursch & Schulte-Hermann, 1987; Bursch *et al.*, 1989; Mihas *et al.*, 1991) and paracetamol (Stachura *et al.*, 1981; Guarner *et al.*, 1988). Hepatic cytoprotection by prostaglandins is apparent not only *in vivo* (Araki & Lefer, 1980; Stachura *et al.*, 1981; Funck-Brentano *et al.*, 1984; Bursch *et al.*, 1989; Ferrari *et al.*, 1989) but also in isolated hepatocytes (Ujhelyi *et al.*, 1984; Guarner *et al.*, 1985; Bursch *et al.*, 1989). This effect in isolated cells shows that prostaglandins can be directly 'cyto'-protective as opposed to 'organo'-protective (Szabo & Szelenyi, 1987), at least in the liver. Thus, at least one mode of action must be a direct effect on cells and not through secondary effects; such as on blood flow. Bursch and colleagues (Bursch & Schulte-Hermann, 1986; 1987; Bursch *et al.*, 1989) have reported that iloprost (ZK36374) can protect rat hepatocytes against the damage induced by carbon tetrachloride and bromobenzene. Iloprost ($0.1\,\mu g\,kg^{-1}\,min^{-1}$ *in vivo* and 6.9×10^{-9} M to 6.9×10^{-15} M in primary hepatocyte cultures) largely preserved normal hepatocellular morphology after intoxication (Bursch & Schulte-Hermann, 1986; 1987). The finding by these authors that iloprost protects cultured rat hepatocytes at concentrations as low as 6.9×10^{-15} M and that the efficacy of the pros-

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tanoid decreased at concentrations above 6.9×10^{-7} M suggests that iloprost is cytoprotective by means other than activation of the previously-characterized prostacyclin receptor. From the data of Bursch and colleagues (Bursch & Schulte-Hermann, 1986; 1987; Bursch *et al.*, 1989) it is not possible to exclude an action of iloprost on toxin metabolism, as protection against bromobenzene or carbon tetrachloride intoxication was obtained following their co-incubation with iloprost.

We have developed a well-defined model of cytotoxicity in which it is possible to study the biochemical events leading to cell injury separate from the generation of toxic metabolites (Tee *et al.*, 1986). In the present study, the effects of iloprost on the toxicity of paracetamol and its reactive metabolite (N-acetyl-*p*-benzoquinoneimine: NABQI) subsequent to the initiation of biochemical events that can lead to cell death have been investigated. Cytoprotection by prostanoids has also been compared with the protection afforded by N-acetyl-L-cysteine, the mechanism of which is relatively well understood.

Methods

Isolation of hepatocytes

Hepatocytes were isolated from male Golden Syrian hamsters (90–110 g) by the collagenase perfusion technique previously described (Tee *et al.*, 1986). However, the calcium and magnesium-free Earle's balanced salt solution (EBSS)-based perfusion media did not contain gentamicin sulphate.

The number of viable cells in the final suspension was determined by counting an aliquot of the suspension in an improved Neubauer counting chamber viewed under phase-contrast illumination. The viability of the cells was assessed by their ability to exclude trypan blue (0.05% w/v in the final volume). The cell suspension was adjusted to 2×10^6 viable cells ml^{-1} and was incubated at 37°C for 30 min with gentle shaking at 25 cycles min^{-1}. The cells were then sedimented at 100 *g* for 1 min and the supernatant fraction, containing dead and damaged cells, was discarded. The cells were resuspended in incubation medium (EBSS containing 8 mM sodium bicarbonate, 10 mM N-2-hydroxy-ethylpiperazine-N'-2-ethanesulphonic acid (HEPES), 12 mM sodium phosphate, 1.05 mM calcium chloride, 0.73 iu ml^{-1} insulin (from bovine pancreas) and 0.1 mM hydrocortisone-21-hemisuccinate) at 4°C before use. This 30 min preincubation aided in the isolation of a cell population of high viability showing minimal blebbing (initial viability 97.6 ± 1.6% $n = 18$).

*N-acetyl-*p*-benzoquinoneimine (NABQI)-synthesis and quantification*

NABQI was synthesized by the oxidation of paracetamol (Aldrich Chemical Company, Gillingham, Dorset) in chloroform (Aristar grade: BDH Limited, Poole, Dorset) with freshly prepared silver oxide (Huggett & Blair, 1983). The solution of NABQI in chloroform was stored under liquid nitrogen until required. NABQI concentrations were measured directly by straight phase high performance liquid chromatography (h.p.l.c.) (Huggett & Blair, 1983) using known standards.

Assay of 'total' intracellular glutathione content

The intracellular glutathione content, comprising reduced glutathione (GSH) and glutathione disulphide (GSSG), was determined by the method of Akerboom & Sies (1981). Intracellular glutathione content has been expressed as nmol GSH per 10^6 cells.

Covalent binding of metabolites of [^{14}C]-paracetamol to proteins of isolated hepatocytes

Ring-labelled [^{14}C]-paracetamol (specific activity: 19.5 mCi $mmol^{-1}$) was obtained from Sigma (Poole, Dorset), with a radiochemical purity of greater than 99%. The covalent binding of the metabolites of [^{14}C]-paracetamol to hepatocyte proteins was determined by the method described previously (Tee *et al.*, 1986). Determination of protein concentration was by the method of Lowry *et al.* (1951). Covalent binding is expressed in nmol of paracetamol equivalents per milligram of protein.

Experimental protocols

Three different experimental protocols for paracetamol toxicity to isolated hepatocytes were employed in these studies; designated protocols A, B, C. In the first two, hepatocytes were preincubated with either iloprost or N-acetyl-L-cysteine, followed by incubation with either NABQI (protocol A), or paracetamol (protocol B). In protocol A, 10 ml of cell suspension (2×10^6 cells ml^{-1}) were preincubated for 30 min with iloprost (10^{-18} M to 10^{-6} M) or with N-acetyl-L-cysteine (1.25 mM). The cells were then incubated with 0.4 mM NABQI, in the continuing presence of the iloprost or N-acetyl-L-cysteine, for 30 min. Aliquots of the cell suspension were removed at various times for assessment of cell viability. In protocol B, 10 ml samples of cell suspension (2×10^6 cells ml^{-1}) were preincubated for 30 min with iloprost or N-acetyl-L-cysteine. The cells were then incubated for 3 h with 2 mM paracetamol in the continuing presence of the iloprost or N-acetyl-L-cysteine. Aliquots of cell suspension were removed at 1, 2 and 3 h for viability assessment. Protocol C was adapted from the two-phase model used by Tee *et al.* (1986) for the study of antidotes to paracetamol toxicity. Ten ml of hepatocytes (2×10^6 cells ml^{-1}) were incubated with 4 mM paracetamol for 90 min (phase 1). The cells were then washed free of paracetamol and metabolites, including NABQI (Tee *et al.*, 1986; 1987), by centrifuging the cells at 100 *g* for 1 min and replacement of the supernatant with fresh incubation medium. This washing of the cells was repeated three times. The cells, in a final volume of 10 ml incubation medium, were then incubated with iloprost (10^{-18} M to 10^{-6} M) or N-acetyl-L-cysteine (1.25 mM) for a further 4.5 h (phase 2). Aliquots of cell suspension were removed at intervals for the assessment of cell viability. Controls contained an appropriate volume of the respective vehicle. Iloprost (ZK36374), in a vehicle buffered to pH 8.3 (1.2 mg Tris base, 8.9 mg sodium chloride, 10 µl of 96% ethanol brought to pH 8.3 with 0.1 N hydrochloric acid and made up to 1 ml with distilled water) was a kind gift of Schering A. G., Berlin. Iloprost was diluted to suitable stock concentrations (10^{-16} M to 10^{-4} M) by serial 1 in 10 dilutions in phosphate buffered saline (PBS), pH 7.4. N-acetyl-L-cysteine was obtained from Sigma (Poole, Dorset) and was dissolved in PBS before use. All cell incubations were carried out at 37°C with gentle shaking at 35 cycles min^{-1}. Hepatocyte suspensions were saturated with 95% O_2: 5% CO_2 every 60 min during incubation.

Assessment of viability

The viability of cells was assessed by trypan blue exclusion or lactate dehydrogenase (LDH) leakage as described previously (Tee *et al.*, 1985). Evaluation of the extent of plasma membrane blebbing was performed in conjunction with the trypan blue exclusion assay. Cells excluding trypan blue which exhibited blebbing were counted. The percentage of blebbed cells was calculated as follows:

$$\%B = \frac{Tb}{Tbe} \times 100$$

where %B = percentage of blebbed cells, Tb = total number of cells excluding trypan blue which were blebbed and Tbe = total number of cells which excluded trypan blue.

The synthesis of prostaglandins PGE_2, $PGF_{2\alpha}$ and PGI_2 by hepatocytes

Hamster isolated hepatocytes in 1 ml of incubation medium were incubated with one of the following for 30 min at 37°C: incubation medium; 10^{-4} M aspirin; 10^{-4} M ibuprofen;

10^{-14} M iloprost or 2 mM paracetamol. After incubation, cells were centrifuged at 1000 *g* for 2 min and the supernatant was removed. Internal standard (2 ng), a mixture of the deuterated prostaglandins, PGE_2, $PGF_{2\alpha}$ and 6-oxo-$PGF_{1\alpha}$, was added to the supernatant. Mixed deuterated prostaglandin standards and non-deuterated standards were a kind gift from Upjohn Company, Michigan, U.S.A. A standard curve for each prostaglandin was constructed by preparing a range of deuterated and non-deuterated standards from 0 to 2000 pg ml^{-1}. Standard curves were constructed by plotting the peak area ratio of the non-deuterated prostaglandin to that of the corresponding deuterated prostaglandin against known concentration. Samples containing internal standard were acidified and extracted with Sep Pak C18 cartridges (Millipore Ltd., Watford). Followed washing of the cartridge with 10 ml distilled water the prostaglandins were eluted with 7 ml ethyl acetate. After evaporation to dryness under nitrogen, samples were transferred to half-dram glass vials in 0.5 ml methanol which was removed under a nitrogen stream.

Samples were converted to their methoxime-trimethylsilyl ether-*bis*trifluoromethylbenzylester derivatives and analysed by capillary column gas chromatography-electron capture mass spectrometry (Barrow & Taylor, 1987). Chromatography was carried out on a CPSil 5 GC column (Chrompak, London) with helium as carrier gas. The column temperature was programmed to increase at 20°C min^{-1} from 200°C to 325°C.

To investigate the effect of inhibitors of prostaglandin synthesis on paracetamol toxicity, hepatocytes were incubated with 2 mM paracetamol for 45 min (phase 1) and, following washing, were incubated with one of the following agents during phase 2: incubation medium; 10^{-4} M aspirin; 10^{-4} M ibuprofen or 10^{-14} M iloprost.

Statistical analysis

The data are presented as means ± s.e. of *n* experiments; each one utilising cells derived from a different hamster. Differences between mean values were tested for statistical significance by one- and two-way analysis of variance. Differences between individual treatment groups were tested by Scheffé's method (Scheffé, 1957), which corrects for multiple comparisons (Armitage & Berry, 1987). The null hypothesis was rejected at $P \leqslant 0.05$.

Results

Pretreatment of hepatocytes with iloprost or N-acetyl-L-cysteine before exposure to NABQI: protocol A

Incubation with any of the respective vehicles was without effect on hepatocyte viability. In the absence of NABQI, treatment of hepatocytes with iloprost alone, at concentrations of up to 10^{-8} M, had no effect on either their viability or degree of plasma membrane blebbing (data not shown). Incubation of cells with NABQI (0.4 mM) alone caused a considerable decrease in total cell viability, to 26.8 ± 3.2% after 30 min of exposure (Figure 1a). Iloprost, at concentrations in the range of 10^{-16} M to 10^{-10} M, significantly reduced the loss of viability induced by NABQI (Figure 1a). Neither 10^{-18} M nor 10^{-8} M iloprost had any significant effect on the loss of viability caused by NABQI (Figure 1a). Pretreatment of cells with N-acetyl-L-cysteine (1.25 mM) for 30 min before exposure to NABQI, significantly ($P < 0.001$) reduced the loss of cell viability (Figure 1a). Following exposure to NABQI (0.4 mM) for 30 min, the remaining viable hepatocytes exhibited a modest degree of plasma membrane blebbing (14.9 ± 3.6%) (Figure 1b). Preincubation of the cells with N-acetyl-L-cysteine (1.25 mM) significantly ($P < 0.005$) reduced the percentage of viable cells which were blebbed after exposure to NABQI (Figure 1b), whilst preincubation with iloprost (10^{-18} M to 10^{-8} M) had no significant effect on the percentage of viable cells exhibiting blebbing (Figure 1b).

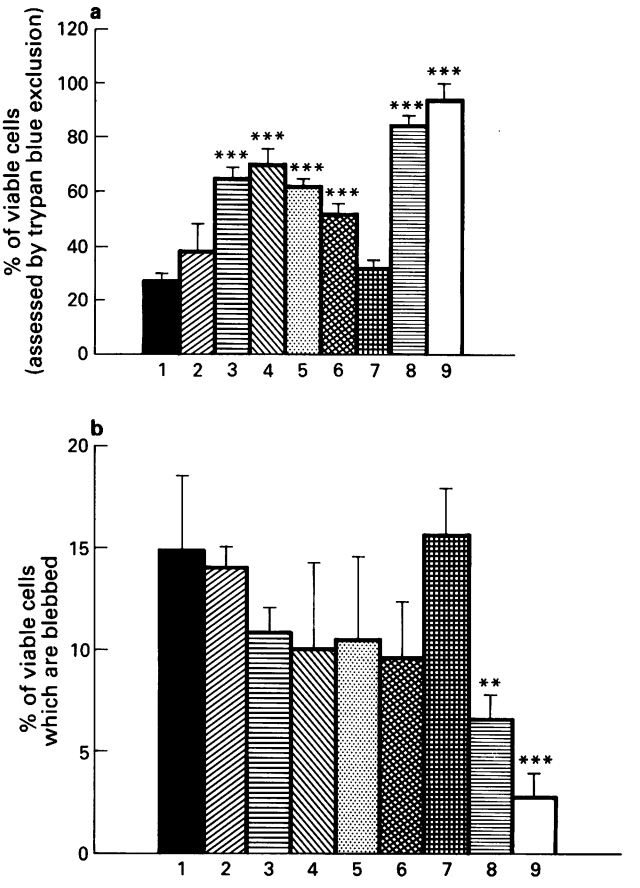

Figure 1 The effect of preincubation of hepatocytes with iloprost (10^{-18} M to 10^{-8} M) or N-acetyl-L-cysteine (1.25 mM) on the toxicity of N-acetyl-*p*-benzoquinoneimine (NABQI) (protocol A): (a) the percentage of viable cells at 30 min assessed by trypan blue exclusion; (b) the percentage of viable cells which were blebbed. All but the control cells (open column, 9) were treated with NABQI (0.4 mM). The treatments were as follows: column (1) NABQI alone; columns (2)–(7) NABQI plus iloprost: 10^{-18} M (2); 10^{-16} M (3); 10^{-14} M (4); 10^{-12} M (5); 10^{-10} M (6); 10^{-8} M (7); column (8) NABQI and N-acetyl-L-cysteine (1.25 mM); column (9) control. Results are expressed as the mean with s.e. shown by vertical bars ($n = 9$). Differences between mean values were tested for statistical significance by two-way analysis of variance. Differences between individual treatment groups were tested by one-way analysis of variance and Scheffe's method. The null hypothesis was rejected at $P \leqslant 0.05$. *** $P < 0.001$; ** $P < 0.005$, compared with cells treated with NABQI only.

Pretreatment of hepatocytes with iloprost or N-acetyl-L-cysteine before exposure to paracetamol: protocol B

Incubation of hepatocytes with paracetamol (2 mM) alone caused a progressive deterioration of cell viability with time, as assessed by trypan blue exclusion (Figure 2a) and LDH leakage (data not shown), and an increase in the degree of cell blebbing (Figure 2b). Iloprost pretreatment of cells significantly reduced the loss of viability caused by incubation with paracetamol. Iloprost had no significant effect on the percentage of viable cells exhibiting blebbing following paracetamol exposure (Figure 2b). Pretreatment of cells for 30 min with N-acetyl-L-cysteine (1.25 mM) before exposure to paracetamol (2 mM) significantly reduced the loss of viability (Figure 2a) and substantially reduced the percentage of viable cells exhibiting plasma membrane blebbing (Figure 2b), compared with cells treated with paracetamol alone.

The glutathione content of control cells was initially 35.8 ± 9.8 nmol per 10^6 hepatocytes and at the end of the experiment it was 32.6 ± 5.9 nmol per 10^6 hepatocytes. The glutathione levels of treated cells are expressed as a percentage

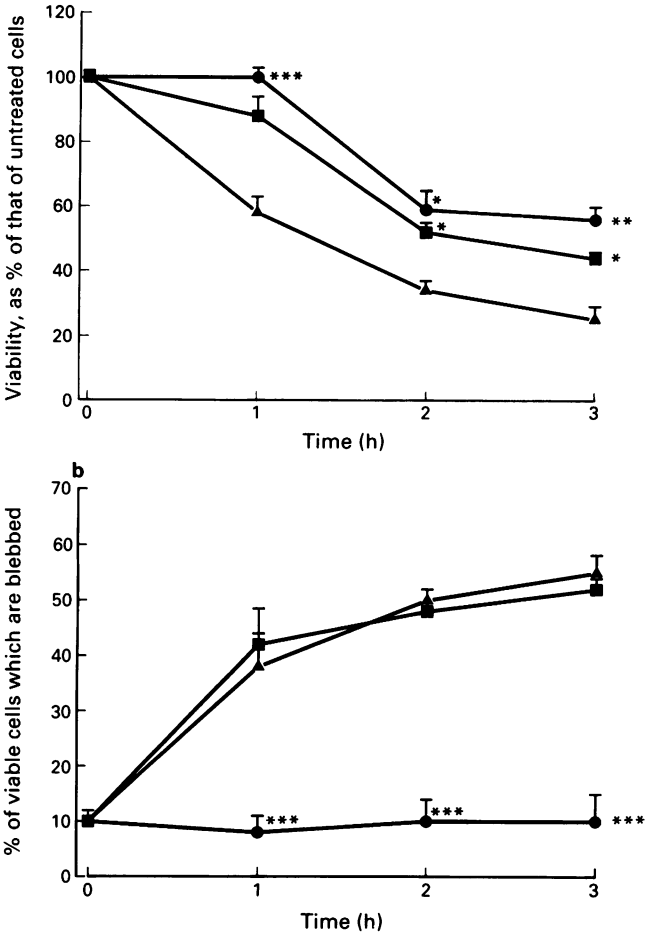

Figure 2 The effect of pretreatment of hepatocytes with iloprost (10^{-14} M) or N-acetyl-L-cysteine (1.25 mM) on the toxicity of paracetamol (protocol B): (a) viability as a percentage of that of untreated cells assessed by trypan blue exclusion: (b) the percentage of viable cells which were blebbed. Cells were treated as follows: paracetamol (2 mM) alone (▲); paracetamol (2 mM) following pretreatment with iloprost (10^{-14} M) (■) and paracetamol (2 mM) following pretreatment with N-acetyl-L-cysteine (1.25 mM) (●). Results are expressed as the mean with s.e. shown by vertical bars ($n = 9$). Differences between mean values were tested for statistical significance by two-way analysis of variance. Differences between individual treatment groups were tested by one-way analysis of variance and Scheffe's method. The null hypothesis was rejected at $P \leqslant 0.05$. *** $P < 0.001$; ** $P < 0.005$; * $P < 0.05$, compared with cells treated with paracetamol only.

of that of control cells. Exposure of hepatocytes to paracetamol resulted in a marked depletion of cellular glutathione to less than 25% of control values (Figure 3). Iloprost alone had no effect on the glutathione content of hepatocytes, at any concentration (Figure 3). Pretreatment with N-acetyl-L-cysteine (protocol B) significantly attenuated the effects of paracetamol on glutathione levels ($P < 0.05$ at 3 h) (Figure 3). Iloprost, at concentrations that were cytoprotective (10^{-14} M or 10^{-12} M), did not prevent the depletion of glutathione caused by incubation with paracetamol (Figure 3).

Following exposure of hepatocytes to [^{14}C]-paracetamol (2 mM) for 3 h there was 0.62 ± 0.03 nmol paracetamol equivalent covalently bound per mg of hepatic protein (Table 1). Pretreatment with N-acetyl-L-cysteine (protocol B) significantly ($P < 0.001$) decreased the covalent binding of [^{14}C]-paracetamol to cellular proteins (Table 1). In contrast, pretreatment of cells with 10^{-14} M, 10^{-12} M or 10^{-9} M iloprost had no significant effect on the extent of covalent binding of [^{14}C]-paracetamol to cellular proteins (Table 1).

Two-phase model of paracetamol toxicity: protocol C

Hamster hepatocytes incubated in incubation medium alone using protocol C, without exposure to paracetamol, maintained their viability well for 6 h (initial viability: 88.8 ± 2.6%; final viability: 68.2 ± 2.4%; $n = 6$; as assessed by trypan blue exclusion). There was a close correlation between viability assessed by LDH leakage and by trypan blue exclusion (Figures 4a,b). Treatment with iloprost alone, at concentrations of up to 10^{-8} M, or with N-acetyl-L-cysteine (1.25 mM) had minimal effects on cell viability (data not shown). After incubation in incubation medium alone for 6 h using protocol C the percentage of viable cells exhibiting plasma membrane blebbing increased slightly, from 14.4 ± 1.5% to 18.7 ± 1.2%.

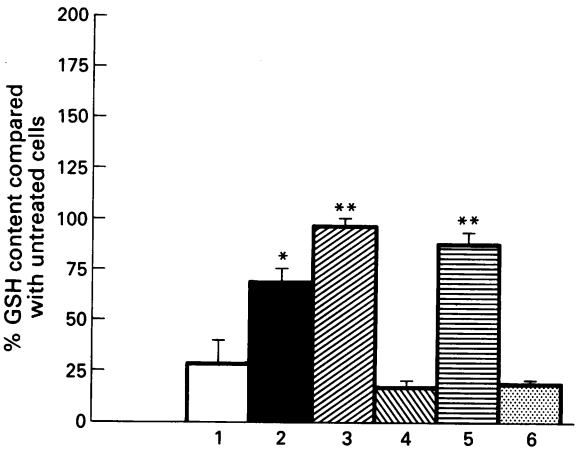

Figure 3 The effect of pretreating hepatocytes with iloprost (10^{-14} M or 10^{-12} M) or N-acetyl-L-cysteine (1.25 mM) followed by paracetamol (protocol B) on the GSH content as a percentage of that of control (untreated) hepatocytes after 3 h of incubation. Hepatocytes were treated with buffer alone, iloprost or N-acetyl-L-cysteine for 30 min prior to incubation in buffer with or without paracetamol. The cells were treated as follows: column (1) paracetamol (2 mM) alone; (2) paracetamol (2 mM) following pretreatment with N-acetyl-L-cysteine (1.25 mM); (3) iloprost (10^{-14} M) alone; (4) paracetamol (2 mM) following pretreatment with iloprost (10^{-14} M) alone; (5) iloprost (10^{-12} M) alone; (6) paracetamol (2 mM) following pretreatment with iloprost (10^{-12} M) alone. Results are expressed as the mean with s.e. shown by vertical bars ($n = 9$). Differences between mean values were tested for statistical significance by two-way analysis of variance. Differences between individual treatment groups were tested by one-way analysis of variance and Scheffe's method. The null hypothesis was rejected at $P \leqslant 0.05$. ** $P < 0.005$; * $P < 0.05$, compared with cells treated with paracetamol only.

Table 1 Effect of iloprost or N-acetyl-L-cysteine in protocol B on the covalent binding of [^{14}C]-paracetamol to cellular proteins of hepatocytes

	Covalent binding of [^{14}C]-paracetamol (nmol mg^{-1} protein)
Paracetamol only	0.62 ± 0.03
Pretreatment with iloprost (10^{-14} M) before exposure to paracetamol	0.49 ± 0.13
Pretreatment with iloprost (10^{-12} M) before exposure to paracetamol	0.56 ± 0.07
Pretreatment with iloprost (10^{-9} M) before exposure to paracetamol	0.52 ± 0.09
Pretreatment with N-acetyl-L-cysteine (1.25 mM) before exposure to paracetamol	0.34 ± 0.01***

Hepatocytes were pretreated with iloprost or N-acetyl-L-cysteine and then incubated with [^{14}C]-paracetamol (2 mM) for 3 h. Covalent binding (nmol mg^{-1} protein) in pretreated samples was compared with that in cells which were treated with paracetamol only. Results have been expressed as the mean ± s.e. ($n = 6$). *** $P < 0.001$ compared with cells treated with paracetamol only, Student's paired t test.

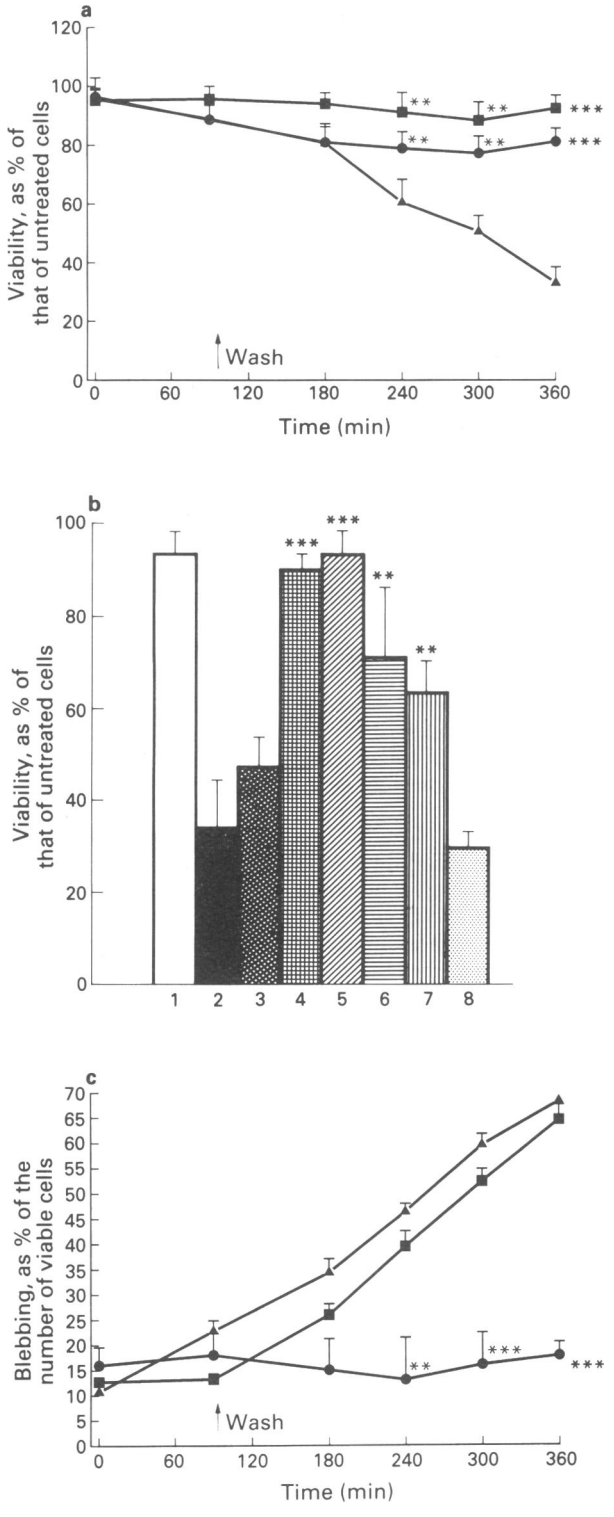

Figure 4 Effects of iloprost in a two-phase model of paracetamol toxicity: protocol C. (a) Viability as a percentage of that of untreated cells, as assessed by lactate dehydrogenase leakage. The cells were treated as follows: paracetamol (4 mM) during phase 1 followed by extensive washing (arrow) and incubation in buffer alone during phase 2 (▲); paracetamol (4 mM) during phase 1 followed by extensive washing (arrow) and incubation in buffer containing N-acetyl-L-cysteine (1.25 mM) during phase 2 (■); paracetamol (4 mM) during phase 1 followed by extensive washing (arrow) and incubation in buffer containing iloprost (10^{-10} M) during phase 2 (●). Results are expressed as the mean with s.e. shown by vertical bars ($n = 6$). Differences between mean values were tested for statistical significance by two-way analysis of variance. Differences between individual treatment groups were tested by one-way analysis of variance and Scheffe's method. The null hypothesis was rejected at $P \leqslant 0.05$. There was a significant difference between treatment with paracetamol alone and treatment with paracetamol followed by either iloprost or N-acetyl-L-cysteine. Exposure of cells to either iloprost (10^{-8} M) or N-acetyl-L-cysteine (1.25 mM) during phase 2 had no significant effect on the percentage of viable cells that were blebbed.

Cells treated with paracetamol (4 mM) during phase 1 maintained their viability throughout this period (Figure 4a), although there was an increase in the percentage of viable cells that were blebbed, beyond that seen in untreated cells (Figure 4c). Following washing of the cells, to remove the paracetamol and its metabolites, there was a progressive loss in cell viability during incubation in incubation medium alone (Figure 4a). The percentage of cells showing blebbing increased throughout the phase 2 incubation (Figure 4c). Treatment of such cells with N-acetyl-L-cysteine (1.25 mM) during phase 2 significantly reduced the loss of cell viability (Figures 4a,b) and the percentage of viable cells exhibiting blebbing (Figure 4c) resulting from prior exposure to paracetamol. Incubation with iloprost (10^{-14} M to 10^{-8} M) during phase 2 significantly reduced the loss of cell viability, assessed by either LDH leakage (Figure 4a) or trypan blue exclusion (Figure 4b), caused by paracetamol. Iloprost, however, at concentrations outside this range was without effect (Figure 4b). At no concentration of iloprost tested was there any change in the percentage of viable cells showing blebbing (Figure 4c).

Synthesis of prostaglandins by hamster isolated hepatocytes

The most abundant of the prostaglandins synthesized by hepatocytes was PGE_2 (208 ± 52 pg ml^{-1}) (Table 2). $PGF_{2\alpha}$ was present at 124 ± 19 pg ml^{-1} but there was negligible 6-oxo-$PGF_{1\alpha}$, a stable rearrangement product of PGI_2. Both aspirin and ibuprofen markedly inhibited prostaglandin synthesis, reducing the levels of all of the prostaglandins to below the limit of detection (< 50 pg ml^{-1}). Iloprost (10^{-14} M) did not affect prostaglandin levels but paracetamol stimulated PGI_2 production ($P < 0.001$).

*** $P < 0.001$; ** $P < 0.005$; * $P < 0.05$, compared with cells treated with paracetamol only. (b) Viability as a percentage of that of untreated cells, as assessed by trypan blue exclusion, at 360 min after the start of phase 1. The cells were treated as follows: column (1) iloprost (10^{-10} M) alone; column (2) paracetamol (4 mM) during phase 1 followed by extensive washing and incubation in buffer alone during phase 2; column (3) paracetamol (4 mM) during phase 1 followed by extensive washing and incubation in buffer containing iloprost (10^{-6} M) during phase 2; column (4) as column (3), but with iloprost (10^{-8} M); column (5) as column (3) but with iloprost (10^{-10} M); column (6) as column (3) but with iloprost (10^{-12} M); column (7) as column (3) but with iloprost (10^{-14} M); column (8) as column (3) but with iloprost (10^{-16} M). Results are expressed as the mean with s.e. shown by vertical bars ($n = 6$). Differences between mean values were tested for statistical significance by two-way analysis of variance. Differences between individual treatment groups were tested by one-way analysis of variance and Scheffe's method. The null hypothesis was rejected at $P \leqslant 0.05$. *** $P < 0.001$; ** $P < 0.005$, compared with cells treated with paracetamol only. (c) The percentage of viable cells which were blebbed. The cells were treated as follows: paracetamol (4 mM) during phase 1 followed by extensive washing (arrow) and incubation in buffer alone during phase 2. (▲); paracetamol (4 mM) during phase 1 followed by extensive washing (arrow) and incubation in buffer containing N-acetyl-L-cysteine (1.25 mM) during phase 2 (●); paracetamol (4 mM) during phase 1 followed by extensive washing (arrow) and incubation in buffer containing iloprost (10^{-10} M) during phase 2 (■). Results have been expressed as the mean with s.e. shown by vertical bars ($n = 6$). Differences between mean values were tested for statistical significance by two-way analysis of variance. Differences between individual treatment groups were tested by one-way analysis of variance and Scheffe's method. The null hypothesis was rejected at $P \leqslant 0.05$. There was a significant difference between treatment with paracetamol alone and treatment with paracetamol followed by either iloprost or N-acetyl-L-cysteine. *** $P < 0.001$; ** $P < 0.005$; * $P < 0.05$, compared with cells treated with paracetamol only.

Table 2 Effect of various treatments on prostaglandin levels of hamster isolated hepatocytes

Treatment	*Prostaglandin concentration* (pg per 10^6 cells) PGE_2	$PGF_{2\alpha}$	6-oxo-$PGF_{1\alpha}$
Control	208 ± 52	124 ± 19	< 50
Aspirin (10^{-4} M)	< 50***	< 50***	< 50
Ibuprofen (10^{-4} M)	< 50***	< 50***	< 50
Iloprost (10^{-14} M)	210 ± 82	102 ± 45	< 50
Paracetamol (2 mM)	265 ± 32	100 ± 65	150 ± 35***

Hepatocytes were incubated with incubation medium (Control) or other treatments for 30 min at 37°C. Results have been expressed as the mean ± s.e. ($n = 8$). *** $P < 0.001$ compared with control cells, Student's t test.

Effect of altering the rate of prostaglandin synthesis on the toxicity of paracetamol

Hepatocytes were incubated with paracetamol (2 mM) for 45 min during phase 1. During phase 2, there was a progressive decrease in the percentage of viable cells, to 32.3 ± 5.2% ($n = 8$) after 3 h (Table 3). Incubation during phase 2 with either aspirin (10^{-4} M) or ibuprofen (10^{-4} M), both of which completely inhibited the synthesis of prostaglandins, exacerbated the loss of viability induced by prior incubation with paracetamol. Simultaneous exposure to aspirin (10^{-4} M) and iloprost (10^{-14} M) during phase 2 provided less protection than iloprost alone (Table 3).

Discussion

There have been a number of reports that iloprost (ZK36374), a stable analogue of prostacyclin with similar anti-aggregatory and vasodilator effects (Schrör *et al.*, 1981), protects cells against the toxicity of a variety of agents. However, the mechanism of action of this effect and the duration over which iloprost is effective are not known. We have now investigated, using freshly isolated hepatocytes, the effects of iloprost on the toxicity of paracetamol and its reactive metabolite NABQI.

Iloprost, when present at the time of addition of toxin to the cells (protocols A and B), significantly reduces the cytotoxicity of both paracetamol and NABQI. In protocol B 2 mM paracetamol was used but, owing to variation in the sensitivity of different groups of hamsters to paracetamol toxicity, 4 mM paracetamol was employed in protocol C. With protocol C it is possible to dissociate the metabolic phase of paracetamol toxicity, during which the compound is activated and initiates those events that ultimately lead to cell death, from a phase of progressive biochemical and morphological deterioration, which leads eventually to irreversible changes and ultimately to loss of viability. Iloprost, added after washing the cells, prevents the loss of viability of paracetamol-pretreated hepatocytes during phase 2 incubation. Iloprost is effective against NABQI, which is directly cytotoxic, and is protective against paracetamol under conditions (protocol C) in which it cannot affect metabolism. Iloprost also does not affect GSH levels, either in the absence or, more significantly, in the presence of paracetamol. Further, iloprost does not affect the covalent binding of paracetamol to cellular protein. Thus, iloprost reduces the loss of cell viability without affecting either the metabolism or detoxication of paracetamol. The reported effectiveness of iloprost against galactosamine-induced injury (Stachura *et al.*, 1981; Noda *et al.*, 1986) provides further evidence that at least one mechanism of action of prostanoids is independent of the metabolism of the toxin.

Table 3 Effect of inhibition of prostaglandin synthesis on the toxicity of paracetamol

Treatment	*Percentage of cells which were viable*
Control	88.0 ± 3.2%***
Paracetamol (2 mM)	32.3 ± 5.2%
Paracetamol (2 mM) + aspirin (10^{-4} M)	18.4 ± 3.1%**
Paracetamol (2 mM) + ibuprofen (10^{-4} M)	20.2 ± 4.6%*
Paracetamol (2 mM) + aspirin (10^{-4} M) + iloprost (10^{-14} M)	72.8 ± 5.2%***
Paracetamol (2 mM) + iloprost (10^{-14} M)	80.4 ± 3.9%***

Hepatocytes, in incubation medium, were incubated with paracetamol (2 mM) or incubation medium for 45 min. Following washing, cells were incubated with one or more of the following for a further 3 h: incubation medium; 10^{-4} M aspirin; 10^{-4} M ibuprofen or 10^{-14} M iloprost. Controls were incubated with incubation medium throughout both phases. After the 3 h incubation samples were taken for the assessment of viability by trypan blue exclusion. Results have been expressed as the mean ± s.e. ($n = 8$). *** $P < 0.001$; ** $P < 0.005$; * $P < 0.01$, compared with cells treated with paracetamol only, Student's t test.

N-acetyl-L-cysteine protects against the toxicity of both paracetamol and NABQI when present at the start of the incubation. Most, if not all, of the protective effect of N-acetyl-L-cysteine against the toxicity of these compounds, when present simultaneously, is by serving as a precursor for GSH synthesis which prevents the escape of the toxic metabolite. In protocol C, N-acetyl-L-cysteine is still protective despite being added after the metabolic phase of toxicity. However, its effect still appears to be mediated via the synthesis of GSH, the levels of which are largely restored to control values following addition of the compound. It is likely that GSH thus formed acts as a thiol-reducing agent, reactivating thiol groups oxidized by NABQI (Bruno *et al.*, 1988).

Whereas N-acetyl-L-cysteine prevents, or even reverses, the plasma membrane blebbing caused by NABQI or paracetamol, iloprost appears only to delay, but not prevent or reverse, plasma membrane blebbing. As iloprost is cytoprotective during phase 2, without affecting the levels of GSH or reversing plasma membrane blebbing, this suggests that whilst it can arrest those events leading to cell death, there must be additional ones leading to plasma membrane blebbing. Both blebbing and loss of viability are probably initiated by a common biochemical event, most likely thiol-group oxidation, as thiol-reducing agents such as dithiothreitol and GSH, synthesized from N-acetyl-L-cysteine, can prevent both processes (Tee *et al.*, 1986). The mechanism of action of iloprost in providing cytoprotection without reversing plasma membrane blebbing is not known.

In agreement with the results of others (Bursch & Schulte-Hermann, 1987; Ferrari *et al.*, 1989) the cytoprotective effects of iloprost are apparent at very low doses of the prostanoid, as little as 10^{-14} M showing significant protection. Given Avogadro's number (6.02×10^{23}) this concentration corresponds to only 30 molecules per hepatocyte. The K_{act} for prostacyclin at its receptor, the receptor through which iloprost exerts its pharmacological effects, has not been determined for hepatocytes. However, for human platelets it is approximately 10^{-8} M (Lombroso *et al.*, 1984). Thus, it is unlikely that iloprost is cytoprotective through activation of this receptor.

The role of endogenous prostaglandins in protection against hepatic injury *in vitro* was investigated by Guarner *et al.* (1985). They demonstrated that inhibition of prostaglandin synthesis by indomethacin led to an increase in the cytotoxicity of carbon tetrachloride. Treatment of mice with OKY1581, an inhibitor of thromboxane synthetase, increased prostacyclin synthesis, and protected the animals against paracetamol toxicity (Guarner *et al.*, 1988). The enhanced toxicity seen in protocol C following exposure of cells to aspirin or ibuprofen supports a role for endogenous prostaglandins in protecting hepatocytes against cytotoxic insult.

Clearly, iloprost is a potent cytoprotective agent in isolated hepatocytes against the toxicity of paracetamol. Its mechanism of action is quite different from that of N-acetyl-L-cyste-

ine. Iloprost does not have any effect on the metabolism or detoxication of paracetamol. The novel mechanism of action of iloprost in providing protection against paracetamol toxicity suggests events in the progression of cell injury to cell death may be open to pharmacological manipulation.

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Endothelium-dependent contractile responses to 5-hydroxytryptamine in the rabbit basilar artery

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1 5-Hydroxytryptamine (5-HT) and 5-carboxamidotryptamine (5-CT) stimulated additional, endothelium-dependent contractions in rabbit isolated basilar arteries which had been submaximally contracted with either histamine or potassium chloride.

2 The additional contractions to 5-HT were not altered by the 5-HT_2 antagonist, ketanserin (1 μM), but were abolished in the presence of the cyclo-oxygenase inhibitor indomethacin (3 μM).

3 The additional smooth muscle contraction stimulated by 5-HT was increased in the presence of the competitive substrate inhibitor for nitric oxide synthase, N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM).

4 Neither of the selective 5-HT agonists, 8-hydroxy-dipropylaminotetralin (8-OH DPAT) or α-methyl 5-HT stimulated endothelium-dependent contraction, but these agonists did reduce the rate at which histamine-induced tension spontaneously declined. This effect represented a direct action on the smooth muscle cells, as it was independent of the presence of endothelial cells.

5 Smooth muscle relaxation was not obtained in response to 5-HT, whether or not indomethacin was present to block endothelium-dependent contraction. None of the other selective 5-HT agonists, 5-CT, 8-OH DPAT or α-methyl 5-HT produced endothelium-dependent smooth muscle relaxation, when applied against a background of contraction.

6 These data show that endothelium-dependent smooth muscle contraction can be produced by stimulating 5-HT receptors in the partially contracted rabbit basilar artery. Similar contraction to 5-CT indicates an involvement by 5-HT_1 receptors. The susceptibility of the contractions to indomethacin suggest they are mediated by a metabolite of arachidonic acid.

Keywords: Vascular smooth muscle; 5-hydroxytryptamine; basilar artery; endothelial cells; endothelium-derived contractile factors (EDCF)

Introduction

The overall action of 5-hydroxytryptamine (5-HT) on blood vessels is complex. Its main action is to stimulate contraction in vascular smooth muscle cells, although a direct relaxant action has been demonstrated (Feniuk *et al.*, 1983). 5-HT can also influence smooth muscle tone via the vascular endothelium. The majority of the evidence which is available has resulted from investigations into the ability of the endothelium to attenuate the constrictor action of 5-HT.

In pre-contracted coronary arteries and jugular veins, 5-HT induces endothelium-dependent relaxation of the smooth muscle cells, which reflects the release of endothelium-derived relaxing factor (EDRF, Cocks & Angus, 1983; Leff *et al.*, 1987). This action of 5-HT is not blocked by ketanserin, and appears to be mediated by a 5-HT_1 receptor. In a number of other arteries, including the canine basilar artery, although removal of the endothelium has been found to potentiate the contractile action of 5-HT, it has not been possible to demonstrate relaxation in pre-contracted arteries with an intact endothelium (Martin *et al.*, 1986; Connor & Feniuk, 1989). One possibility, is that the increased contraction to 5-HT which follows removal of the endothelium in these arteries can be explained by a high spontaneous release of EDRF. In the rabbit basilar artery, removal of the endothelium increases the contractile response to 5-HT, indicating that part of the response to 5-HT in this artery is mediated via the endothelium (Garland, 1987).

In addition to an endothelium-dependent inhibitory action on vascular smooth muscle cells, there is some evidence to suggest that 5-HT can stimulate endothelium-dependent smooth muscle contraction in the aorta of spontaneously hypertensive rats, and *in vivo*, in mouse cerebral arteries (Luscher & Vanhoutte, 1988; Rosenblum & Nelson, 1988).

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We have now investigated the cerebrovascular action of 5-HT and some related agonists, using rabbit isolated, pre-contracted basilar arteries, to show to what extent constrictor responses to 5-HT are mediated by the endothelium. Some of these results have been presented in preliminary form to the British Pharmacological Society (Clark & Garland, 1990).

Methods

Preparation of isolated arteries

New Zealand white rabbits (2–4 kg) of either sex were anaesthetized with an intravenous injection of sodium pentobarbitone (60 mg kg^{-1}), and killed by rapid exsanguination. The brain was removed, placed in physiological salt solution (PSS; see Garland, 1987 for composition) at room temperature, and the basilar artery carefully dissected and removed. In some cases, the endothelial cells were destroyed at this stage, by carefully rubbing the intimal surface with a blunt-ended syringe needle. This procedure abolished relaxation to the subsequent addition of acetylcholine (1–100 μM). After some experiments, artery segments were examined histologically, to confirm that the endothelial cells had been destroyed. The artery was then cut into 2 mm cylindrical segments, which were placed in individual organ baths (5 ml) containing PSS bubbled with 95% O_2:5% CO_2. The segments were each suspended between 2 L-shaped stainless steel wire supports (each of 0.14 mm diameter) inserted into the lumen. One support was connected to an isometric force transducer (Grass, FT03), the other to a micrometer. Segments were then equilibrated at a predetermined optimal resting tension of 500 mg for at least 1 h (Garland, 1987). The response to a submaximal concentration of histamine (1 μM) or potassium chloride (30 mM) was then determined in each tissue. These concentrations of histamine and potassium gave contractions of around 50% tissue maximum.

Concentration-response determinations

Cumulative concentration-response curves to agonists were obtained by direct addition of agonists to the bathing fluid, and allowing sufficient time for the effects of each concentration to become fully established before adding a higher concentration. In experiments on pre-contracted segments, four preparations from the same animal were mounted in parallel and precontracted with submaximal concentrations of either histamine or potassium chloride. In three of the baths, cumulative concentration-response curves to agonists were established on top of the background contraction. The fourth segment served as a time control to detect 'fade' of background contraction. No more than two concentration-response curves were determined in each segment. The concentration-response curves were reproducible under these conditions. In the experiments studying the effect of indomethacin and N^G-nitro-L-arginine methyl ester (L-NAME), these compounds were added 30 and 5 min before contraction was induced with histamine, respectively.

Drugs

The compounds used in this study were: 5-hydroxytryptamine creatinine sulphate (5-HT), histamine dihydrochloride, N^G-nitro-L-arginine methyl ester (Sigma). 5-Carboxamidotryptamine (5-CT), 8-hydroxy-dipropylaminotetralin hydrobromide (8-OH DPAT), α-methyl 5-hydroxytryptamine (α-methyl 5-HT) Research Biochemicals. Ketanserin tartrate was a generous gift from Janssen U.K.

Statistical analysis

Data are expressed as the mean ± s.e.mean. The significance of differences between curves and mean values was calculated with either one or two-way analysis of variance or Students *t* test. Values of $P < 0.05$ were taken as significant.

Results

Effect of 5-hydroxytryptamine on precontracted arteries

In segments of basilar artery submaximally contracted with 1 μM histamine (13.8 ± 0.8 mN; $n = 44$ arteries) and with a functional endothelium, both 5-HT and 5-CT (1 nM–100 μM) produced additional, concentration-dependent increases in smooth muscle tension (Figures 1 and 2). The additional contractions were stimulated with agonist concentrations in excess of 1 nM, and reached a maximum with 1 μM in the case of 5-HT, and 100 μM with 5-CT. The contraction in response to 5-HT represented an increase above the histamine contraction of 22.3 ± 3.6% ($n = 6$), and with 5-CT an additional contraction of 19.1 ± 5.5% ($n = 9$). In pre-contracted arteries in which the endothelial cells had been destroyed, additional contraction was not produced by the addition of 5-HT (Figure 1). Removal of the endothelium did not significantly affect the contraction produced in response to 1 μM histamine (12.8 ± 2.0 mN; $n = 40$), nor did it alter the time-dependent fade in the histamine-induced contraction, indicating that the smooth muscle cells were not damaged by the procedure employed to remove the endothelium. In the absence of endothelial cells, only the highest concentration of 5-CT, 100 μM, induced any additional contraction. This contraction had a similar maximum (19.1 ± 5.1%; $n = 9$) to the experiments on segments with a functional endothelium (Figure 2). Although neither agonist produced any additional contraction in the absence of functional endothelial cells, apart from 100 μM 5-CT, the spontaneous decline in the histamine contraction was reduced, indicating direct smooth muscle stimulation. In the presence of contraction produced by raised extracellular potassium (30 mM; 14.0 ± 1.2 mN, $n = 9$) rather than histamine, the additional contraction produced in response to 5-HT was not significantly altered (Figure 3). Smooth muscle relaxation was not obtained with either 5-HT or 5-CT in any of the precontracted artery segments.

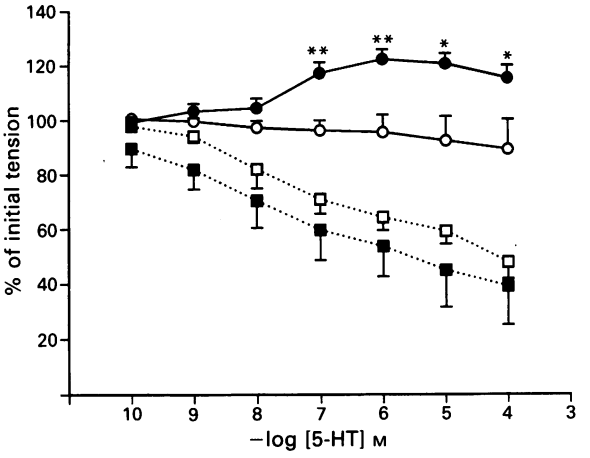

Figure 1 Additional contraction to 5-hydroxytryptamine (5-HT) in segments of basilar artery submaximally contracted with 1 μM histamine. Additional changes in tension are expressed relative to the histamine response (100%). Points are the mean from 6 experiments with s.e.mean shown by vertical bars. Arteries with (●) and without (○) a functional endothelium; time controls from segments with (■) and without (□) an endothelium. $*P < 0.05$; $**P < 0.01$: differences between arteries with and without an endothelium.

Effect of ketanserin, indomethacin and N^G-nitro-L-arginine methyl ester

The additional contraction produced in response to 5-HT was not modified by the presence of ketanserin, in concentrations ranging from 10 nM to 1 μM ($n = 4$ separate experiments with each concentration of ketanserin). With each of these concentrations of ketanserin, the concentration-response curves for additional contraction to 5-HT were superimposable. In some of these experiments, ketanserin did slightly reduce the initial contraction to histamine. However, in these tissues the concentration of histamine was increased, to ensure that the level of smooth muscle contraction was similar in control segments and segments equilibrated with either 10 nM, 100 nM or 1 μM ketanserin. The level of contraction induced with histamine

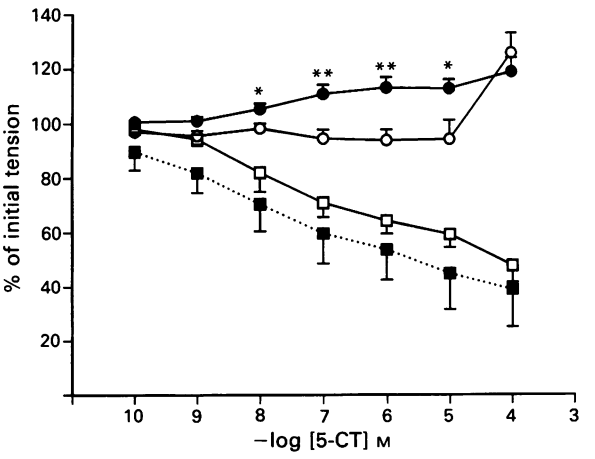

Figure 2 Additional contraction to 5-carboxamidotryptamine (5-CT) in segments of basilar artery submaximally contracted with 1 μM histamine. Additional changes in tension are expressed relative to the histamine response (100%). Points are the mean from 9 experiments with s.e.mean shown by vertical bars. Arteries with (●) and without (○) a functional endothelium; time controls from segments with (■) and without (□) an endothelium. $*P < 0.05$; $**P < 0.01$; differences between arteries with and without an endothelium.

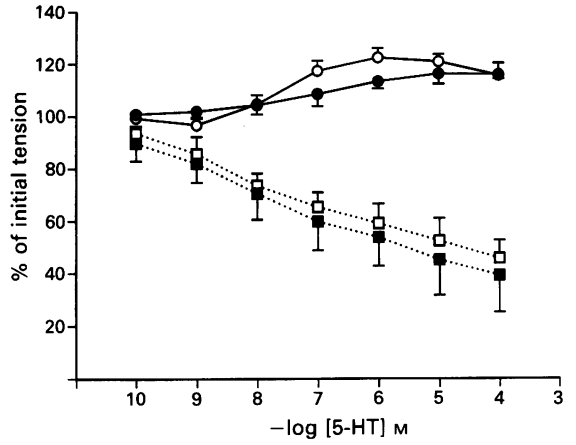

Figure 3 Additional contraction produced in response to 5-hydroxytryptamine (5-HT) in arteries submaximally contracted with either 1 μM histamine (○) or 30 mM potassium (●). In both series the endothelium was functional, and the additional contractions in the presence of either histamine or potassium were not significantly different: $P > 0.05$; (□) and (■) represent the corresponding time controls. Values represent the mean from 7 experiments with s.e.mean shown by vertical bars.

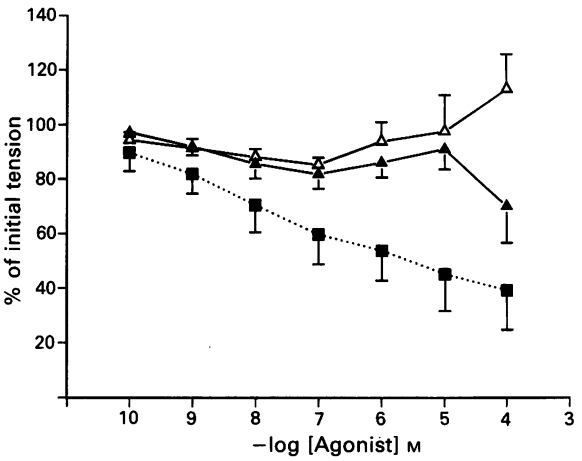

Figure 5 Effect of α-methyl 5-hydroxytryptamine (△, $n = 8$) and 8-hydroxy dipropylaminotetralin (▲, $n = 7$) in segments of basilar artery submaximally contracted with 1 μM histamine; Points are means (s.e.mean shown by vertical bars) from arteries with a functional endothelium, and are expressed relative to the contraction produced with histamine (100%); (■) represent the values of the corresponding time controls.

immediately before the addition of 5-HT was 12.8 ± 1.9 mN, 12.7 ± 1.6 mN, 11.1 ± 1.5 mN and 12.2 ± 2.1 mN, respectively ($n = 4$ in each case).

Indomethacin (3 μM) abolished the additional contractions to 5-HT, across the entire concentration-range (Figure 4). Although no additional smooth muscle contraction was produced to 5-HT when indomethacin was present, the background contraction was maintained. This indicated that the direct constrictor action of 5-HT had not been altered—compare Figure 4 with the time control shown in Figure 1. Indomethacin did not modify the initial contraction in response to histamine. In this series, 1 μM histamine induced a contraction of 12.4 ± 1.4 mN, which was not different from control values of 13.8 ± 0.8 mN.

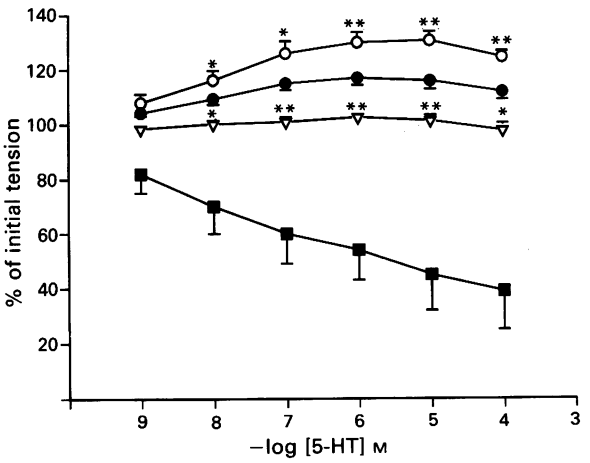

Figure 4 Responses to 5-hydroxytryptamine (5-HT) in segments of basilar artery submaximally contracted with 1 μM histamine. Indomethacin (3 μM) abolished additional contraction to 5-HT (▽), whereas the nitric oxide synthase substrate inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) significantly increased the contractions (○). Control responses in the absence of inhibitor (●); time control showing spontaneous fade of contraction (■). The inhibitors did not significantly modify the time control. Changes in tension are expressed relative to the histamine response (100%). Points are the mean from 5 separate experiments in artery segments with a functional endothelium; vertical bars show s.e.mean. $*P < 0.05$; $**P < 0.01$; differences from control values in the absence of either indomethacin or L-NAME.

L-NAME, which is a competitive substrate inhibitor for nitric oxide synthase (Rees *et al.*, 1990), had the opposite effect to indomethacin. In the presence of L-NAME, the additional contractions to 5-HT were increased (Figure 4). As with indomethacin, L-NAME was effective over the range of 5-HT concentrations tested. L-NAME was applied before histamine, and did not increase smooth muscle tone.

Effect of α-methyl 5-HT and 8-hydroxy-dipropylaminotetralin on precontracted arteries

The other agonists tested on precontracted basilar arteries were 8-OH DPAT and α-methyl 5-HT (Figure 5). Only α-methyl 5-HT produced any significant additional contraction, but in much higher concentrations than was the case with either 5-HT or 5-CT. Although the contraction in response to 100 μM α-methyl 5-HT (13.0 ± 3.0%) was similar to the maximum contraction with both 5-HT and 5-CT, this action of α-methyl 5-HT was not dependent on the presence of an intact endothelium, as very similar responses occurred in artery segments in which the endothelium had been destroyed ($n = 7$). Both 8-OH DPAT and α-methyl 5-HT significantly reduced the spontaneous fade of contraction to histamine, indicating a direct constrictor action on the vascular smooth muscle cells. Both α-methyl 5-HT and 8-OH DPAT failed to induce endothelium-dependent relaxation in the basilar artery, although concentrations of 8-OH DPAT in excess of 10 μM did, in some experiments, directly stimulate smooth muscle relaxation. The response of precontracted arteries to 8-OH DPAT was not modified in the presence of either indomethacin ($n = 3$) or L-NAME ($n = 4$).

Discussion

The finding that 5-HT and 5-CT, but not the other 5-HT agonists, 8-OH DPAT and α-methyl 5-HT, produced additional, endothelium-dependent smooth muscle contraction, indicates that the contraction is specifically receptor linked. Further, the results suggest that the receptor which mediates this response is probably of the 5-HT_1 type, but not 5-HT_{1A}, as endothelium-dependent contraction was stimulated with 5-CT but not 8-OH DPAT. The lack of effect with both α-methyl 5-HT and ketanserin also argues against the involvement of 5-HT_2 receptors. The most likely explanation for the contraction, is that the endothelial cells release a vasoconstrictor agent in response to stimulation with either 5-HT or 5-CT.

The release of endothelium-derived contractile factors (EDCF), stimulated in various ways, has been reported in a number of vascular preparations. Studies with cerebral arteries have concentrated on arteries from the dog, cat and sheep, and show that noradrenaline, acetylcholine, arachidonic acid, ATP, ADP, stretch, hypoxia and increases in transmural pressure can all produce endothelium-dependent contraction (Usui *et al.*, 1987; Shirahase *et al.*, 1987; Katusic *et al.*, 1988; Shirahase *et al.*, 1988a,b; Katusic *et al.*, 1987; Katusic & Vanhoutte, 1986; Klaas & Wadsworth, 1989; Harder, 1987). Reports that 5-HT can produce endothelium-dependent contraction are limited to two observations, the first in the aorta of spontaneously hypertensive rats and the second in mouse cerebral arteries *in vivo* (Luscher & Vanhoutte, 1986; Rosenblum & Nelson, 1988). In the latter the action of 5-HT on pial arteries was reversed from contraction to vasodilatation by localized, laser-induced damage to the endothelium.

However, it is of interest to note that additional contraction to 5-HT has been reported in partially contracted canine basilar and guinea-pig iliac arteries with 5-HT and 5-HT_1 agonists (Connor & Feniuk, 1989; Sahin-Erdemli *et al.*, 1991). In neither case was the endothelium-dependence of the contraction assessed. In addition, in human umbilical arteries, under some conditions, a component of the contraction to 5-HT mediated by 5-HT_1 receptors, involved a product of cyclo-oxygenase activity (MacLennan *et al.*, 1989). Although the endothelial cells were apparently not functional in this study, the experimental basis for this conclusion was not reported. It is therefore not clear whether these responses to 5-HT depended in any way on the endothelium.

In only a small number of studies has the release of a diffusible constrictor factor(s) from endothelial cells been demonstrated directly (Rubanyi & Vanhoutte, 1985; Harder *et al.*, 1989). However, the use of various inhibitors indicates that in the majority of cases the endothelium-mediated constrictor action involves one or more metabolites of arachidonic acid. In some cases this metabolite appears to be thromboxane A_2. The ability of indomethacin to abolish the endothelium-dependent contraction to 5-HT in the present study, conforms with the observations made with other agonists and vascular preparations. If the additional contraction to 5-HT in the basilar artery does involve the release of a constrictor factor, the mechanism of release apparently does not require a change in the membrane potential of the endothelial cells, as similar responses to 5-HT followed sub-maximal contraction induced with either histamine or potassium. Not all endothelium-dependent contractions can be abolished with inhibitors of cyclo-oxygenase. The contraction of cerebral arteries, which follows an increase in transmural pressure, although endothelium-dependent and involving a diffusible factor, is resistant to cyclo-oxygenase inhibitors. This suggests that in cerebral arteries at least, the release of different factors, each of which can cause smooth muscle contraction, can be controlled independently (Harder *et al.*, 1989).

In addition to an endothelium-dependent constrictor action in the basilar artery, these cells also in some way reduce the direct smooth muscle contractile action of 5-HT (Garland, 1987). This attenuation is similar to the influence exerted by endothelial cells in the canine basilar artery, where it was suggested the attenuation may reflect a high basal release of EDRF (Connor & Feniuk, 1989). The fact that additional contraction to 5-HT was increased following inhibition of the arginine-nitric oxide pathway, provides evidence that nitric oxide has a role in responses of the basilar artery to 5-HT, but does not indicate whether contraction is reduced by basal or stimulated release of nitric oxide. Likewise, failure to demonstrate relaxation to 5-HT, in the presence of sub-maximal contraction and indomethacin to block endothelium-dependent contraction, does not mean that 5-HT cannot stimulate the release of EDRF/NO. To determine this we would need to block selectively the direct action of 5-HT on the vascular smooth muscle cells. Despite using a variety of available 5-HT antagonists (methiothepin, spiperone, cyanopindolol, ketanserin, mianserin and 1-(1-naphthyl)piperazine) we were unable to find one that was selective. In fact only methiothepin depressed contractions to 5-HT, but was not sufficiently selective to allow investigation of a possible relaxant action of 5-HT.

In summary, the present work is the first demonstration of endothelium-dependent contraction to 5-HT in isolated cerebral arteries, and indicates that the response is mediated by a 5-HT_1-like receptor. Whether or not 5-HT has a similar action in cerebral arteries from other species requires clarification.

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Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery

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1 Acetylcholine caused a concentration-dependent smooth muscle hyperpolarization and relaxation in rat small mesenteric arteries (diameter at 100 mmHg 250–450 mm) stimulated with noradrenaline (3 μM).

2 Nitric oxide (NO), generated from either NO-gas or from acidified sodium nitrite, also induced smooth muscle hyperpolarization but only in the absence of active force. However, unlike the hyperpolarizations to acetylcholine, those to NO were abolished either by prior smooth muscle depolarization caused by noradrenaline, or by the K^+ channel blocker, glibenclamide (3 μM).

3 Hyperpolarization and relaxation to acetylcholine were unaffected by prior exposure of the mesenteric artery to either the cyclo-oxygenase inhibitor, indomethacin (10 μM), or the nitric oxide synthase inhibitor, N^G-nitro-L-arginine (L-NNA, 100 μM).

4 Haemoglobin (1.5 μM), which binds and inactivates NO, blocked the hyperpolarizing and vasorelaxant response to NO, but did not alter either response to acetylcholine.

5 These data show that, in the rat small mesenteric artery, membrane hyperpolarizations to NO and acetylcholine are mediated by different mechanisms, and that the hyperpolarization induced by NO is not involved in the responses to acetylcholine. In addition, they provide evidence that the acetylcholine responses in this artery, which are endothelium-dependent, are not mediated by the release of NO.

Keywords: Acetylcholine hyperpolarization; endothelium-derived hyperpolarizing factor (EDHF); rat small mesenteric artery; K^+ channels; nitric oxide

Introduction

Acetylcholine and related cholinomimetics cause both vasorelaxation and hyperpolarization in vascular smooth muscle cells by an endothelium-dependent mechanism (Bolton *et al.*, 1984; Chen *et al.*, 1988; Feletou & Vanhoutte, 1988; McPherson & Angus, 1991; Rand & Garland, 1991). Smooth muscle relaxation follows the stimulation of soluble guanylate cyclase by a diffusible factor which is released from endothelial cells (EDRF; see Angus & Cocks, 1989). EDRF is thought to be nitric oxide (NO) or a derivative, as the relaxation to acetylcholine is blocked in the presence of compounds such as haemoglobin, which 'capture' NO (Angus & Cocks, 1989). Hyperpolarization to acetylcholine also appears to be mediated, at least in part, by the release of a diffusible factor (Feletou & Vanhoutte, 1988). However, in contrast to the relaxation induced by acetylcholine, hyperpolarization is not blocked by either oxyhaemoglobin or methylene blue, which has led to the suggestion that a factor(s) distinct from NO is also released from the endothelium. This factor(s) has been termed endothelium-derived hyperpolarizing factor (EDHF; Chen *et al.*, 1988; Huang *et al.*, 1988; Taylor & Weston, 1988).

The idea that acetylcholine-induced hyperpolarization reflects an endothelial cell action distinct from the release of NO, is supported by the failure to demonstrate hyperpolarization to NO in rabbit cerebral and canine coronary arteries (Brayden, 1990; Komori *et al.*, 1988). However, this is not a consistent observation, as Tare *et al.* (1990) have demonstrated a clear hyperpolarization and relaxation to NO in uterine arteries. These data suggest an important role for NO in both membrane and tension responses of the uterine artery to acetylcholine.

To date, the majority of studies indicate that an increase in membrane permeability to K^+ is responsible for the acetylcholine-induced hyperpolarization, although the type of K^+-channel involved is not clear (see Longmore & Weston, 1990). Standen *et al.* (1989) showed in the rabbit middle cerebral artery, that the hyperpolarizations to acetylcholine, and a number of other vasodilators, were sensitive to glibenclamide, a compound which they showed could inhibit ATP-sensitive K^+ channels opened by cromakalim in vascular smooth muscle cells. However, in isolated small mesenteric arteries from the rat we have recently shown (McPherson & Angus, 1991) that acetylcholine causes a marked glibenclamide-insensitive, endothelium-dependent hyperpolarization and relaxation, suggesting different mechanisms of action of acetylcholine in the two vascular segments.

The purpose of the present study was to investigate the possibility that the release of NO from endothelial cells was responsible for the smooth muscle hyperpolarization to acetylcholine in the rat small mesenteric artery. We showed that NO can produce hyperpolarization, but the mechanism underlying this hyperpolarization is different from that in response to acetylcholine. Further, the experiments indicate that NO does not contribute to either the acetylcholine-induced hyperpolarization or relaxation in this artery.

Methods

Isolation of resistance blood vessels

Wistar Kyoto (WKY) rat were killed by CO_2 asphyxia. The mesentery was rapidly removed and placed in ice cold Krebs solution (composition in mM: NaCl 119, KCl 4.7, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, $CaCl_2$ 2.5 and glucose 11) gassed with 5% CO_2 in O_2. A segment of rat small mesenteric artery, 2 mm in length, corresponding to a third to fourth order branch from the superior mesenteric artery, was mounted in a small vessel myograph as previously described (Angus *et al.*, 1988). Briefly, two 40 μm wires were threaded through the lumen of the vessel segment. One wire was attached to a stationary support driven by a micrometer, while the other was attached to an isometric force transducer. Data were recorded on a dual-channel flat bed recorder

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(W&W Scientific Instruments, model 320) and on computer disk. Vessels were allowed to equilibrate under zero force for 30 min. Using the diameter of the vessel, calculated from the distance between the two mounting wires, a passive diameter-tension curve was constructed as previously described (Mulvany & Halpern, 1977). From this curve the effective transmural pressure was calculated. The vessel was set at a tension equivalent to that generated at 0.9 times the diameter of the vessel at 100 mmHg. Non-linear curve fitting of the passive diameter-tension curve was achieved by use of custom written programmes for the IBM PC (NORMALIZE, GA McPherson) which uses the Marquart-Levenberg modification of the Gauss-Newton technique (McPherson, 1985). Vessel diameters at an equivalent transmural pressure of 100 mmHg (D_{100}) are given in the text.

Electrophysiology

In experiments where the intracellular membrane potential was monitored, the vessel was mounted as described above. A conventional glass electrode (1 mm blanks, World Precision Instruments Inc., New Haven, U.S.A.) filled with 0.5 M KCl (tip resistance approximately 100 MΩ) was used to impale a single smooth muscle cell. The microelectrode was positioned by a Burleigh Inchworm motor driven by a 6000 series controller (Burleigh, U.S.A.). The microelectrode was advanced in 0.5 μm steps until a stable impalement was achieved.

The bath containing the vessel (7 ml volume) was part of a 25 ml recirculating system which contained a jacketed organ bath, where the Krebs solution was warmed and oxygenated. Drugs could also be added at this site. This design allowed cumulative steady-state concentration-effect curves to be constructed when assessing the electrophysiological effects of acetylcholine. In some experiments the vessel was activated with a sub-maximal concentration of noradrenaline (1–3 μM). In these experiments changes in membrane potential and active tension were recorded simultaneously. Concentration-relaxation curves to acetylcholine showed a time dependent decrease in sensitivity of about 3 fold between the first and second curves. Subsequent curves (up to four) could be superimposed on the second. The second concentration-response curve to acetylcholine was therefore used as the control response in all studies.

NO containing solutions were prepared from either NO gas or from acidified sodium nitrite. In the text these are referred to as NO_g and NO_a respectively (see later). In view of the unstable nature of NO, quantities of either were added to the inlet port of the tissue bath from a gas tight syringe. The amount added was expressed in terms of μmol injected in the port. In some experiments infusions (Terfusion STC-521 syringe pump) of NO_a were made into the inlet port of the bath to simulate a steady-state application of the compound.

Data collection and analysis

Force and membrane potential data were captured by use of a programme, DIGISCOPE (GA McPherson), custom written for the IBM PC. This programme uses a DASH16 A/D card (Metrabyte, U.S.A.) which collected and displayed data at 200 Hz. Data were saved on hard disk and reproduced on a Hewlett Packard 7470A plotter.

Concentration-effect curves were analysed by graphical procedures. Results in the text are the mean ± s.e.mean for the specified number of experiments.

Drugs

The following drugs were used: acetylcholine bromide, haemoglobin, (−)-noradrenaline (+)-bitartrate (arterenol), N^G-nitro-L-arginine (Sigma); glibenclamide (Hoechst); sodium nitrite (BDH, Analar grade). Nitric oxide and helium (research grade) were both purchased from CIG Australia.

Nitric oxide stock solutions were prepared by injecting NO gas into sealed vials containing cold distilled water which had been degassed (30 min) with helium (Palmer *et al.*, 1987). Subsequent dilutions were made in sealed vials with a gas-tight syringe. NO gas was injected into the stock solutions in quantities sufficient to produce a saturated solution based on the solubility constant for NO in water at 1 ATM and 0°C (7.4 ml/100 ml; Tracey *et al.*, 1990). NO solutions were also prepared from acidified sodium nitrite as previously described (Cocks & Angus, 1990). A 100 mM solution of sodium nitrite was prepared in a sealed container in ice cold acidified (pH less than 2 with HCl) distilled water. The resultant solution was allowed to stand for 10 min. Dilutions were made with the same acidified distilled water solution. In this case the concentration of NO was assumed to be equivalent to the final concentration of sodium nitrite in the solution.

Oxyhaemoglobin was prepared by reducing methaemoglobin in the presence of sodium dithionite, and then separating and collecting oxyhaemoglobin on a Sephadex column. Oxyhaemoglobin was then quantified spectrophotometrically, separated into aliquots and stored until required (for up to 14 days) at −20°C (Martin *et al.*, 1985).

Results

Electrophysiological and tension responses to acetylcholine and nitric oxide (NO)

Acetylcholine Smooth muscle cells in the rat small mesenteric artery (D_{100} = 337 ± 10 mm, n = 22) had a resting membrane potential of −57.0 ± 1.2 mV (n = 42 cells, 16 different vessels). In the absence of active tone, acetylcholine (0.01–10 μM) hyperpolarized these cells towards a membrane potential of −69 ± 1 mV; an effect that was not sensitive to the presence of glibenclamide (3 μM) (Figure 1). Noradrenaline (3 μM) caused membrane depolarization to −35 ± 2 mV (n = 12 cells) in association with an increase in active force. Figure 1 also shows simultaneous traces of changes in tension and membrane potential in response to acetylcholine, in a noradrenaline precontracted vessel (D_{100} = 336 μm). Acetylcholine caused a concentration-dependent hyperpolarization to −67 ± 3 mV in the presence of the highest concentration of acetylcholine used in this study (3 μM), representing hyperpolarization of approximately 30 mV. This hyperpolarization was associated with over 90% reversal of the contractile response. The absolute value for membrane potential attained in the presence of acetylcholine (3 μM) was similar whether

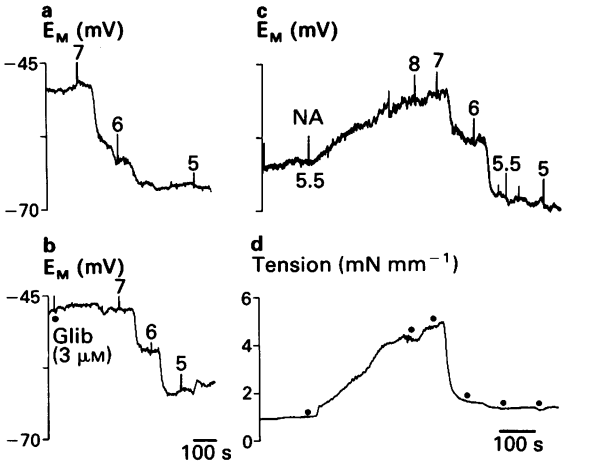

Figure 1 Effect of acetylcholine on rat small mesenteric artery in a non-activated (a,b) and activated vessel. (a,b) Concentration-dependent hyperpolarization to acetylcholine (−log M) in the absence (a) and in the presence (b) of glibenclamide (Glib, 3 μM). (c,d) The effect of acetylcholine on membrane potential (c) and tension development (d) in a vessel preactivated with noradrenaline (NA, 3 μM).

recorded in the absence or in the presence of depolarization induced by noradrenaline (i.e. −69 versus −67 mV respectively).

Nitric oxide In the absence of active tone, bolus applications of NO_g (0.1–1 μmol) caused a transient hyperpolarization of between 3 and 9 mV ($n = 16$) which was dependent on the dose injected into the bath (Figure 2a). A similar effect could be mimicked by NO_a generated from acidified sodium nitrite solution. The hyperpolarization observed with NO_a occurred over a similar concentration-range (0.05–5 μmol) and varied in amplitude to a maximum of 8.5 ± 0.7 mV ($n = 8$) (Figure 2b). The hyperpolarization to NO_g or NO_a was reversibly blocked by glibenclamide (3 μM, Figure 2). If NO_g or NO_a were applied against a background of noradrenaline-induced depolarization greater than approximately 20 mV, hyperpolarization to these compounds did not occur (Figure 3), although there was a pronounced smooth muscle relaxation. Concentration-relaxation curves constructed to either NO_g or NO_a (Figure 3), in the absence and presence of glibenclamide (3 μM), were not significantly different.

Because of the unstable nature of NO, the majority of the studies described involved applying bolus injections close to the vascular preparation. We wished to determine whether similar results where obtained under steady state conditions using a constant infusion of NO. Consequently, studies were repeated with a constant infusion of NO_a (2–16 μmol min^{-1}). Under these conditions NO_a caused a sustained hyperpolarization which, like the more transient responses to bolus injections of NO_a, could be blocked with glibenclamide (3 μM). (Figure 2c).

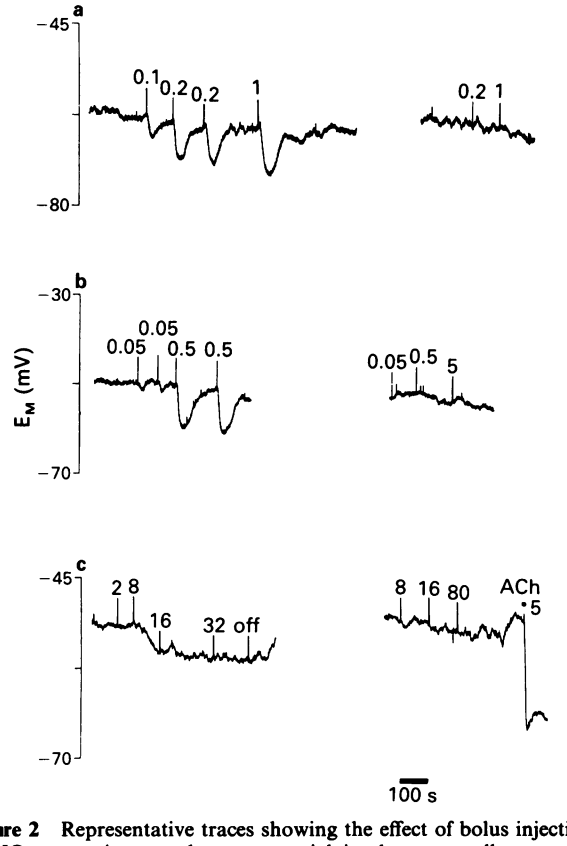

Figure 2 Representative traces showing the effect of bolus injections of NO on resting membrane potential in the rat small mesenteric artery. Responses were obtained in the absence (left side) and presence (right side) of glibenclamide (3 μM). (a) Dose-dependent hyperpolarizations to NO prepared from NO gas (NO_g; μmol). (b) Dose-dependent hyperpolarizations to NO generated from acidified $NaNO_2$ (NO_a; μmol). (c) Hyperpolarization produced by infusions of acidified $NaNO_2$. In this case quantities of NO_a are given as μmol min^{-1}. In this experiment the effect of acetylcholine (ACh 10 μM) was also assessed at the end of the experiment.

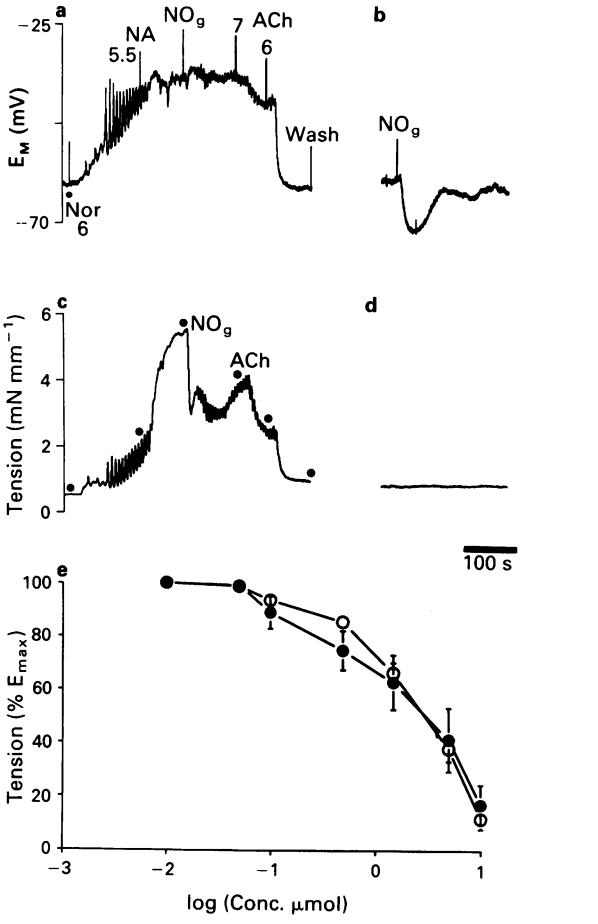

Figure 3 (a,b,c,d) Representative trace showing the responses to NO_g in the absence and presence (a,c) of noradrenaline (NA, 3 μM) to induce active force: (a,b) are the membrane potential and (c,d) tension. In the presence of active tone (a,c) NO_g caused a marked relaxation (c) in the absence of a change in membrane potential (a), while the application of acetylcholine (ACh) was followed by a marked hyperpolarization. Conversely, acetylcholine (−log M) caused a concentration-dependent hyperpolarization and relaxation. In the absence of active force (b,d) NO_g caused a marked hyperpolarizing response (b). (e) Mean dose-relaxation response curves constructed in rat small mesenteric arteries assessing the ability of nitric oxide (NO_a) to cause relaxation of tone induced by noradrenaline (3 μM). Results were obtained in the absence (○) or presence (●) of glibenclamide (3 μM). Results are the mean obtained from 6 separate experiments; s.e.mean shown by vertical bars.

Several control experiments were also performed to insure that the mechanical and electrophysiological effect observed with NO were not the result of the diluents, particularly in the case of NO_a which was made in acidified distilled water. Figure 4 shows the result of one such experiment examining the effect of NO_a on membrane potential. The injection of unacidified sodium nitrite or acidified distilled water were without effect, while bubbling of the acidified sodium nitrite solution (with 5% CO_2 in O_2 for 5 min at room temperature) markedly attenuated the hyperpolarizing response to NO_a (Figure 4).

Effect of N^G-nitro-L-arginine, indomethacin and oxyhaemoglobin on responses to acetylcholine

N^G-nitro-L-arginine (L-NNA) (100 μM) applied 20–60 min before noradrenaline and present during the subsequent application of acetylcholine, did not alter the depolarization to noradrenaline (membrane potential −39 ± 4 mV). Hyperpolarization and relaxation in response to acetylcholine was unaffected by the presence of L-NNA (Figure 5).

Indomethacin (10 μM), applied 10 min before noradrenaline and present throughout the subsequent application of acetyl-

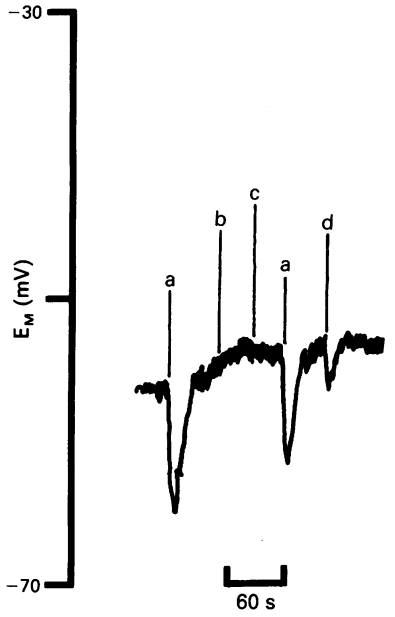

Figure 4 Representative trace showing the effect of diluents used to make the acidified nitrite-derived NO_a. Responses were obtained to acidified nitrite solution (50 μl of a 100 μM stock solution which is equivalent to a dose of 5 μmol) (a), non-acidified nitrite solution (50 μl of a 100 μM stock) (b) and acidified distilled water (50 μl) used to make up the acidified nitrite (c). In addition the response to acidified nitrite (5 μmol), which had been bubbled for 5 min at room temperature with 5% CO_2 in O_2, is also given (d).

choline, did not modify either the electrophysiological or the functional responses to noradrenaline or acetylcholine (Figure 5). This was the case if indomethacin was present alone or in the presence of L-NNA (100 μM). Noradrenaline depolarized (-34 ± 2 mV cf. -34 ± 5 mV) and contracted (2.3 ± 0.1 mN mm^{-1} cf. 2.3 ± 0.4 mN mm^{-1}) the rat small mesenteric artery in the absence and presence of indomethacin respectively, indicating that indomethacin did not alter the conditions from which the actions of acetylcholine were assessed.

Incubation of segments of mesenteric artery with oxyhaemoglobin (1.5 μM for 10 min) did not modify the vasorelaxant response ($n = 8$) to acetylcholine (Figure 6). Electrophysiological studies ($n = 3$) showed that the hyperpolarizing effects of acetylcholine were not sensitive to oxy-

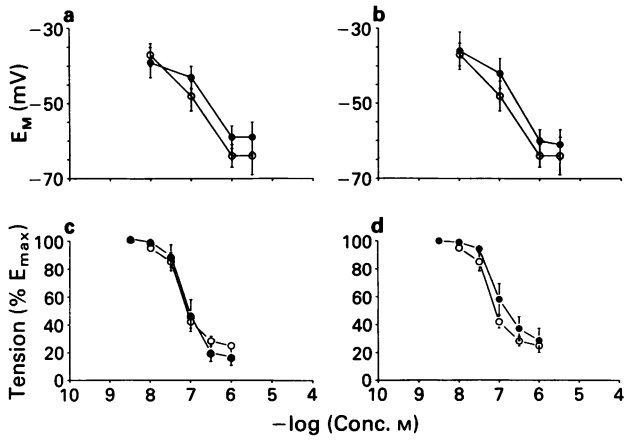

Figure 5 Mean concentration-effect curves constructed to acetylcholine in the rat small mesenteric artery in the absence (○) and presence (●) of (a,c) N^G-nitro-L-arginine (100 μM) or (b,d) indomethacin (10 μM). (a,b) Membrane potential changes induced by acetylcholine. Points are means of 8–14 observations in 6–8 experiments. (c,d). Relaxation of noradrenaline-induced contraction with acetylcholine in a separate series experiments. Results are mean from 6–8 separate experiments; s.e.mean shown by vertical bars.

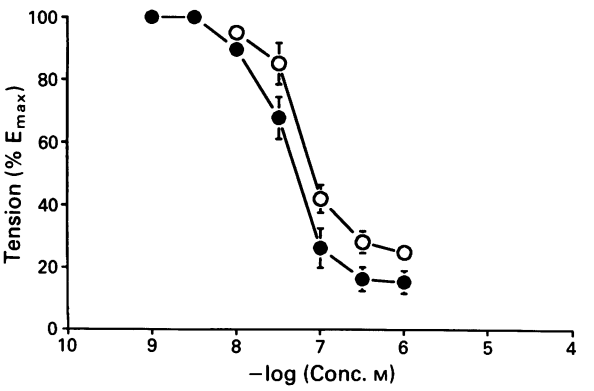

Figure 6 Mean concentration-response curve constructed to acetylcholine examining its ability to relax noradrenaline (3 μM) precontracted rat small mesenteric artery in the absence (○) and in the presence (●) of oxyhaemoglobin (1.5 μM). Results are the mean from 8 experiments; s.e.mean shown by vertical bars.

haemoglobin. Figure 7 shows an original trace where the effects of acetylcholine and NO_a on a noradrenaline (3 μM)-preactivated blood vessel were examined. While acetylcholine caused a marked relaxation and hyperpolarization, the pronounced relaxation to NO_a observed in absence of oxyhaemoglobin (Figure 3) was not observed in its presence (Figure 7b). In the absence of active tone, where NO_a caused a membrane hyperpolarizing effect, NO_a responses were also sensitive to the actions of oxyhaemoglobin (1.5 μM) (Figure 8).

Discussion

There are two main conclusions from this work. First, that in the rat small mesenteric artery, hyperpolarizations to acetylcholine and NO are caused by different mechanisms and second, that NO does not have an obligatory role in the endothelium-dependent responses to acetylcholine. The first conclusion is based on the ability to affect the hyperpolarization to either acetylcholine or NO differentially. The

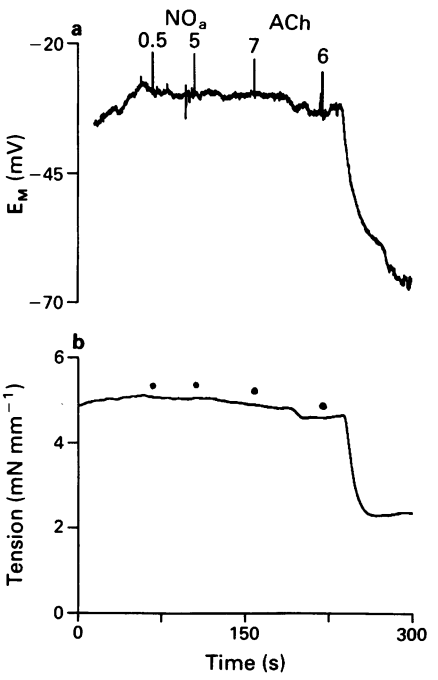

Figure 7 (a) Representative trace showing the effect of NO_a (0.5 and 5 μmol) and acetylcholine (ACh, 0.1 and 1 μM) on membrane (a) and tension (b) recorded simultaneously in a noradrenaline (3 μM)-precontracted rat small mesenteric artery pretreated with oxyhaemoglobin (1.5 μM).

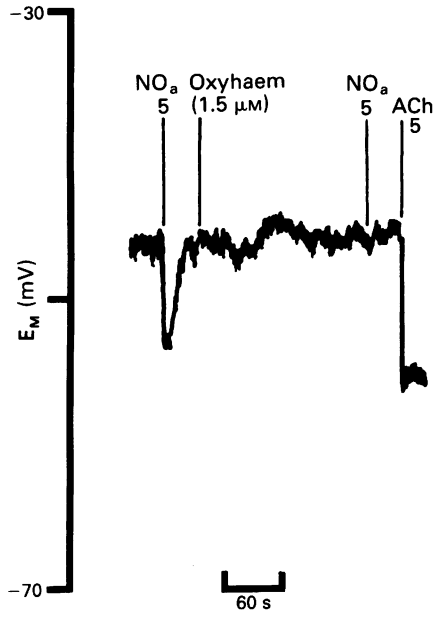

Figure 8 Representative trace showing the effect of NO_a on membrane potential in a rat small mesenteric artery. The experiment was performed in the absence of active tone. Responses to NO_a (5 μmol) were obtained in the absence and presence of oxyhaemoglobin (1.5 μM).

second is based on the persistence of both the hyperpolarization and relaxation to acetylcholine, after treatment of the mesenteric artery with compounds that affect NO synthesis or action (NO-synthase substrate inhibitor, oxyhaemoglobin or glibenclamide).

The hyperpolarization caused by NO, but not acetylcholine, could be blocked either by prior smooth muscle depolarization or in the presence of the potassium channel blocker glibenclamide. Why membrane depolarization should block the NO-induce hyperpolarization is not clear, but it is similar to the effect of depolarization on the responses to NO in the guinea-pig uterine artery (Tare *et al.*, 1990). In this artery, smooth muscle depolarization with phenylephrine, to around −30 mV, reduced the hyperpolarization to NO by over 70%. However, in contrast to the present study, relaxation to NO was also markedly reduced, as were both the hyperpolarization and relaxation to acetylcholine. Tare and coworkers (1990) suggested that their observations could be explained by an increase in membrane conductance, which is responsible for the depolarization, being sufficient to swamp the NO-induced potassium conductance. The similar depression they observed in both the acetylcholine-induced hyperpolarization and relaxation was one piece of evidence indicating that NO mediated the action of acetylcholine in the uterine artery. An alternative explanation is that prior membrane depolarization prevents the NO-activated K^+-channels from opening. In this situation, the channels would be similar to those comprising the potassium selective inward rectifier found in arteriolar smooth muscle (Edwards & Hirst, 1988). However, although hyperpolarization was blocked at membrane potentials which would prevent the opening of inwardly rectifying potassium channels, the channels are not identical to those in guinea-pig submucosal arterioles, as these latter channels were blocked in the presence of millimolar concentrations of barium (Edwards & Hirst, 1988) which failed to affect the acetylcholine-induced hyperpolarization in rat small mesenteric artery (data not shown). ATP-dependent K^+ channels in other cell types show inward rectification (Rorsman & Traube, 1990), which together with the glibenclamide-sensitivity of the hyperpolarizations to NO in this study, might indicate an involvement by this type of K^+ channel. Whatever the precise explanation for the reduction in hyperpolarization to NO, we did not observe a voltage-dependent block of either the relaxation to NO, or both the hyperpolarization and relaxation to acetylcholine. The magnitude of the hyperpolarizations to acetylcholine was in fact increased, as would be predicted by depolarization shifting the membrane potential away from the potassium equilibrium potential. These observations indicate that different mechanisms are responsible for the NO- and acetylcholine-induced hyperpolarizations in the mesenteric artery, and also that the hyperpolarization to NO is not an important mechanism in the relaxation response.

This idea is also supported by the different effect that glibenclamide displayed against the hyperpolarization to either NO or acetylcholine in the rat small mesenteric artery. In this and previous studies we have found that the hyperpolarization and relaxation to acetylcholine are not blocked by glibenclamide, although glibenclamide could block similar responses to cromakalim (McPherson & Angus, 1991). In addition, we now show that, in contrast to the hyperpolarization to acetylcholine, that to NO in this artery can be blocked with glibenclamide. Apart from indicating that acetylcholine and NO responses may be mediated by different membrane mechanisms, the result may also indicate that the NO-induced hyperpolarization occurs after activation of ATP-dependent K^+ channels. In cell types other than vascular smooth muscle, glibenclamide is known to block ATP-dependent K^+ channels (see Rorsman & Trube, 1990). ATP-dependent K^+-channels, which can be activated by cromakalim and blocked by glibenclamide, have been described in excised patches from rabbit and rat mesenteric artery. It has been suggested that these channels could provide an important mechanism for producing hyperpolarization and subsequent relaxation in vascular smooth muscle (Standen *et al.*, 1989). Support for this idea comes from experiments with the rabbit middle cerebral artery, where glibenclamide antagonizes both the hyperpolarization and relaxation to acetylcholine (Brayden, 1990). Some caution is needed however, before assuming that because hyperpolarization in vascular smooth muscle can be induced by cromakalim and blocked by glibenclamide, it is necessarily mediated by ATP-dependent K^+ channels. For example, glibenclamide is several orders of magnitude less potent (μM) at blocking hyperpolarization and relaxation in vascular smooth muscle than it is in stimulating the release of insulin from pancreatic β-cells, by blocking ATP-dependent K^+ channels (nM region, Sturgess *et al.*, 1988; Zunkler *et al.*, 1988). This suggests that in these two cell types, the channels are not identical. In addition in experiments using cells dispersed from the rabbit portal vein, cromakalim can activate a K^+ channel which is not ATP-dependent, and which closely resemble the K^+ channels that are responsible for delayed rectification (Beech & Bolton, 1989).

It would also appear that, in the rat mesenteric artery of the size we studied (approximately 350 μm), NO contributes very little in the responses to acetylcholine. Both L-NNA and oxyhaemoglobin had no significant effect on either the relaxation or the hyperpolarization to acetylcholine although the results from these studies should be viewed with some caution. While L-NNA has been shown to inhibit potently NO-synthase present in endothelial cells, both *in vitro* and *in vivo* (Rees *et al.*, 1990), one assumption that we have made is that L-NNA can enter the rat small mesenteric artery endothelial cell and that it is a substrate for the NO-synthase in this cell. From the present studies we cannot confirm this. However the results with oxyhaemoglobin also suggests that NO has a small role in the acetylcholine mediated relaxation and hyperpolarization in the rat small mesenteric artery. We found oxyhaemoglobin failed to effect the actions of acetylcholine at concentrations (1.5 μM) that abolished the effects of NO.

That acetylcholine-mediated hyperpolarization is oxyhaemoglobin-insensitive has been reported previously by Chen *et al.* (1988). The finding that the relaxation response shows a similar resistance is somewhat unusual and is suggestive of another factor (apart from NO) mediating either one or both of the effects of acetylcholine. In contrast to our findings,

previous work on rat mesenteric arterial preparations has provided some evidence that NO is at least partially involved in the relaxation response to acetylcholine. For example Furchgott and co-workers (Furchgott *et al.*, 1987), using a perfused rat mesenteric preparation, showed acetylcholine relaxation responses to be partially sensitive to haemoglobin (10 μM). In addition, other studies have shown acetylcholine responses in a blood-perfused mesenteric preparation, are sensitive to methylene blue (Randall & Hiley, 1988). The reasons for these differences are unclear at present. We have subsequently repeated some experiments with haemoglobin (4 μM, data not shown), the maximum concentration practically achievable in our system, and have found that vasorelaxation and hyperpolarizing responses to acetylcholine are resistant. The vessels characterized using the perfused mesenteric bed would be of a smaller diameter than those used by us. One possibility is that these smaller vessels use NO to a greater extent to cause the vasorelaxant action. We are currently testing this hypothesis. In any case, the finding that the concentration of haemoglobin used in the majority of our studies successfully abolished responses to NO suggests the concentration of haemoglobin was sufficient.

While it is likely that the acetylcholine-mediated hyperpolarization of the rat small mesenteric artery is not the result of NO liberation, it is currently unclear what factor or mechanism is responsible for the phenomenon. Previous studies have shown that the hyperpolarization and relaxation responses to acetylcholine can be blocked differentially (see introduction) by oxyhaemoglobin and methylene blue, leading to the suggestion that at least two factors are released from the endothelium on stimulation with acetylcholine (Chen *et al.*, 1988; Feletou & Vanhoutte, 1988; Nishiye *et al.*, 1989; Taylor & Weston, 1990). One causes hyperpolarization (EDHF) and the other relaxation (EDRF). The results from this study suggest the possibility of a third factor/mechanism which does not rely on NO as the signal carrier. The fact that indomethacin did not reduce hyperpolarization to acetylcholine in this and other studies (Chen *et al.*, 1988; Feletou & Vanhoutte, 1988), indicates that any unidentified hyperpolarizing factor is probably not a product of cyclo-oxygenase activity. An alternative or additional possibility, is that acetylcholine may directly hyperpolarize endothelial cells, and this hyperpolarization may spread electrotonically through the arterial media, closing voltage-operated calcium channels and inducing relaxation (Mekata, 1986). Such a process would, by definition, operate through voltage-dependent K^+ channels. Support for this idea comes from the finding that acetylcholine can directly hyperpolarize endothelial cells, and the knowledge that areas of close contact, which might support electrical transmission, are present between endothelial and vascular smooth muscle cells (Busse *et al.*, 1988; Spagnoli *et al.*, 1982; Taugner *et al.*, 1984). The only study to date which has directly investigated this possibility involved simultaneous intracellular recording from an endothelial cell and a neighbouring smooth muscle cell in pig coronary artery (Beny, 1990). Although bradykinin hyperpolarized both the endothelial and smooth muscle cell, this appeared to be, in both cells, a direct effect of bradykinin, as the injection of either current or lucifer yellow into endothelial cells, failed to provide any evidence for coupling between these cells. However, this does not mean that such coupling is not important in other vessels, particularly smaller resistance arteries. A variation in coupling between different arteries may go some way towards explaining the variation in both the size and duration of hyperpolarization caused by acetylcholine.

Lastly, some other technical considerations are worth considering. First we generated NO from two separate sources, NO gas and acidified nitrite, because we were concerned that the results obtained with NO generated from acidified nitrite, may be complicated by the presence of other nitro compounds. However, results obtained examining the actions of both NO preparations on the rat small mesenteric artery were similar, suggesting this was not the case. In addition we showed acidified distilled water and unacidified sodium nitrite, both major contaminants, were without effect on the rat small mesenteric artery. We were also concerned that NO liberated from the endothelium by acetylcholine has characteristics differing from NO injected near the vessel using a flow through perfusion system. In the first case, the smooth muscle cells underlying the endothelium would be exposed to a steady state level of NO released in close apposition to the muscle by acetylcholine. However, when injected into the bath the tissue would be exposed to a transient amount of NO. We feel this was not a complicating issue since, in experiments where NO was applied under steady state conditions via an infusion pump, the responses showed the same characteristics as when applied in a bolus fashion.

In summary, these data show that NO can hyperpolarize smooth muscle cells in the rat mesenteric artery, but by a different membrane mechanism to acetylcholine. Further, they indicate that any NO which is released by acetylcholine stimulating the endothelium has an insignificant role in the subsequent pronounced smooth muscle hyperpolarization and relaxation. The mechanisms underlying these responses are not clear and require further investigation.

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Nedocromil sodium prevents *in vivo* generation of the eosinophilotactic substance induced by PAF but fails to antagonize its effects

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1 The intrathoracic injection of platelet activating factor (PAF) into rats induced a decrease in the pleural leucocyte numbers within 15 min, accompanied by a marked exudation, maximal 1 h later. After 6 h, concomitantly with the reduction of exudation, a marked increase in the number of mononuclear cells, neutrophils and eosinophils was observed. Within 24 h, the pleural eosinophil accumulation peaked and persisted up to 96 h.

2 Topical treatment with nedocromil sodium affected pleural exudation by PAF under conditions where systemic meclizine was ineffective. Nedocromil sodium blocked, dose-dependently, the increase in the pleural content of mononuclear cells, neutrophils and eosinophils, observed 6 h after PAF administration, as well as the eosinophilia 24 h later. Moreover, the co-incubation of peritoneal eosinophils with nedocromil sodium did not interfere with the migration triggered by PAF.

3 The transfer of the 6 h-PAF pleural washings from donor to recipient rats caused a selective pleural eosinophilia, which was clearly inhibited when nedocromil sodium was administered to donor, but not to recipient animals, showing that this drug interferes with the generation rather than with the expression of the eosinophilotactic activity(ies).

4 These findings indicate that the nedocromil sodium interferes with PAF-induced exudation and leucocyte accumulation, by a mechanism other than its ability to reduce the local effects of histamine and which may relate to suppression of the eosinophilotactic principle generation.

Keywords: Nedocromil sodium; PAF; pleurisy; eosinophils

Introduction

Eosinophils are important participants in human allergy, particularly asthma (Frigas & Gleich, 1986). They are found in excess in blood and in the bronchoalveolar lavage fluid (BALF) after immune provocation in man and animals. Eosinophil invasion also occurs in lungs of guinea-pigs after systemic administration of platelet activating factor PAF or antigen (Lellouch-Tubiana *et al.*, 1988). We have recently demonstrated a marked eosinophil migration following the intrapleural administration of PAF (Silva *et al.*, 1989), allergen (Lima *et al.*, 1990) or compound 48/80 (Martins *et al.*, 1991) to rats. Eosinophilia was independent of acute inflammation, i.e., of early exudation and neutrophil-monocyte invasion of the pleural cavity, since it persisted when the acute inflammatory process was over (Silva *et al.*, 1989). In addition, eosinophil invasion was suppressed by the *in situ* co-injection of protein synthesis inhibitors with (PAF) (Silva *et al.*, 1991), under conditions where the other signs of inflammation including neutrophil invasion, were unaffected (Silva *et al.*, unpublished data). Furthermore, the transfer of pleural washings from PAF-injected rats to the pleural cavity of recipient animals also led to a selective eosinophil invasion, unaccompanied by acute inflammation. Generation of this eosinophilic principle was suppressed by protein synthesis inhibitors although such drugs failed to inhibit the eosinophilia in recipient animals, after the pleural fluid transfer (Silva *et al.*, 1991). This led us to postulate the existence of a protein synthesis-dependent mediator formed locally in the pleural cavity of PAF-injected rats, which was capable of stimulating *in vivo* and *in vitro* the migration of eosinophils (Silva *et al.*, 1989). Since PAF is claimed to participate in allergy and particularly in eosinophil migration after the i.v. administration of antigen to passively sensitized guinea-pigs (Lellouch-Tubiana *et al.*, 1987), we have investigated whether the anti-allergic drug, nedocromil sodium (Ruggieri & Patalano, 1989) interferes with the effects of PAF and/or with the generation of this eosinophilotactic factor, as nedocromil sodium did inhibit *in vivo* eosinophil recruitment following antigen challenge of sensitized guinea-pigs (Pretolani *et al.*, 1990).

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Methods

Animals

Wistar rats of either sex weighing 180–200 g, obtained from Charles River and Oswaldo Cruz Foundation breeding were used.

Induction of pleurisy

The reaction was produced in ether-anaesthetized rats by intrathoracic (i.t.) injections (0.1 ml) of PAF (1 μg per cavity) diluted with 0.9% NaCl solution (saline), containing 0.01% bovine serum albumin (BSA), and histamine (200 μg per cavity) diluted with saline. Control animals received the same volume of the vehicle. After different time intervals, the animals were killed with an overdose of ether, the pleural cavity was opened and rinsed with 3 ml of heparinized saline (5 iu ml^{-1}). The pleural washing was recovered and the volume measured in a graduated syringe. In the rare case in which a haemorrahge was noted in the pleural cavity, the animal was not included in subsequent studies.

Total protein quantification

The fluid recovered from the pleural cavity was centrifuged for 10 min at 2000 r.p.m., and the total protein content was quantified from the supernatant by the Biuret technique in a spectrophotometer (Shimadzu Corporation).

Leucocyte analysis

Total leucocytes were counted in a Coulter counter ZBI and expressed as cells $\times 10^6$ per cavity. Differential analyses were performed in cytocentrifuged smears stained with May-Grunwald-Giemsa dye under oil immersion objective, by means of a light microscope.

Transfer procedure

Six hours after the i.t. injection of PAF into donor animals, the pleural cavity was washed with 0.5 ml of sterile saline, and the pleural fluid was centrifuged (2500 r.p.m.) at 4°C for 15 min (Silva *et al.*, 1991). The supernatant was injected into the pleural cavity of recipient rats in a final volume of 0.2 ml and the analysis of eosinophil counts was made 24 h later.

In vitro *eosinophil chemotaxis assay*

Eosinophils were obtained from the peritoneal cavity of normal rats and were 'purified' by means of a discontinuous gradient of metrizamide, according to Martins *et al.* (1989). To test the potential effect of nedocromil sodium on eosinophil migration promoted by PAF (10^{-8} and 10^{-6} M), the cells were pre-incubated with nedocromil sodium (10^{-6} and 10^{-4} M), or its vehicle, for 10 min at 37°C. Migration experiments were performed in 48-Well microchemotaxis chamber and Toyo cellulose nitrate filters (3 μm pore). The latter were fixed and stained as previously described by Richards & McCullough (1984).

Treatment

Nedocromil sodium (7.5–30 mg kg^{-1}) and meclizine (30 mg kg^{-1}) were given by the i.p. route, 1 h before the provocation. In some experiments nedocromil sodium was administered i.t. at 10 and 30 μg per cavity. Nedocromil sodium was diluted with sterile saline and meclizine was dissolved in Tween 80 and further diluted with saline. All drugs were prepared immediately before use.

Drugs

PAF (1-O-hexadecyl-2-acetyl- sn-glyceryl-3- phosphorylcholine) was purchased from Bachem (Switzerland); histamine and meclizine were from Sigma (U.S.A.); nedocromil sodium (disodium 6,9,dihydro-9-ethyl-4,6,-dioxo- 10-propyl-4H-pyrano [3,2-g]-guinoline-2,8-dicarboxylate) was kindly provided by Dr A. Norris, from Fisons plc (U.K.).

Statistical analysis

Data are expressed as mean ± standard error mean (s.e.mean) and analysed statistically by means of the analysis of variance (ANOVA) followed by the Newman-Keuls Student's test. In some particular cases the difference in means was analysed by unpaired Student's *t* test. *P* values of 0.05 or less were considered significant.

Results

Inflammatory effects following the intrathoracic administration of PAF

As seen in Table 1, the i.t. administration of PAF (1 μg per cavity) was followed by a marked exudation within 1 h, which was accompanied by a 6 fold increase in the protein extravasation. Fifteen minutes after PAF, a significant reduction in the total leucocyte counts was observed, represented by a decrease of mono- and polymorphonuclear leucocyte counts (Figure 1a,b). The same figure shows that leucocyte numbers were restored to the basal levels 1 h after PAF stimulation. By 6 h, when exudation and protein extravasation were halved (Table 1), the total number of leucocytes recovered from the pleural cavity was at its height ($P < 0.001$) and was represented by an increase of mononuclear cells ($P < 0.02$), eosinophils ($P < 0.001$) and mainly neutrophils ($P < 0.003$) (Figure 1 a,b). Total leucocyte counts (Figure 1) and exudation (Table 1) were back to basal values at 24 h, whereas the eosinophil counts remained significantly increased up to 96 h ($P < 0.001$, d.f. = 81) (Figure 1b).

Table 1 Temporal kinetics of exudate volume and extravasated protein induced by the i.t. injection of PAF (1 μg per cavity)

Groups	*Time* (h)	*Exudation volume* (ml)	*Total protein* (mg)
Sal	1 h	0.08 ± 0.03	3.19 ± 0.39
PAF		0.68 ± 0.03*	19.14 ± 1.53*
Sal	6 h	0.04 ± 0.02	3.04 ± 0.23
PAF		0.40 ± 0.06*	6.22 ± 1.07*
Sal	24 h	0.09 ± 0.03	1.91 ± 0.58
PAF		0.12 ± 0.03	2.32 ± 0.64

Each value represents the mean ± s.e.mean from at least 7 animals; * $P < 0.001$ statistically significant as compared to saline-injected control group by Student's *t* test.

Interference of nedocromil sodium with the effects of PAF and histamine

As illustrated in Table 2, the local treatment with nedocromil sodium (30 μg per cavity) significantly inhibited the pleural exudation and the increase in the total protein content induced by PAF and histamine (200 μg per cavity), 1 h later. In addition, the H_1 anti-histaminic, meclizine, failed to modify PAF-induced exudation under conditions where pleurisy

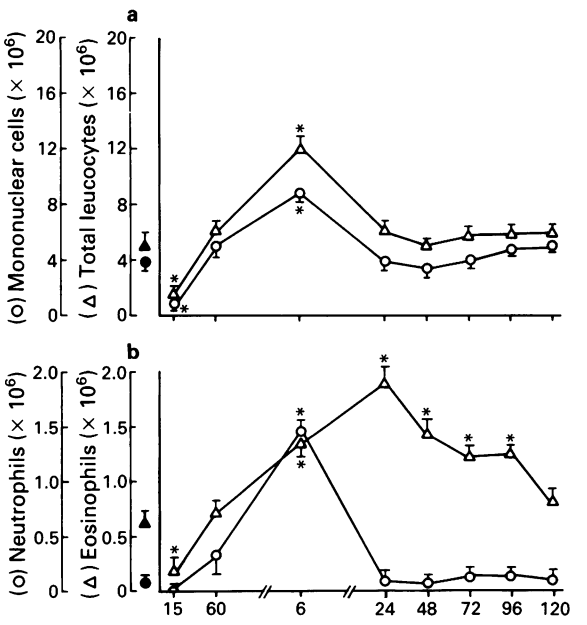

Figure 1 Time course of pleurisy induced by PAF (1 μg per cavity). Total leucocyte and mononuclear cells (a) as well as neutrophil and eosinophil counts (b) are represented in number of cells per pleural cavity. Each point represents the mean from at least 7 animals; vertical bars show s.e.mean. The closed symbols are the mean of all values (at least 40) obtained from saline-injected animals. Statistically significant differences are indicated by an asterisk (* $P < 0.01$).

Table 2 Interference of nedocromil sodium (30 μg per cavity, Ned) and meclizine (30 mg kg^{-1}, Mec) with the exuded volume and extravasated protein caused by PAF and histamine

	Exudate volume (ml)		*Total protein* (mg)	
Treatment	*PAF*	*Hist*	*PAF*	*Hist*
None	0.68 ± 0.03†	0.76 ± 0.07†	19.14 ± 1.53†	20.08 ± 2.32†
Ned	0.42 ± 0.03*	0.38 ± 0.06*	7.31 ± 0.67*	10.88 ± 1.47*
Mec	0.72 ± 0.04	0.11 ± 0.06*	19.51 ± 2.23	4.44 ± 0.84*

Values obtained from saline-injected rats of 0.08 ± 0.03 for exudate volume and of 3.19 ± 0.39 for total protein were considered as basal. Each value represents the mean ± s.e.mean from at least 7 animals. † $P < 0.001$ from the saline-injected group and * $P < 0.001$ from PAF or histamine groups by Student's t test.

induced by histamine was markedly suppressed by its respective antagonist.

Treatment with nedocromil sodium was also effective in inhibiting the increased number of mononuclear cells ($P < 0.001$, d.f. = 21, Figure 2b, neutrophils ($P < 0.001$, d.f. = 17, Figure 2c) and eosinophils ($P < 0.005$, d.f. = 21, Figure 2d) observed 6 h after PAF injection. In addition, as shown in Table 3, nedocromil sodium given i.p., unlike the H_1 blocking agent meclizine, also abolished in a dose-dependent manner the late eosinophil accumulation caused by PAF.

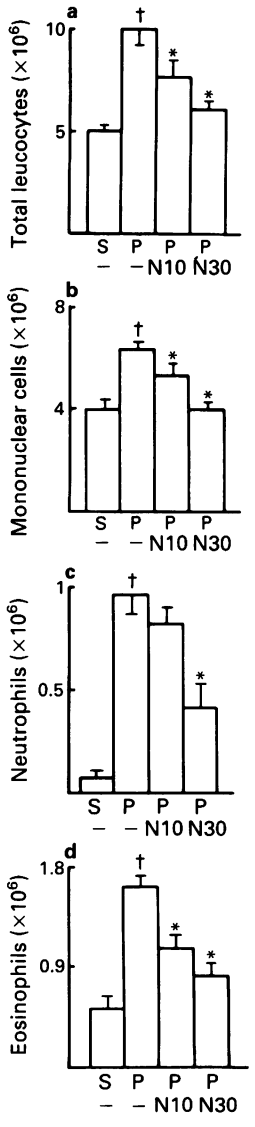

Figure 2 Interference of topical administration of nedocromil sodium (10–30 μg per cavity) with the leucocyte accumulation 6 h after PAF stimulation. Each column represents the mean from at least 7 animals with s.e.mean shown by vertical bars; † $P < 0.001$ from the saline-injected group by Student's t test and * $P < 0.01$ from PAF-injected group by Newman-Keuls Student's test.

Interference of nedocromil sodium with in vitro *eosinophil chemotaxis*

As shown in Table 4, the pre-incubation of rat peritoneal eosinophils with nedocromil sodium (10^{-6} and 10^{-4} M), for

Table 3 Interference of nedocromil sodium with the eosinophil infiltration (24 h) induced by PAF (1 μg per cavity)

Stimulus	*Treatment*	*Dose* (mg kg^{-1})	*Eosinophils per site* (× 10^{-6})	*Inhibition* (%)
Sal	None	—	0.84 ± 0.06	—
PAF	None	—	1.98 ± 0.12†	—
PAF	Ned	7.5	1.72 ± 0.09	22.8
PAF	Ned	15.0	1.48 ± 0.08*	43.8
PAF	Ned	30.0	1.09 ± 0.06*	78.0
PAF	Mec	30.0	1.81 ± 0.09	14.9

Nedocromil sodium (Ned) and meclizine (Mec) were administered i.p., 1 h before the lipid, at the indicated doses. Each value represents the mean ± s.e.mean from at least 8 animals; † $P < 0.001$ from the saline-injected group by Student's t test and * $P < 0.001$ from PAF-injected group by Newman-Keuls Student's test.

Table 4 Interference of nedocromil sodium with the *in vitro* eosinophil chemotaxis induced by PAF

Stimulus	*Treatment* (M)	*Eosinophils/15HPF*
Vehicle	—	25.0 ± 3.5
PAF (10^{-8} M)	—	82.3 ± 2.7
	10^{-6}	78.5 ± 2.4
	10^{-4}	92.3 ± 4.6
PAF (10^{-6} M)	—	115.4 ± 8.6
	10^{-6}	113.5 ± 5.3
	10^{-4}	114.3 ± 3.6

The eosinophils were pre-incubated with nedocromil sodium, for 10 min at 37°C. Each value represents the mean ± s.e.mean from 3 assays. HPF = high power field.

Table 5 Interference of nedocromil sodium (30 μg per cavity) with eosinophil influx in the pleural cavity of recipient, after the treatment of donor or recipient rats

		Treated groups	
Stimulus	*Treatment*	*Donor*	*Recipient*
SPW	Saline	0.58 ± 0.09	0.50 ± 0.06
PPW	Saline	1.54 ± 0.18†	1.20 ± 0.07†
PPW	Nedocromil	0.57 ± 0.07*	1.12 ± 0.16

Numbers represent eosinophil counts (× 10^6) per cavity. The analyses were performed in recipient animals 24 h after the transfer of saline pleural washings (SPW) or PAF pleural washings (PPW). Each value represents the mean ± s.e.mean from at least 7 animals; † $P < 0.05$ from the SPW group and * $P < 0.007$ from PPW group by Student's t test.

10 min at 37°C, did not inhibit the eosinophil migration promoted by PAF (10^{-8} and 10^{-6} M), as shown by 48-Well microchemotaxis chamber assay.

Interference of nedocromil sodium with the generation or expression of eosinophilia from PAF-injected rats to recipient animals

When pleural washings from PAF prestimulated rats were transferred to normal recipient animals, the number of eosinophils recovered from their pleural cavity was doubled within 24 h ($P < 0.05$, Table 5). The topical administration of nedocromil sodium into the pleural cavity of donor rats, followed within 6 h by the transfer of the washing to recipients, suppressed the late eosinophilia (Table 5). In contrast, when nedocromil sodium was administered to recipients, no inhibition of the eosinophil infiltration was observed (Table 5).

Discussion

Nedocromil sodium is the disodium salt of a pyranoquinoline dicarboxylic acid which, though being structurally quite different, shares several properties with the classic anti-allergic drug, sodium cromoglycate (Auty & Holgate, 1989). It has been demonstrated that nedocromil sodium impairs the release of inflammatory mediators from activated mast cells (Riley *et al.*, 1987), and accordingly its efficacy has been attributed to this ability. It has also been shown that nedocromil sodium prevents early and late allergic reactions and allergen-induced bronchial hyperresponsiveness in experimental animals and man (Altounyan *et al.*, 1986; Hutson *et al.*, 1988; Pretolani *et al.*, 1990).

Our findings confirm those of Tarayre *et al.* (1986) and Martins *et al.* (1989), that the intrathoracic injection of PAF into rats leads to acute protein extravasation, maximal at 1 h, declining thereafter. This phenomenon was accompanied by an initial reduction in the pleural leucocyte content, which was over within 6 h. At this time, a significant increase in the number of mononuclear cells, eosinophils and mainly neutrophils was noted. In contrast, 24 h after PAF stimulation, only the eosinophil content was augmented, remaining elevated for up to 96 h.

The topical administration of nedocromil sodium significantly reduced the acute cellular exudate (40%) and protein extravasation (61.8%) induced by PAF, under conditions where the H_1-antagonist, meclizine, was ineffective. Nedocromil sodium thus inhibits PAF-induced exudation by a mechanism other than antagonism of histamine receptors or of inhibition of amine release. Furthermore, the cellular influx into the pleural cavity at 6 h, as well as the late eosinophilia induced by PAF, were significantly suppressed by topical treatment with nedocromil sodium. Since the latter's efficacy has also been demonstrated against cellular activation (Moqbel *et al.*, 1986), we hypothesized that nedocromil sodium might inhibit the cellular influx by acting directly upon the leucocytes. As previously reported (Wardlaw *et al.*, 1986; Martins *et al.*, 1989), PAF is also chemotactic for eosinophils *in vitro*. However, in our experiments, the preincubation of the eosinophils with nedocromil sodium at 10^{-6} and 10^{-4} M, did not prevent the chemotaxis induced by the lipid. These results rule out the possibility that the effectiveness of nedocromil sodium in abrogating the late eosinophil recruitment might be related to a direct effect on eosinophils.

We have recently demonstrated that the eosinophil accumulation triggered by PAF, at 24 h, depends on the synthesis of an intermediate factor, probably a protein which is completely abrogated by its co-administration with protein synthesis inhibitors (Silva *et al.*, 1991). After the transfer of the pleural washing from donors stimulated with PAF to recipients, the latter presented a 2 fold increase in the pleural eosinophil content after 24 h (Silva *et al.*, 1989). In order to clarify the mode of action of nedocromil sodium, we pretreated donor or recipient animals with this drug before transferring the pleural fluid. Under these conditions, nedocromil sodium inhibited the transferred eosinophilia when administered to the donor, and no protection was observed when the recipient animals were treated. These results are consistent with the interpretation that the efficacy of nedocromil sodium relates to its ability to inhibit the generation of the eosinophilotactic factor rather than the effects of this material.

In conclusion, our findings indicate that nedocromil sodium prevents PAF-induced pleurisy in rats by a mechanism independent of its recognized property of antagonizing H_1-histamine receptors and possibly via its ability to impair the formation of the eosinophilotactic activity generated by PAF.

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Pharmacological characterization of a receptor for calcitonin gene-related peptide on rat, L6 myocytes

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1 The L6 myocyte cell line expresses high affinity receptors for calcitonin gene-related peptide (CGRP) which are coupled to activation of adenylyl cyclase. The biochemical pharmacology of these receptors has been examined by radioligand binding or adenosine 3':5'-cyclic monophosphate (cyclic AMP) accumulation.

2 In intact cells at 37°C, human and rat α- and β-CGRP all activated adenylyl cyclase with EC_{50}s of about 1.5 nM. A number of CGRP analogues containing up to five amino acid substitutions showed similar potencies. In membrane binding studies at 22°C in 1 mM Mg^{2+}, the above all bound to a single site with IC_{50}s of 0.1–0.4 nM.

3 The fragment CGRP(8–37) acted as a competitive antagonist of CGRP stimulation of adenylyl cyclase with a calculated K_d of 5 nM. The K_d determined in membrane binding assays was lower (0.5 nM).

4 The N-terminal extended human α-CGRP analogue Tyr^0-CGRP activated adenylyl cyclase and inhibited [^{125}I]-iodohistidyl-CGRP binding less potently than human α-CGRP (EC_{50} for cyclase = 12 nM, IC_{50} for binding = 4 nM).

5 The pharmacological profile of the L6 CGRP receptor suggests that it most closely resembles sites on skeletal muscle, cardiac myocytes and hepatocytes. The L6 cell line should be a stable homogeneous model system in which to study CGRP mechanisms and pharmacology.

Keywords: Calcitonin gene-related peptide receptors; CGRP(8–37); amylin; ligand binding; adenylyl cyclase

Introduction

Calcitonin gene-related peptide (CGRP) is a widely-distributed 37 amino acid peptide localized to peripheral sensory and motor nerves (Goodman & Iversen, 1986). CGRP, a very potent vasodilator (in part due to its direct effect on vascular smooth muscle, Brain *et al.*, 1985; Greenberg *et al.*, 1987), also has a number of documented actions on skeletal muscle. At the biochemical level, it can activate adenylyl cyclase, both in isolated muscles and primary myocytes (Laufer & Changeux, 1987; Miles *et al.*, 1989). At the functional level, CGRP stimulates the synthesis and expression of nicotinic acetylcholine receptors (New & Mudge, 1986; Fontaine *et al.*, 1986); enhances the rate of desensitization of this receptor (Mulle *et al.*, 1988) and inhibits insulin-stimulated glycogen synthesis (Leighton & Cooper, 1988) and/or insulin-stimulated glucose transport (Hothersall *et al.*, 1990). The metabolic effects of CGRP on skeletal muscle are shared with amylin, a 37 amino acid peptide which has 46% homology with CGRP. Amylin, originally identified in amyloid plaques in the pancreatic islets of Type II diabetics, may be a circulating β-cell hormone controlled by blood glucose levels. Moreover, amylin itself has been implicated in the pathogenesis of Type II (insulin-resistant) diabetes (Cooper *et al.*, 1988; Ohsawa *et al.*, 1989; Nishi *et al.*, 1990). Thus, an emerging issue is whether amylin and CGRP act on common or distinct receptors, possibly with some degree of cross-talk (Morishita *et al.*, 1990; Nishi *et al.*, 1990). Although the pharmacology of CGRP receptors has been approached before (Chiba *et al.*, 1989; Dennis *et al.*, 1989; Zaida *et al.*, 1990) it is timely to address this in detail because certain CGRP fragments can act as antagonists (Chiba *et al.*, 1989; Dennis *et al.*, 1990), opening the way to a more thorough pharmacological characterization of receptors than has been previously possible. Indeed, these putative antagonists may distinguish between subtypes of CGRP/amylin receptors.

One of the best ways of simplifying the biology of ligand-receptor interactions is to investigate these events in a clonal cell line (Hanley, 1987). Responses can be studied in a single cell type under defined conditions and this may be the only practical way to study phenomena which require methods such as chronic labelling. A preliminary characterization of a CGRP receptor on L6 skeletal muscle cells has been published by Kreutter *et al.* (1989). We have independently identified the CGRP receptor on this cell line, and present a detailed characterization including its structural requirements for ligand recognition and closely-coupled signalling events. Moreover, as L6 cells express many differentiated properties of skeletal muscle, they may prove a model for both CGRP receptor biology and CGRP actions on developing and mature muscle.

Methods

Cell culture

L6 myocytes (European Culture Collection, Porton) were maintained as monolayers in antibiotic-free Dulbecco's modified Eagle's Medium (DMEM)/10% (v/v) foetal calf serum passaging every 3–4 days (~80% confluence). They were grown to confluence in 6 or 24 well plates (cyclic AMP accumulation in intact cells) or 15 cm Petri dishes (membrane preparations). Other cell lines were from either the European Culture Collection or the American Type Culture Collection, and were maintained according to the suppliers instructions.

Adenosine 3':5'-cyclic monophosphate (cyclic AMP) accumulation

Cells were stimulated for 5 min with peptide in DMEM/10% foetal calf serum, at 37°C. Incubations were terminated by putting the cells on ice, and replacing the medium with an equal volume of ice-cold 20 mM Tris, 5 mM EDTA, pH 7.7. The cells were boiled in this for 5 min, and then 50 μl aliquots were

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taken for assay of cyclic AMP. This was done following the method of Gilman (1970). Briefly, the sample was mixed with 50 μl of 20 nM [^{3}H]-cyclic AMP and 100 μl of 0.1 mg ml^{-1} of cyclic AMP-dependent protein kinase and incubated for 24 h. Protein-bound [^{3}H]-cyclic AMP was removed by addition of 100 μl of a 5% (w/v) charcoal suspension. Following centrifugation to pellet the charcoal, 185 μl of the supernatant was removed and counted to measure the radioligand remaining in solution. A standard curve was constructed with cyclic AMP solutions varying in concentration from 10 nM to 1 nM.

To measure stimulation of adenylyl cyclase in membranes, cells were homogenized by twelve up and down strokes with a teflon pestle, in 30 mM KCl, 4.5 mM $MgSO_4$, 25 mM Tris, 0.01% bovine serum albumin (BSA), pH 7.6 (10 ml buffer/15 cm plate). The homogenate was spun at 35 000 g for 30 min, and the pellet was resuspended by six up and down strokes with a teflon pestle in 1/10th the volume of the above buffer. From the resuspended pellet, 25 μl was taken and added to a final volume of 100 μl containing 3 mM ATP, 10 mM $MgCl_2$, 2 mM phosphocreatine, 50 u ml^{-1} creatine kinase and 2 mM 3-isobutyl-1-methyl-xanthine (IBMX). This was incubated with drugs for 15 min at 37°C, and then boiled for 5 min. Following this, 50 μl aliquots were assayed for cyclic AMP as above.

Radioligand binding

Membranes were prepared from confluent plates of L6 cells by homogenizing the cells in ice-cold 20 mM Tris pH 7.6 (7.5 ml/15 cm plate) and centrifuging at 35 000 g for 30 min. The pellet was resuspended in an equal volume of 1 mM $MgCl_2$, 0.3% (w/v) protease-free BSA, 20 mM Tris pH 7.6 and 0.5 ml was incubated with 10 pM [^{125}I]-iodohistidyl-CGRP for 10 min at room temperature together with competing ligands, in Eppendorf microcentrifuge tubes. Incubations were terminated by centrifugation (Beckman microcentrifuge, 5 min), and rinsing pellets twice with water. Bound radioactivity was determined by γ-counting. Non-specific binding was defined with 1 μM unlabelled human α-CGRP. Certain experiments indicated in the text were carried out at 37°C with 40 pM [^{125}I]-iodohistidyl-CGRP, incubated with membranes for 10 min in 10 mM $MgCl_2$, 10 mM NaCl, 0.3% BSA, 20 mM Tris pH 7.6. Other details were the same as described above for experiments done at 25°C.

Inositol phosphate accumulation

This was carried out as described by Jackson & Hanley (1989). Briefly cells were labelled overnight in inositol-free DMEM, with 10 nCi ml^{-1} of [^{3}H]-inositol (Amersham). Cells were preincubated for 10 min with 10 mM LiCl and then challenged with drugs for 30 min. Incubations were terminated with 20% trichloroacetic acid, the supernatants neutralised with freon: tri-*n*-octylamine, and inositol phosphates separated on Dowex columns.

Handling of calcitonin gene-related peptide

CGRP is readily adsorbed onto plastic. To minimize losses in handling, all dilutions were made into buffers containing 0.3% protease-free BSA. All plasticware used in handling CGRP (i.e. pipette tips, Eppendorf tubes) were pretreated with Sigmacote to prevent adsorption. It was particularly important to treat the Eppendorf tubes used in the binding assays to prevent non-specific binding to the plastic. The CGRP solutions were stored as frozen aliquots at −20°C and were rethawed two or three times before being discarded.

Data handling

Both binding and cyclic AMP accumulation data were initially fitted to a logistic (Hill) function of the form bound (effect) = $B_{max}X^{n_H}/(X^{n_H} + C^{n_H})$ where B_{max} is the capacity (or maximum effect), C is the IC_{50} (or EC_{50}), n_H is the Hill coefficient and X is the free drug concentration. For radioligand displacement studies the equation was modified to include non-specific binding (Aceves *et al.*, 1985). Best values ± standard errors of the parameters were obtained from non-linear regression analysis using the Harwell library routine VB∅1A. It was assumed that these parameters were normally distributed. Statistical evaluation of data was by t tests. Within any single experiment, each measurement of bound CGRP or cyclic AMP was made in duplicate. Each experiment was repeated 3 to 5 times.

Drugs

[^{125}I]-iodohistidyl-human α-CGRP, 2000 Ci mmol^{-1}, was obtained from Amersham, Bucks. Human α-CGRP, CGRP(8–37), CGRP analogues referred to in the text were gifts from Celltech Ltd. Other peptides were obtained from Bachem (amylin, human β-CGRP), or Peninsula (salmon calcitonin, rat α/β-CGRP, Tyr^0-CGRP). Protease free bovine serum albumin, Sigmacote and cyclic AMP-dependent protein kinase were obtained from Sigma. IBMX was from Aldrich, and guanylyl imidodiphosphate (GppNHp) was from Boehringer Mannheim.

Results

Evaluation of established cell lines for functional CGRP receptors

A variety of cell lines were challenged with 10^{-8} M human α-CGRP and changes in cyclic AMP or inositol phosphate levels were evaluted. The AR4-2J (rat exocrine pancreas), TE 671 (human rhabdomyosarcoma, skeletal muscle-like), A7r5 (rat vascular smooth muscle), BC3-H1 (mouse vascular smooth muscle) and H9c2 (rat heart myoblast) cell lines showed no biochemical response to human α-CGRP. However, in the L6 cell line (rat skeletal muscle) CGRP stimulated an increase in cyclic AMP, suggesting the presence of a functional CGRP receptor coupled to activation of adenylyl cyclase.

Characterization of the cyclic AMP response

The elevation of cyclic AMP by human α-CGRP was demonstrated both in intact L6 cells, and washed membranes (Figures 1 and 7), indicating that activation of adenylyl cyclase is a primary response to the agonist-receptor interaction. In both washed membranes and intact cells the maximal response to CGRP is similar in magnitude to the maximum produced by addition of isoprenaline, a β-adrenoceptor agonist. In intact cells CGRP increased the cyclic AMP to approximately 200 pmol per 10^6 cells from a basal level of 10 pmol per 10^6 cells. The dose-response curve to human α-CGRP in intact cells had a steep slope (Hill coefficient 1.9) with an EC_{50} of 1.54 nM (Table 1). In membranes the dose-response curve was less steep ($n_H = 0.83 \pm 0.13$), and human α-CGRP was slightly less potent ($EC_{50} = 4.7 \pm 1.1$ nM). The accumulation of cyclic AMP in intact cells reaches a maximum by 5 min, which is followed by a slow decline lasting several hours (data not shown).

Binding of [^{125}I]-iodohistidyl-CGRP

Preliminary experiments indicated that the specific binding of [^{125}I]-iodohistidyl-CGRP (radioiodinated from human α-CGRP) was linear with increasing membrane protein concentration and was destroyed by boiling for 10 min. Specificity of binding was investigated by examining the ability of a range of peptides to displace [^{125}I]-iodohistidyl-CGRP. Bradykinin, vasopressin, angiotensin II, substance P, endothelin, atrial natriuretic peptide and galanin were all inactive at

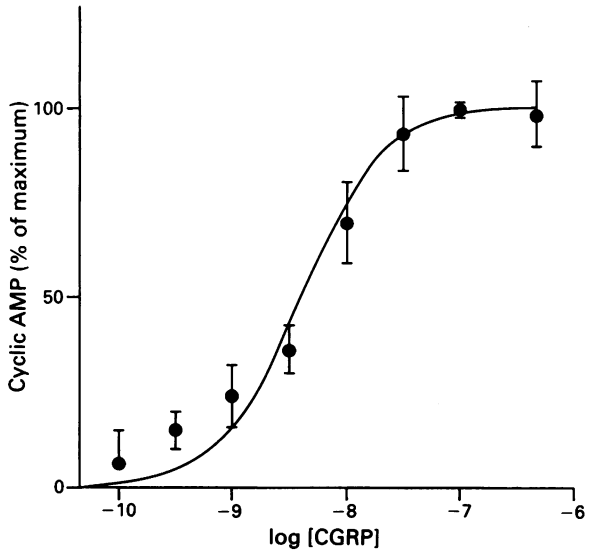

Figure 1 Dose-dependence of adenylyl cyclase stimulation by human α-calcitonin gene-related peptide (α-CGRP) in washed cell membranes (●) at 37°C in 10 mM $MgCl_2$. Responses have been normalised to percentages of maximum (10 μM human α-CGRP). Each point represents mean of 3 separate experiments; s.e.mean shown by vertical bars. Fitted parameters were $EC_{50} = 4.7 \pm 1.1$ nM, $n_H = 0.83 \pm 0.13$. At 100%, the absolute activity was 80 ± 15 pmol cyclic AMP formed mg^{-1} protein $5min^{-1}$. Basal cyclic AMP levels were less than 10 pmol cyclic AMP mg^{-1} protein. Isoprenaline produced a maximal activity of 100 pmol cyclic AMP formed mg^{-1} protein $5min^{-1}$.

1 μM, whereas salmon calcitonin (1 μM) inhibited 63 ± 3% of the specific binding of the radioligand. Specific binding was optimal in 1 mM $MgCl_2$, 20 mM Tris: replacement of the Mg^{2+} by 10 mM EDTA reduced this by 62 ± 10%. The specific binding was sensitive to increasing the Mg^{2+} concentration (5 mM Mg^{2+} reduced binding by 57 ± 12%), or addition of NaCl (100 mM decreased binding by 53 ± 2%). The kinetics of binding of 10 pM radioligand in 1 mM $MgCl_2$ is shown in Figure 2. The association and dissociation were rapid, with $t_{1/2}$ values of approximately 1–2 min. Binding was reversible and stable for up to 30 min at 25°C in the absence of protease inhibitors (Figure 2). Routine assays were accordingly carried out for 10 min. Under these conditions a typical binding assay (with 0.5–0.2 mg of protein) would have approximately 5000–3000 d.p.m. total binding, of which 40% would be non specific (as defined by 1 μM unlabelled human α-CGRP).

The direct binding of [^{125}I]-iodohistidyl-CGRP is shown in Figure 3. Binding could not be measured at ligand concentrations greater than 200 pM, due to high (75% of total) non-specific binding. The binding curve has a Hill coefficient of 0.89 ± 0.10 implying that the binding at these concentrations of iodohistidyl-CGRP is to a single site, with a K_d of

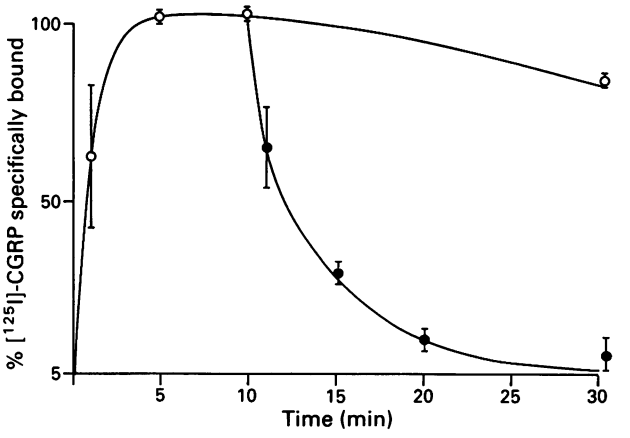

Figure 2 Time course of association (○) and dissociation (●) of 10 pM [^{125}I]-iodohistidyl-human α-calcitonin gene-related peptide (α-CGRP) to L6 membranes in 1 mM $MgCl_2$, 20 mM Tris, pH 7.6 at 25°C. After 10 min, the incubation was split, and the dissociation of the radioligand was measured after the addition of 1 μM unlabelled CGRP to one portion. Each point represents mean of 3 separate experiments with s.e.mean shown by vertical bars. At 100% specific binding, the total d.p.m. bound per assay was 5100, of which 2200 was due to non-specific binding.

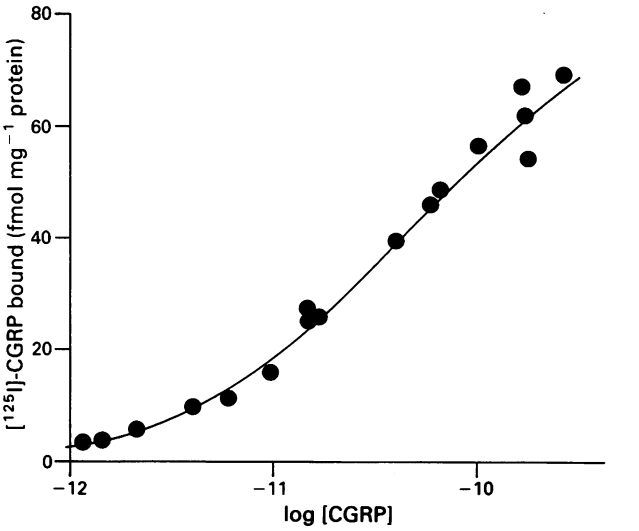

Figure 3 Binding of [^{125}I]-iodohistidyl-human α-calcitonin gene-related peptide (α-CGRP) to L6 membranes in 1 mM $MgCl_2$, 20 mM Tris, pH 7.6 at 25°C. Points were fitted to a curve with $K_d = 43 \pm 14$ nM, Hill coefficient 0.84 ± 0.10, and capacity of 75 ± 8 pmol receptor mg^{-1} protein. Each point represents the mean of a duplicate determination, and the data are from four separate experiments.

Table 1 Potencies of calcitonin gene-related peptide (CGRP) forms and CGRP(8-37) on stimulation of adenylyl cyclase and inhibition of radioligand binding

		Stimulation of adenylyl cyclase		*Inhibition of radioligand binding*	
Form	*Sequence*	EC_{50} (nM)	n_H (*n*)	IC_{50} (nM)	n_H (*n*)
Human α	Ala_1-Asp_3-Val_{22}-Asn_{25}-Lys_{35}	1.54 ± 0.16	1.92 ± 0.16 (3)	0.12 ± 0.01	1.03 ± 0.15 (3)
Human β	Asn_3-Met_{22}-S_{25}	1.29 ± 0.15	1.80 ± 0.15 (3)	0.20 ± 0.04	0.84 ± 0.08 (4)
Rat α	Ser_1-Asn_3-Asp_{25}-Glu_{35}	1.41 ± 0.17	1.63 ± 0.19 (4)	0.30 ± 0.08	0.91 ± 0.16 (3)
Rat β	Ser_1-Asn_3-Asp_{25}	1.36 ± 0.13	1.59 ± 0.11 (4)	0.27 ± 0.02	0.81 ± 0.04 (4)
CGRP(8-37)		$K_d = 4.1$ nM (4)		0.70 ± 0.19	0.90 ± 0.09 (5)

Structures show amino acid changes relative to human α-CGRP, Ala-Cys-Asp-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-PheNH_2. n_H = Hill coefficient. Since CGRP(8–37) is an antagonist, the K_d value is quoted instead of the EC_{50}. Stimulation of adenylyl cyclase was measured in intact cells at 37°C, and radioligand binding to membranes was measured at 22°C. Each value represents the mean ± s.e.mean of *n* separate experiments.

43 ± 14 pM, and a capacity of 75 ± 8 pmol receptor mg^{-1} protein. Competition against radioligand (10 pM) by varying concentrations of human α-CGRP was also investigated (Figure 5). This curve also had a Hill slope close to unity (1.03 ± 0.15), with an IC_{50} of 126 ± 14 pM, which after correction for receptor occupancy gives a K_d of 102 pM. This is close to the value obtained for the iodinated radioligand in the direct binding experiments, suggesting that iodination increased the affinity of CGRP for its receptor only slightly. At 37°C, in a buffer containing 10 mM Mg^{2+}, the potency of human α-CGRP was decreased (IC_{50} = 6.6 nM) (Figure 4); these conditions are comparable to those used to measure adenylyl cyclase activation in broken membranes where the EC_{50} for CGRP was 4 nM. The involvement of a G-protein in modulating ligand binding was investigated by adding 100 μM GppNHp. This reduced the binding of 10 pM [^{125}I]-CGRP by 38 ± 5%, suggesting that the high affinity binding was in part due to the formation of a receptor-G-protein complex.

Characterization of the receptor by CGRP fragments and analogues

The structure-activity relationships of CGRP and a variety of CGRP fragments and analogues were examined. The results are shown in Figures 5 and 6, Tables 1 and 2. Effects on radioligand binding and cyclic AMP accumulation were compared.

The effects of four naturally-occurring CGRP forms (rat and human α and β forms) on cyclic AMP accumulation are shown in Table 1. These forms differ from each other by at most five amino acid variations. They are all full agonists with steep dose-response curves for cyclic AMP elevation (Hill coefficients: 1.6–1.9), and similar potencies (EC_{50}: 1.3–1.7 nM). The CGRP forms behave very similarly in binding studies (IC_{50}: 0.1–0.3 nM; Hill coefficients: 0.8–1.03).

Other CGRP analogues were tested in both assays. These contain up to five amino acid substitutions relative to human α-CGRP. Tyr°-CGRP contains an extra tyrosine residue on the N-terminus. Most of the rest can be considered as rat and human α-β hybrids, although chick CGRP was also tested. Their structures relative to human α-CGRP, together with their potencies in stimulating adenylyl cyclase and inhibiting [^{125}I]-iodohistidyl-CGRP binding are shown in Table 2. Tyr°-CGRP showed the larger changes, with the IC_{50} for radioligand displacement reduced nearly 40 fold (4 nM), and the EC_{50} for stimulation of the cyclase reduced to 12 nM. The other analogues all had similar potencies to human or rat CGRP in both assays. (Figure 6, Table 2). To extend these studies, a series of more extensively modified CGRP analogues and fragments were examined.

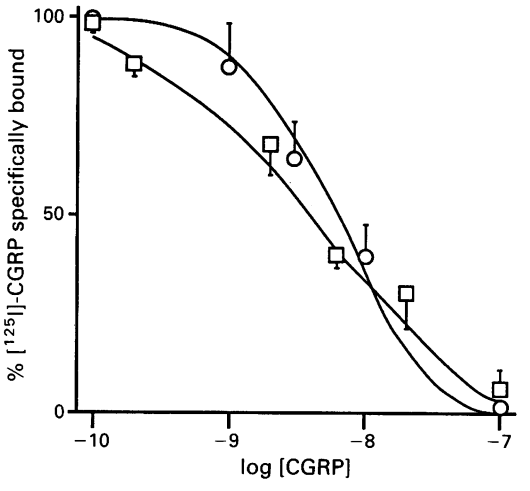

Figure 4 Inhibition of the binding of 40 pM [^{125}I]-iodohistidyl-human α-calcitonin gene-related peptide (α-CGRP) to L6 membranes in 10 mM $MgCl_2$, 10 mM NaCl, 20 mM Tris, pH 7.6 at 37°C by human α-CGRP (○) and CGRP(8–37) (□). Fitted parameters were: human α-CGRP IC_{50} = 6.6 ± 1.2 nM, n_H = 1.2 ± 0.2 and CGRP(8–37) IC_{50} = 4.0 ± 0.4 nM, n_H = 0.71 ± 0.05. Each point represents the mean of 3 separate experiments; vertical bars show s.e.mean. 100% specific binding represents 900 d.p.m. bound per assay with 600 d.p.m. non-specific binding.

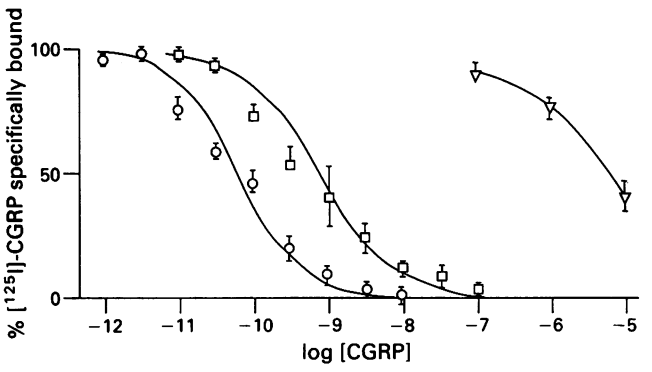

Figure 5 Inhibition of the binding of 10 pM [^{125}I]-iodohistidyl-human α-calcitonin gene-related peptide (α-CGRP) to L6 membranes in 1 mM $MgCl_2$, 20 mM Tris, pH 7.6 at 25°C by human α-CGRP (○), CGRP(8–37) (□), and CGRP (13–25) (△). Fitted values were: human α-CGRP IC_{50} = 0.12 ± 0.01 nM, n_H = 1.03 ± 0.15 and CGRP(8–37) IC_{50} = 0.70 ± 0.19 nM, n_H = 0.90 ± 0.09. The data for CGRP(13–25) were fitted by eye. Each point represents the mean of 3–5 separate experiments; s.e.mean shown by vertical bars. 100% specific binding represents 1800 d.p.m. per assay, with 1200 d.p.m. per assay as non-specific binding.

Table 2 Potencies of calcitonin gene-related peptide (CGRP) analogues on adenylyl cyclase and inhibition of radioligand binding

	Stimulation of cyclase	*Inhibition of [^{125}I]-CGRP binding*	
Structure	EC_{50} (nM)	IC_{50} (nM)	n_H
Ser_1	5.0 ± 0.9	0.26 ± 0.04	0.77 ± 0.05
Asn_3	1.0 ± 0.1	0.46 ± 0.07	0.82 ± 0.07
Met_{22}	1.3 ± 0.2	0.24 ± 0.06	0.82 ± 0.10
Ser_{25}	1.0 ± 0.1	0.15 ± 0.04	0.88 ± 0.15
Gly_{35}	1.9 ± 0.1	0.17 ± 0.06	0.88 ± 0.23
Asn_3-Met_{22}	1.0 ± 0.1	0.32 ± 0.04	0.84 ± 0.04
Asn_3-Ser_{25}	0.5 ± 0.1	0.15 ± 0.04	0.95 ± 0.09
Met_{22}-Ser_{25}	2.3 ± 0.4	0.17 ± 0.03	0.73 ± 0.09
Asn_3-Ile_{22}-Ser_{25}	3.0 ± 0.2	0.12 ± 0.02	1.13 ± 0.12
Asp_{14}-Phe_{15}	3.1 ± 0.5	0.23 ± 0.03	0.72 ± 0.11
Tyr°-	12.0 ± 0.5	4.0 ± 1.2	0.72 ± 0.07
Des Phe-NH_2	> 1 μM	> 1 μM	
Des Ala-Phe-NH_2	> 1 μM	> 1 μM	

Each value represents mean ± s.e.mean of data from 3 (binding) or 4 (cyclase) experiments. Radiolabelled CGRP was present at 10 pM. Structures show amino acid changes relative to human α-CGRP. The fragments CGRP(13–25), CGRP(28–37), CGRP(29–37), CGRP(30–37) at concentrations of 1 μM did not stimulate adenylyl cyclase nor did they antagonize the action of 10 nM CGRP on adenylyl cyclase activity. Their IC_{50}s in competition with 10 pM [^{125}I]-iodohistidyl-human α-CGRP at binding to L6 membranes were greater than 1 μM.

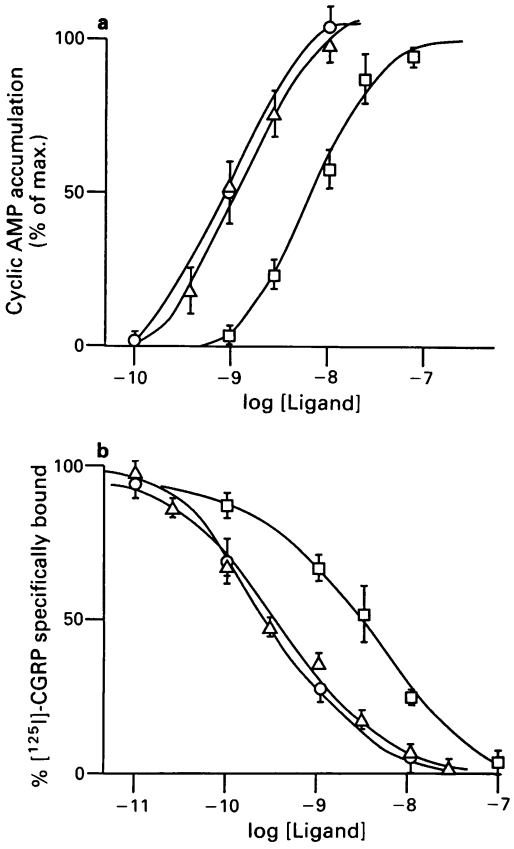

Figure 6 (a) Activation of adenylyl cyclase in intact cells in DMEM at 37°C by Asn_3-Met_{22}-CGRP (○), Tyr^0-CGRP (□) and rat α-CGRP (△). Cyclic AMP accumulation was measured after 5 min stimulation with drug. At 100% the absolute accumulation was 200 pmol cyclic AMP formed per 10^6 cells. Basal cyclic AMP levels were no more than 10 pmol per 10^6 cells. Fitted parameters were: Asn_3-Met_{22}-CGRP $EC_{50} = 1.0 \pm 0.1$ nM, $n_H = 1.5 \pm 0.2$; Tyr^0-CGRP $EC_{50} = 12.0 \pm 0.5$ nM, $n_H = 1.8 \pm 0.2$ and rat α-CGRP $EC_{50} = 1.4 \pm 0.2$ nM, $n_H = 1.6 \pm 0.2$. Each point represents mean of 4 separate experiments; s.e.mean shown by vertical bars. (b) Inhibition of the binding of 10 pM [^{125}I]-iodohistidyl-human α-CGRP to L6 membranes in 1 mM $MgCl_2$, 20 mM Tris pH 7.6 at 25°C by Asn_3-Met_{22}-CGRP (○), Tyr^0-CGRP (□), and rat α-CGRP = (△). Fitted parameters were: Asn_3-Met_{22}-CGRP $IC_{50} = 0.32 \pm 0.04$ nM, $n_H = 0.84 \pm 0.04$; Tyr^0-CGRP $IC_{50} = 4.0 \pm 1.2$ nM, $n_H = 0.72 \pm 0.07$ and rat α-CGRP $IC_{50} = 0.30 \pm 0.08$, $n_H = 0.91 \pm 0.16$. Each point represents the mean of 3 separate experiments; s.e.mean shown by vertical bars. 100% specific binding in a typical experiment represents 2000 d.p.m., with 1000 d.p.m. non-specific binding.

Three batches of human amylin (Bachem) were inactive at stimulating adenylyl cyclase, and inhibiting CGRP binding, despite showing activity in independent amylin bioassays (relaxation of porcine coronary artery, B. Hughes and R. Foulkes, personal communication; stimulation of adenylyl cyclase in CHO cells, C. D'Santos and D. Poyner, unpublished observations). A single batch from Bachem caused a weak stimulation of adenylyl cyclase ($EC_{50} = 67 \pm 15$ nM; maximal effect, 60% of that seen with 10 nM CGRP) and inhibited [^{125}I]-CGRP binding only at high concentration ($IC_{50} = 320 \pm 10$ nM). Despite batch variation, human amylin is unequivocally much lower in potency than CGRP molecular forms on the L6 cell CGRP-sensitive receptor.

Four classes of human α-CGRP fragments were used: 13–25 corresponds to the middle of the molecule; 28–37, 29–37 and 30–37 are C-terminal fragments; des Ala-NH_2 and des Phe-Ala-NH_2 lack the last one and two C-terminal amino acids respectively; and 8–37 lacks the N-terminus. These were all tested in both binding and cyclase assays, with results shown in Tables 1 and 2, Figures 5 and 7. None was able to stimulate adenylyl cyclase, and CGRP(13–25), and the C-terminal fragments and truncations were poor inhibitors of [^{125}I]-iodohistidyl-CGRP binding. However, CGRP(8–37) was able to act as a competitive antagonist of CGRP on the functional assay of adenylyl cyclase activation; (Schild plot slope close to unity: 0.96 ± 0.07; estimated $K_d = 5$ nM, see Figure 7). CGRP-(8–37) was also a potent inhibitor of [^{125}I]-iodohistidyl-CGRP binding, (IC_{50} 0.7 nM; $K_d = 0.55$ nM; Hill coefficient: 0.90 ± 0.09). At 37°C in 10 mM Mg^{2+}, the IC_{50} (4.0 ± 0.4 nM), was nearly identical to the K_d estimated from results obtained with intact cells in physiological saline at 37°C (Figure 4). These results confirm that CGRP (8–37) is a competitive antagonist of CGRP-sensitive sites and responses, and interacts with a single class of sites in L6 muscle cells.

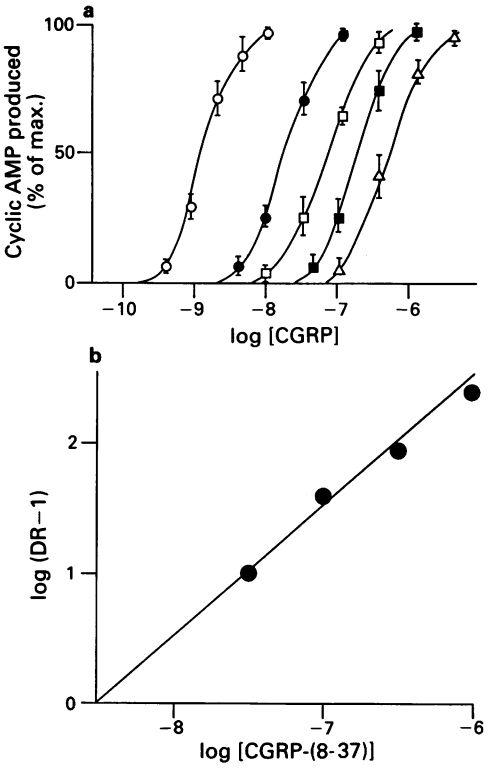

Figure 7 Activity of calcitonin gene-related peptide (8–37) (CGRP-(8–37)). (a) Action of human α-CGRP on intact L6 cells in DMEM at 37°C to activate adenylyl cyclase. Cyclic AMP accumulation was measured after 5 min. At 100%, the absolute activity was 150 pmol cyclic AMP formed per 10^6 cells, with basal levels not more than 10 pmol per 10^6 cells. Human α-CGRP present alone (○); 10^{-8} M CGRP(8–37) present (●); 3×10^{-8} M CGRP(8–37) present (□); 10^{-7} M CGRP(8–37) present (■); 3×10^{-7} M CGRP(8–37) present (△). Each point represents mean of 3 or 4 separate experiments; s.e.mean shown by vertical bars. (b) Data of (a) presented as a Schild plot, with a fitted line of the form $y = (0.96 \pm 0.07) \times +(8.3 \pm 0.4)$. Assuming competitive antagonism, so that the slope can be regarded as unity, this leads to an estimate for the pA_2 value of 8.3 ± 0.4 (i.e. $K_d = 5$ nM).

Binding of [^{125}I]-iodohistidyl-CGRP to intact cells

It is possible to demonstrate the binding of 100 pM [^{125}I]-iodohistidyl-CGRP to intact cells. After a 1 h incubation in phosphate buffered saline supplemented with 0.3% BSA at 35°C, approximately 3000 d.p.m. total binding was observed per 10^6 cells. Of this, 1500 d.p.m. was non-specific. However, the affinity of human α-CGRP is low as assessed by inhibition studies ($IC_{50} \sim 100$ nM), suggesting the receptors in their native environment may be preferentially in a low-affinity state.

Discussion

It is recognised that the rat L6 cell line has characteristics in common with skeletal muscle myoblasts. Recently L6 cells

have been reported (Kreutter et al., 1989) to bind [^{125}I]-CGRP and to respond to CGRP by activation of adenylyl cyclase. We have confirmed and extended these observations, and have focused on the structure-function requirements of CGRP peptides, including comparisons with salmon calcitonin and amylin.

CGRP activation of adenylyl cyclase implies this is the primary transduction event following receptor recognition and is not secondary to a change in another messenger system. It is likely therefore that the receptor is coupled to one form of Gs, the GTP binding protein (G-protein) responsible for stimulating adenylyl cyclase. This notion is supported by radioligand binding being partially inhibited by a non-hydrolysable GTP analogue, guanylyl-imidodiphosphate (GppNHp); which would be expected to dissociate receptor-G-protein complexes, leading to loss of high affinity agonist binding. The inhibition of this binding by the cation chelator EDTA and 100 mM NaCl are also recognised characteristics of a receptor-G-protein complex (Hulme et al., 1983; Poyner, 1990). The CGRP binding site of rat liver plasma membranes has been shown to be sensitive to GppNHp (Yamaguchi et al., 1988a). Further work is needed to examine the receptor-G-protein interaction.

The affinity constants for CGRP binding and the EC_{50}s for activation of adenylyl cyclase lie within the range (0.01–10 nM) obtained in other tissues e.g. liver (Yamaguchi et al., 1988b), gastric smooth muscle (Maton et al., 1988). In most cases the radioligand appears to bind to a single population of sites although Yoshizaki et al. (1987) demonstrated high ($K_d \sim 10^{-11}$ M) and low ($K_d \sim 10^{-8}$ M) affinity binding sites in membranes made from rat brain and heart ventricles. Agonist binding to G-protein coupled receptors is known to be complex because measured affinity constants are sensitive to ionic conditions, which can modulate receptor agonist affinity either by acting at the level of the receptor or G-protein. Additionally the state of G-protein coupling may be subject to tissue-specific regulation. Nevertheless, the L6 cell receptor is identifiably similar to other CGRP receptors as regards its interactions with the naturally-occurring CGRP forms, and therefore can be properly regarded as a simplified, homogeneous and stable model of CGRP receptors and cognate signalling pathways.

The fragment CGRP(8–37) antagonizes the effects of CGRP on rat liver plasma membranes (Chiba et al., 1989), where it appears to have a K_d of about 10 nM (although in binding studies it has a K_i of 98 nM), antagonizes the actions of CGRP on the rat mesenteric vascular bed (Han et al., 1989), and antagonizes *in vivo* haemodynamic effects of CGRP (Han et al., 1989; Gardiner et al., 1990). The related fragment CGRP(12–37) binds with high affinity to brain and spleen membranes, antagonizes the actions of CGRP in the guinea-pig right atrium but has no effects on the rat vas deferens (Dennis et al., 1989). Assuming a similar spectrum of activities for these antagonists, this implies that CGRP fragments may subdivide 'CGRP receptors' into at least two classes. By this criterion the L6 CGRP receptor most resembles those found on hepatocytes, neurones, cardiac, skeletal and vascular smooth muscle, but is distinct from CGRP receptors found on extravascular smooth muscle. The fragment CGRP(28–37) was inactive in this study. It is interesting that the related fragment Tyr^0-CGRP(28–37) at 1 μM can antagonize the actions of CGRP on the opossum internal anal sphincter (Chakder & Rattan, 1990).

A further pharmacological feature which distinguishes the L6 receptor is that it recognises the extended CGRP analogue Tyr^0-CGRP with significantly lower affinity than native CGRP. Binding studies have shown that cardiac and hepatocyte receptors also show this discrimination, but not receptors in the brain, spleen or vas deferens (Yamaguchi et al., 1988b; Dennis et al., 1989). Thus by this second criterion, the L6 CGRP receptor may be classed as distinct from the neuronal CGRP receptor, even though both bind CGRP(8–37) with high affinity. Salmon calcitonin usually binds only weakly to CGRP receptors (e.g. Yamaguchi et al., 1988b). However, Sexton et al. (1988) have reported a CGRP binding site in neuronal tissue which binds salmon calcitonin with similar affinity to CGRP. Clearly the L6 CGRP receptor is distinct from this site since it binds salmon calcitonin approximately 100 times less potently than human α-CGRP.

Several features relating to the structure activity relationship of CGRP are apparent from the effects of the peptide fragments and analogues. The binding activity of the peptide is not perturbed by several amino acid substitutions. On the other hand, the C-terminal amide is essential for binding. The middle and C-terminal portions of the molecule but not the N-terminus are required for high affinity binding. However, the N-terminus is essential for biological activity. These results are consistent with the data of Dennis et al. (1989), discussed above, and also of both Zaida et al. (1990) and Maggi et al. (1990) who examined the cardiovascular effects of CGRP fragments. It is unclear whether the N-terminus has a direct role in receptor activation or merely stabilizes the rest of the peptide in a 'productive' conformation. The functional importance of the N-terminus is emphasized by the result that its extension by a single tyrosine decreased potency by 10–40 fold.

The actions of the related peptide, amylin, merit special comment. Although we observed significant batch variation, human amylin was at best weakly active, if at all, on L6 cells. The major sequence differences between amylin and CGRP are in the middle of the sequence, reinforcing the conclusion that this stretch of the CGRP peptide is essential for high affinity binding to this receptor. However, amylin has a powerful anti-insulin effect on glycogen synthesis, which is virtually equipotent with CGRP (Leighton & Cooper, 1988). Clearly amylin cannot be exerting its actions through the subtype of CGRP receptor found on L6 cells. Thus skeletal muscle may possess a distinct, high-affinity amylin selective receptor.

In conclusion, the L6 myocytes have a functional CGRP receptor which is linked to stimulation of adenylyl cyclase, via a G-protein. It can be competitively antagonized by the CGRP (8–37) fragment, and resembles CGRP receptors found in hepatocytes, cardiac, skeletal and vascular smooth muscle sites. Further work is needed, both to establish in more detail the relationship between the L6 receptor and those found in other tissues, and also to examine the mechanisms mediating endpoint effects of CGRP, such as cell proliferation (Haegerstrand et al., 1990).

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Capsaicin-induced bronchoconstriction in the guinea-pig: contribution of vagal cholinergic reflexes, local axon reflexes and their modulation by BW443C81

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1 The objective of the study was to investigate the central vagal and local axon reflex components of bronchoconstrictor responses evoked by inhalation of capsaicin aerosol in anaesthetized guinea-pigs. This was accomplished by comparing the effects of bilateral vagotomy, atropine and the peripherally-acting polar enkephalin analogue, BW443C81, on bronchoconstrictor responses evoked by capsaicin. The effects of codeine were also determined.

2 Aerosols of capsaicin were generated from a 0.9 $\mu g\,ml^{-1}$ solution. Inhalation of capsaicin aerosol in 5, 10 and 15 breaths evoked dose-related bronchoconstrictor responses. The responses were immediate in onset and of extended duration.

3 Capsaicin-induced bronchoconstrictor responses were significantly inhibited following bilateral vagotomy or atropine (0.3 $mg\,kg^{-1}$, i.v.) pretreatment by 46% ± 14% ($P < 0.05$) and 59% ± 13% ($P < 0.01$), respectively.

4 Administration of BW443C81 by intravenous infusion (3, 30 and 100 $\mu g\,kg^{-1}\,min^{-1}$) caused a significant inhibition of capsaicin-induced bronchoconstrictor responses which achieved a greater maximum than either bilateral vagotomy or atropine. Codeine (100 $\mu g\,kg^{-1}\,min^{-1}$, i.v.) did not significantly inhibit the bronchoconstrictor responses.

5 Inhibition of capsaicin-induced bronchoconstrictor responses by BW443C81 (30 $\mu g\,kg^{-1}\,min^{-1}$, i.v.) was significantly ($P < 0.05$) reduced by the peripherally-acting opioid antagonist N-methyl nalorphine (100 $\mu g\,kg^{-1}\,min^{-1}$, i.v.).

6 These results show that capsaicin-induced bronchoconstrictor responses are mediated by at least two mechanisms, a vagal and/or cholinergic reflex pathway and a non-cholinergic pathway. BW443C81, but not codeine, significantly inhibited ($P < 0.005$) both mechanisms of capsaicin-induced bronchoconstriction probably by an action on peripheral opioid receptors located on vagal sensory nerves.

Keywords: Reflex bronchoconstriction; inhalation; capsaicin; sensory nerves

Introduction

Stimulation of C-fibre afferents in the lung may contribute to a variety of airway reflexes including cough (Winning *et al.*, 1986; Karlsson *et al.*, 1988; Fuller *et al.*, 1988), bronchoconstriction (Forsberg & Karlsson, 1986), mucus secretion (Davis *et al.*, 1982) as well as changes in breathing patterns (Forsberg *et al.*, 1988).

In addition to evidence that strongly supports the role of C-fibres in reflex bronchoconstriction (Coleridge & Coleridge, 1986; Widdicombe, 1988) it has been demonstrated that activation of airway C-fibres causes the release of neuropeptides from the peripheral afferent nerve endings in the lung via a possible local axon reflex mechanism. These neuropeptides can cause bronchoconstriction, mucus secretion and oedema. Substance P (SP), was until recently, the only neuropeptide evident in the lung, known to evoke these responses (Lundberg & Saria, 1982), but biochemical and immunohistochemical evidence shows that many neuropeptides such as calcitonin gene-related peptide (CGRP) (Gibbins *et al.*, 1985), neurokinin A, neurokinin B and neuropeptide K coexist with SP within the C-fibres (Hua *et al.*, 1985).

Much of the evidence which supports the role of C-fibres in airway reflexes comes from studies using capsaicin, the purported 'selective' C-fibre stimulant. Administration of capsaicin produces responses which are species-related. Intravenous or intra-arterial administration of capsaicin induces a predominantly non-cholinergic bronchoconstriction in the guinea-pig (Lundberg & Saria, 1982; Biggs & Goel, 1985) which is not affected by vagotomy. In contrast, in dog and cat, intravenous administration of capsaicin provokes bronchoconstriction which is dependent on a vagal cholinergic reflex (Russell & Lai Fook, 1979; Adcock, 1989). Aerosol administration of capsaicin in cats also evokes a vagally-mediated cholinergic reflex bronchoconstriction, with no apparent non-cholinergic component (Adcock & Smith, 1989), whilst in man, inhaled capsaicin causes cough and a transient, mainly cholinergic, bronchoconstriction (Fuller *et al.*, 1985).

It has been shown that in both vagal C-fibres and Aδ-fibres innervating the respiratory tract of the cat, impulse activity was attenuated by intravenous infusion of the opioid peptide BW443C81 (Adcock *et al.*, 1987, Adcock & Smith, 1987). In addition, in cats, bronchoconstrictor responses evoked by inhalation of capsaicin were attenuated by both intravenous and aerosolized BW443C81 (Adcock & Smith, 1989).

The present study was designed to elucidate the mechanism by which capsaicin evokes bronchoconstriction in guinea-pigs when administered by aerosol. This was carried out by comparing the effects of atropine, bilateral vagotomy and the peripherally-acting opioid peptide BW443C81 (Tyr.D.Ala.Gly.Phe(4-NO_2).Pro.NH_2 diacetate) on the bronchoconstrictor responses evoked by inhaled capsaicin. Since codeine is a drug of choice in the clinical treatment of cough, results with this 'classical' opiate are included for comparison with BW443C81.

A preliminary account of this work has been presented to the British Pharmacological Society (Buchan *et al.*, 1989).

Methods

Male Dunkin Hartley guinea-pigs (400–450 g) were anaesthetized with 2% halothane in oxygen at a flow rate of 2 l min^{-1}. The right external jugular vein was cannulated with pp50 tubing (Portex) and anaesthesia was subsequently maintained by the intravenous administration of chloralose (100–

150 mg kg^{-1}; 10 ml kg^{-1}). Although it has been shown that halothane can stimulate/sensitize lung C-fibre endings (Coleridge & Coleridge, 1984), in our laboratory this effect on C-fibres is short lived in anaesthetized cats. Further, baseline airway tone is no different in guinea-pigs anaesthetized with halothane compared to animals anaesthetized with barbiturate (unpublished observations). The left external jugular vein was cannulated with pp60 tubing (Portex) for bolus administration of atropine or infusion of either BW443C81 or codeine. The trachea was cannulated and animals were artificially ventilated at a rate of 50 breaths min^{-1} with laboratory air, 10 ml kg^{-1} (Palmer Bioscience ventilator). With this ventilation regime blood gases and pH were satisfactorily maintained at physiological levels. Pulmonary inflation pressure (PIP) was measured with a Statham P23 pressure transducer attached to a side-arm of the tracheal cannula. The left carotid artery was cannulated with pp60 tubing (Portex) containing heparinised saline (125 u ml^{-1}), to record arterial blood pressure (Statham P23 transducer) and heart rate was derived from the arterial pulse with a cardiotachometer. Cardiovascular and pulmonary variables were recorded continuously on a Beckman R611 recorder. Body temperature was maintained at 37–38°C with a heated operating table.

Experimental protocol

Animals were allowed to stabilize for a minimum of 20 min before administration of aerosol. Capsaicin, (5 ml, 0.9 μg ml^{-1}) was added to the well of a modified DeVilbiss ultrasonic nebuliser (Lees & Payne, 1986) placed in the air inflow circuit from the ventilator. Each animal received separate administrations of capsaicin 5, 10 and 15 breaths.

In some experiments, atropine (0.3 mg kg^{-1}), was administered as an intravenous bolus, 10 min prior to the first capsaicin inhalation. This dose of atropine caused complete abolition of the bronchoconstrictor response to intravenous administration of acetylcholine (10 μg kg^{-1}) for the duration of the experiment of approximately 60 min. In other experiments, before administration of capsaicin, bilateral vagotomy was performed by sectioning the vago-sympathetic bundles. BW443C81 (3, 30, 100 μg kg^{-1} min^{-1}), codeine or N-methyl nalorphine at 100 μg kg^{-1} min^{-1} were administered by intravenous infusion, which started 10 min before capsaicin administration was begun. The total administered dose of BW443C81 and codeine at 100 μg kg^{-1} min^{-1}, was 6 mg kg^{-1} over the 1 h infusion period. This protocol minimized the cardiovascular effects of the two drugs and maintained effective BW443C81 plasma concentrations (Adcock, 1989). A peristaltic cassette pump (202U Watson Marlow) was used to infuse drugs at a rate of 0.1 ml min^{-1}.

Data analysis

Bronchoconstrictor responses to capsaicin were determined by measurement of the change between areas of response (mm^2) on the pulmonary inflation pressure (PIP) traces for a duration of 5 min before and 5 min after capsaicin administration. The duration of the bronchoconstrictor response, especially at 15 breaths, was quite prolonged and thus the area of response was more representative of capsaicin action rather than the magnitude or peak height of response. The areas were calculated using an Apple IIe computer with graphics tablet. Statistical significance of drug treatments was determined by comparing mean difference in areas (Δ areas) of each capsaicin response in drug-treated animals with those in saline-treated animals by Student's t test for unpaired data.

Drugs

Drugs and chemicals were obtained from the following sources: BW443C81, synthesized by Dr L.A. Lowe and N-methyl nalorphine, synthesized by the late Dr S. Wilkinson (Wellcome Research Laboratories); codeine phosphate (Wellcome Research Laboratories); atropine sulphate (BDH Chemicals Ltd); α-chloralose (Koch-Light Ltd); capsaicin (Fluka AG); ethanol (BDH Chemicals Ltd); Euthesate and Halothane (May & Baker Ltd); heparin (Evans Medical Ltd); Tween 80 (Sigma). Capsaicin was dissolved in an ethanol (0.2 ml), Tween 80 (0.2 ml) and saline (9.6 ml) mixture to 1 mg ml^{-1} and diluted with saline to 0.9 μg ml^{-1} of free base. All other drugs were dissolved in saline and doses are expressed as free base.

Results

Effect of inhalation of capsaicin

Inhalation of aerosols of the vehicle for capsaicin had no effect on the cardiopulmonary variables measured in these experiments. In preliminary experiments (results not shown) tachyphylaxis developed to bronchoconstrictor effects of capsaicin aerosol if either multiple dose-response curves were attempted or large doses were administered repeatedly. Inhalation from a 0.3 μg ml^{-1} solution of capsaicin, in most animals, produced a threshold response whilst 3.0 μg ml^{-1} produced respiratory and cardiovascular distress. Therefore, only one dose-response curve was carried out in each animal using 5, 10 and 15 breaths of a 0.9 μg ml^{-1} solution which produced a dose-related increase in PIP. This increase in PIP was completely reversed by 2 breath hyperinflations. A typical response to capsaicin (15 breaths), showing the extended duration of bronchoconstriction, is shown in Figure 1.

Effect of bilateral vagotomy and atropine

Capsaicin-induced bronchoconstrictor responses were significantly, but not totally inhibited by bilateral vagotomy (46% ± 14% at 10 breaths capsaicin, $P < 0.05$, $n = 13$) or the administration of atropine 0.3 mg kg^{-1} i.v. bolus (59% ± 13% at 10 breaths capsaicin, $P < 0.01$, $n = 14$) (Figure 2).

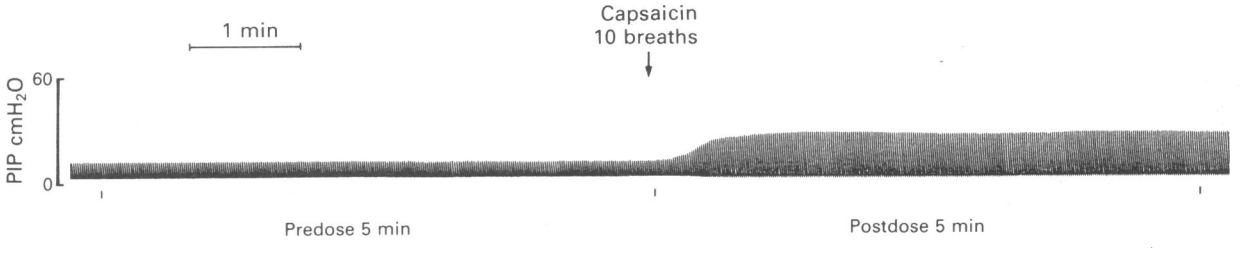

Figure 1 A typical trace (pulmonary inflation pressure, PIP) of the bronchoconstrictor response evoked by inhalation of capsaicin (15 breaths, 0.9 μg ml^{-1}) in an artificially ventilated anaesthetized guinea-pig.

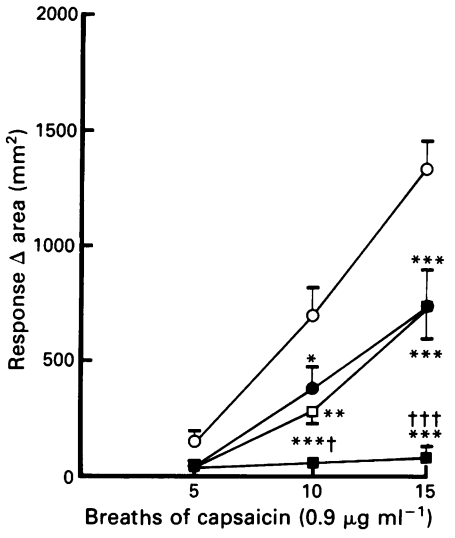

Figure 2 Bronchoconstrictor responses in artificially ventilated anaesthetized guinea-pigs following inhalation of capsaicin (5, 10, 15 breaths, 0.9 $\mu g ml^{-1}$): saline, 0.1 ml min^{-1} (○, $n = 20$); atropine 0.3 mg kg^{-1}, i.v. (□, $n = 14$); bilateral vagotomy (●, $n = 13$); BW443C81 30 $\mu g kg^{-1} min^{-1}$, i.v. (■, $n = 6$). The results are expressed as areas of response (postdose-predose) and represent mean with s.e.mean of areas (mm^2) shown by vertical bars. Statistical significance between treated groups and control groups was determined by Student's t test for unpaired data and shown by * $P < 0.05$; ** $P < 0.01$ *** $P < 0.005$; BW443C81 compared to either atropine or vagotomy is shown by † $P < 0.05$; ††† $P < 0.005$.

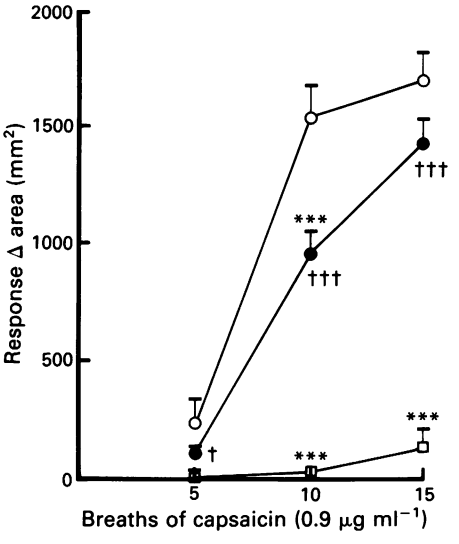

Figure 4 Effect of N-methyl nalorphine (N-MeNal) on the inhibition by BW443C81 of bronchoconstrictor responses evoked by inhalation of capsaicin (5, 10, 15 breaths, 0.9 $\mu g ml^{-1}$) in artificially ventilated anaesthetized guinea-pigs: N-MeNal 100 $\mu g kg^{-1} min^{-1}$, i.v. (○, $n = 5$); BW443C81 30 $\mu g kg^{-1} min^{-1}$, i.v. (□, $n = 6$) or BW443C81 30 $\mu g kg^{-1} min^{-1}$, i.v. + N-MeNal 100 $\mu g kg^{-1} min^{-1}$, i.v. (●, $n = 6$). The results are expressed as areas of response (postdose-predose) and represent mean with s.e.mean of areas (mm^2) shown by vertical bars. Statistical significance between treated groups and control groups was determined by Student's t test for unpaired data and shown by *** $P < 0.005$; N-MeNal compared to BW443C81 is shown by † $P < 0.05$; ††† $P < 0.005$.

Effect of BW443C81

Administration of BW443C81 by intravenous infusion (3, 30, 100 $\mu g kg^{-1} min^{-1}$) significantly ($P < 0.005$) attenuated capsaicin-induced bronchoconstrictor responses, (77% ± 14%, $n = 8$, 96% ± 3%, $n = 13$ and 92% ± 2%, $n = 14$ respectively; Figure 3). In comparative experiments, BW443C81 (100 $\mu g kg^{-1} min^{-1}$) caused a greater inhibition of bronchoconstriction than either bilateral vagotomy or atropine pretreatment (Figure 2).

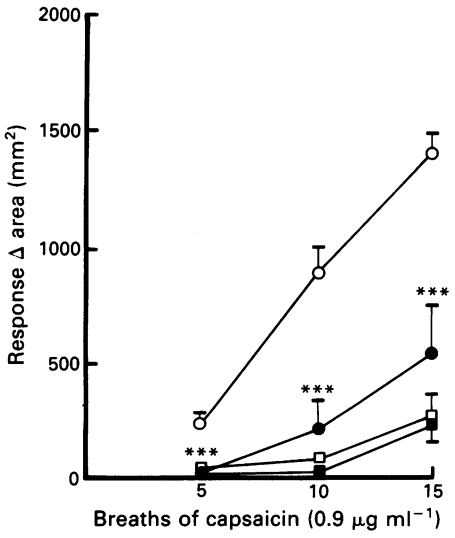

Figure 3 Inhibition by BW443C81 of bronchoconstrictor responses evoked by inhalation of capsaicin (5, 10, 15 breaths, 0.9 $\mu g ml^{-1}$) in artificially ventilated anaesthetized guinea-pigs: saline, 0.1 ml min^{-1} (○, $n = 27$); BW443C81 3 $\mu g kg^{-1} min^{-1}$ i.v. (●, $n = 8$); BW443C81 30 $\mu g kg^{-1} min^{-1}$, i.v. (■, $n = 13$) or BW443C81 100 $\mu g kg^{-1} min^{-1}$, i.v. (□, $n = 14$). The results are expressed as areas of response (postdose-predose) and represent mean with s.e.mean of areas (mm^2) shown by vertical bars. Statistical significance between treated groups and control groups was determined by Student's t test for unpaired data and shown by *** $P < 0.005$.

In separate experiments, inhibition of capsaicin (at 10 breaths)-induced bronchoconstrictor responses by intravenous infusion of BW443C81 (30 $\mu g kg^{-1} min^{-1}$) was significantly ($P < 0.005$) reduced from 98% ± 1% inhibition of the control group response (which received N-methyl nalorphine (N-MeNal) infusion 100 $\mu g kg^{-1} min^{-1}$ alone), to 38% ± 6% when the quaternary opioid antagonist N-MeNal and BW443C81 were infused concomitantly, as shown in Figure 4.

BW443C81 (100 $\mu g kg^{-1} min^{-1}$, i.v.) infusion caused a significant ($P < 0.0001$) increase in blood pressure from baseline systolic/diastolic values of 47.4 ± 2.7/28.8 ± 2.1, ($n = 12$) to plateau values of 68.3 ± 2.6/46.8 ± 1.9, ($n = 12$), within 4 min. Heart rate was unaffected by the infusion of BW443C81.

Effect of codeine

Codeine administered by intravenous infusion (100 $\mu g kg^{-1} min^{-1}$) caused a small, apparent, but non-significant reduction (40% ± 15% at 10 breaths capsaicin, $n = 8$) of the capsaicin-induced bronchoconstrictor responses. However, codeine (300 $\mu g kg^{-1} min^{-1}$, i.v.) caused a significant ($P < 0.005$) reduction (64% ± 14% at 10 breaths capsaicin, $n = 7$) of the capsaicin-induced bronchoconstrictor responses.

Discussion

Administration of capsaicin aerosol to anaesthetized guinea-pigs evoked bronchoconstrictor responses which were significantly inhibited by about 50% after bilateral vagotomy or pretreatment with atropine. These results demonstrate, therefore, that there is a vagal and cholinergic reflex component and a non-cholinergic component that contributes to capsaicin-evoked bronchoconstriction when adminstered by inhalation to guinea-pigs.

Capsaicin evokes vagal cholinergic reflex bronchoconstrictor responses when administered by aerosol to cats, and these responses are abolished by bilateral vagotomy or

atropine pretreatment (Adcock & Smith, 1989). Furthermore, intravenously administered capsaicin in dogs evokes reflex bronchoconstriction which is abolished after bilateral vagotomy (Russell & Lai-Fook, 1979). Thus in feline and possibly in canine airways, in contrast to guinea-pig airways, capsaicin-evoked bronchoconstrictor responses are dependent solely on a vagal cholinergic reflex pathway.

The non-cholinergic component of capsaicin-evoked bronchoconstriction observed in the present experiments in the guinea-pig following inhalation may be similar to that observed following intravenous administration of capsaicin in the same species (Biggs & Goel, 1985). Following intravenous administration in guinea-pigs, capsaicin evokes bronchoconstrictor responses that are not mediated by a vagal and/or cholinergic reflex but most probably by stimulation of sensory nerves to release bronchoconstrictor neuropeptides via a local axon reflex (Biggs & Goel, 1985). The mechanism of action of intravenous or intra-arterial capsaicin was investigated (Biggs & Goel, 1985) by administration of the antagonist [D-Arg^{1} · D-Pro^{2} · D-Trp^{7,9} · Leu^{11}]SP. It was shown that the antagonist reduced SP- and capsaicin-induced increases in resistance but had no effect on increase in dynamic thoracic elastance. It was therefore suggested that parenteral capsaicin-induced bronchospasm did not occur primarily via release of SP from sensory nerve endings (Lundberg *et al.*, 1983), but possibly via, as yet, unidentified peptide mediators not blocked by the SP antagonist.

The apparent difference observed in the nature of bronchoconstrictor responses to capsaicin by different routes of administration in this species may simply reflect the access to the sensory receptors involved in the two components of bronchoconstriction. Since capsaicin is purported to be a 'selective' stimulant of C-fibre receptors which mediate both vagal cholinergic and non-cholinergic excitatory responses (Coleridge & Coleridge, 1984; Lundberg *et al.*, 1988), capsaicin could stimulate pulmonary C-fibres when administered intravenously and bronchial C-fibres when administered by inhalation. However, this seems unlikely, since intra-arterial administration of capsaicin, which will stimulate bronchial C-fibres receptors, also failed to evoke vagal and/or cholinergic reflex bronchoconstrictor responses in guinea-pigs (Biggs & Goel, 1985). Furthermore, there is no evidence that either vagal cholinergic or non-cholinergic excitatory responses are exclusive to one type of C-fibre. One possible explanation for the difference could be that when administered systemically in guinea-pigs, capsaicin may evoke opposing bronchodilator reflexes. This is supported by evidence that capsiacin, when administered via a left ventricular injection in dogs, in contrast to right atrial injection, failed to evoke a bronchoconstrictor response but may have evoked a slight bronchodilation (Russell & Lai-Fook, 1979). However, it is unlikely that such a mechanism could selectively mask the vagal reflex bronchoconstriction and not the non-cholinergic component. Alternatively, capsaicin may activate an inhibitory 'gating' mechanism on the vagal reflex, since capsaicin has also been shown to attenuate mechanically-induced cough reflexes in anaesthetized cats following intravenous administration (Tatar *et al.*, 1988). Inhalation of capsaicin in guinea-pigs may evoke reflex bronchoconstriction by stimulation of a sub-population of C-fibre receptors or other type of sensory receptor which are not stimulated by capsaicin when administered intravenously or intra-arterially. Indeed we have shown that inhalation of capsaicin at doses that cause reflex bronchoconstriction in cats are not selective for C-fibre receptors but also stimulate irritant receptors (Mohammed *et al.*, 1990).

In the present study the peripherally-acting μ-opioid receptor agonist, BW443C81, attenuated both components of capsaicin-induced bronchoconstriction, since the inhibition obtained was greater than with either bilateral vagotomy or atropine pretreatment. It is unlikely that this effect is due to a bronchodilator action of BW443C81 since neither histamine-evoked bronchoconstrictor responses in anaesthetized cats (Adcock, 1989), nor histamine-evoked contraction of guinea-pig trachea *in vitro* (Adcock unpublished observations) are affected by BW443C81. Codeine was considerably less potent than BW443C81 in inhibiting the capsaicin-induced bronchoconstrictor responses. In a similar study, however, intravenously administered codeine inhibited reflex bronchoconstriction evoked by inhalation of citric acid in conscious guinea-pigs (Karlsson *et al.*, 1990). The peripherally acting quaternary opioid receptor antagonist, N-methyl nalorphine, significantly reduced the inhibition by BW443C81 of capsaicin-evoked bronchoconstrictor responses, confirming that the effects of BW443C81 are mediated by activation of opioid receptors. BW443C81 also abolished bronchoconstrictor responses evoked by inhalation of capsaicin in anaesthetized cats, a response that is entirely dependent on a vagal cholinergic reflex in this species (Adcock & Smith, 1989).

Although opioid peptides have been shown to inhibit acetylcholine release from postganglionic parasympathetic efferent nerve fibres in canine airways (Russell & Simons, 1985) it is unlikely that BW443C81 is acting in this way in the present study, since BW443C81 does not significantly inhibit cholinergic contractions of guinea-pig isolated bronchi evoked by electrical field stimulation (Shankley *et al.*, 1989). Furthermore, bradycardia evoked by electrical stimulation of the vagal efferent nerves in anaesthetized cats is unaffected by doses of BW443C81 used in this study (Adcock & Chapple, unpublished observations). A more likely explanation for inhibition of the vagal and/or reflex component is modulation of impulse activity in vagal sensory/afferent nerves from sensory receptors in the respiratory tract. Indeed, recent findings demonstrate that BW443C81 inhibits spontaneous and capsaicin-evoked impulse activity in pulmonary and bronchial C-fibres from endings located in the respiratory tract of anaesthetized cats (Adcock & Smith, 1987). The same is true for spontaneous and histamine-evoked impulse activity in Aδ-fibres from intrathoracic irritant receptors (Adcock *et al.*, 1987; Adcock & Smith, 1987).

It is now well established that opioids, including BW443C81, inhibit non-cholinergic bronchoconstrictor responses in guinea-pig airways *in vitro* (Frossard & Barnes, 1987; Shankley *et al.*, 1989) and *in vivo* (Belvisi *et al.*, 1988; Shankley *et al.*, 1989). Non-cholinergic bronchoconstriction may be due to the release of neuropeptides such as SP and neurokinins (NK) from sensory nerve endings (Saria *et al.*, 1984; 1988), since it is inhibited by tachykinin antagonists (Lundberg *et al.*, 1983), and is absent in animals pretreated with capsaicin which destroys, predominantly, SP immunoreactive nerves (Lembeck & Holzer, 1979). Furthermore, opioids have been shown to inhibit SP release from sensory nerves in rat hind paw (Smith & Buchan, 1984) and guinea-pig bronchus (Bartho *et al.*, 1987). Opioid receptors have been identified in the sensory fibres of the vagus nerve (Atweh *et al.*, 1978; Young *et al.*, 1980). Furthermore, a reduction in opioid binding sites on unmyelinated afferent nerves was demonstrated after pretreatment with capsaicin (Laduron, 1984) which suggests that opioid receptors may be localized to primary afferent nerves. Thus BW443C81 may inhibit capsaicin-evoked bronchoconstrictor responses by two mechanisms: firstly, by attenuating impulse traffic evoked by capsaicin stimulation of sensory nerve endings and therefore decreasing the vagal/cholinergic reflex and the local axon reflex; secondly, by modulating local axon reflexes by acting prejunctionally to inhibit the release of sensory neuropeptides and thus the excitatory non-cholinergic responses.

In conclusion, it appears that vagal/cholinergic and non-cholinergic, local axon reflex mechanisms contribute to the bronchoconstrictor response evoked by capsaicin and that these mechanisms are evidently species- and route-dependent. Capsaicin-induced bronchoconstrictor responses following aerosol administration in guinea-pigs appears to involve both of these mechanisms, which can be inhibited by activation of opioid receptors on sensory nerves. Exaggerated vagal reflex mechanisms, resulting from increased responsiveness of sensory nerve endings, may be the underlying cause of non-

specific airway hyperreactivity in asthmatics (Nadel, 1973; Boushey *et al.*, 1980). Thus agents, like BW443C81, may be useful probes to evaluate the contribution of sensory reflexes, if any, to non-specific airway hyperreactivity that occurs in diseases of the airway in man.

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Effects of the P_2-purinoceptor antagonist, suramin, on human platelet aggregation induced by adenosine 5'-diphosphate

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1 The effects of suramin, a trypanocidal drug which has been reported to be a P_2-purinoceptor antagonist on smooth muscle, were investigated in human platelets, where adenosine 5'-diphosphate (ADP) induces aggregation by acting on a subtype of purinoceptors which has been called P_{2T}.

2 Suramin (100 μM) had no inhibitory effect on ADP-induced platelet aggregation in plasma, even after 40 min incubation in the presence of bacitracin, a peptidase inhibitor, and did not affect the ability of adenosine 5'-triphosphate (ATP) (40 μM) to inhibit competitively ADP-induced aggregation. This lack of effect of suramin on platelets in plasma is probably due to its extensive binding to plasma proteins.

3 In washed platelets, suramin (50–400 μM) acted as an apparently competitive antagonist, causing parallel shifts to the right of the log concentration-response curve to ADP. No depression of the maximal response to ADP was observed at concentrations of suramin (50–150 μM) for which full log concentration-response curves to ADP could be obtained, but the slope of the Schild plot was around 2, indicating that this antagonism was not simply competitive. The apparent pA_2 value for suramin, taken from this Schild plot, was 4.6.

4 Suramin (200–400 μM) also noncompetitively inhibited aggregation induced by U46619 (a thromboxane receptor agonist) or by 5-hydroxytryptamine in the presence of adrenaline (100 μM), and caused a depression of the maximal response to these agonists. This nonspecific effect of suramin may explain the high Schild plot slope obtained against ADP.

5 These results provide evidence that the ADP receptor on human platelets is indeed similar to the P_2-purinoceptors responding to adenine nucleotides on smooth muscle and other tissues, and show that suramin cannot distinguish between the proposed subtypes of the P_2-purinoceptors.

Keywords: Human platelets; purinoceptors; adenine nucleotides; suramin

Introduction

The adenine nucleotides adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP) have potent extracellular effects on many tissues which are mediated by specific receptors known as P_2-purinoceptors. Two subclasses of these, P_{2X} and P_{2Y}, have been proposed to exist on smooth muscle causing contraction and relaxation respectively, with ADP and ATP being equipotent as agonists on these subclasses (Burnstock & Kennedy, 1985). Two other subclasses, P_{2Z} and P_{2T}, have been identified on immune cells and on platelets respectively, and differ from the P_{2X} and P_{2Y} subclasses in that on P_{2Z}-purinoceptors the agonist is ATP^{4-} and ADP is inactive, whereas on P_{2T}-purinoceptors ADP is the agonist and ATP is a competitive antagonist (Gordon, 1986). By use of synthetic analogues of adenine nucleotides, different structure-activity relationships have also been found for these four subclasses of P_2-purinoceptor, which supports the proposed subdivision. The structure-activity relationships for the P_{2T} subtype are more similar to those for the P_{2Y} subtype than to those for the P_{2X}, as 2-substituted analogues of ADP and ATP are more potent than the parent nucleotides, whereas methylene phosphonate analogues are less potent (Cusack & Hourani, 1990). Until recently no selective, reversible, competitive P_2 antagonists have been found, so this subclassification can only be provisional. Although ATP and its analogues are antagonists at P_{2T}-purinoceptors (Cusack & Hourani, 1982), they are agonists on the other subclasses and are therefore not useful for receptor classification.

Recently it has been reported that the trypanocidal drug suramin is a selective, competitive antagonist at P_{2X} and P_{2Y} receptors on vascular and visceral smooth muscle preparations, although it does not distinguish between these two receptor subtypes, having a pA_2 value of around 5 in each case (Dunn & Blakeley, 1988; Den Hertog *et al.*, 1989a, b; Hoyle *et al.*, 1990; Leff *et al.*, 1990; Von Kugelgen *et al.*, 1990). Suramin also selectively and competitively inhibits the effects of ATP on PC12 phaeochromocytoma cells with similar potency, the reported pA_2 value being 4.52 (Nakazawa *et al.*, 1990; Inoue *et al.*, 1991). As well as antagonizing P_2-purinoceptors, suramin is also known to inhibit a number of other proteins with nucleotide binding sites, for example yeast hexokinase (Wills & Wormall, 1950), erythrocyte membrane Na^+/K^+-ATPase (Fortes *et al.*, 1973), firefly luciferase (Fortes *et al.*, 1973), vacuole-type H^+-ATPases (Moriyama & Nelson, 1988; Calcaterra *et al.*, 1988), smooth muscle ecto-nucleotidases (Hourani & Chown, 1989), protein kinase C (Mahoney *et al.*, 1990), mitochondrial adenine nucleotide exchanger (Calcaterra *et al.*, 1988), the GTPase activity associated with G_i (Butler *et al.*, 1988) and various polynucleotide synthesizing enzymes (Broder *et al.*, 1985; Ono *et al.*, 1988; Offensperger *et al.*, 1988).

In this study we report the effects of suramin on the P_{2T}-purinoceptors on human platelets, in which ADP induces a change in shape, aggregation and the release of mediators from storage granules (Born, 1962).

Methods

Platelet aggregation

Venous blood was drawn from healthy human volunteers into one sixth of its volume of Acid-Citrate-Dextrose anticoagulant (trisodium citrate dihydrate 25 $g\,l^{-1}$, citric acid monohydrate 15 $g\,l^{-1}$, glucose 20 $g\,l^{-1}$), and centrifuged at 290 g for 20 min. Volunteers denied taking aspirin for 10 days before the experiment. The platelet-rich plasma (PRP) was removed and the platelets isolated by centrifugation at 680 g for 20 min in the presence of prostacyclin (1 μM). The supernatant was discarded and the platelets resuspended at a density of $10^8\,ml^{-1}$ in

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HEPES-saline buffer of the following composition (mM): NaCl 145, KCl 5, $MgCl_2$ 1, HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) 10, glucose 10 and bovine serum albumin (BSA) 2 mg ml^{-1}, adjusted to pH 7.4 with 1 M NaOH. Aggregation and shape change were followed photometrically (Born, 1962) with a Chrono-log Lumi-Aggrometer, and aggregation was quantified as the maximal rate of change in light transmission (expressed as arbitrary units min^{-1}) through a stirred sample (500 μl) at 37°C on addition of agonist. Human fibrinogen (0.3 mg ml^{-1}) was added to all samples 20 s before addition of agonist, and calcium (1 mM, as calcium chloride) was added at the same time as suramin or at least 3 min before addition of agonist. Suramin was added either simultaneously with the agonist or preincubated with the platelet suspension for various times at 37°C. The agonists used were ADP (0.1–300 μM), U46619 (11α,9α-epoxymethanoprostaglandin H_2) (0.3–30 μM) and 5-hydroxytryptamine (5-HT) (0.1–100 μM). In the case of 5-HT, adrenaline (100 μM) was added simultaneously to potentiate its effects and result in a measurable aggregation response.

In studies on platelets in plasma, blood was drawn into one ninth of its volume of trisodium citrate (38 g l^{-1}) and centrifuged at 290 g for 20 min, and the PRP was removed. Aggregation was quantified as above in 500 μl samples of PRP, without addition of calcium or fibrinogen.

EC_{50} values were obtained by regression analysis of the linear portion of the log concentration-response curve to ADP.

Drugs

ADP, ATP, prostacyclin, U46619, adrenaline, 5-HT, fibrinogen (fraction 1 from human plasma, essentially plasminogen-free) and bacitracin were obtained from Sigma Chemical Co., Poole. Suramin was a generous gift from Bayer, UK, and all other chemicals were AnalaR Grade from BDH, Poole. Prostacyclin was dissolved at 100 μg ml^{-1} in 10 mM NaOH and U46619 was dissolved initially at 30 mM in absolute ethanol then diluted to 10 mM with distilled water and both drugs were stored frozen. All other drugs were dissolved in distilled water and the bacitracin, suramin, fibrinogen, 5-HT and adrenaline were made up freshly each day while the nucleotides were stored frozen.

Results

In citrated human plasma, ADP induced platelet aggregation with an EC_{50} value of 1.1 μM, and this aggregation was competitively inhibited by simultaneous addition of ATP (40 μM), with a K_B value of 10 μM derived from the shift in the ADP concentration-response curve. Suramin (100 μM) added simultaneously had no effect on the ADP-induced aggregation or on the inhibition of this by ATP (Figure 1a). Preincubation with suramin (100 μM) for 40 min at 37°C did not inhibit ADP-induced aggregation (Figure 1b), even in the presence of the peptidase inhibitor bacitracin (2 units ml^{-1}) (Figure 1c). Suramin alone did not induce aggregation or shape change at concentrations up to 1 mM (results not shown).

In washed platelets, ADP induced aggregation with an EC_{50} value of 4.3 μM, and suramin added simultaneously with ADP caused a dose-dependent parallel shift to the right of the log concentration-response curve (Figure 2a). Schild analysis of these data gave a slope of 1.82 ± 0.21, which was significantly greater than unity ($P < 0.005$, Student's t test), and an apparent pA_2 value (the negative log of the concentration causing a dose-ratio of 2) of 4.62 (Figure 2b). Under these conditions ATP also caused dose-related parallel rightward shifts of the log concentration-response curve for ADP, but gave Schild plots with slopes close to unity and pA_2 values around 5 (results not shown). Incubation of washed platelets with suramin for 10 or 40 min at 37°C before addition of ADP also yielded Schild plots with slopes significantly greater than unity (2.01 ± 0.16 and 2.06 ± 0.01 respectively), and with increasing time of incubation there was a reduction in the potency of suramin (apparent pA_2 values of 4.43 and 4.05 after 10 and 40 min respectively) (Figure 2c).

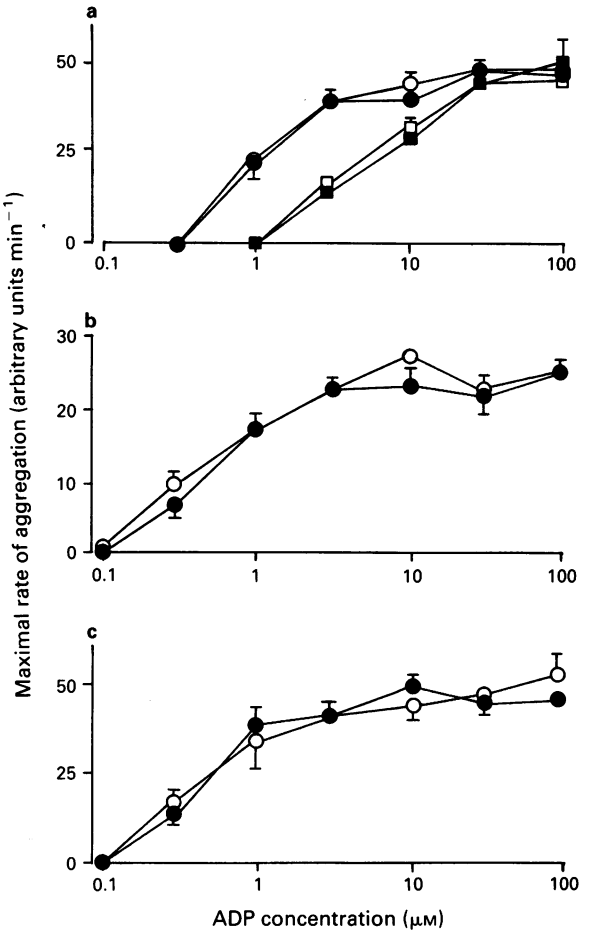

Figure 1 Effects of suramin on ADP-induced human platelet aggregation in citrated plasma. (a) Aggregation induced by ADP alone (○) or in the presence of suramin (100 μM) (●), ATP (40 μM) (□) or both suramin (100 μM) and ATP (40 μM) (■), added simultaneously with ADP. (b) Aggregation induced by ADP after preincubation of platelet-rich plasma for 40 min at 37°C with distilled water (○) or suramin (100 μM) (●). (c) Aggregation induced by ADP after preincubation of platelet-rich plasma for 40 min at 37°C with bacitracin (2 units ml^{-1}) (○) or with bacitracin (2 units ml^{-1}) and suramin (100 μM) (●). Each point is the mean of 3 determinations, and vertical bars show the s.e.mean.

Washed platelet aggregation induced by U46619 or by 5-HT (in the presence of 100 μM adrenaline) was also inhibited by suramin at concentrations of 200 or 400 μM, but this inhibition was not competitive and suramin caused a marked reduction in the maximal response to these agonists. ATP (100 μM) also reduced the maximal response to U46619, the effect being similar to that of 200 μM suramin, but did not affect aggregation induced by 5-HT (Figure 3).

Discussion

In this study we have shown that suramin acts as an antagonist of ADP-induced aggregation of human platelets in buffer but has no effect on platelets in plasma. The lack of effect of suramin in plasma was unlikely to be due to degradation of the antagonist by plasma enzymes, as varying the incubation time between 0 and 40 min even in the presence of the peptide inhibitor bacitracin did not affect the results. Indeed, in pharmacokinetic studies *in vivo* suramin has been shown not to be significantly metabolised but does bind non-specifically and

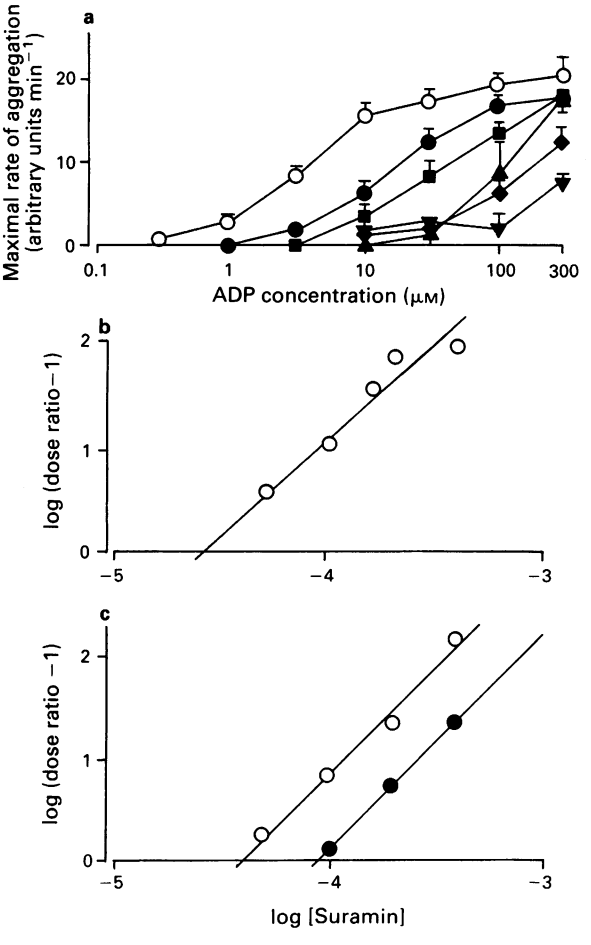

Figure 2 Effects of suramin on ADP-induced washed human platelet aggregation. (a) Aggregation induced by ADP alone (○) or in the presence of suramin at 50 μM (●), 100 μM (■), 150 μM (▲), 200 μM (◆) or 400 μM (▼). These results are the pooled data derived from a number of separate experiments on blood from several donors. Each point is the mean of at least 3 determinations, and the vertical bars show s.e.mean. (b) Schild plot of the data presented in (a). (c) Corresponding Schild plots obtained when platelets were preincubated with suramin for 10 (○) or 40 (●) min before addition of ADP.

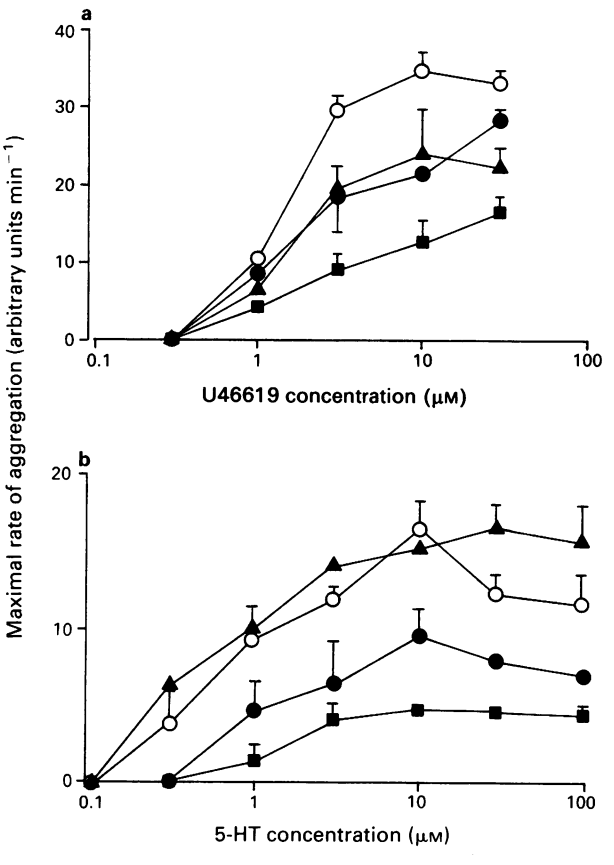

Figure 3 Effects of ATP and suramin on washed human platelet aggregation induced by (a) U46619 or (b) 5-hydroxytryptamine (5-HT) in the presence of adrenaline (100 μM), alone (○) or in the presence of suramin (200 μM (●) or 400 μM (■)) or ATP (100 μM) (▲). Each point is the mean of 3 determinations and the vertical bars show s.e.mean.

with high capacity to plasma proteins, greater than 99% of the drug being protein-bound (Collins *et al.*, 1986). It is likely therefore that the lack of effect of suramin on platelets in plasma is due to this extensive binding to plasma proteins.

In washed platelets, suramin antagonized the aggregation induced by ADP in an apparently competitive manner, causing parallel, dose-dependent shifts to the right of the log concentration-response curve to ADP. However, Schild analysis (Arunlakshana & Schild, 1959) of these data revealed that suramin was not acting as a pure competitive antagonist as the slope of the Schild plot was around 2. In a detailed study of the antagonism by suramin of the effect of ATP on P_{2X}-purinoceptors in the rabbit ear artery, Leff *et al.* (1990) also reported a Schild slope significantly greater than unity, and attributed this to slow equilibration of suramin with the receptors, as increasing the incubation times for low concentrations of suramin reduced the Schild slope to unity. From an analysis of the time-dependence of the inhibitory effect of suramin, Leff *et al.* (1990) concluded that very long incubation times, up to 220 min, were necessary to reach equilibrium, but in our study incubation times longer than 40 min could not be used because responses to ADP could not be reliably obtained. However, in platelets slow equilibration with the receptors is unlikely to be the explanation for the steep Schild plot, as increasing the incubation time with suramin from 0 to 40 min did not affect the slope of the Schild plot but merely reduced the potency of suramin, possibly due to slow binding of suramin to the BSA which was routinely present in the assay buffer. In addition, in a stirred suspension of platelets access of suramin to the receptors is likely to be more rapid and complete than in smooth muscle preparations, and long incubation times are not necessary to achieve Schild slopes of unity with other ADP antagonists in platelet-rich plasma (Cusack & Hourani, 1982).

Other possible explanations for a Schild slope greater than unity are that more than one molecule of the antagonist is binding to the receptor, that the antagonist is acting on a heterogeneous population of receptors, that it is acting non-competitively or that it is having multiple effects on the response (Kenakin, 1987). A Schild slope near 2 could imply that on average 2 molecules of suramin must bind to the receptor, but although from our results it is not possible to draw firm conclusions about the stoichiometry of the interaction, there is no evidence that any other ADP antagonist exhibits multiple binding interactions (Cusack & Hourani, 1982). The dimeric structure of suramin may, however, confer different binding characteristics from those of previously tested ADP antagonists, which are analogues of AMP or of ATP. The second possibility, that there is a heterogeneous population of ADP receptors on platelets, has been suggested by Colman *et al.* (1980; see also Colman 1990), although this suggestion is not consistent with results from antagonist studies (Cusack & Hourani, 1982) or from radioligand binding studies, in which only one site was detected (Macfarlane *et al.*, 1982; 1983; for discussion see Macfarlane, 1987; Hourani & Cusack, 1991). In any case, of the two receptor types proposed by Colman *et al.* (1980), only one was thought to mediate aggregation, the other being proposed to mediate the inhibition by ADP of adenylate cyclase, and as in our study we have investigated only aggregation this would not explain the steep Schild slope obtained for suramin. Although the third

possibility, that suramin could be acting non-competitively, cannot be ruled out, the log concentration-response curves to ADP were shifted to the right with no apparent depression of the maximal response, at least at concentrations of suramin (50–150 μM) for which full log concentration-response curves to ADP could be obtained. Suramin, however, clearly had non-specific effects in addition to its antagonism of ADP, as it non-competitively inhibited the aggregation induced by U46619 or by 5-HT, which act at thromboxane ('TP') and 5-HT$_2$ receptors respectively on platelets (Hourani & Cusack, 1991). Given its avid binding to plasma proteins, it is possible that at high concentrations, suramin may bind to fibrinogen and make it unavailable for aggregation, resulting in non-competitive inhibition. The fourth explanation for the steep Schild slope, that suramin is having multiple effects, is therefore the most likely. Although ATP at high concentrations also non-competitively inhibited aggregation induced by U46619, this is probably due to its inhibition of the effect of released ADP, as ATP did not inhibit aggregation induced by 5-HT, which is a weaker platelet aggregating agent than U46619 and does not induce release of stored nucleotides from platelets. Indeed, to achieve aggregations to 5-HT comparable with those induced by ADP and U46619, it was necessary to potentiate the effects of 5-HT with a fixed concentration of adrenaline, as either agonist alone did not induce reliable aggregations.

Although the pA$_2$ values for suramin we obtained in this study have to be treated with caution because of the steep slope of the Schild plot and consequent uncertainty as to the mechanism of action of suramin, they do correspond closely to those obtained in other studies. As an inhibitor of ADP-induced aggregation suramin had an apparent pA$_2$ value between 4.62 and 4.05 depending on the incubation time, which is close to the values ranging from 4.5 to 5.4 which have been reported for the inhibition of the effects of ATP on smooth muscle preparations and phaeochromocytoma cells (Hoyle *et al.*, 1990; Leff *et al.*, 1990; Von Kugelgen *et al.*, 1990; Inoue *et al.*, 1991). In those cases in which no pA$_2$ value has been reported, the concentrations which have shown an inhibitory effect against ATP are generally in the micromolar range, and are also therefore consistent with these values (Den Hertog *et al.*, 1989a, b; Hoiting *et al.*, 1990). The similarity of the apparent pA$_2$ values for suramin found for the inhibition of ADP-induced aggregation to that found for suramin for antagonism of ATP on other tissues, suggests that the platelet ADP receptor is indeed similar to P$_2$-purinoceptors elsewhere, and that suramin is unable to distinguish between the subtypes of P$_2$-purinoceptors. The concentrations of suramin required for antagonism at P$_2$-purinoceptors are also similar to the IC$_{50}$ or K_i values reported for inhibition by suramin of the various purine-binding enzymes, which are generally in the micromolar range (Wills & Wormall, 1950; Fortes *et al.*, 1973; Butler *et al.*, 1988; Calcaterra *et al.*, 1988; Moriyama & Nelson, 1988; Ono *et al.*, 1988; Mahoney *et al.*, 1990), suggesting that these binding sites may be structurally related.

In conclusion, our results have shown that suramin is an antagonist of the ADP receptor mediating aggregation of human platelets, although it is not effective in plasma, it is not specific for ADP and its antagonism is not simply competitive. The pA$_2$ values are consistent with values found for P$_2$-purinoceptors elsewhere, showing that although it is unique in that ATP is an antagonist rather than an agonist, the platelet ADP receptor is indeed a type of P$_2$-purinoceptor.

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Blockade of delayed rectifier K^+ currents in neuroblastoma × glioma hybrid (NG 108-15) cells by clofilium, a class III antidysrhythmic agent

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1 The whole-cell patch-clamp technique was used to examine the effects of the class III antidysrhythmic agent, clofilium, on voltage-activated delayed rectifier K^+ currents (IK_v) in undifferentiated mouse neuroblastoma × rat glioma hybrid (NG 108-15) cells. Ca^{2+}-activated K^+ currents also seen in these cells were abolished by bath application of 4 mM Co^{2+}.

2 Bath application of clofilium (0.3 to 70 μM) caused dose-dependent, irreversible inhibition of IK_v in these cells. Under control conditions, activated currents were sustained during 200 ms depolarizing steps, but in the presence of clofilium, or after its wash-out, currents were reduced in amplitude and showed a time-dependent decay.

3 Clofilium blockade of IK_v was voltage-dependent: the degree of current inhibition increased with increasing depolarizations. The transient nature of IK_v seen in the presence of clofilium was also more apparent at higher test potentials.

4 The effects of clofilium were use-dependent: when cells were left unstimulated during drug application, and then depolarizations were resumed, several pulses were required for clofilium blockade to reach a steady level. Similar results were obtained post-clofilium, when cells were unstimulated during application and then removal of clofilium, suggesting that although the blocking action of the drug was use-dependent, it bound to the closed, delayed rectifier K^+ channel.

5 High concentrations (100 or 300 μM) of sotalol, another class III antidysrhythmic agent, were without discernible effects on IK_v in NG 108-15 cells.

6 The effects of clofilium on a neuronal IK_v described here, and its possible mechanism of action, are compared with previously reported effects of clofilium on the cardiac IK_v.

Keywords: K^+ current; delayed rectifier; sotalol; clofilium; class III antidysrhythmic; neuroblastoma × glioma (NG 108–15) cells

Introduction

The activity of K^+ channels is fundamental to the control of membrane potential in both excitable and non-excitable cells. In the heart, many K^+ channel types exist, and their complicated interactions contribute to shaping the cardiac action potential waveform (see Noble, 1984, for review). Their importance in such events has been exploited in the search for agents which can correct dysrhythmias. Class III antidysrhythmic agents exert their stabilizing effects by prolonging the cardiac action potential and hence the effective refractory period (they are particularly useful in severe dysrhythmia e.g. ventricular tachycardia and fibrillation; Osterrider & Waterfall, 1990). These compounds are structurally diverse, and the mechanisms by which they prolong the cardiac action potential are complex and not fully understood. However, one commonly found feature is that they inhibit the voltage-gated delayed rectifier K^+ current (IK_v) which contributes to the repolarization of cardiac myocytes following each action potential (Cook & Quast, 1990).

IK_v is also present in neuronal tissue, where it contributes to the termination of the neuronal action potential. Although IK_v activates at a much greater rate in neuronal tissue compared with cardiac tissue, the currents from the two tissue types show several other pharmacological and kinetic similarities (Adams *et al.*, 1980; Noble, 1984; Halliwell, 1990). The original aim of this study was to examine the effects of the two class III antidysrhythmic agents clofilium and sotalol, which have both been demonstrated to inhibit cardiac IK_v (Carmeliet, 1985; Arena & Kass, 1988), on a neuronal IK_v, in the undifferentiated neuroblastoma × glioma hybrid cell line NG 108-15. While sotalol was without effect on neuronal IK_v, clofilium was seen to have a potent inhibitory effect. Further experiments indicated that its mechanism of blockade is quite different from that reported on cardiac IK_v. A preliminary account of some of these findings has appeared in abstract form (Reeve & Peers, 1991).

Methods

Mouse neuroblastoma × rat glioma hybrid (NG 108-15) cells (Hamprecht, 1977) were continuously grown in culture flasks containing Dulbecco's modified Eagle's medium (DMEM) with 10% foetal calf serum, 1 × HAT (0.1 mM hypoxanthine, 0.4 μM aminopterin and 160 μM thymidine), penicillin (100 iu ml^{-1}), streptomycin (100 μg ml^{-1}) and L-glutamine (2 mM). They were maintained in a humidified incubator at 37°C (10% CO_2). No chemicals were added to promote or induce cell differentiation. When required for electrophysiological study, cells were removed from their culture flasks with gentle mechanical agitation and plated onto polylysine-coated coverslips in 35 mm culture dishes. They were left to adhere for between 3 and 5 days, in the same culture medium and incubating conditions.

On each experimental day, fragments of coverslip with attached cells were transferred to a continually perfused (1 ml min^{-1}) low-volume (approximately 80 μl) recording chamber. The perfusate was composed of (in mM): NaCl 135, KCl 5, $MgSO_4$ 1.2, $CaCl_2$ 2.5, HEPES 10, glucose 10 (pH 7.4, 21–24°C). Whole-cell patch-clamp recordings (Hamill *et al.*, 1981) were made from cells by use of patch electrodes filled with a solution of composition (in mM): KCl 117, EGTA 11, HEPES 11, $CaCl_2$ 1, $MgSO_4$ 2, NaCl 10, ATP 2 (pH 7.2).

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When filled with this solution, electrodes had resistances of between 3 and 10 MΩ. Cells were voltage-clamped at −70 mV, and 200 ms step changes in the membrane potential were applied at a frequency of 0.2 Hz. Capacitive transients were minimized by analogue means. Data were stored on computer, and current amplitude measurements were subsequently obtained using VCAN software (J. Dempster, Strathclyde University) following leak subtraction, which was performed by the appropriate scaling and subtraction of the mean current amplitude evoked by small depolarizing and hyperpolarizing steps. Currents recorded under control conditions were sustained during 200 ms step depolarizations (i.e. showed no time-dependent inactivation), and so their amplitudes were measured by averaging the last 20 to 30 ms of the evoked current. In the presence of clofilium, currents showed a time-dependent decay (see Results), and so were measured at their peak (I_{peak}) and also over the final 20 to 30 ms of the step depolarization, as for controls (I_{end}). Current-voltage (I–V) relationships were constructed and all analysis of current amplitudes was performed after leak subtraction, but as the correction changed values of current amplitude by only a maximum of approximately 5%, and was rarely completely successful in removing capacitive transients, the example current traces shown are unsubtracted. Statistical comparisons were made by the paired, two-tailed Student's t test, unless otherwise indicated.

Results

Isolation of the delayed rectifier K^+ current in NG 108–15 cells

Figure 1a shows current-voltage (I–V) relationships obtained from an undifferentiated NG 108-15 cell. Under control conditions K^+ currents activated at between −30 mV and −10 mV, and increased with increasing test potential. In chemically-differentiated NG 108-15 cells these currents are known to be composed of both Ca^{2+}-dependent K^+ currents (IK_{Ca}) and Ca^{2+}-independent, delayed rectifier K^+ currents (IK_v; Brown & Higashida, 1988). The Ca^{2+}-dependent component relies upon Ca^{2+} influx via voltage-dependent Ca^{2+} channels for activation, and so can be indirectly inhibited with Ca^{2+} channel blockers (Brown & Higashida, 1988). In undifferentiated NG 108-15 cells used here, we found that the Ca^{2+} channel blocker, Co^{2+}, was effective in inhibiting IK_{Ca} (e.g. Figure 1a). For example, at a test potential of +20 mV, 4 mM Co^{2+} reduced K^+ currents by 39.8 ± 3.2% ($n = 6$, $P < 0.005$) and at +60 mV by 31.9 ± 4.3% ($P < 0.02$). In the presence of 4 mM Co^{2+}, a brief delay in the activation of the residual K^+ current was apparent (e.g. Figure 1b), characteristic of IK_v. A concentration of 4 mM Co^{2+} was maximal in inhibiting IK_{Ca}, because application of 0.2 mM Cd^{2+} in the continued presence of 4 mM Co^{2+} did not produce further inhibition ($n = 3$; e.g. Figure 1a). Cd^{2+} alone could not be used to isolate IK_v as it is toxic to cells after several minutes exposure. Further increases in the Co^{2+} concentration only produced a parallel shift of the I–V curve to the right (not shown), indicating the membrane surface charge screening effect of high doses of divalent cations (Hille, 1984). Indeed, currents recorded in the presence of Co^{2+} only began to activate at around −10 mV (see Figure 1a), indicating a possible screening effect of the cation at this dose, similar to that reported in pancreatic β cells (Smith *et al.*, 1990). We also found that the currents recorded in the presence of 4 mM Co^{2+} were inhibited in a concentration-dependent manner by bath application of tetraethylammonium (TEA, not shown), with an IC_{50} of approximately 1.5 mM. This further supports the idea that the Ca^{2+}-independent K^+ currents in these cells are delayed rectifier (IK_v) currents, as this IC_{50} value for TEA is similar to that reported on IK_v in other preparations (Adams *et al.*, 1980; Stanfield, 1983). All subsequent experiments were designed to study the actions of clofilium and sotalol on IK_v alone, and were therefore carried out in the presence of 4 mM Co^{2+}.

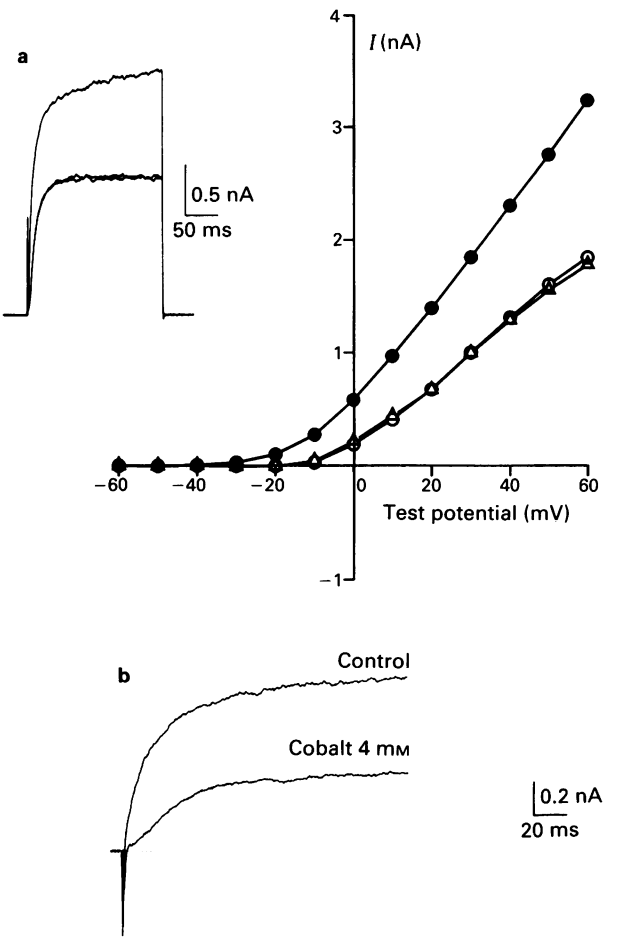

Figure 1 (a) Current-voltage (I–V) relationships all obtained from the same undifferentiated NG 108-15 cell under control conditions (●), in the presence of Co^{2+} 4 mM (○) and in the presence of Co^{2+} 4 mM and Cd^{2+} 0.2 mM (△). Inset shows examples of traces of K^+ currents recorded under these conditions, all at the test potential of +40 mV. (b) Superimposed currents, both obtained at a test potential of +10 mV, showing the effects of Co^{2+} 4 mM. Note the delay in activation of the current in the presence of Co^{2+}, characteristic of the delayed rectifier current.

Effects of clofilium on IK_v in NG 108-15 cells

The effects of bath application of 10 μM clofilium on IK_v recorded in a NG 108-15 cell are shown in Figure 2a. In the presence of the drug, currents were reduced in amplitude as compared with controls and became increasingly transient with increasing depolarizations, showing a time-dependent decay during 200 ms depolarizing test depolarizations. This contrasted with the currents recorded under control conditions, which were sustained through the 200 ms depolarizations (Figure 2a). However, the initial rising phase, or upstroke, of IK_v was unaffected by clofilium (e.g. Figure 2b). The current-voltage relationship from this cell is plotted in Figure 2c and shows that blockade of IK_v is voltage-dependent: the degree of blockade increased with increasing test potential. Also, the progressively more transient appearance of the currents seen at more positive test potential values is indicated by the differences in the current amplitudes measured at their peak and near the end of the test depolarization. This information is averaged for each concentration of clofilium tested (0.3 to 70 μM) in Figure 3, which plots the percentage reduction in current amplitudes as measured at their peak (I_{peak}; Figure 3a) and near the end of the step depolarization

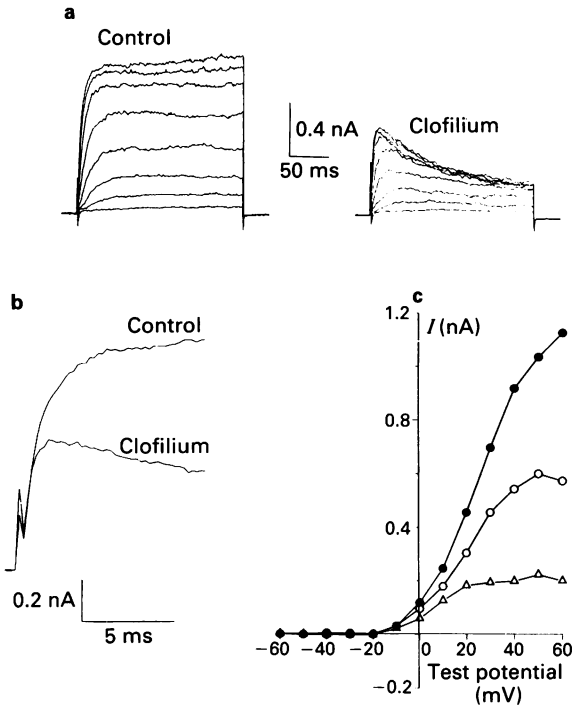

Figure 2 (a) Superimposed families of currents obtained from the same NG 108-15 cell under control conditions and in the presence of clofilium 10 μM. Currents were evoked by step changes in the membrane potential from −70 mV to between −10 mV and +60 mV, in 10 mV increments. (b) Superimposed currents shown on a fast timebase, indicating the lack of effect of clofilium on the initial part of the upstroke or activation of IK_v. Currents were obtained from the same cell as in (a), using a test potential of +60 mV. (c) *I–V* relationship obtained from the same cell as in (a) and (b), under control conditions (●), and in the presence of 10 μM clofilium, when currents were measured at their peak (○) and at the end of the test depolarization (△).

(I_{end}; Figure 3b) versus test potential. At all concentrations examined, clofilium reduced both peak and end current amplitude in a manner that increased with increasing test potential. At higher concentrations (10 μM or greater) the voltage-dependence of inhibition of I_{end} (Figure 3b) was steeper than that for I_{peak} (Figure 3a), indicating the increasingly transient nature of the currents with test potential. Due to the voltage-dependence of clofilium blockade of IK_v in NG 108-15 cells (Figure 3), and its effects on changing the time-course of activated currents, a dose-response curve has not been constructed. However, if the values of percentage current inhibition as measured at the end of depolarizations to +40 mV are considered, an IC_{50} value of approximately 4 μM is obtained. The effects of TEA, measured identically, yielded an IC_{50} value of approximately 1.5 mM.

Mechanism of action of clofilium on IK_v in NG 108-15 cells

Time series experiments (in which the cell is repeatedly depolarized from −70 mV to +40 mV, pulse duration 200 ms, 0.2 Hz) were performed to investigate the mechanism by which clofilium (at a concentration of 30 μM) causes blockade of IK_v in NG 108-15 cells. For comparison, we also investigated the effects of 10 mM TEA, which gave a similar, final degree of current inhibition. The rate of reduction in current amplitude caused by clofilium was markedly slower than that for TEA; in 6 cells tested, the reductions in current amplitude caused by 10 mM TEA were 50% complete after 10.9 ± 0.4 s (mean ± s.e.mean) following change of the bath solution for solution containing the drug. However, the effects of 30 μM clofilium were only half-maximal after 33.6 ± 2.1 s exposure (n = 5 cells), a significantly slower effect ($P < 0.0001$, unpaired

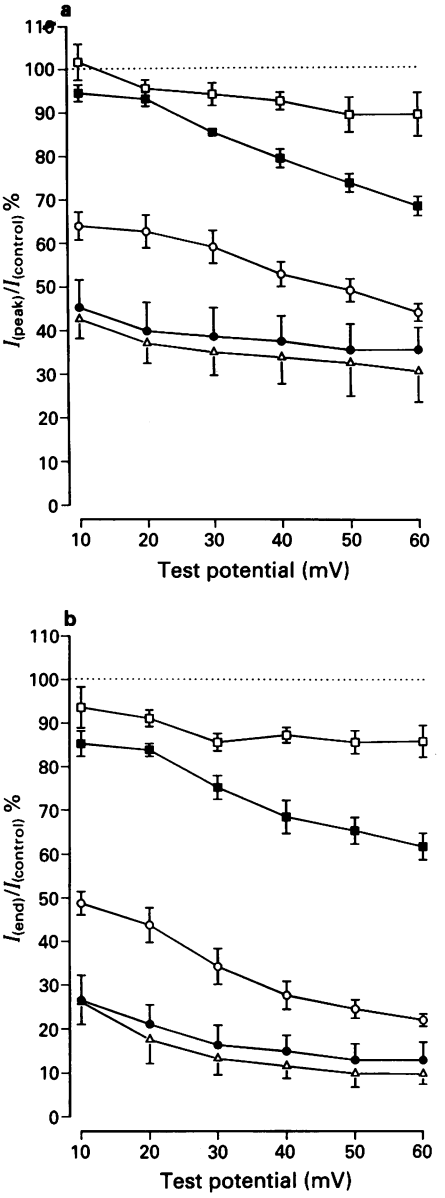

Figure 3 Plots of percentage reductions in IK_v caused by clofilium versus test potential. Currents were measured at their peak (I_{peak}; 3a) and at the end of the step depolarizations (I_{end}; 3b) in the presence of different doses of clofilium: 0.3 μM (□; n = 3 cells), 3 μM (■, n = 4), 10 μM (○, n = 5), 30 μM (●, n = 4) and 70 μM (△, n = 6). Points plotted are mean values, with vertical bars showing s.e.mean.

t test). This observation suggested that some factor(s) other than bath exchange time (which limits the rate of blockade by TEA) slowed the blockade of IK_v by clofilium.

One possible factor was that the action of clofilium was use-dependent i.e. blockade depended on repeated activation of IK_v in these cells. Typical examples of experiments designed to investigate this possibility are shown in Figure 4. As for experiments described above, time-series studies were used, where cells were repeatedly depolarized from −70 mV to +40 mV. Figure 4a illustrates the time course of blockade caused by 30 μM clofilium, when the cell was not depolarized during exchange of the bath solution for the solution containing the drug (a period of 45 s). It can be seen that, on resuming depolarizations, clofilium did not cause an immediate inhibition of the current. Indeed, several depolarizations were required for clofilium to reach a steady level of inhibition of IK_v in this cell. Similar results were obtained in a total of 6 cells in which this protocol was used. This effect was in marked contrast to blockade caused by 10 mM TEA. Figure 4b shows an experiment which followed the same protocol as

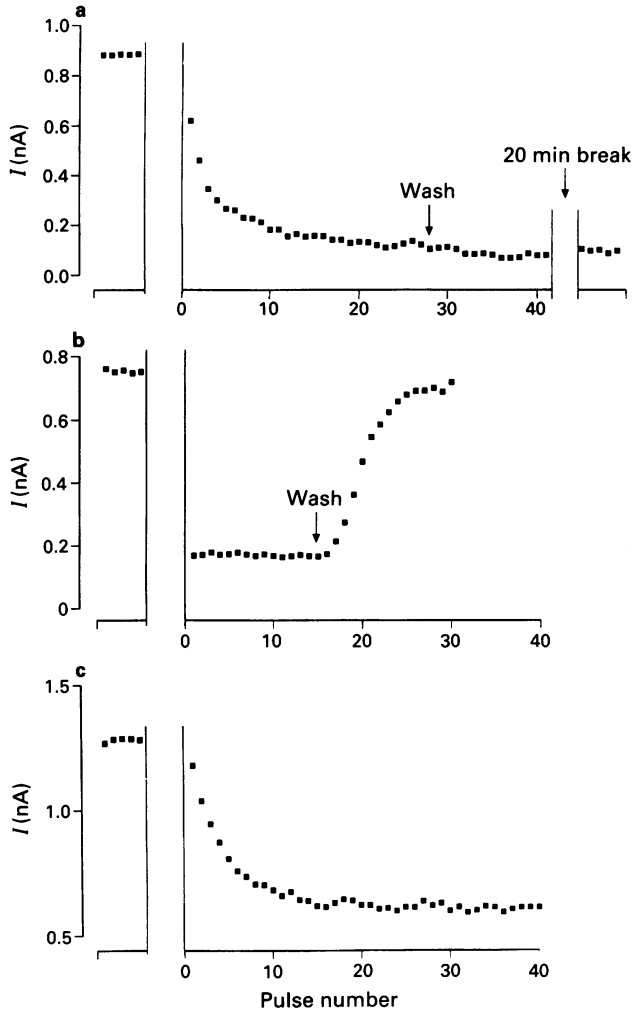

Figure 4 Time-series plots obtained from three different NG 108-15 cells by repeatedly stepping the membrane potential from −70 mV to +40 mV. (a) First break indicates a period of 45 s during which the cell was held at −70 mV without applying step depolarizations, and 30 μM clofilium was applied to the bath perfusate. Note the irreversibility of the drug, as indicated by the lack of recovery of current amplitudes following a 20 min break after the drug was washed out (depolarizations were continued during this period). (b) Break indicates 45 s period during which the cell was not depolarized (as in (a)), but 10 mM tetraethylammonium (TEA) was applied to the perfusate. Note the immediate reduction of current amplitude following resumption of depolarizations, and the full recovery of current amplitude following washout of TEA. (c) Break indicates 45 s period of bath application of 30 μM clofilium followed by 3 min period of washout of the drug, during which the cell was not depolarized.

in Figure 4a, but using TEA rather than clofilium as the test drug. On resuming cell depolarizations, the effects of TEA were immediate: the first current evoked in its presence was strongly inhibited as compared with controls, and subsequent depolarizations showed no further inhibition of IK_v. These effects of TEA were consistently observed in 5 cells. Furthermore the block induced by TEA but not clofilium, was reversible on wash-out of the drug.

Figure 4c shows an experiment (representative of 5 cells examined) in which a similar protocol to that in Figure 4a was used, except that during the period when the cell was not depolarized, clofilium (30 μM) was applied for 45 s and then washed off for 3 min. On resuming cell depolarization, it can be seen that currents were progressively reduced in amplitude and, as in Figure 4a, several pulses were required for clofilium to reach its steady level of inhibition of IK_v. As this progressive reduction in amplitude occurred in the absence of unbound clofilium in the perfusate, it appears that clofilium can bind to the closed channel, does not readily unbind, and repeated channel activation is required for blockade to become maximal i.e. although blockade is use-dependent, drug binding to the channel (or a closely associated site) is not.

Lack of effect of sotalol IK_v *in NG 108-15 cells*

The effects of another class III antidysrhythmic agent, sotalol, which has also been shown to block cardiac IK_v (Carmeliet, 1985) was tested on the neuronal IK_v in NG 108-15 cells. Bath application of sotalol at doses of 100 μM ($n = 13$ cells) or 300 μM ($n = 6$; data not shown) were without any discernible effect on IK_v throughout the range of activating test potentials studied, indicating that sotalol exhibits at least a degree of tissue-selectivity, as such concentrations cause profound electrophysiological changes in cardiac myocytes (Carmeliet, 1985).

Discussion

IK_v contributes to the repolarization of both cardiac and neuronal tissue following action potential generation. Although IK_v in the two tissue types show a number of differences (particularly in their rates of activation), they have a number of common features; most notably they have a similar pharmacological profile (e.g. Cook, 1988). The most common and important feature of the actions of class III antidysrhythmic agents is their ability to inhibit the cardiac IK_v, and the studies presented here were prompted by the lack of information concerning the tissue-selectivity of these compounds. The two examples chosen in this study, clofilium and sotalol, were shown to be profoundly different in their actions on neuronal IK_v, as recorded in NG 108-15 cells.

Sotalol, which has been shown to prolong the cardiac action potential in human and guinea-pig cardiac tissue (e.g. Campbell, 1987), has been demonstrated, by use of conventional voltage-clamp recordings, to cause dose-dependent, reversible inhibitions of guinea-pig cardiac IK_v which were half-maximal at a concentration of 10 μM (Carmeliet, 1985). In contrast to these observations, we found no effect of sotalol at concentrations up to 300 μM on neuronal IK_v in NG 108-15 cells, a finding which is consistent with the idea that sotalol exhibits tissue selectivity in its actions on IK_v.

In marked contrast to the lack of observed effect of sotalol, we found clofilium to be a potent inhibitor of IK_v in NG 108-15 cells (Figures 2–4). However, the blocking effects of clofilium on IK_v in NG 108-15 cells are quite different from its blocking effects reported in guinea-pig isolated ventricular myocytes (Arena & Kass, 1988). In the cardiac tissue, clofilium blockade of IK_v is irreversible, voltage-independent and does not appear to change the kinetics of the activated current. Furthermore, the drug is ineffective at a concentration of 10 μM and causes only 50% inhibition at a concentration of 50 μM (Arena & Kass, 1988). Blockade by clofilium of IK_v in NG 108-15 cells reported here was also irreversible (e.g. Figure 4a), but was voltage-dependent and greatly altered the kinetics of the activated current (e.g. Figure 2a; although was without effect on current activation; Figure 2b). Clofilium was also seen to be a more potent inhibitor of the neuronal than the cardiac IK_v; a concentration of 10 μM, which is without effect in cardiac tissue (see above) caused strong inhibition in NG 108-15 cells (Figures 2 and 3).

Such differences in the effects of clofilium in neuronal tissue, as compared with cardiac tissue (see above), strongly suggest a different mechanism of action of the drug in the two tissue types. For this reason, we performed experiments designed to investigate the mechanism of clofilium blockade of IK_v in NG 108-15 cells, using protocols similar to those of Lee & Tsien (1983), who investigated the mechanism of action of organic Ca^{2+} channel antagonists on cardiac Ca^{2+} currents. Such studies revealed a number of features of clofilium blockade of IK_v in NG 108-15 cells. Firstly, the effects of clofilium were shown to be use-dependent (Figure 4a), repeated depolar-

izations were required for the blockade by clofilium to reach a steady level. This effect was in marked contrast to the effects of TEA, where the first depolarization following its application evoked a much smaller current, which did not diminish further during subsequent depolarizations (Figure 4b). Secondly, it appears that clofilium blocks the open delayed rectifier K^+ channel in NG 108-15 cells; the upstroke, or activation of currents was initially unchanged in the presence of the drug (Figure 2b), but the peak was reduced and the current showed marked time-dependent decay during test depolarizations (Figure 2), suggesting that the drug was only effective when channels were activated, or opened. However, a third feature of the action of clofilium in these cells was that its irreversible binding to the delayed rectifier channel (or a site intimately related to the functioning of the channel) was not dependent on channel activation; a similar pattern of use-dependent inhibition of IK_v was found after application and then washout of unbound clofilium (compare Figures 4a and 4c).

Although the effects of clofilium described here are different from the action of the drug on cardiac IK_v, they are similar in some respects to the recently reported actions of clofilium on batrachotoxin-activated skeletal muscle Na^+ channels incorporated into planar lipid bilayers (Nettleton *et al.*, 1991). These Na^+ channels are blocked by clofilium in a concentration – and strongly voltage-dependent manner, and clofilium appears to act as an open channel blocker in a similar manner to local anaesthetics (Nettleton *et al.*, 1991).

In summary, data presented here demonstrate that clofilium can block neuronal delayed rectifier K^+ channels, but its effects are markedly different from those previously described on cardiac IK_v (Arena & Kass, 1988), suggesting a different mechanism of action. The actions of clofilium are voltage-dependent, changes occur in the kinetics of the activated IK_v in NG 108-15 cells, and the drug is a more potent inhibitor of neuronal IK_v than cardiac IK_v. However, sotalol, another class III antidysrhythmic agent, was without effect on IK_v in NG 108-15 cells. Such findings suggest these compounds show differential tissue selectivity and it would be of interest to examine the actions of other examples of this diverse class of compounds in tissues other than the heart.

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Reduced α_1- and β_2-adrenoceptor-mediated positive inotropic effects in human end-stage heart failure

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1 α_1-Adrenoceptor (phenylephrine in the presence of propranolol) and β_2-adrenoceptor (fenoterol)-mediated positive inotropic effects were investigated in human ventricular preparations isolated from five nonfailing (prospective organ donors) and from eight explanted failing hearts with end-stage idiopathic dilative cardiomyopathy (NYHA IV).

2 For comparison, the nonselective β-adrenoceptor agonist isoprenaline, the phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methylxanthine (IBMX), the cardiac glycoside dihydroouabain, and calcium were studied.

3 Furthermore, the influence of IBMX on adenosine 3':5'-cyclic monophosphate (cyclic AMP) PDE activity as well as total β-adrenoceptor density, β_1- and β_2-adrenoceptor subtype distribution, and α_1-adrenoceptor density were compared in nonfailing and failing human heart preparations. The radioligands (−)-[^{125}I]-iodocyanopindolol for β-adrenoceptor binding and [^{3}H]-prazosin for α_1-adrenoceptor binding were used.

4 The inotropic responses to calcium and dihydroouabain in failing human hearts were unchanged, whereas the maximal α_1- and β_2-adrenoceptor-mediated positive inotropic effects were greatly reduced. The inotropic effects of the other cyclic AMP increasing compounds, i.e. isoprenaline and IBMX, were also reduced to about 60% of the effects observed in nonfailing controls. The potency of these compounds was decreased by factors 4–10.

5 The basal PDE activity and the PDE inhibition by IBMX were similar in nonfailing and failing preparations.

6 The total β-adrenoceptor density in nonfailing hearts was about 70 fmol mg^{-1} protein. In failing hearts the total number of β-adrenoceptors was markedly reduced by about 60%. The β_1/β_2-adrenoceptor ratio was shifted from about 80/20% in nonfailing to approximately 60/40% in failing hearts which was due to a selective reduction of β_1-adrenoceptors. The β_2-adrenoceptor population remaining unchanged. α_1-Adrenoceptor density was increased from about 4 fmol mg^{-1} protein in nonfailing to 10 fmol mg^{-1} protein in failing hearts.

7 Changes in PDE activity and adrenoceptor downregulation cannot completely explain the reduced positive inotropic effects of α_1- and β_2-adrenoceptor agonists in failing human hearts. This supports the hypothesis that impairment of other processes such as the coupling between receptor and effector system, i.e. the respective G-proteins, are equally important in end-stage heart failure.

Keywords: α_1-Adrenoceptor; β_2-adrenoceptor; positive inotropic effect; human heart; end-stage heart failure; idiopathic dilated cardiomyopathy

Introduction

Heart failure is characterized by a reduced effect of sympathomimetic agents on forces of contraction. In isolated cardiac preparations a reduced responsiveness to agents acting on β-adrenoceptors like isoprenaline has been reported (Bristow *et al.*, 1982; 1984; Ginsburg *et al.*, 1983; Schmitz *et al.*, 1987; Böhm *et al.*, 1988a; Brodde, 1991). Likewise the effects of compounds that increase adenosine 3':5'-cyclic monophosphate (cyclic AMP) by inhibition of phosphodiesterase are reduced in failing human heart preparations (Wilmshurst *et al.*, 1984; Brown *et al.*, 1986; Feldman *et al.*, 1987; Schmitz *et al.*, 1987). In patients with idiopathic dilated cardiomyopathy the number of β_2-adrenoceptors was similar in nonfailing and failing human hearts while the amount of β_1-adrenoceptors was selectively reduced (Bristow *et al.*, 1986; 1990; Brodde *et al.*, 1989; Böhm *et al.*, 1989a; Brodde, 1991; Steinfath *et al.*, 1991). It is possible, therefore, that the functional response to β_2-adrenoceptor stimulation in the failing hearts is unimpaired in comparison to nonfailing controls. This could explain the beneficial effect of fenoterol (Aggestrup *et al.*, 1980; Mügge *et al.*, 1985; Brodde *et al.*, 1986) and zinoterol (Bristow *et al.*, 1986) which preferentially act on β_2-adrenoceptors in heart failure. However, the positive inotropic effects of fenoterol have not been compared in nonfailing and failing human cardiac preparations. Compounds that act independently of cyclic AMP like cardiac glycosides or calcium are known to increase force of contraction to a similar extent in preparations from nonfailing and failing human hearts (Brown *et al.*, 1986; Feldman *et al.*, 1987; Schmitz *et al.*, 1987). Hence, it was of interest to study the effects of other cyclic AMP-independent agents, namely α_1-adrenoceptor agonists. It is conceivable that an unimpaired or even enhanced positive inotropic response mediated through α_1- and/or β_2-adrenoceptors may in part compensate for a reduced β_1-adrenoceptor-mediated effect of catecholamines.

Therefore, we investigated the effects of various positive inotropic agents in right venticular preparations isolated from nonfailing and failing human hearts, i.e. the positive inotropic effects of the β_2-adrenoceptor agonist, fenoterol, the α_1-adrenoceptor agonist, phenylephrine, the nonselective β-adrenoceptor agonist, isoprenaline, the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX), the cardiac glycoside dihydroouabain, and calcium. In order to get an insight

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into the possible mechanisms underlying the reduced positive inotropic effects of some of these agents in the failing human heart, we compared phosphodiesterase activity as well as total β- and α_1-adrenoceptor density and β_1/β_2-adrenoceptor subtype distribution in nonfailing and failing human ventricular preparations.

Methods

Patients

Five nonfailing hearts were obtained from transplant donors (2 female, 3 male; mean age: 36.3 years, range 27 to 54 years; death due to cerebral hemorrhage) whose hearts could not be used for surgical reasons or immunological incompatibility. Eight failing hearts were obtained from patients undergoing orthotopic heart transplantation due to end-stage idiopathic dilated cardiomyopathy (NYHA IV). Clinical data of heart transplant recipients are given in Table 1. None of these patients was treated with β-adrenoceptor antagonists or received catecholamines during the last three weeks before operation. Patients were treated with nitrates, diuretics, calcium antagonists, angiotensin converting enzyme inhibitors and digitalis glycosides alone or in combination. General anaesthesia was performed with neuroleptic combinations (droperidol, fentanyl, and nitrous oxide). Written informed consent was obtained from the families of all donors of nonfailing hearts and from all patients undergoing cardiac transplantation before explantation of the heart. After explantation, the hearts were transferred to the laboratory in ice-cold aerated bathing solution (composition see below). Contraction experiments were started immediately, whereas radioligand binding studies and cyclic nucleotide phosphodiesterase isolation were carried out on tissue samples frozen in liquid nitrogen and stored at -80°C until use.

Contraction experiments

Contraction experiments were performed on electrically driven trabeculae carneae isolated from right ventricles of five nonfailing and eight failing human hearts. The trabeculae (diameter less than 1.0 mm, length 6–8 mm) were dissected in bathing solution at room temperature. The bathing solution was a modified Tyrode solution containing (mmol l^{-1}) NaCl 119.8, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 22.6, Na_2EDTA 0.05, ascorbic acid 0.28 and glucose 5.0, continuously gassed with 95% O_2 and 5% CO_2; pH 7.4. Several preparations were obtained from each heart, attached to a bipolar platinum stimulating electrode (field stimulation) and suspended individually in 10 ml glass tissue chambers for recording isometric contractions as described previously (Böhm *et al.*, 1984). The temperature in the organ bath was 35°C. A release of endogenous catecholamines due to the field stimulation is not likely to play an important role because the β-adrenoceptor antagonist propranolol (1 μmol l^{-1}, 30 min) decreased force of contraction by about 10% only. Force of contraction was measured with an inductive force transducer (W. Fleck, Mainz, Germany) attached to a Hellige Helco Scriptor recorder. Each muscle was stretched to the length at which force of contraction was maximal. The resting force (approximately 5 mN) was kept constant throughout the experiment. Preparations were electrically paced at 0.5 Hz with rectangular pulses of 5 ms duration (Grass stimulator SD 9) and a voltage about 10–20% above threshold. All preparations were equilibrated in drug-free bathing solution until complete mechanical stabilization (at least 90 min). Effects of the drug were investigated after individual equilibration by exposure to cumulatively increasing contractions. First, a concentration-response curve for calcium was established. After complete washout, a concentration-response curve for isoprenaline was performed. Then, alternatively, the effects of IBMX or fenoterol were studied. Effects of phenylephrine were recorded in the presence of 1 μmol l^{-1} propranolol given 30 min before phenylephrine. Finally, the effects of dihydroouabain were recorded on all preparations. The incubation times for each concentration of the drugs were 5 min for calcium, 5 min for isoprenaline, 15 min for IBMX, 10 min for fenoterol, 10 min for phenylephrine, and 30 to 45 min for dihydroouabain until the effect was stable.

Cyclic nucleotide phosphodiesterase (PDE) activity

PDE activity from five nonfailing or eight failing human hearts was separated into three isoenzymes by DEAE-cellulose anion exchange chromatography as described previously by Brunkhorst *et al.* (1988) with minor modifications. Frozen samples of ventricular tissue were thawed in 30 ml ice-cold PDE isolation buffer ($MgCl_2$ 2 mmol l^{-1}, dithioerythritol 1 mmol l^{-1}, Tris-HCl 10 mmol l^{-1}; pH 7.5). All subsequent procedures were carried out at 4°C. The tissue was minced with scissors and homogenized with a Polytron-homogenizer (PT 10–35, Kinematica, Littau-Luzern, Switzerland) and then with a glass-teflon potter (Colora, Lorch, Germany). The homogenate was centrifuged at 3,0000 *g* for 20 min. After gauze filtration, the resulting supernatant was applied to a DEAE-cellulose column (40 × 1.6 cm, bed volume about 30 ml) equilibrated with freshly prepared buffer (sodium acetate 70 mmol l^{-1}, 2-mercaptoethanol 5 mmol l^{-1}; pH 6.5). After washing the column with 2–3 bed volumes of this buffer, the phosphodiesterases were eluted from the column using a continuous 70–1000 mmol l^{-1} sodium acetate gradient (containing 2-mercaptoethanol 5 mmol l^{-1}, pH 6.5, total volume 400 ml, flow rate approximately 25 ml h^{-1}). Fractions of 8 ml were collected and immediately assayed for cyclic AMP and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) PDE activity (substrate concentration 1 μmol l^{-1}) in the presence or absence of 2 units of calmodulin and $CaCl_2$ 10 μmol l^{-1}. The cyclic AMP PDE was additionally assayed in the presence of cyclic GMP 0.5 μmol l^{-1} and with a substrate concentration of 25 μmol l^{-1} cyclic AMP. Appropriate

Table 1 Haemodynamic characteristics of heart transplant recipients with idiopathic dilated cardiomyopathy (NYHA-stage IV)

Patient No.	*Sex*	*Age*	*CO*	*CI*	*PAP*	*RV*	*PCP*
1	m	34	4.29	2.2	38	55	27
2	m	47	4.37	2.0	28	40	10
3	m	57	1.96	n.d.	40	46	32
4	f	66	3.30	1.9	38	45	30
5	m	52	3.00	1.6	42	57	35
6	m	46	4.00	2.1	36	ND	19
7	f	58	3.00	1.9	35	ND	25
8	m	51	ND	2.1	25	35	18
mean ± s.e.mean		51.4 ± 3.4 (n = 8)	3.42 ± 0.3 (n = 7)	2.0 ± 0.1 (n = 7)	35.3 ± 2.1 (n = 8)	46.3 ± 3.5 (n = 6)	24.5 ± 3.0 (n = 8)

CO = cardiac output (1 min^{-1}), CI = cardiac index (1 min^{-1} m^2), PAP = mean pulmonary artery pressure (mmHg), RV = systolic right ventricular pressure (mmHg), PCP = mean pulmonary capillary pressure (mmHg), m = male, f = female, ND = not determined

peak fractions (referred to as PDE I, II, and III) were pooled and concentrated to 14% of the original volume by ultrafiltration (Amicon cell, fitted with a PM 10 membrane; Amicon, Witten, Germany). The PDE isoenzymes were characterized by specific properties as given by Brunkhorst *et al.* (1988). PDE I and III are low K_m enzymes, PDE II is a high K_m enzyme. PDE I and II hydrolyze both cyclic AMP and cyclic GMP while PDE III hydrolyzes only cyclic AMP to an appreciable degree. PDE I is stimulated by Ca^{2+}/calmodulin, PDE II is stimulated and PDE III is inhibited by 0.5 μmol l^{-1} cyclic GMP. Then, the protein was diluted to 65% with ethylene glycol monoethyl ether and stored at −20°C. Under these conditions, hydrolytic activity was stable for 2–3 weeks. Phosphodiesterase activity was determined in a two-step procedure as described previously (Brunkhorst *et al.*, 1988). The reaction mixture consisted of $MgCl_2$ 5 mmol l^{-1}, Tris-HCl 40 mmol l^{-1}, (pH 8.0), cyclic AMP or cyclic GMP 1 μmol l^{-1}, [^{3}H]-cyclic AMP or [^{3}H]-cyclic GMP (20,000–30,000 c.p.m.) and enzyme preparation (diluted in 0.1% bovine serum albumin) in a final volume of 200 μl. By suitable enzyme dilution, the substrate conversion was kept below 20% in order to be in the linear range of the enzyme reaction. The incubation time was 10 min at 30°C. Protein concentrations were determined by Bio-Rad Protein Assay according to Bradford (1976).

Radioligand binding experiments

For determination of β-adrenoceptors, tissues (150–200 mg) were thawed and membranes were prepared as described by Brodde *et al.* (1984) with minor modification. In brief: tissues were minced and homogenized for 10 s and 2 × 20 s in ice-cold $KHCO_3$ 1 mmol l^{-1} with a Ultra-Turrax (Jahnke and Kunkel, Staufen, Germany) and a polytron-homogenizer (PT 10–35 Kinematica, Luzern, Switzerland). Homogenates were centrifuged at 150 *g* for 20 min at 4°C. The supernatant was filtered through four layers of gauze and centrifuged at 50,000 *g* for 20 min at 4°C. Pellets were resuspended in Tris-HCl 10 mmol l^{-1}, NaCl buffer 154 mmol l^{-1} – pH 7.4 containing ascorbic acid 0.55 mmol l^{-1} and homogenized for 10 s. For determination of total β-adrenoceptor density membranes were incubated with six different concentrations of (−)-[^{125}I]-iodocyanopindolol (ICYP) ranging from 5 to 200 pmol l^{-1} for 1 h at 37°C (Brodde *et al.*, 1986). Nonspecific binding of ICYP was defined as binding to membranes that was not displaced by a high concentration of the nonselective β-adrenoceptor antagonist (±)-CGP 12177 (1 μmol l^{-1}; 4-[3-tertiary-butylamino-2-hydroxypropoxy]-benzimidazole-2-on). Specific binding of ICYP was defined as total binding minus nonspecific binding; it amounted to 70–80% at 50 pmol l^{-1} of ICYP. To determine the relative amount of β_1- and β_2-adrenoceptors membranes were incubated with ICYP (50 pmol l^{-1}) in the presence and absence of 25 different concentrations of the β_1-adrenoceptor antagonist CGP 20712A (1-[2-(3-carbamoyl-4-hydroxy)phenoxyethylamino]-3-[4-(methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol methanesulphonate). The $\beta1/\beta2$-adrenoceptor ratio of the CGP 20712A competition curves were analysed by the iterative curve-fitting programme Graph-PAD InPlot (GraphPAD Software, San Diego, California, U.S.A.).

For determination of α_1-adrenoceptors, membranes were prepared according to the method of Bevilacqua *et al.* (1987) with minor modification. Frozen left ventricular samples were thawed in ice-cold homogenization buffer (KCl 100 mmol l^{-1}, Tris-HCl 25 mmol l^{-1}, $MgCl_2$ 2 mmol l^{-1}, EDTA 1 mmol l^{-1}, sucrose 8.55%; pH 7.5). All subsequent procedures were carried out at 4°C. Myocardial tissues were minced with scissors and homogenized twice with an 'Ultra-turrax'. After gauze filtration the membrane preparation was centrifuged (5 min at 600 *g*), the pellet discarded and the supernatant recentrifuged (35 min at 48,000 *g*). The resulting pellet was suspended in the same buffer and again centrifuged (35 min at 48,000 *g*). The resulting pellet was suspended in buffer without KCl (Tris-HCl 25 mmol l^{-1}, $MgCl_2$ 2 mmol l^{-1}, EDTA 1 mmol l^{-1}, sucrose 8.55%; pH 7.5) and homogenized first with a glass-teflon potter (Colora, Lorch, Germany) and with a 'Polytron-homogenizer'. This suspension was centrifuged (15 min at 13,000 *g*), and the resulting supernatant recentrifuged (45 min at 160,000 *g*). The pellet was finally resuspended in incubation buffer (Tris-HCl 50 mmol l^{-1}, $MgCl_2$ 10 mmol l^{-1}, EDTA 1 mmol l^{-1}; pH 7.5). For determination of α_1-adrenoceptors, membranes were incubated with 6 different concentrations of [^{3}H]-prazosin ranging from 0.1 to 5.0 nmol l^{-1} for 60 min at 37°C. Nonspecific binding was defined with phentolamine 5 μmol l^{-1}.

Drugs

The following drugs were used: (±)-isoprenaline HCl, phenylephrine HCl, fenoterol HBr (all from Boehringer, Ingelheim, Germany), (±)-propranolol HCl (Rheinpharma, Heidelberg, Germany), phentolamine HCl (Ciba-Geigy, Basel, Switzerland), 3-isobutyl-1-methyl-xanthine (IBMX; EGA-Chemie, Steinheim, Germany), dihydroouabain (Hommel, Adliswil, Switzerland), calmodulin from bovine heart (Sigma, Deisenhofen, Germany), ethylene glycol monoethyl ether (Fluka, Neu-Ulm, Germany), DEAE-cellulose (Sephacel; Pharmacia LKB, Freiburg, Germany), Lab-trol-E (Merz + Dade, Düdingen, Switzerland), [^{3}H]-cyclic AMP, [^{3}H]-cyclic GMP, [^{3}H]-prazosin, (−)-[^{125}I]-iodocyanopindolol (all from NEN, Dreieich, Germany). (±)-CGP 12177 and (±)-CGP 20712A were gifts from Ciba-Geigy, Basel, Switzerland. All other chemicals were of analytical or best commercial grade available. Deionized and twice distilled water was used throughout.

Statistics

Data are expressed as arithmetic means ± s.e.mean. Significant differences between means were assessed by Student's *t* test for paired and unpaired observations. A *P* value of less than 0.05 was considered significant. EC_{50} and IC_{50} values are given as geometric means with 95% confidence limits. The equilibrium dissociation constant (K_D) and the maximal number of binding sites (B_{max}) were calculated from plots according to Scatchard (1949) and by the computer programme GraphPAD InPlot. The two methods yielded identical results.

Results

Contraction experiments

The effects of various positive inotropic agents on force of contraction of trabeculae carneae isolated from nonfailing and failing human hearts were compared (Table 2, Figure 1). The predrug values of the nonfailing preparations were 1.0 ± 0.1 mN (n = 119), and the predrug values of the failing preparations were 1.7 ± 0.1 mN (n = 148; legend to Figure 1). The maximal contractile activity of the preparations was assessed by calcium which acts independently of adrenoceptor activation and cyclic AMP concentration. It was about 600% of the predrug value at about 12 mmol l^{-1} in nonfailing and failing preparations (Table 2). Contractures occurred without further increase in force of contraction at higher concentrations. Thus, the effects at about 12 mmol l^{-1} were defined as maximum. The EC_{50} values for calcium also were similar in nonfailing and failing preparations (about 8 mmol l^{-1}; Table 2). Furthermore, the maximum response (500–600% at 5 μmol l^{-1}) and EC_{50} values (about 1.5 μmol l^{-1}) obtained for ouabain were comparable in nonfailing and failing preparations (Table 2). At higher concentrations of ouabain, a slight increase in force of contraction was observed and contractures occurred. The maximum inotropic response was

Table 2 Maximum change in force of contraction (FC) and EC_{50} values for different agents in trabeculae carneae from nonfailing and failing human hearts

Nonfailing	*Change in FC* (mN)	*EC_{50}-value* (μmol l^{-1})	n
Calcium	5.91 ± 0.62	8200 (5900–14,200)	32
Ouabain	5.02 ± 0.70	1.80 (1.6)–2.1)	32
Isoprenaline	4.62 ± 0.51	0.019 (14.8–24.5)	24
Fenoterol	3.88 ± 0.42	0.34 (0.28–0.42)	11
IBMX	3.98 ± 0.78	8.70 (6.2–12.3)	12
Phenylephrine	1.80 ± 0.77	12.3 (7.1–21.4)	7
Failing	*Change in FC* (mN)	*EC_{50}-value* (μmol l^{-1})	n
Calcium	5.78 ± 0.50	8000 (5700–12,300)	45
Ouabain	6.58 ± 1.12	1.27 (1.15–1.39)	43
Isoprenaline	3.00 ± 0.34*	0.14 (0.13–0.15)*	29
Fenoterol	2.52 ± 0.32*	3.34 (2.63–4.17)*	11
IBMX	1.26 ± 0.18*	36.3 (30.2–43.6)*	19
Phenylephrine	ND	ND	7

ND = no detectable positive inotropic effect.
*$P < 0.05$ versus nonfailing.
IBMX = isobutylmethylxanthine.

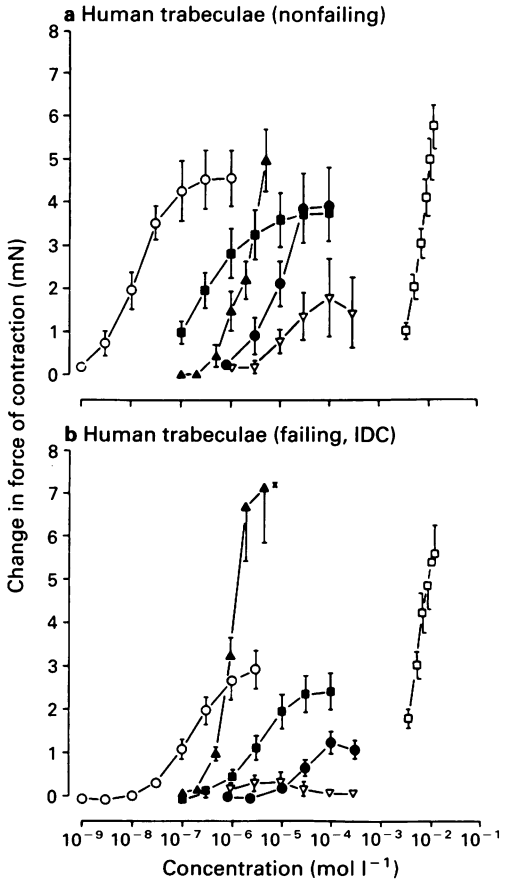

Figure 1 Cumulative concentration-response curves for the effects of isoprenaline (○; a: $n = 24$; b: $n = 29$), 3-isobutyl-1-methylxanthine (IBMX ●; a: $n = 12$; b: $n = 19$), dihydroouabain (▲; a: $n = 22$–35; b: $n = 20$–35), fenoterol (■; a: $n = 11$; b: $n = 11$), phenylephrine in the presence of 1 μM propranolol (▽; a: $n = 6$; b: $n = 13$), and calcium (□; a: $n = 34$; b: $n = 41$) on force of contraction in electrically driven (0.5 Hz) human trabeculae (n = number of trabeculae from 4 nonfailing (a) and 5 failing hearts (b), respectively). x = concentration of dihydroouabain at which contracture occurred in some preparations. Ordinates: increase in force of contraction in mN. Abscissae: drug concentration in mol l^{-1}. The predrug values of force of contraction were 1.0 ± 0.1 mN (a: $n = 119$) and 1.7 ± 0.1 mN (b: $n = 148$), respectively.

assessed at the highest concentration of ouabain without contractures. The maximum positive inotropic effects of isoprenaline, fenoterol, and IBMX were similar to the effects of ouabain and calcium (about 400 to 500% of predrug value) in nonfailing preparations. The β_2-adrenoceptor agonist, fenoterol, was less potent than the nonselective β-adrenoceptor agonist, isoprenaline. In failing heart preparations the maximal inotropic responses for isoprenaline, fenoterol, as well as IBMX were markedly reduced as compared with nonfailing preparations. In addition, the EC_{50} values for isoprenaline, fenoterol, and IBMX were significantly greater in failing then in nonfailing hearts (Table 2). The maximal positive inotropic effect of the α_1-adrenoceptor agonist, phenylephrine, was about 180% of the predrug value at 100 μmol l^{-1} (Table 2) in nonfailing preparations which is about 45% of the maximal effect of isoprenaline. In failing hearts, phenylephrine produced no marked positive inotropic effects. Thus, the maximal inotropic effects of all compounds acting via an increased cyclic AMP level, namely isoprenaline, IBMX, as well as fenoterol, and of the cyclic AMP-independent acting α_1-adrenoceptor agonist, phenylephrine, were reduced in failing human hearts as compared with the effects observed in nonfailing hearts and with the unchanged effects of calcium and ouabain.

Cyclic nucleotide phosphodiesterase activity

Cyclic nucleotide phosphodiesterase (PDE) activity in failing and nonfailing hearts was determined in order to elucidate a possible involvement of PDE in the development of heart failure. Basal activities of all three isoenzymes (legend of Figure 2) did not differ in nonfailing and failing human hearts. The effects of the PDE inhibitor IBMX on cyclic AMP PDE activity of the separated PDE isoenzymes PDE I–III are shown in Figure 2. In nonfailing human ventricular preparations, IBMX inhibited all three PDE isoenzymes nonselectively with similar potencies (IC_{50} about 10–30 μmol l^{-1}; Figure 2a). No difference in PDE inhibition by IBMX could be observed in preparations from failing and nonfailing hearts (Figure 2b).

Radioligand binding experiments

Total β-adrenoceptor density, β_1/β_2-adrenoceptor subtype distribution, and α_1-adrenoceptor density in nonfailing and failing human left ventricular myocardium are summarized in Table 3. In nonfailing hearts, the total β-adrenoceptor density was found to be 70.4 ± 6.2 fmol mg^{-1} protein including a

Table 3 Binding of (−)-[^{125}I]-iodocyanopindolol ([^{125}I]-ICYP) to β-adrenoceptors and [^{3}H]-prazosin to α_1-adrenoceptors in membrane preparations obtained from nonfailing and failing human left ventricular myocardium

β-Adrenoceptor binding	*Nonfailing* ($n = 5$)	*Failing* ($n = 8$)
B_{max} (fmol mg^{-1} protein)	70.4 ± 6.2	29.9 ± 3.4*
β_1/β_2-ratio (%)	79/21	60/40*
β_1-subtype density (fmol mg^{-1} protein)	55.6 ± 3.2	17.9 ± 2.4*
β_2-subtype density (fmol mg^{-1} protein)	14.8 ± 3.2	12.0 ± 2.4
K_D (pmol l^{-1})	11.4 ± 2.9	14.1 ± 3.2
α_1-Adrenoceptor binding	*Nonfailing* ($n = 5$)	*Failing* ($n = 7$)
B_{max} (fmol mg^{-1} protein)	3.9 ± 1.3	10.3 ± 2.5*
K_D (nmol l^{-1})	0.34 ± 0.04	0.47 ± 0.13

The relative amount of β_1- and β_2-adrenoceptors was determined by inhibition of [^{125}I]-ICYP binding by the selective β_1-adrenoceptor antagonist CGP 20712A and analysis of the resulting competition curves by the interactive curve fitting programme GraphPAD InPlot. *$P < 0.05$ vs. nonfailing.

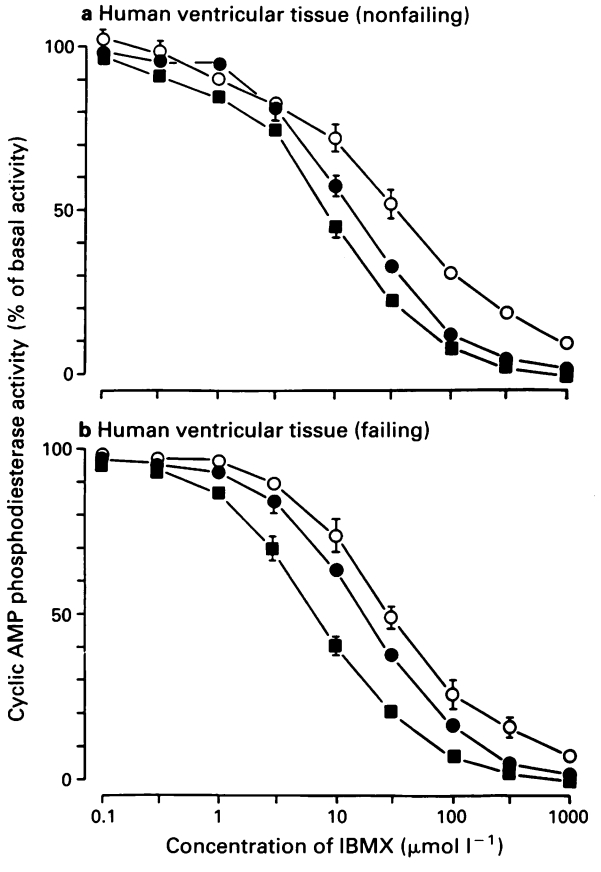

Figure 2 Influence of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) on the activity of the cyclic AMP phosphodiesterase (PDE) isoenzymes, PDE I (●), PDE II (○), and PDE III (□) in ventricular tissue isolated from nonfailing (a: $n = 3$) or failing (b: $n = 3$) human hearts. Ordinates: cyclic AMP PDE activity as percentage of basal activity. Abscissae: concentration of IBMX in μmol l^{-1}. Incubation time was 10 min at 30°C. Substrate concentration was 1 μmol l^{-1} cyclic AMP. Basal activities of PDE isoenzymes (pmol cyclic AMP mg^{-1} protein min^{-1}): a: PDE I: 1472 ± 677 ($n = 3$), PDE II: 246.0 ± 38.6 ($n = 3$), PDE III: 2736 ± 1239 ($n = 3$); (b) PDE I: 1696 ± 755 ($n = 3$), PDE II: 329 ± 26 ($n = 3$), PDE III: 5624 ± 395 ($n = 3$). IC_{50} values are given as geometric means with 95% confidence intervals. IC_{50} values (μmol l^{-1}, $n = 3$ each) determined in nonfailing human heart tissues: PDE I 13.7 (7.9–23.7); PDE II 33.6 (12.6–89.4); PDE III 8.23 (5.7–11.8). IC_{50}-values (μmol l^{-1}, $n = 3$ each) determined in failing human heart tissues: PDE I 17.6 (15.6–19.8); PDE II 29.0 (14.3–58.8); PDE III 6.64 (4.0–11.0).

β_1-adrenoceptor subtype population of 78.9 ± 2.9%. In failing heart preparations the total number of β-adrenoceptors was markedly reduced by about 60% as compared with nonfailing controls (29.9 ± 3.4 fmol mg^{-1} protein). In these failing hearts the β_1/β_2-adrenoceptor ratio shifted to about 60/40% due to a selective reduction of β_1-adrenoceptors. The β_2-adrenoceptor population was unchanged. The α_1-adrenoceptor density, which was 3.9 ± 1.3 fmol mg^{-1} protein in nonfailing hearts, increased to 10.3 ± 2.5 fmol mg^{-1} protein in failing hearts ($P < 0.05$). The binding affinity (K_D) was unchanged in nonfailing and failing hearts for β-adrenoceptor binding and α_1-adrenoceptor binding, respectively.

Discussion

The positive inotropic effect of nonselective β-adrenoceptor agonists like isoprenaline has been shown to be reduced in failing human myocardium as compared with nonfailing human cardiac preparations in the present and in previous studies (Ginsburg *et al.*, 1983; Bristow *et al.*, 1984; Schmitz *et al.*, 1987; Böhm *et al.*, 1988a; Brodde, 1991). In addition, the contractile response to the β_2-selective adrenoceptor agonist and to the phosphodiesterase inhibitor IBMX was diminished to a similar extent in failing preparations.

Since the common mechanism of action of these positive inotropic agents is an increase in cyclic AMP, one can assume that an insufficient rise in myocardial cyclic AMP is involved in the decreased responsiveness of failing heart muscle preparations. Indeed, a reduced adenylate cyclase activity (Bristow *et al.*, 1982; 1984; 1988; Denniss *et al.*, 1989) and a greatly attenuated increase in cyclic AMP content after stimulation with isoprenaline or phosphodiesterase inhibitors (Danielsen *et al.*, 1989; Meyer *et al.*, 1989; Schmitz *et al.*, 1989) in failing as compared with nonfailing human heart preparations have recently been shown. It has also been suggested that the decreased positive inotropic response to nonselective β-adrenoceptor agonists like isoprenaline is due to a reduced number of β_1-adrenoceptors in the failing myocardium (Ginsburg *et al.*, 1983; Bristow *et al.*, 1984; 1986; 1988; Zerkowski *et al.*, 1986). However, a lower density of β_1-adrenoceptors cannot easily explain a reduced positive inotropic effect of the phosphodiesterase inhibitor IBMX which inhibits cyclic AMP breakdown bypassing cardiac cell surface receptors. Another explanation for the diminished contractile response to cyclic AMP increasing agents could be an altered phosphodiesterase activity in the failing myocardium. However, the present findings that the sensitivity of the different myocardial cyclic AMP phosphodiesterase isoenzymes to IBMX, as well as the basal activities of these enzymes are the same in preparations from failing and nonfailing hearts, argue against the possibility that an enhanced breakdown of cyclic AMP in failing as compared with nonfailing myocardium is responsible for the attenuated positive inotropic effects of phosphodiesterase inhibitors or β-adrenoceptor agents. Another explanation, however, could be provided by the recent finding that an inhibitory regulatory sarcolemmal GTP-binding protein (G_i-protein) is increased in failing human myocardium (Feldman *et al.*, 1988; Neumann *et al.*, 1988; Böhm *et al.*, 1989b; 1990). The increased G_i-protein might keep adenylate cyclase activity at a reduced level so that cyclic AMP production even in response to β-adrenoceptor agents or phosphodiesterase inhibitors is insufficient to induce a maximal positive inotropic effect. Another G-protein which stimulates adenylate cyclase activity (G_s-protein) has been shown to be unchanged in failing hearts and therefore is probably not involved in the reduced responsiveness to cyclic AMP increasing agents (Feldman *et al.*, 1988).

The present finding that the positive inotropic effect of the β_2-selective adrenoceptor agonist, fenoterol (Mügge *et al.*, 1985; Bristow *et al.*, 1989) is also diminished in the failing heart to an extent comparable to the reduced effect of the nonselective β-adrenoceptor agonist isoprenaline or the phosphodiesterase inhibitor, IBMX, supports the hypothesis that the G_i-protein is involved. It is worth noting that the diminished β_2-adrenergic response cannot be explained completely by a more reduced myocardial β_2-adrenoceptor density because the number of β_2-adrenoceptors was found to be unchanged in failing heart preparations. One might argue that the crude membrane preparations used in this and other studies may also contain non-myocardial cell membrane fractions (e.g. from endothelial, neuronal or smooth muscle cells) which might contribute to the overall receptor number measured. This could obscure the relation between changes in myocyte function and changes in receptor number. However, as yet viable myocytes from human hearts free of non-myocardial cells cannot be isolated in amounts sufficient for radioligand binding studies. Although earlier studies suggested that the β_2-adrenoceptor-mediated positive inotropic effect was not significantly reduced in failing human hearts (Bristow *et al.*, 1986), a recent article suggested that the β_2-adrenergic response is also blunted (Bristow *et al.*, 1989). Thus, the present data do not support the hypothesis (Bristow *et al.*, 1986; 1988; Brodde *et al.*, 1986) that an unaffected

β_2-adrenergic responsiveness may help maintain cardiac contractility if the β_1-adrenergic response is diminished, at least not in idiopathic dilated cardiomyopathy. Another possibility, at least for the β-adrenoceptor agonists and IBMX is that cellular events initiated by cyclic AMP are less sensitive in the failing heart. This phenomenon is a matter of controversy. Böhm *et al.* (1988) did not find a depression in dibutyryl cyclic AMP (db cyclic AMP) response in studies of human tissue from failing hearts, whereas Jones *et al.* (1990) have reported a depression in db cyclic AMP response seen in failing human isolated myocytes, indicating a reduced sensitivity for cyclic AMP. This discrepancy cannot be explained at present and requires further investigation.

It has been demonstrated repeatedly that agents acting independently of cyclic AMP, namely cardiac glycosides and calcium, are still effective in the failing heart (Brown *et al.*, 1986; Feldman *et al.*, 1987; Schmitz *et al.*, 1987) indicating that in end-stage heart failure the contractile myofilaments are not detectably compromised. Hence, it is reasonable to assume that other positive intropic agents acting independently of cyclic AMP might share the ability to increase force of contraction in the failing heart when cyclic AMP generating compounds like β-adrenoceptor agents or phosphodiesterase inhibitors fail to be effective. α_1-Adrenoceptors have been shown to be independent of cyclic AMP. They are thought to activate phospholipase C and thereby cause an increase in diacylglycerol and inositoltrisphosphate (Brown *et al.*, 1985; Otani *et al.*, 1988; Scholz *et al.*, 1988; Kohl *et al.*, 1989). In nonfailing human cardiac preparations, the α_1-adrenoceptor-mediated effect was 43% of the effect of isoprenaline in the present study. Therefore, in the nonfailing human heart, α_1-adrenergic stimulation may contribute to a physiologically relevant extent to the positive inotropic effects of endogenous catecholamines. In contrast, in failing preparations no significant α_1-adrenoceptor-mediated positive inotropic effect was observable. However, α_1-adrenergic positive inotropic effects have been detected also in preparations derived from diseased cardiac tissue (Schümann *et al.*, 1978; Brückner *et al.*, 1984; Schmitz *et al.*, 1987; Böhm *et al.*, 1988b; Jakob *et al.*, 1988). This discrepancy may be explained by the preparations used in the respective studies. Schümann *et al.* (1978) investigated atrial tissue. In previous reports from our laboratory, tissue from less severely failing hearts was studied (Brückner *et al.*, 1984; Schmitz *et al.*, 1987). Jakob *et al.* (1988) described only a marginal effect of α_1-adrenoceptor stimulation which could be the result of using tissue from severely diseased patients. These findings underline the necessity to compare failing and nonfailing human cardiac preparations. Our present results argue against the effectiveness of cyclic AMP-independently acting α_1-adrenoceptor agonists to sustain force of contraction in severe heart failure. The loss of responsiveness to α_1-adrenergic stimulation might even aggravate the clinical course of the disease, although the physiological relevance of an α_1-adrenoceptor-mediated positive inotropic effect in man *in vivo* is as yet unclear. A reduction of α_1-adrenoceptors cannot explain the diminished α_1-adrenergic positive inotropic effect in end-stage heart failure. Instead, we observed an increase in α_1-adrenoceptor density beside the reduced positive inotropic effect. However, it should be mentioned that in nonfailing human hearts, the α_1-adrenoceptor density was very low (about 4 fmol mg^{-1} protein) so that the determination was performed close to the detection limit of the method used. Therefore, one should be careful in interpreting this apparent increase in α_1-adrenoceptors in failing hearts. Nevertheless, it seems to be very likely that there is no decrease of α_1-adrenoceptor number in failing human hearts. In accord with this, α_1-adrenoceptor density has been reported not to be changed significantly in heart failure (Bristow *et al.*, 1988; Böhm *et al.*, 1988b) even if it tended to be higher in failing hearts (Bristow *et al.*, 1988). In a more recent study (Vago *et al.*, 1989), an increase in α_1-adrenoceptors in failing human left ventricular preparations was also observed. However, the authors did not measure force of contraction.

Thus, as with the β_2-adrenoceptor system, there is a discrepancy between inotropic responsiveness and number of α_1-adrenoceptors in failing as compared with nonfailing hearts. There is evidence that α_1-adrenoceptors also exert their effects via an as yet unidentified G-protein (Vago *et al.*, 1989). Hence, it is possible that like the β-adrenoceptor system, the amount or function of G-proteins transducing α_1-adrenergic effects might be altered in heart failure. Thus, the coupling via G-proteins to the effector systems might be an important biochemical defect responsible for the compromised adrenergic regulation of force of contraction in human end-stage heart failure.

In summary, there was a pronounced α_1- and β-adrenoceptor-mediated positive inotropic effect in nonfailing human myocardium. The comparison of contractile response in failing and nonfailing human cardiac preparations revealed a reduction of both, α_1- and β-adrenoceptor-mediated positive inotropic effects in end-stage heart failure. The reduced β-adrenergic response was also observed with the β_2-adrenoceptor agent fenoterol. The impaired inotropic responsiveness was not accompanied by a downregulation of the β_2-adrenoceptors. In addition, the positive inotropic effect of the phosphodiesterase inhibitor, IBMX, was also markedly reduced in failing heart preparations but phosphodiesterase was inhibited similarly in failing and nonfailing preparations. These findings support the hypothesis that additional mechanisms other than receptor downregulation, namely altered amounts or function of the respective G-proteins, are of importance in failing human ventricular myocardium.

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4-Aminopyridine and low Ca^{2+} differentiate presynaptic inhibition mediated by neuropeptide Y, baclofen and 2-chloroadenosine in rat hippocampal CA1 *in vitro*

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1 Presynaptic inhibition is mediated by several receptors at the stratum radiatum-CA1 synapse of rat hippocampus. We tested whether the same mechanism is activated by neuropeptide Y (NPY), baclofen and 2-chloroadenosine (2-CA), reasoning that if the receptors all activated the same process, then they should all respond to indirect manipulations of transmitter release in the same manner.

2 The effects on presynaptic inhibition by the potassium channel blocker, 4-aminopyridine (4-AP) and low extracellular concentrations of Ca^{2+} in the presence of 4-AP were compared using evoked population excitatory postsynaptic potentials (p.e.p.s.p.) responses in the rat hippocampal slice *in vitro*.

3 Log concentration-effect relationships for the inhibition of excitatory transmission were constructed for all 3 drugs in normal saline, and in the presence of 30 and 100 μM 4-AP. 4-AP reduced the inhibition mediated by all three substances, 100 μM 4-AP was only slightly more effective than 30 μM.

4 Lowering extracellular Ca^{2+} from 1.5 to 0.75 mM in the presence of 30 μM 4-AP restored the presynaptic inhibition caused by all effective concentrations of NPY and baclofen. By contrast, inhibition caused by 2-CA was not restored by lowering Ca^{2+}, except at concentrations of 2-CA greater than 10 μM.

5 The results are consistent with the hypothesis that presynaptic NPY Y_2 and $GABA_B$ receptors both inhibit transmitter release by the inhibition of voltage-dependent Ca^{2+} influx, but that the A_1 adenosine receptor may activate a different presynaptic mechanism.

Keywords: Neuropeptide Y; $GABA_B$ receptor; rat hippocampal slice; presynaptic inhibition; presynaptic mechanisms; 4-aminopyridine; adenosine A_1 receptor; 2-chloroadenosine; baclofen, NPY Y_2 receptor

Introduction

Several neurotransmitters and neuromodulators are known to mediate the presynaptic inhibition of neurotransmitter release in the mammalian CNS. In hippocampal area CA1, at least 5 receptors are known to mediate inhibition of excitatory amino acid neurotransmitter release, presumably glutamate, from the terminals of stratum radiatum fibres that form synaptic connections with dendrites of CA1 pyramidal neurones. These receptors include: (1) a γ-aminobutyric acid ($GABA_B$) receptor (Ault & Nadler, 1982); (2) a muscarinic receptor (Hounsgaard, 1978); (3) an unusual glutamate receptor, at which L-2-amino-4-phosphonobutyrate (AP4) is an agonist (Forsythe & Clements, 1990); (4) an adenosine receptor (A_1-like; Dunwiddie & Fredholm, 1989) and (5) a neuropeptide Y (NPY) Y_2 receptor (Colmers *et al.*, 1988; 1991). Of the known endogenous substances mediating presynaptic inhibition in area CA1 of hippocampus, NPY is the only one that has no postsynaptic effects (Colmers *et al.*, 1987).

Since the first description of presynaptic inhibition, a debate has continued as to the mechanism by which transmitter substances prevent release of transmitter (cf. Starke, 1981). The precise mechanism of presynaptic action in hippocampus is not known for any of these substances although there is evidence that they all activate receptors coupled to guanosine 5'-triphosphate (GTP) binding proteins. Nearly all of these substances have been shown to inhibit calcium influx into cultured or acutely dissociated neurones (Dolphin & Scott, 1986; Macdonald *et al.*, 1986; Wanke *et al.*, 1987; Walker *et al.*, 1988), and most are known to activate potassium conductances in cell bodies (Newberry & Nicoll, 1984; Egan & North, 1986; Greene & Haas, 1985; Zidichouski *et al.*, 1990). Because of technical limitations, it has not been possible to examine the actions of these substances on evoked transmitter release by direct measurements on presynaptic terminals of CNS. The question of how NPY acts at the presynaptic terminal has been indirectly addressed by the use of 4-aminopyridine (4-AP), which blocks potassium conductances at presynaptic terminals (Buckle & Haas, 1982). 4-AP at concentrations well below those needed to affect the somatic 'A' current has been shown to block the presynaptic actions of NPY in hippocampal CA1 (Colmers *et al.*, 1988); higher concentrations block presynaptic inhibition mediated by adenosine in guinea-pig olfactory cortex (Scholfield & Steel, 1988). Lowering extracellular calcium concentrations in the presence of 4-AP restored NPY-induced inhibition, suggesting the inhibition of a presynaptic terminal calcium conductance by NPY (Colmers *et al.*, 1988).

To examine whether the presynaptic inhibition mediated in hippocampus by NPY, baclofen and adenosine could be via the same mechanism, we compared the effects of 4-AP and low calcium on the concentration-response relationships for all three substances. If inhibition of transmitter release by all three receptors is mediated by the same mechanism, the inhibition by all three should be equally affected by 4-AP and low Ca^{2+}, since neither manipulation should alter the binding of the agonists to their receptors. The data indicate that, although baclofen and NPY are similar in their response to these manipulations, adenosine differs from the others.

Methods

Transverse hippocampal slices (450 μm thick) were prepared from male Sprague-Dawley rats (75–150 g), and incubated at 34 ± 0.5°C, submerged and constantly perfused (2.5–3 ml min^{-1}) with saline bubbled with 95% O_2: 5% CO_2 (pH 7.35) (Colmers *et al.*, 1988; 1991). Composition of the saline was (in mM): NaCl 124, KCl 1.8, $MgSO_4$ 2, KH_2PO_4 1.25, $CaCl_2$ 1.5 and glucose 10. Low Ca^{2+} solutions were prepared from Ca^{2+}-free stock solution to which appropriate amounts of $CaCl_2$ were added. Stimuli were delivered to stratum radiatum of area CA1 via a shielded bipolar, etched-tungsten electrode connected to a stimulus isolation unit (AMPI). Orthodromic population field potentials evoked by stimuli (3–40 V, 50–200 μs, 0.1 Hz) were recorded in stratum radiatum of CA1 with a glass micropipette (4–15 MΩ, 2M NaCl; Figure 1)

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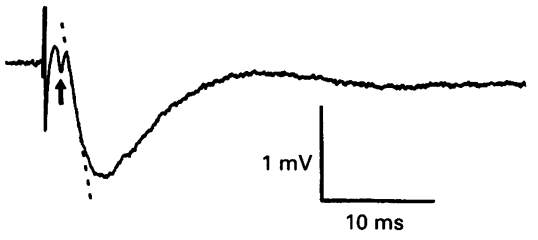

Figure 1 Population e.p.s.p. (digital average of three traces) recorded in stratum radiatum of area CA1. Dashed line indicates slope of p.e.p.s.p., arrow indicates presynaptic volley, which was resolved in most, but not all preparations.

connected to the headstage of an Axoclamp 2A amplifier. For each data point, three successive field potentials were digitized at 50 μs/point, and averaged using a virtual averaging paradigm by a digital storage oscilloscope (Nicolet 4094; Model 4562 plug-in) and were stored on diskette. The slope of the initial, linear portion of the population e.p.s.p. (p.e.p.s.p.) was measured as the difference in voltage and time between the extremes of the linear portion (determined by inspection) of the p.e.p.s.p., using the oscilloscope. Once stable responses had been obtained, stimulus-response curves were constructed, and a stimulus intensity was chosen to elicit a response on the linear portion of the stimulus-response curve (Andersen *et al.*, 1978). Generally, representative responses were chosen which were of similar size (±20%) to those observed in control for each preparation; however, they were always on the linear portion of the stimulus-response curve. Stimulus-response curves were constructed under each basal condition, and stimulus intensities chosen using the same criteria as for control conditions. In most cases, the responses evoked in a given preparation by a given stimulus in control and in the presence of 4-AP/low Ca^{2+} were similar in slope and amplitude, while 4-AP at both concentrations caused a much greater response, requiring lower stimulus intensities to elicit the same responses. Stimulus-response curves were also obtained during drug effects and upon washout.

Test substances were made freshly in saline of appropriate composition. Synthetic porcine NPY (Richelieu Biotechnologies), was made up at 100 μM in distilled water and kept frozen until use. 2-Chloroadenosine (2-CA; Research Biochemicals Inc.) and (±)-baclofen (Lioresal: gift of Ciba-Geigy) were made up as concentrated stock solutions (dilutions of 1:100 or greater in final use) and kept frozen or refrigerated, respectively, in the dark until use.

Data are expressed as percentage inhibition of control values. Preparations served as their own controls; all data are from preparations which showed significant recovery upon washout. Statistical comparisons were performed by a Student's *t* test. Statistical differences were considered significant at $P \leq 0.05$. Concentrations of drugs somewhat above the EC_{50} were selected for comparison in the figures, and for representative statistical comparisons. In addition, ligand concentrations over which significant differences between treatments were observed are given, where appropriate.

Results

Data were obtained from 56 preparations, with a total of 361 drug applications. Initial p.e.p.s.p. slope measurements in control conditions varied from preparation to preparation, and ranged from 0.12 to 1.26 mV ms^{-1}. Although we attempted to perform concentration-response curves on each preparation under each condition, this was not always possible.

Bath application of 1 μM NPY reversibly reduced the p.e.p.s.p. slope in CA1 by 63.15 ± 1.94% ($n = 4$; Figure 2). NPY was significantly effective at concentrations above 300 nM (Figure 3); concentrations of NPY higher than 3 μM were not tested. Baclofen (10 μM) also reduced the p.e.p.s.p. slope, by 71.53 ± 2.88 ($n = 6$; Figure 2). Baclofen significantly

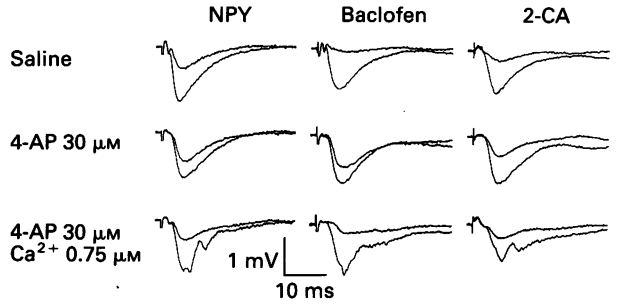

Figure 2 Effects of 4-aminopyridine (4-AP) and 4-AP/low Ca^{2+} on inhibition of p.e.p.s.p. by representative concentrations of neuropeptide Y (NPY), baclofen and 2-chloroadenosine (2-CA). Upper row shows superimposed traces in control and during peak drug effects in normal saline (1.5 mM Ca^{2+}) for 1 μM NPY (left column), 10 μM baclofen (centre column), and 1 μM 2-CA (right column). Centre row shows the effects of these drugs, superimposed on controls, in the presence of 30 μM 4-AP. Bottom row shows the effects of these drugs, relative to controls, in 30 μM 4-AP with 0.75 mM Ca^{2+}. Data illustrated for a given drug were obtained from the same preparation. All preparations recovered from inhibition upon washout (not illustrated). All traces shown are digital averages of three successive field potentials (0.1 Hz). In all cases, the smaller response represents the effect of the drug tested.

inhibited p.e.p.s.p. slope at concentrations above 300 nM (Figure 3). 2-CA, 1 μM, inhibited p.e.p.s.p. slope by 65.86 ± 6.03% ($n = 6$; Figure 2). 2-CA was effective at concentrations of 100 nM or greater (Figure 3).

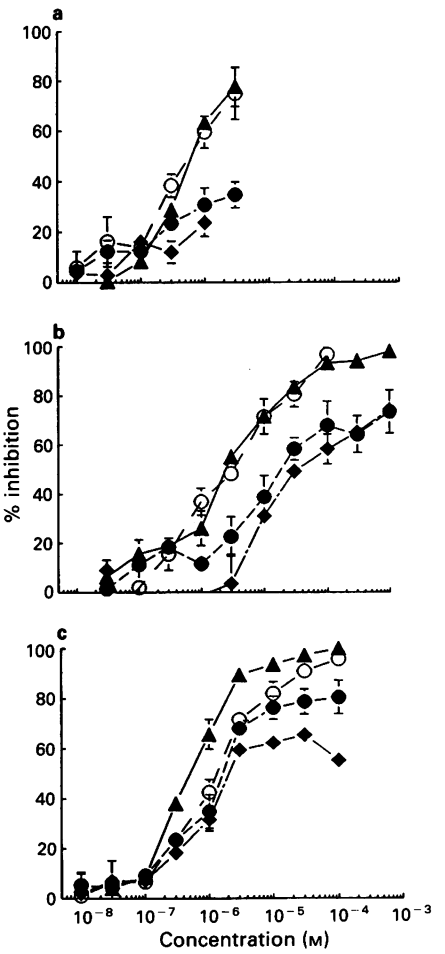

Figure 3 Dose-response relationships for inhibition of p.e.p.s.p. by neuropeptide Y (a), baclofen (b) and 2-chloroadenosine (c) in control (1.5 mM Ca^{2+}) saline (▲), and in the presence of 30 μM 4-aminopyridine (4-AP, ●), 100 μM 4-AP (◆), and 30 μM 4-AP, 0.75 mM Ca^{2+} (○). Data shown are means for $n \geq 3$ for each point, s.e.mean shown by vertical bars.

Application of 30 μM 4-AP caused a significant increase in orthodromic synaptic responses (Figure 2). At initial stimulus intensities, p.e.p.s.ps were prolonged compared with control, and often demonstrated multiple late components. Recording in the cell body layer revealed multiple population spikes and often spontaneous epileptiform discharges were observed. To study the actions of the presynaptic inhibitors under conditions similar to those of control, stimulus-response relationships were again constructed, and a stimulus amplitude chosen which evoked p.e.p.s.p. of approximately the same slope and amplitude as in control (see Methods). Once stable responses had been obtained, dose-response curves to the inhibitory substances were again constructed.

As reported previously for population spike and intracellularly recorded e.p.s.p. (Colmers *et al.*, 1988), 4-AP also caused a significant ($P < 0.05$) reduction in the inhibition of p.e.p.s.p. slope by NPY at concentrations above 300 nM. At 30 μM, 4-AP reduced the inhibition of p.e.p.s.p. by 1 μM NPY from 63.15 $\pm$ 1.94% ($n = 4$) to 30.60 $\pm$ 6.66% ($n = 3$) of control. The inhibition of p.e.p.s.p. slope by 10 μM baclofen was reduced from 71.53 $\pm$ 2.88% ($n = 6$) to 38.78 $\pm$ 8.69% ($n = 6$) of control (Figure 2); the 4-AP effect was significant for baclofen concentrations above 1 μM. The inhibition by 1 μM 2-CA was reduced from 65.86 $\pm$ 6.03% ($n = 6$) to 34.82 $\pm$ 6.81% ($n = 6$) of control; 4-AP effects were significant at 2-CA concentrations above 100 nM.

A higher concentration (100 μM) of 4-AP caused only a slight, and generally insignificant, further reduction in p.e.p.s.p. inhibition by all three drugs, indicating that 30 μM 4-AP was near but slightly below the maximal effective concentration for 4-AP at these terminals. However, an interesting effect of increasing concentrations of 4-AP was a significant, progressive depression of the maximal inhibition of p.e.p.s.p. by 2-CA (Figure 3). The maximal inhibition by baclofen was also significantly depressed by 30 μM 4-AP, but was not further reduced by 100 μM 4-AP. It was not possible to observe this effect with NPY, as maximal concentrations could not be tested.

As has been previously observed (Colmers *et al.*, 1988), reduction of extracellular Ca^{2+} in the presence of 4-AP caused a reduction in evoked synaptic responses. Thus, in 30 μM 4-AP/0.75 mM Ca^{2+}, p.e.p.s.p. slopes were reduced, as expected from the lower levels of extracellular Ca^{2+} available to the presynaptic terminals. Once responses had stabilized, and the stimulus intensity was re-adjusted so responses were comparable with controls (see Methods), drug effects were again tested. For both NPY and baclofen, low Ca^{2+}/4-AP significantly restored the ability of the ligands to inhibit transmitter release. Indeed, no significant difference was observed between control saline and low Ca^{2+}/4-AP for inhibition mediated by NPY or baclofen. For 2-CA, by contrast, low Ca^{2+}/4-AP caused no significant restoration of inhibition at drug concentrations less than 10 μM (Figure 2). Experiments could not be conducted in the presence of 0.75 mM Ca^{2+} alone since, in the absence of 4-AP, this Ca^{2+} concentration did not support synaptic transmission (not illustrated).

To test whether there was a significant postsynaptic component to the observed reduction in p.e.p.s.p. slope, we applied a supramaximal concentration of the 5-hydroxytryptamine (5-HT) agonist 5-carboxamidotryptamine (5-CT; Beck, 1989). 5-CT activates the postsynaptic 5-HT_{1A} receptors in hippocampus (Beck, 1989) that cause an increase in the same potassium conductance as does baclofen (Andrade *et al.*, 1986). At 1 μM, 5-CT had no significant effect on p.e.p.s.p. slope ($n = 5$; Figure 4).

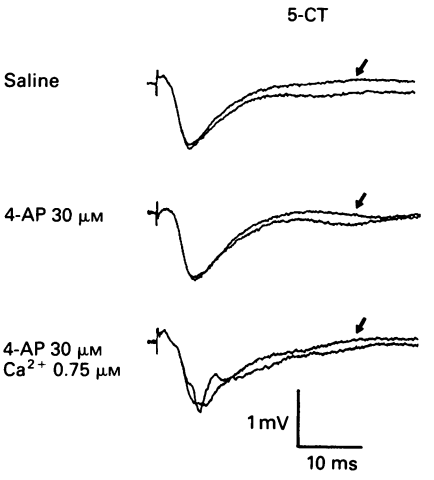

Figure 4 5-Carboxamidotryptamine (5-CT; 1 μM) does not affect p.e.p.s.p. slope in control (upper traces), in 30 μM 4-aminopyridine (4-AP, middle traces) or in 30 μM 4-AP, 0.75 mM Ca^{2+}. Traces in control and during 5-CT application are shown superimposed. Arrows indicate traces obtained during 5-CT application. Data are all from the sample preparation. All traces are digital averages of three successive field potentials.

Discussion

NPY, baclofen and 2-CA all inhibit evoked glutamatergic synaptic transmission in area CA1 of the rat hippocampus by presynaptic actions in stratum radiatum. While all of the receptors mediating presynaptic inhibition in hippocampus have also been shown to inhibit Ca^{2+} influx into neuronal cell bodies, an unequivocal demonstration that this is the mechanism whereby they inhibit transmitter release at presynaptic terminals remains elusive. We have asked a related but somewhat different question: does the presynaptic inhibition mediated at the same population of terminals by different receptors respond in the same manner to two manipulations which should only indirectly affect processes inhibiting transmitter release? Because neither of the manipulations should influence the binding of the ligands to their receptors or significantly affect the coupling of the receptors to their G-proteins, these manipulations should have equal effects on responses to all three ligands if they share a common mechanism. The evidence presented here indicates that this is not entirely the case.

All three substances tested inhibit synaptic transmission in hippocampus, with EC_{50}s of between 500 nM and 2.8 μM. The physiological measure chosen, the slope of the p.e.p.s.p., is relatively insensitive to the postsynaptic actions of neuromodulators (Harrison *et al.*, 1991). However, it has been demonstrated that postsynaptic $GABA_B$ receptors reduce synaptic NMDA conductances evoked at higher stimulus levels (Morrisett *et al.*, 1991). Here, application of a supramaximal concentration of 5-CT, an agonist at the 5-HT_{1A} receptors (Beck, 1989) that activates the same postsynaptic K^+ conductances in CA1 pyramidal cells as are activated by $GABA_B$ receptors (Andrade *et al.*, 1986), had no significant inhibitory effect on p.e.p.s.p. slopes when applied either alone or in the presence of 30 μM 4-AP, although a later component of the response was somewhat reduced (Figure 4). The absence of an effect of 5-CT on the slope of the p.e.p.s.p. is in agreement with other observations (Harrison, 1990), and suggests that the postsynaptic potassium conductance activated by both 5-HT_{1A} or $GABA_B$ receptors (Andrade *et al.*, 1986) has no significant effect on the modest levels of excitatory transmission elicited in these experiments. Furthermore, NPY has no demonstrable postsynaptic actions in hippocampus (Colmers *et al.*, 1987; 1988). Therefore, we are confident that the only significant inhibition measured in the present experiments is at a presynaptic site.

The action of 4-AP suggests, at first glance, that the three compounds cause presynaptic inhibition by increasing a K^+ conductance at the presynaptic terminal, as has been hypothesized for the action of adenosine in guinea-pig olfactory cortex (Scholfield & Steel, 1988). However, the effects of the low-Ca^{2+}/4-AP treatment on NPY and baclofen responses are not consistent with an increase in K^+ conductance. The inhibition by all effective concentrations tested of both substances was

restored to control levels by lowering extracellular Ca^{2+} in the presence of 4-AP. This restoration militates against either 4-AP or low Ca^{2+} affecting the binding of the drugs to their receptors or the coupling of the receptors to their effectors. In fact, the observed decrease in receptor – G-protein coupling caused by lowered Ca^{2+} concentrations (Gilman, 1987) would be expected to produce the opposite of the results observed here. Because the restoration of inhibition by low Ca^{2+} occurs throughout the concentration-effect curve to both substances, it also argues against a significant component of the observed inhibition by NPY and baclofen being mediated directly by another mechanism, such as an increase of K^+ conductance in the presynaptic terminal (Colmers *et al.*, 1988). Thus, the present evidence is entirely consistent with an inhibitory action by presynaptic NPY and $GABA_B$ receptors on Ca^{2+} influx underlying the effect on synaptic transmission.

However, the response of the 2-CA-mediated inhibition to 4-AP/low Ca^{2+} is not as clearly consistent with an exclusive effect at presynaptic Ca^{2+} channels. Most importantly, lowering extracellular Ca^{2+} did not significantly restore the inhibition, except at concentrations of 2-CA greater than 10 μM, (a supramaximal concentration in control; Figure 3). Clearly, this differs significantly from the response of NPY and baclofen; the lack of response to low Ca^{2+} is also not consistent with a mechanism involving predominantly the reduction of Ca^{2+} influx, at least at concentrations of 2-CA lower than 10 μM. At the neuromuscular junction, it has been proposed that adenosine inhibits transmitter release at a step subsequent to the entry of Ca^{2+} (Silinsky, 1985). Recent evidence indicates that, in rat hippocampal cultures, adenosine inhibits quantal release of glutamate by a mechanism independent of Ca^{2+} influx (Scholz & Miller, 1991). Although it is difficult to reconcile a mechanism involving a decrease in Ca^{2+} sensitivity by the release process with the present data, it appears that adenosine receptors are capable of controlling the release process in more than one fashion (Silinsky, 1985). The data admit of the explanation that, at low concentrations, adenosine inhibits transmitter release largely by a process independent of voltage-dependent Ca^{2+} influx, although there may be an additional effect on Ca^{2+} influx at higher concentrations.

The present results suggest that there are at least two different mechanisms of presynaptic inhibition mediated by the three receptors studied here. We consider it unlikely that all three receptors utilize three altogether different mechanisms. Although the nature of the present and similar experiments limits the conclusions about presynaptic processes which can be obtained (Milner *et al.*, 1986) it is nevertheless possible to demonstrate under identical experimental conditions that the events which underlie presynaptic inhibition are not identical for all receptors.

The results indicate that presynaptic inhibitions mediated by NPY and baclofen in stratum radiatum of hippocampal area CA1 *in vitro* respond in a comparable manner to the application of 4-AP and reduced extracellular Ca^{2+} in the presence of 4-AP. The response is consistent with a mechanism of presynaptic inhibition involving the inhibition of Ca^{2+} influx into the presynaptic terminals that release glutamate. However, the inhibition mediated by 2-CA does not respond in the same manner, suggesting that it may thus act by a predominantly different mechanism to inhibit transmitter release.

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Extracellular ATP and UTP exert similar effects on rat isolated hepatocytes

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1 Extracellular UTP and ATP show obvious similarities in their control of several metabolic functions of rat isolated hepatocytes.

2 They have a similar time-course and concentration-dependency for the activation of glycogen phosphorylase, the generation of inositol trisphosphate (IP_3), the inhibition of glycogen synthase and the lowering of adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels.

3 There is a similar synergism of the nucleotides with glucagon in activating phosphorylase.

4 They undergo a similar inhibition by phorbol myristic acid of their glycogenolytic effect.

5 The ATP and UTP effect on IP_3 levels are not additive.

6 It is tentatively concluded that UTP and ATP use a common receptor.

Keywords: ATP; UTP; rat hepatocytes; glycogen metabolism

Introduction

Extracellular adenine nucleotides regulate many important cell functions in several different tissues through an interaction with P_2-purinoceptors, which can be subdivided into P_{2X} and P_{2Y} receptors according to the rank order of potency of several ATP analogues (Burnstock & Kennedy, 1985). P_{2Y}-purinoceptors are characterized by the rank order of potency: 2-methylthio-ATP $\gg$ ATP = ADP > α,β-methylene-ATP. P_{2X}-receptors are more specific for α,β-methylene-ATP than for ATP and 2-methylthio-ATP. Moreover the P_{2X} effect of ATP can be desensitized by pretreatment with α,β-methylene-ATP (see Gordon, 1986, for a review).

UTP and other uracyl nucleotides also regulate various functions in many different cell types, as reviewed by Seifert & Schultz (1989). These authors presented evidence that in several tissues and cell types the pyrimidine nucleotides exert their effects by interacting with a specific pyrimidinoceptor rather than by interacting with the purinoceptor. This view has recently been refined by O'Conner *et al.* (1991). Taking into account all nucleotide effects these authors propose that the nucleotide receptors can be subdivided into three classes: P_{2Y}-purinoceptors with 2-methylthio-ATP $\gg$ ATP = ADP > UTP, 'Nucleotide' receptors with UTP = ATP > ADP > 2-methylthio-ATP and a 'mixed' type of receptor with 2-methylthio-ATP > ATP = UTP = ADP as their respective rank order of potencies. The relative densities and coupling affinities of these receptors can, according to the authors, vary considerably from tissue to tissue.

Rat liver parenchymal cells possess purinoceptors activated by extracellular ATP. Binding of the nucleotide to these receptors leads to the activation of phospholipase C, generating diacylglycerol and inositol-(1,4,5)-trisphosphate (IP_3). IP_3 induces a rise in cytosolic calcium (Berridge & Irvine, 1989) leading to the activation of glycogen phosphorylase, which is responsible for glycogen breakdown. Diacylglycerol stimulates the activity of protein kinase C which results, among other effects, in the inactivation of glycogen synthase, in combination with the inhibition by calcium of glycogen synthase phosphatase (Mvumbi *et al.*, 1985).

Based on the rank order of the glycogenolytic potency of different ATP analogues, we (Keppens & De Wulf, 1986) and others (Gordon, 1986) have proposed that the purinoceptor involved in glycogenolysis belongs to the P_{2Y}-subclass in the nomenclature of Burnstock & Kennedy (1985). However, our results recently obtained with 2-methylthio-ATP (Keppens & De Wulf, 1991) suggest some heterogeneity for the P_{2Y}-purinoceptor. Indeed although 2-methylthio-ATP has been shown to be a more potent glycogenolytic agonist than ATP, some other effects were not compatible with the existence of a common receptor for both nucleotides: (a) 2-methylthio-ATP shows little affinity for the P_{2Y}-receptor characterized with ATPα[^{35}S]; (b) 2-methylthio-ATP has little effect on the levels of IP_3, and (c) it has no effect on the levels of cyclic AMP increased previously by glucagon (Keppens & De Wulf, 1991).

So far, the only information about effects of UTP on liver comes from the work of Häussinger *et al.* (1987, 1988). These authors used the perfused rat liver to study the effects of both ATP and UTP and reported a different time-course for the glucose release from the liver on perfusion with either ATP or UTP. Differences in portal vein pressure, K^+ uptake, calcium and thromboxane release and in oxygen consumption were also observed in their studies. Although they suggested that the effects of ATP were mediated by P_{2Y}-purinoceptors and those of UTP by a different receptor (Häussinger *et al.*, 1987) they were conscious of the fact that there is a complex interaction between hepatic parenchymal and non-parenchymal cells (Häussinger *et al.*, 1988).

The present study compares several effects of ATP and UTP at the level of the liver parenchymal cell, avoiding contribution by other substances, possibly released by ATP or UTP from non-parenchymal cells. The aims of this study were to substantiate the glycogenolytic effect of extracellular UTP on hepatocytes and to compare these effects of UTP with those induced by ATP.

Methods

Adult male Wistar-strain albino rats (200–250 g body wt.) fed *ad libitum* with a standard laboratory chow were used.

Isolation and incubation of the hepatocytes were done as previously described (Vandenheede *et al.*, 1976). Briefly, the liver was removed from the animal, cleared of blood and then perfused at 37°C for about 10 min with a Krebs-Henseleit bicarbonate buffer without calcium. Subsequently, collagenase (30 mg 100 ml^{-1} of perfusion buffer) and calcium (2.5 mM) were added and the perfusion was continued for another 25–30 min. The hepatocytes were harvested and incubated at 37°C (or at 10°C when indicated) in a Krebs-Henseleit buffer containing 10 mM glucose in closed plastic vials saturated with 95% O_2, 5% CO_2 (v/v). Cyclic AMP and IP_3 concentrations were

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determined with a competitive protein-binding technique, the former based on the procedure described by Gilman (1970), using an assay kit from the Radiochemical Centre (Amersham, Bucks., U.K.) and the latter based on the method described by Bredt *et al.* (1989) with slight modifications. Cerebellar membranes, known to contain high amounts of specific IP_3 binding protein (Worley *et al.*, 1987) were prepared by homogenization and repetitive centrifugation of rabbit cerebellum. The incubation medium contained about 50 μg of these membranes, 1 nM [^{3}H]-IP_3 and 10 μl cell extract in a final volume of 100 μl. Bound and free ligand were separated after 10 min of incubation by filtration through Whatman GF/A filters, which were washed three times with 7 ml 50 mM Tris-HCl (pH 8.4) and 1 mM EDTA. Radioactivity was then determined by liquid scintillation counting.

UTP and ATP were from Sigma Chemical Co., St. Louis, MO, U.S.A.; [^{3}H]-inositol-(1,4,5)-trisphosphate was from Amersham International, Amersham, Bucks., U.K.; glucagon was from Novo Laboratories, Copenhagen, Denmark. Suramin was a gift from Bayer Belgium, Pharma division, Brussels, Belgium.

The curve fitting programme 'Enzfitter' (Leatherbarrow, 1987) was from Elsevier Biosoft (Cambridge, U.K.) and was used to fit the experimental data to a Michaelean-type of equation (concentration-dependent effects).

Results

To compare the effects of ATP and UTP we measured their capacity to activate glycogen phosphorylase, to increase IP_3 levels, to inhibit the rise in cyclic AMP levels after glucagon and to inactivate glycogen synthase.

First we determined the kinetics of these effects of ATP and UTP (Figure 1). The activation of glycogen phosphorylase by 5 μM UTP and ATP was rapid, reached maximal levels of phosphorylase a within 20 s and declined afterwards. The effect of 10 μM ATP and UTP on the levels of IP_3 was even more rapid and transient than the activation of phosphorylase, reaching maximal levels after 5 s. Finally, the inactivation of glycogen synthase by 5 μM ATP and UTP and the cyclic AMP-lowering effect of 1 mM ATP and UTP were maximal within 1 min and again no differences between ATP and UTP were seen.

Next we determined the concentration-dependencies of these four parameters. Figure 2 illustrates the effects of increasing concentrations of ATP and of UTP on the activation of phosphorylase (at 20 s), on the increase of IP_3 levels (at 5 s), on the decrease of cyclic AMP levels (at 1 min) and on the inactivation of synthase (at 1 min). The curves on the figure and the concentrations at which half-maximal effects were obtained (K-values, see below) were computer-generated by applying a Michaelean-type of equation to the data. The data show a complete similarity between ATP and UTP effects on the liver parenchymal cell. Indeed, both nucleotides were equipotent in activating phosphorylase (K_a = 1.1 μM), increasing the levels of IP_3 (K_{IP} = 40 μM), lowering the cyclic AMP levels (K_c = 15 μM) and inactivating synthase (K_s = 5 μM).

In order to compare further ATP and UTP effects on the liver parenchymal cell, we checked whether UTP, like ATP, could act synergistically with glucagon. For ATP it has already been shown that glucagon increases the ability of ATP to augment the levels of cytosolic calcium (Charest *et al.*, 1985). Figure 3 illustrates that a synergism can also be demonstrated by measuring the activation of phosphorylase at low temperatures (10°C) where there is only a very moderate effect of glucagon and of ATP or UTP separately. The combination of either ATP or UTP with glucagon resulted in a pronounced and similar activation of phosphorylase, presumably due to an increase in the cytosolic calcium levels.

We next used the P_2-purinoceptor antagonist, suramin (Dunn & Blakely, 1988) and procion blue, a P_{2Y}-antagonist (Burnstock & Warland, 1987). Figure 4 shows that the glycogenolytic effect of ATP, UTP or vasopressin was not inhibited by low concentrations (20 μM) of either P_2-antagonist. Figure 4 further illustrates that the inhibitory effect of higher concentrations of the antagonists (400 μM) is non-specific since the glycogenolytic effect of vasopressin (Figure 4c) was equally well counteracted. Even the glycogenolytic effect of glucagon was inhibited by these high concentrations of suramin and procion-blue (not shown).

To check further the possibility that UTP (or ATP) might interact with a P_{2X} type of receptor, we used α,β-methylene-ATP, which, in other tissues, is able to induce a desensitization of the P_{2X}-mediated effect (Burnstock & Kennedy, 1985). Pretreatment of the hepatocytes for 5 min with 200 μM

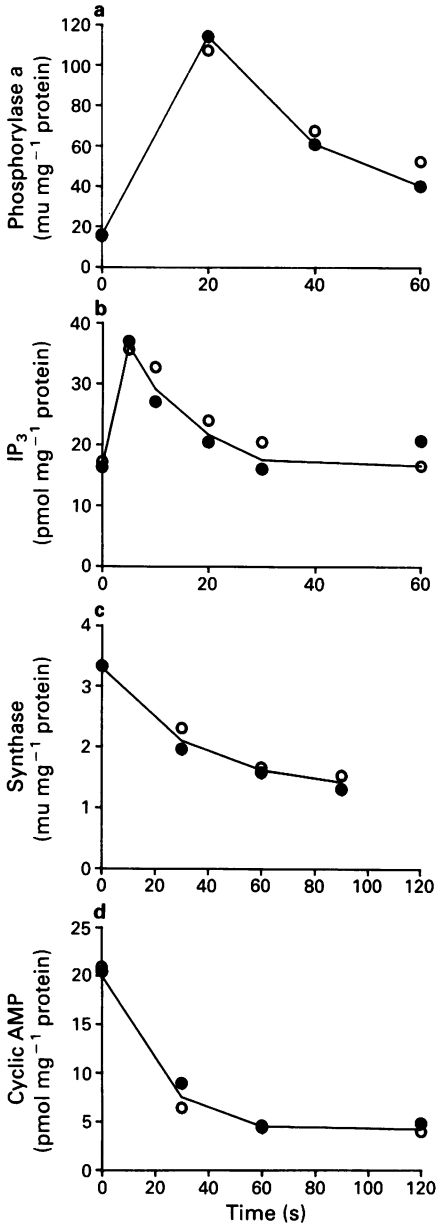

Figure 1 Comparison of the time-dependent activation of glycogen phosphorylase, increase of inositol trisphosphate (IP_3) levels, inactivation of glycogen synthase and inhibition of the rise in cyclic AMP levels. Hepatocytes were pre-incubated for 20–30 min in the presence of 10 mM glucose and were then challenged with ATP (○) or UTP (●) (both at 5 μM) for the effects on phosphorylase and synthase, at 10 μM for the increase in IP_3 levels and at 1 mM for lowering cyclic AMP levels. Samples were taken at the indicated times. (a) Phosphorylase activation; (b) IP_3 increase; (c) inactivation of synthase and (d) lowering of cyclic AMP levels in hepatocytes previously treated for 1 min with 20 nM glucagon. Data shown are representative of 3–5 independent experiments.

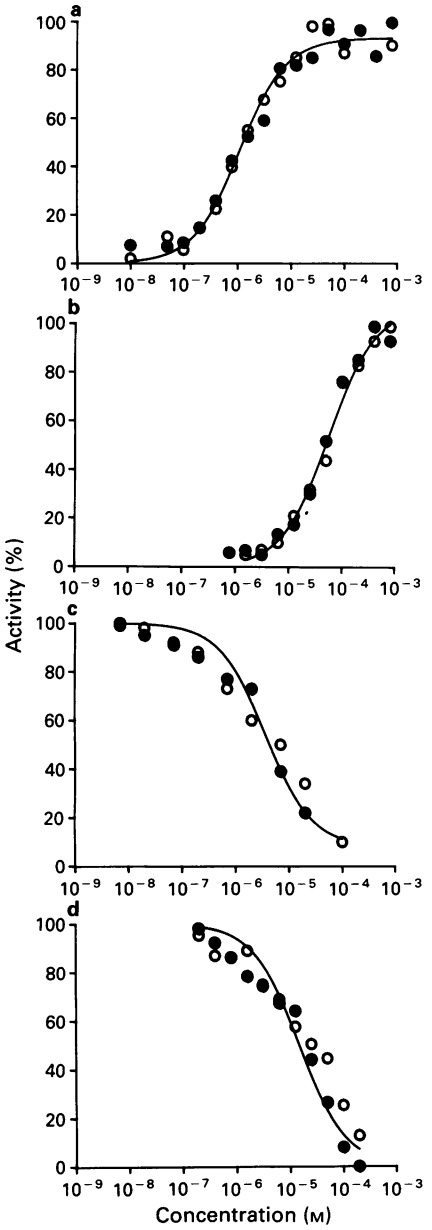

Figure 2 Comparison of the concentration-dependencies of ATP and UTP for the activation of glycogen phosphorylase, the increase in inositol trisphosphate (IP_3) levels, the inactivation of glycogen synthase and the inhibition of the rise in cyclic AMP levels. Hepatocytes were pre-incubated with 10 mM glucose for 20–30 min and then challenged with increasing concentrations of either ATP (○) or UTP (●). (a) Phosphorylase *a* levels after 20 s; (b) IP_3 levels after 5 s; (c) synthase *a* levels after 1 min and (d) cyclic AMP levels after 1 min (glucagon (20 nM) was added 1 min prior to the addition of the nucleotides). Data, expressed as percentage changes from control values, are mean values of at least 5 independent experiments; s.e.mean did not exceed 10%.

of α,β-methylene-ATP did not desensitize the glycogenolytic effect of UTP (or of ATP).

The effect of both agonists on phosphorylase activation was equally counteracted by phorbol myristic acid. Indeed, pretreatment of the hepatocytes for 5 min with 1.6 μM of the phorbol ester increased the K_a value 2 to 3 fold.

Finally we tested whether the ATP and UTP effects were additive. Since the activation of glycogen phosphorylase, the lowering of cyclic AMP levels after glucagon and the inactivation of synthase were all maximal, we looked for a possible additivity of ATP and UTP effects on the levels of IP_3. Figure 5 shows the IP_3 levels obtained after addition of supramaximal concentrations of ATP, UTP or of vasopressin either

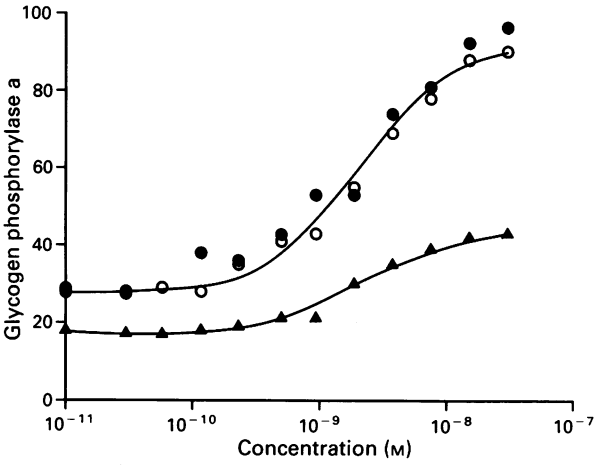

Figure 3 Synergistic effect between ATP or UTP and glucagon on the activation of glycogen phosphorylase. Hepatocytes were pre-incubated for 20 min at 37°C with 10 mM glucose. They were then brought to 10°C and kept at this temperature for at least 15 min before adding increasing concentrations of glucagon either alone (▲) or with 5 μM ATP (○) or UTP (●). Samples were taken 10 min later for the determination of the levels of phosphorylase *a*.

alone or in combination with each other. It is clear that the effect of ATP and UTP on the levels of IP_3 were not additive, in contrast to the additive effects of these nucleotides with vasopressin.

Discussion

It is clear from these data that ATP and UTP exert very similar effects on isolated hepatocytes. Indeed, both the time- and concentration-dependent activation of phosphorylase by UTP and ATP are completely identical (Figure 1, see also Keppens & De Wulf, 1985, and Charest *et al.*, 1985). Since the activation of phosphorylase by ATP has been shown to be linked to increased levels of IP_3 (Charest *et al.*, 1985) a similar situation might exist for UTP. Figures 1 and 2 indeed show a similar pattern of IP_3 generation after ATP and UTP. The time- and concentration-dependence for the increase in IP_3 levels after ATP, although measured in a different way, is similar to the results presented by Charest and co-workers (1985). Indeed they also reported a very rapid (maximal after 5 s) effect of ATP on the levels of IP_3 with a comparable concentration-dependence. The observation that higher concentrations of the nucleotides are needed to increase IP_3 levels (K_{IP} = 50 μM) than to activate phosphorylase (K_a = 1.1 μM) is in accordance with what is known for other cyclic AMP-independent, calcium-mediated agonists, for example vasopressin, for which the K_{IP} is about 50 fold higher than the K_a (Lynch *et al.*, 1985). A very small increase in IP_3 seems to be sufficient to initiate the release of calcium ions into the cytosol leading eventually to the activation of phosphorylase. Since overall effects of UTP and ATP on IP_3 levels are identical a very similar interaction of ATP and UTP with phospholipase C is suggested. In addition to these similarities, both nucleotides also inactivate glycogen synthase and lower the levels of cyclic AMP in an identical way (Figure 2). Furthermore they are, in combination with glucagon, equally potent in synergistically activating glycogen phosphorylase (Figure 3). Four other experiments further illustrate the similarity between ATP and UTP. First, phorbol myristic acid, previously shown to increase the K_a for the activation of phosphorylase by ATP (Keppens & De Wulf, 1991) equally increases the K_a for the activation of phosphorylase by UTP. Second, α,β-methylene-ATP, described as a P_{2X}-desensitizing agent (Burnstock & Kennedy, 1985) has no effect on the glycogenolytic potency of either UTP or ATP. Third, the use of a P_2-antagonist

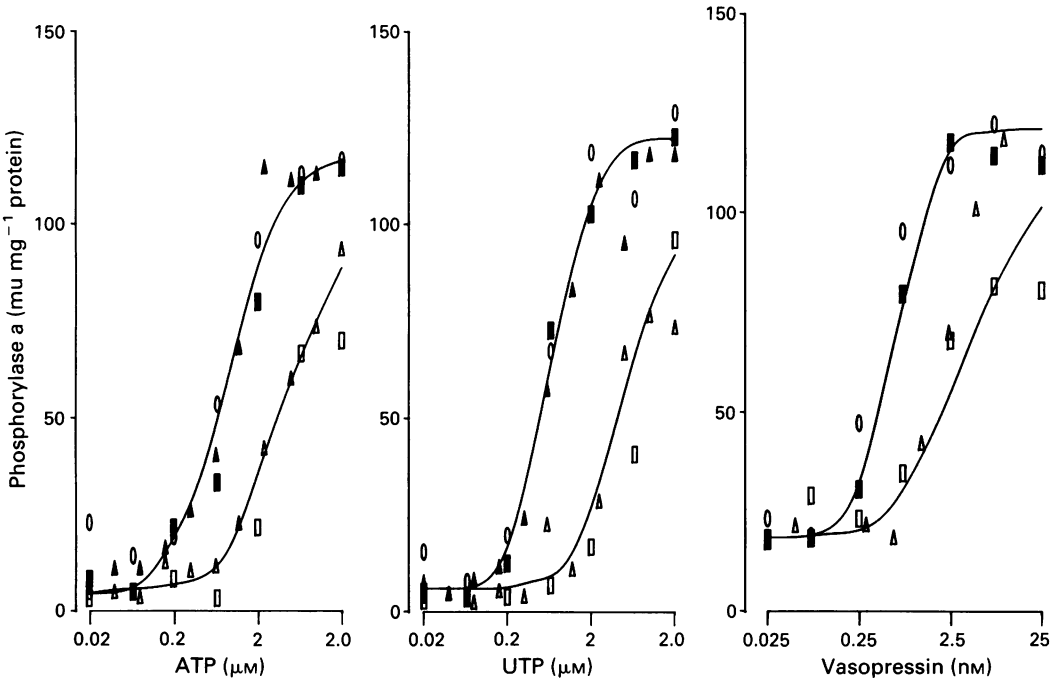

Figure 4 The effect of suramin and procion-blue on the glycogenolytic potency of ATP, UTP and vasopressin Hepatocytes were pre-incubated for 20 min at 37°C with 10 mM glucose. They were then further incubated as such (control, ○) or challenged with suramin (20 μM ■, 400 μM □) or procion blue (20 μM ▲, 400 μM △). One min later the cells were treated with the indicated concentrations of ATP (a), UTP (b) or vasopressin (c). The activity of glycogen phosphorylase was determined 20 s after the addition of ATP and of UTP, or 1 min after vasopressin. Data shown are from one representative experiment.

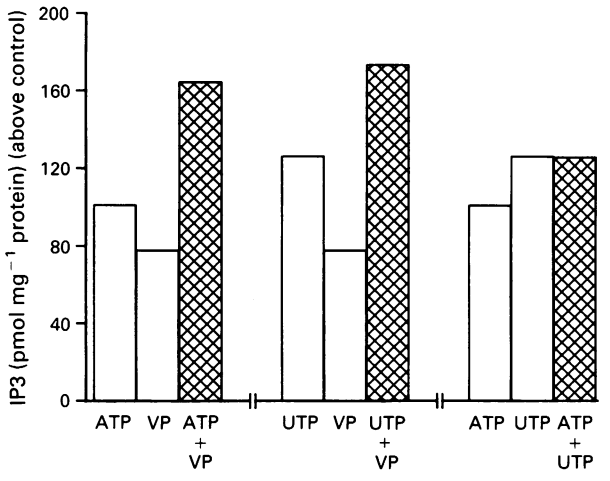

Figure 5 The non-additivity of the effects of ATP and UTP on the levels of inositol trisphosphate (IP_3). Hepatocytes were pre-incubated for 20 min at 37°C with 10 mM glucose. They were then challenged with supramaximal concentrations of ATP (1 mM), UTP (1 mM) or vasopressin (1 μM), either alone or in combination with each other. IP_3 levels were estimated 5 s later. Data shown are the means of two independent experiments, done in duplicate. Control value for IP_3 was 18.1 pmol mg^{-1} of protein.

(suramin) and a P_{2Y}-antagonist (procion blue) did not succeed in discriminating between the effects of ATP and UTP on glycogenolysis. Finally, there was no additivity of the effect of ATP and UTP on the levels of IP_3, in clear contrast to the additivity of vasopressin with either ATP or UTP (Figure 5). The overall conclusion to be drawn from these data is that no difference exists between the effects of ATP and UTP. In the light of the recently proposed receptor sub-classification by O'Conner *et al.* (1991) the liver would belong to those tissues with a heterogeneous 'mixed' receptor population, characterized by the rank order of glycogenolytic potency: 2-methylthio-ATP > ATP = UTP = ADP.

Our data showing a complete similarity between the effects of ATP and UTP on the liver parenchymal cell do not necessarily contradict the results reported by the group of Häussinger (1987, 1988) using the liver perfusion technique. They speculated that the differences observed between ATP and UTP might be due to their respective capacities to induce the release of thromboxane from non-parenchymal cells (Häussinger *et al.*, 1988). Thromboxane on its own induces glycogenolysis in hepatocytes, thereby obscuring a direct effect of the nucleotides on the parenchymal cell. Our results clearly show that with a suspension of parenchymal cells, no difference between ATP and UTP is detected, suggesting a close similarity (or identity) between the ATP and UTP receptors. These data are in accord with those of Pfeilschifter (1990) on the effects of extracellular ATP and UTP on rat renal mesangial cells and those of Davidson and co-workers (1990) on pituitary cells. The authors concluded that there are probably no separate purino- and pyrimidino-ceptors on these cells. A similar situation might therefore exist for the liver parenchymal cells with ATP and UTP using a common receptor.

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Interaction of pinaverium (a quaternary ammonium compound) with 1,4-dihydropyridine binding sites in rat ileum smooth muscle

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1 The interaction of pinaverium bromide, a quaternary ammonium compound, with binding sites for (L-type) calcium channel blockers was investigated in rat ileum smooth muscle.

2 Pinaverium inhibited [^{3}H]-(+)-PN200-110 ([^{3}H]-(+)-isradipine) specific binding to tissue homogenates incompletely (K_i 0.38 μM; maximal inhibition 80%). In contrast, binding to single cell preparations (obtained by collagenase treatment) and to saponin-treated homogenates was completely inhibited. These data are compatible with the view that, in untreated homogenates, 20% of [^{3}H]-(+)-isradipine binding sites are not accessible to pinaverium because it is associated with sealed inside-out vesicles.

3 Pinaverium bromide increased the apparent K_D of [^{3}H]-(+)-isradipine binding to saponin-treated homogenates but did not significantly affect the B_{max} value. Moreover, the dissociation rate constant of [^{3}H]-(+)-isradipine binding was not changed by pinaverium. These data suggest that pinaverium interacts with the dihydropyridine binding site in a competitive manner. However, in contrast to uncharged dihydropyridine calcium antagonists, pinaverium inhibited, rather than stimulated, [^{3}H]-diltiazem binding to rat brain membranes (at 30–37°C).

4 Although B_{max} values of [^{3}H]-(+)-isradipine were similar in homogenates prepared from tissue and cells (collagenase-treated), the K_D value was significantly higher in cell homogenates (166 vs 95 pM). Similarly, the K_i value of pinaverium was higher in cell preparations than in tissue homogenates (0.77 vs 0.38 μM). Thus, collagenase can significantly modify the dihydropyridine recognition site.

5 The competitive interaction of pinaverium, a permanently charged drug, with [^{3}H]-(+)-isradipine bound to intact cells and its absence of interaction with [^{3}H]-(+)-isradipine bound to sealed inside-out vesicles imply that the dihydropyridine receptor lies near the external surface of the plasma membrane.

Keywords: Pinaverium bromide; collagenase; dihydropyridine Ca^{2+} antagonists; ileum smooth muscle; diltiazem

Introduction

Pinaverium bromide, a quaternary ammonium compound, has been recommended for the treatment of hypermotility disorders of the intestine, especially irritable bowel syndrome (for review see Christen, 1990). Earlier studies on smooth muscle tissue suggest that pinaverium exerts its spasmolytic action mainly by blocking voltage-dependent Ca^{2+} channels and has little effect on receptor-operated channels and intracellular Ca^{2+} stores (Droogmans *et al.*, 1983; Baumgartner *et al.*, 1985; Beech *et al.*, 1990). Its selectivity for the intestinal tract is attributable to its low absorption by the gut and its marked hepatobiliary excretion (Jacquot *et al.*, 1989).

In this paper, we have characterized the interaction of pinaverium bromide with specific binding sites for L-type channel blockers (Godfraind *et al.*, 1986). We carried out competition experiments on homogenates of longitudinal smooth muscle from rat ileum and on dissociated 'intact' cells obtained after treatment with collagenase; we have also investigated the influence of collagenase on binding parameters. The results show that pinaverium bromide interacts with dihydropyridine binding in an apparently competitive manner at concentrations reported to be active on Ca^{2+} channel currents. Analysis of this interaction suggests that pinaverium bromide, a positively charged drug, has access to the dihydropyridine binding site from the outside of the cell.

Methods

Single cell preparations

Single smooth muscle cells from rat ileum were prepared by the method of Momose & Gomi (1978) modified by Takayanagi *et al.* (1986). Wistar rats (250–350 g) were killed by decapitation and the ileum was quickly removed. The longitudinal muscle layers were peeled manually from the underlying circular muscle and incubated at 37°C for 90 min in a medium (mM: NaCl 137, KCl 2.7, $MgCl_2$ 1.0, $CaCl_2$ 0.18, glucose 5.6, HEPES 4.2; pH 7.4) bubbled with a gas mixture of 95% O_2: 5% CO_2. Tissue pieces were then incubated in a medium containing 0.1% purified collagenase (Sigma Ia) and 1% bovine serum albumin at 37°C for 30 min. The incubation was terminated by washing tissue fragments twice by centrifugation (500 *g* for 30 s). Pellets were resuspended in the incubation medium at 37°C and single cells could then be separated by gently pipetting the suspension through a wide-bore Pasteur pipette. The suspension of single cells was filtered through a nylon mesh (0.18 mm).

Membrane preparation

Freshly prepared tissue or single cells were suspended in about 10 volumes of an ice-cold solution containing 0.25 mM sucrose, 0.1 mM phenylmethylsulphonylfluoride and 5 mM Tris-HCl (pH 7.4), and homogenized with three strokes of the pestle rotating at 1500 rev min^{-1} in an all-glass Potter–Elvehjem-type grinder (Braun, Melsungen, Germany) kept at 2°C. Protein concentration was estimated according to Lowry *et al.* (1951).

In some experiments, tissue homogenates were treated by collagenase in the same way as were the muscle strips used for cell isolation. The incubation was then terminated by centrifugation at 200 000 *g* for 30 min and the pellet was resuspended with a Dounce homogenizer in ice-cold 0.25 mM sucrose buffered at pH 7.4 with 5 mM Tris-HCl.

Binding studies

The specific binding of [^{3}H]-(+)-isradipine was measured, as described previously (Wibo *et al.*, 1988), by incubating frac-

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Table 1 [^{3}H]-(+)-isradipine binding parameters in homogenates from rat ileum smooth muscle: effect of collagenase

	K_D (pM)	B_{max} (fmol mg^{-1} prot.)
Tissue homogenates (4)	95.2 ± 8.8	102.3 ± 15.5
Cells homogenates (3)	165.7 ± 6.4*	95.8 ± 6.8
Collagenase-treated tissue homogenates (3)	145.9 ± 3.0*	100.2 ± 10.2

Values are means ± s.e.mean from n (in parentheses) membrane preparations. [^{3}H]-(+)-isradipine binding parameters were estimated by linear regression (EBDA programme). *Significantly higher than the tissue homogenate value ($P < 0.01$, t test).

tions with the radioligand for 60 min at 37°C in 2.5 ml of a buffered salt solution of the following composition (mM): NaCl 145, KCl 5, $MgCl_2$ 1.25, $CaCl_2$ 2.5, Tris 20; pH 7.4 at 37°C. Non-specific binding was estimated in the presence of 1 μM nifedipine. After incubation, membranes collected on Whatman GF/F filters were washed twice with 10 ml of ice-cold 0.9% NaCl.

[^{3}H]-ouabain binding was measured in a 'Mg–Pi' medium (37°C, 60 min) as previously described (Noël & Godfraind, 1984). Non-specific binding was estimated in the presence of 1 mM ouabain. Membranes were collected on GF/F filters and washed twice with 10 ml of chilled 10 mM Tris-HCl (pH 7.4). In all binding assays, filters were immersed in Picofluor 15/ toluence (1/3, v/v) and radioactivity was counted with an efficiency of 40–45%.

For [^{3}H]-diltiazem binding studies, rat cerebral cortex membranes were prepared according to the method of Schoemaker *et al.* (1985). Membranes (50 μg of protein in a final volume of 0.5 ml) were incubated in 50 mM Tris-HCl buffer (pH 7.4) with 5 nM [^{3}H]-diltiazem for 60 min at 30° or 37°C. After incubation, membranes were harvested by centrifugation (10000 g for 10 min). The supernatant was removed by aspiration and the pellet was rapidly washed with 100 μl of ice-cold Tris-HCl buffer. The pellet was then solubilized in 100 μl of Soluene and its radioactivity was measured in the presence of 10 ml of Picofluor 15/toluene (1/3, v/v), with an efficiency of 40–45%.

Drugs

[^{3}H]-(+)-PN200-110 ([^{3}H]-(+)-isradipine, isopropyl 4-(2,1,3-benzoxadiozol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridinecarboxylate; 85.9 Ci mmol^{-1}), [^{3}H]-diltiazem (77 Ci mmol^{-1}) and [^{3}H]-ouabain (15 Ci mmol^{-1}) were obtained from NEN Research Products (Boston, MA, U.S.A.). Pinaverium bromide (4-(6-bromoveratryl)-4-[2-{2-(10 norpinan-2-yl)ethoxy}ethyl] morpholinium bromide) was provided by Latema Sarbach (Suresnes, France). Nifedipine and nitrendipine were gifts from Bayer AG (Leverkusen, Germany) and (+)-*cis*-diltiazem from Synthelabo (Paris, France). Ouabain was from Merck (Darmstadt, Germany), and collagenase (type Ia) from Sigma Chemical Co (St Louis, MO, USA). Other chemicals used were of analytical grade. All the experiments with 1,4-dihydropyridines were carried out under yellow light to prevent their degradation.

Statistical analysis

Results of the experiments are expressed as means ± s.e.mean. Tests of significance have been made by Student's t test, P values smaller than 0.05 being considered significant.

In binding experiments, saturation isotherms were analysed by Scatchard and Hill plots. The dissociation constant (K_D) and B_{max} were calculated by linear regression. In competitive experiments, displacement curves were analysed by a sigmoid curve fitting programme (Munson & Rodbard, 1980). An iterative technique gave estimate of IC_{50} (concentration of displacer inhibiting 50% of specific binding) and the K_i value was calculated according to Cheng & Prusoff (1973).

Results

The specific binding of [^{3}H]-(+)-isradipine to tissue homogenates was saturable in the concentration range of 25–250 pM. As shown in Table 1, the values of K_D and B_{max} calculated from the Scatchard plots ($n = 4$) were 95.2 ± 8.8 pM and 102.3 ± 15.5 fmol mg^{-1} of protein, respectively. B_{max} values were similar in homogenates prepared from intact tissue and dissociated cells, unlike K_D values which were increased by 75% in cell homogenates. Since cell preparations had been submitted to the action of collagenase, we treated tissue homogenates with collagenase to study its eventual influence on [^{3}H]-(+)-isradipine binding. The results showed an increase of the K_D value by 50% and no significant variation of the B_{max} value (Table 1).

Pinaverium bromide inhibited [^{3}H]-(+)-isradipine specific binding to tissue homogenates incompletely (80% inhibition at 10–100 μM, Figure 1). Slopes of Hill plots (not shown) were close to 1. The K_i value derived from the concentration for half-maximal inhibition (IC_{50}) according to Cheng & Prusoff (1973) was 0.38 ± 0.10 μM (Table 2). The incomplete inhibition of [^{3}H]-(+)-isradipine binding by pinaverium bromide might

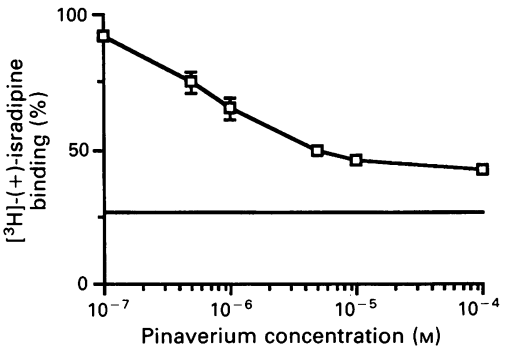

Figure 1 Inhibition by pinaverium of [^{3}H]-(+)-isradipine binding to homogenates of ileum smooth muscle. Competition experiments were performed with [^{3}H]-(+)-isradipine at a concentration near the K_D, 0.1 nM, and increasing concentrations of pinaverium. Total binding (specific + nonspecific) is expressed as a percentage of the value obtained in the absence of pinaverium. Data are means from 6 determinations (2 different preparations); vertical bars show s.e.mean when they are larger than symbols. Non-specific binding (horizontal line) was determined in the presence of 1 μM nifedipine and was significantly lower ($P < 0.01$; *t test*) than the value of total binding at each pinaverium concentration.

Table 2 Comparison of pinaverium binding parameters in tissue homogenates and 'intact' cells from rat ileum smooth muscle

	IC_{50} (μM)	K_i (μM)	*Hill coeff.*
Tissue homogenates (3)	0.68 ± 0.19	0.38 ± 0.10	0.96 ± 0.07
'Intact' cells (3)	1.33 ± 0.02*	0.77 ± 0.01*	0.98 ± 0.09

Values are means ± s.e.mean from n (in parentheses) preparations. IC_{50} values were estimated from displacement curves by a sigmoid curve-fitting iterative technique (EBDA programme). K_i values were calculated according to Cheng & Prusoff (1973).
* Significantly higher than the tissue homogenate values ($0.01 < P < 0.05$, t test).

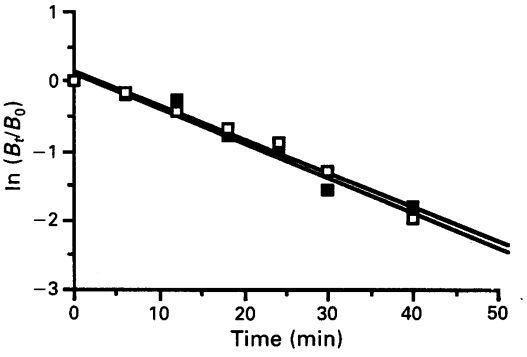

Figure 2 Effect of pinaverium bromide on the rate of dissociation of [^{3}H]-(+)-isradipine binding. After incubation with 0.1 nM [^{3}H]-(+)-isradipine for 60 min at 37°C, dissociation was induced by addition of 1 μM nifedipine in the presence (■) or absence (□) of 10 μM pinaverium. B_0 and B_t are specific [^{3}H]-(+)-isradipine bound at time 0 and time t, as indicated on the abscissa scale. Data are means from 6 determinations; s.e.means were smaller than symbols.

suggest that this drug allosterically interacts with the dihydropyridine recognition site, as previously found for other drugs (Murphy *et al.*, 1983; Glossmann *et al.*, 1985). However, in dissociation experiments (Figure 2), we did not observe any significant effect of pinaverium bromide (10 μM), the dissociation rate constant (k_{-1}) of [^{3}H]-(+)-isradipine being equal to 0.049 ± 0.009 min^{-1} and 0.048 ± 0.006 min^{-1} in the presence or absence of pinaverium, respectively. Moreover, in saturation experiments pinaverium at concentrations up to 10 μM did not significantly affect the B_{max} of [^{3}H]-(+)-isradipine binding (Figure 3).

The incomplete inhibition of [^{3}H]-(+)-isradipine binding by pinaverium bromide in tissue homogenates, might be explained by the presence of sealed vesicles, in which dihydropyridine binding sites are inaccessible to pinaverium, a positively charged drug. Indeed, in samples preincubated with saponin (0.5–1.0 mg mg^{-1} protein), the specific binding of [^{3}H]-(+)-isradipine was completely inhibited by pinaverium bromide (Table 3). Additional experiments showed that the addition of saponin did not alter [^{3}H]-(+)-isradipine binding up to a saponin/protein concentration ratio of 1 (results not shown). The existence of sealed vesicles in tissue homogenates was supported by the results of binding experiments with [^{3}H]-ouabain (Figure 4). In the presence of saponin (0.5 mg mg^{-1} protein), the K_D of [^{3}H]-ouabain binding was not modified (98 vs 96.5 nM), whereas the B_{max} increased from 452 to 557 fmol mg^{-1} protein.

Competition experiments were also carried out on single cells preparations that had not been homogenized. As shown

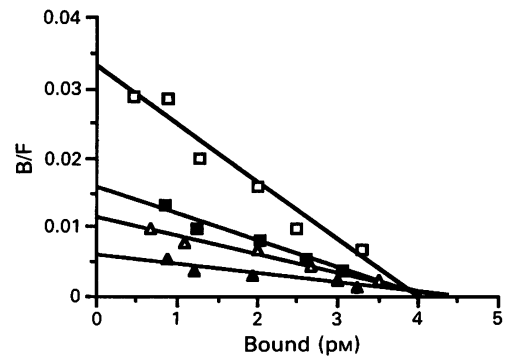

Figure 3 Effect of pinaverium on saturation curves of [^{3}H]-(+)-isradipine binding to homogenates of ileum smooth muscle. Saturation experiments were performed with pinaverium bromide at concentrations of 0.3 μM (■), 1 μM (Δ) and 10 μM (▲), or without drug (□). Results are shown as Scatchard plots. Samples were preincubated with saponin (0.5 mg mg^{-1} prot.) for 20 min at room temperature before incubation with [^{3}H]-(+)-isradipine. Each value is the mean of 3–6 determinations; s.e.means were smaller than symbols.

Table 3 Relationship between saponin concentration and inhibition of [^{3}H]-(+)-isradipine binding by 100 μM pinaverium

Saponin/protein ratio (w/w)	*Inhibition* (%)
No saponin	80.1 ± 0.8
1/3	88.7 ± 1.5
1/2	98.9 ± 0.1
1/1	99.0 ± 0.1

Tissue homogenates (750 μg protein ml^{-1}) were preincubated with various concentrations of saponin, as indicated (20 min at room temperature). Samples (200 μl) were then further incubated at 37°C for 60 min with 0.1 nM [^{3}H]-(+)-isradipine, in a final volume of 2.5 ml (see Methods). Data are means ± s.e.mean from 3 different preparations.

in Figure 5, pinaverium completely inhibited [^{3}H]-(+)-isradipine specific binding to intact cells in the absence of saponin treatment. Pinaverium IC_{50} and K_i values in single cells preparations were about twice as high as those found in tissue homogenates (Table 2).

It is well known that diltiazem, a benzothiazepine inhibitor of L-type Ca^{2+} channels, stimulates [^{3}H]-dihydropyridine binding at 30°–37°C, and that conversely, some dihydropyridines enhance the binding of [^{3}H]-diltiazem (Schoemaker & Langer, 1985). To gain further insight into the interaction of pinaverium with the dihydropyridine binding site, we compared its effect on [^{3}H]-diltiazem binding with that of nitrendipine. This experiment was carried out on a membrane

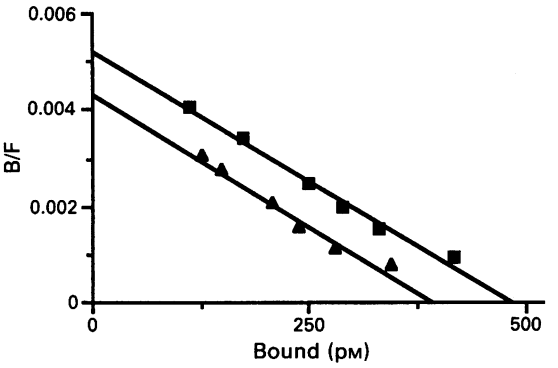

Figure 4 Effect of saponin on saturation curve of [^{3}H]-ouabain binding to homogenates of ileum smooth muscle. After 20 min preincubation at room temperature in the presence (■) or absence (▲) of saponin (0.5 mg mg^{-1} prot.), membranes were incubated at 37°C for 60 min with 100 nM [^{3}H]-ouabain and increasing concentrations of cold ouabain. Non-specific binding was determined in the presence of 1 mM ouabain. Results are shown as Scatchard plots; each value is the mean of 6 determinations.

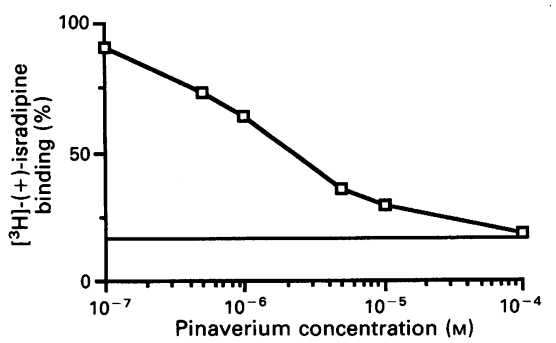

Figure 5 Inhibition by pinaverium of [^{3}H]-(+)-isradipine binding to cells isolated from ileum smooth muscle. Competition experiments were performed as described in legend of Figure 1, except that isolated cells were used instead of tissue homogenates. Data are means from 6 determinations (2 different preparations); s.e.means were smaller than symbols. Non-specific binding (horizontal line) was not significantly different from total binding at a pinaverium concentration of 100 μM.

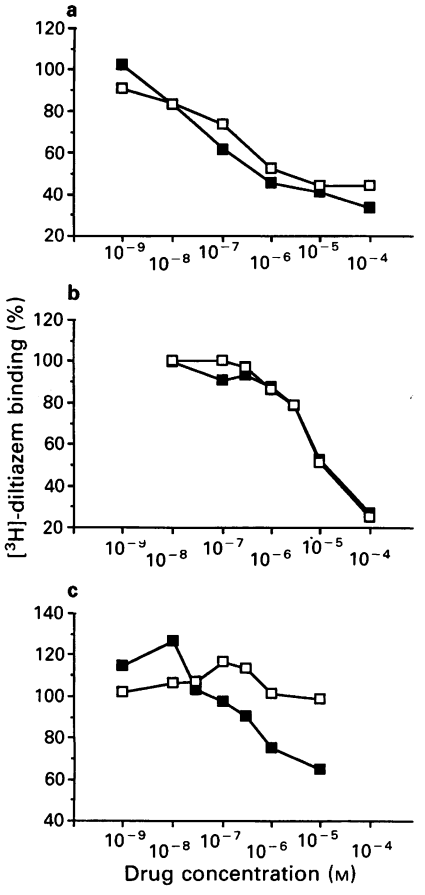

Figure 6 Effects of (+)-*cis*-diltiazem, pinaverium and nitrendipine on the binding of [^{3}H]-diltiazem to rat cerebral cortex membranes. Binding was measured at 30°C (■) or 37°C (□) as described in Methods, with 5 nM [^{3}H]-diltiazem and increasing concentrations of competitor: (a) (+)-*cis*-diltiazem; (b) pinaverium; (c) nitrendipine. Total binding (without non-specific binding subtracted) is expressed as a percentage of the value obtained in the absence of unlabelled ligand. Data are means of 3–6 determinations. The s.e.means (not shown) do not exceed 5% of the mean values.

fraction from rat brain, because we met with difficulties when using membranes from intestinal muscle (data not shown). As shown in Figure 6, (+)-*cis*-diltiazem inhibited [^{3}H]-diltiazem binding with an IC_{50} of 128 nM at 37°C and 42 nM at 30°C (Hill coefficient 0.6–0.7), in agreement with previous findings (Schoemaker & Langer, 1985). At 37°C, nitrendipine stimulated [^{3}H]-diltiazem binding at concentrations of 10–300 nM, whereas, at 30°C, it stimulated binding at 1–10 nM and inhibited binding at concentrations higher than 100 nM. Pinaverium had no detectable stimulatory effect, but inhibited binding at concentrations of 1–100 μM, and the curves at 30°C and 37°C were superimposable. The IC_{50} estimated from these curves by nonlinear regression was 5–6 μM, a value distinctly higher than that measured in [^{3}H]-(+)-isradipine displacement experiments (Table 2).

Discussion

As shown recently by Beech *et al.* (1990), pinaverium bromide is able to block voltage-dependent, L-type Ca^{2+} channels in rabbit intestinal smooth muscle cells. In binding experiments, pinaverium bromide appears to interact competitively at the dihydropyridine receptor, and the K_i value that we measured with 'intact' cells (0.77 μM) is in good agreement with the inhibitory potency in patch-clamp experiments (IC_{50} 1.5 μM). That the inhibition of dihydropyridine binding is competitive is supported by the following arguments. (1) [^{3}H]-(+)-isradipine specific binding could be completely inhibited by pinaverium bromide in 'intact' cells and saponin-treated homogenates and the Hill coefficients were close to 1. (2) The effect of pinaverium bromide on equilibrium binding parameters was compatible with a competitive inhibition, since, at concentrations up to 10 μM, this drug increased the apparent K_D of [^{3}H]-(+)-isradipine binding but did not significantly affect B_{max} (Figure 3). The average K_i calculated from the apparent K_D ($K_{D,app}$) values at 4 different concentrations of pinaverium by the equation: $K_{D,app} = K_D(1 + [\text{pinaverium}]/K_i)$, is 0.69 μM, in good agreement with the values deduced from the analysis of displacement curves (Table 2). (3) The dissociation rate constant of [^{3}H]-(+)-isradipine binding was not changed by 10 μM pinaverium bromide (Figure 2). Nevertheless, some subtle differences may be noted between the effects of pinaverium and dihydropyridines, a finding which is not unexpected in view of the different chemical structures involved and the presence of a positively charged group in pinaverium. In contrast to most dihydropyridine calcium antagonists, which stimulate [^{3}H]-diltiazem binding in a temperature-dependent manner, pinaverium inhibited [^{3}H]-diltiazem binding and its effect was identical at 30°C and 37°C. Interestingly, amlodipine, a predominantly charged dihydropyridine, enhances [^{3}H]-diltiazem binding (at 37°C) over a narrow concentration range and inhibits binding at concentrations higher than 1 μM (Burges *et al.*, 1989). Its behaviour is thus intermediary between those of pinaverium and uncharged dihydropyridines. Moreover, calcium channel blockade by pinaverium is not appreciably voltage-dependent, in contrast to the blockade evoked by dihydropyridine calcium antagonists (Beech *et al.*, 1990).

The complete inhibition of [^{3}H]-(+)-isradipine binding by pinaverium bromide in intact cells suggests that this permanently charged drug has access to the dihydropyridine binding site from the outside of the cell. The incomplete inhibition of [^{3}H]-(+)-isradipine binding in tissue homogenates is probably due to the presence of sealed inside-out vesicles, in which dihydropyridine binding sites are accessible to [^{3}H]-(+)-isradipine (uncharged) but not to pinaverium (positively charged). Saponin removed a permeability barrier in inside-out vesicles, which accounted for some 20% of [^{3}H]-(+)-isradipine binding sites in homogenates. This interpretation is confirmed by the effect of saponin on [^{3}H]-ouabain binding (Figure 3). It is well-known that ouabain binding sites are exposed on the outside of the plasma membrane and are, therefore, inaccessible to ouabain in sealed inside-out vesicles. The percentage of sealed inside-out vesicles as determined in this manner (18.8%), is in good agreement with the proportion of the [^{3}H]-(+)-isradipine binding sites that appear to be inaccessible to pinaverium bromide (20%). Thus, our findings support the view that the site of the calcium channel that interacts with dihydropyridines lies near the external surface of the plasma membrane, as previously proposed by Kass *et al.* (1989) from their study on the effect of external pH on the interaction of amlodipine (pK_a 8.6) with the calcium channel, and more recently, from the finding that a permanently charged (quaternary ammonium) dihydropyridine derivative inhibits L-channel current when applied extra-, but not intracellularly (Kass *et al.*, 1991). However, a recent study that used photoaffinity labelling to locate the dihydropyridine binding site in the Ca channel α_1 subunit of skeletal muscle claimed that this site has a cytosolic localization (Regulla *et al.*, 1991).

An additional conclusion that can be drawn from our results is that collagenase might be deleterious for the voltage-dependent Ca^{2+} channels. Indeed, the K_D of [^{3}H]-(+)-isradipine and the K_i of pinaverium bromide were both appreciably increased in cells obtained by collagenase digestion and in tissue homogenates treated with collagenase. These observations support the view, expressed by Rusch & Hermsmeyer (1988), that 'dissociating methods for cell dispersion cannot be regarded as innocent of influencing calcium channel viability'.

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Different patterns of release of endothelium-derived relaxing factor and prostacyclin

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1 Release of endothelium derived relaxing factor (EDRF) and prostacyclin (PGI_2) from endothelial cells (EC) cultured from bovine aortae was measured by bioassay and radioimmunoassay, respectively, during infusions (10 min) of bradykinin (BK), adenosine diphosphate (ADP), arachidonic acid (AA), alkaline buffers and the free-bases (FB) of L-arginine or D-arginine. Release of EDRF from the luminally perfused rabbit aorta was also measured during infusions (10 min) of acetylcholine (ACh), substance P and ADP.

2 Bradykinin (10 or 30 nM) infused through the column of EC induced release of both EDRF and PGI_2, neither of which was maintained for the duration of the infusion.

3 ADP (1.6 or 4 μM) infused through the column of EC induced release of a EDRF which was maintained for the duration of the infusion and a release of PGI_2 which lasted for a much shorter period.

4 Arachdonic acid (30 or 90 μM) infused through the column of EC caused a sustained release of EDRF and PGI_2, both of which outlasted the infusion of AA.

5 L-Arginine FB, D-arginine FB or alkaline buffer infused through the column of EC released EDRF, but only small amounts of PGI_2. The release of EDRF outlasted the period of infusion and was due to an increase in the pH of the Krebs solution perfusing the EC.

6 Infusions of ACh (0.25–1 μM) or ADP (4–16 μM) caused a sustained release of EDRF from the luminally-perfused rabbit aorta, whereas infusion of substance P (3.3–10 μM) caused only a transient release of EDRF.

7 These results show that distinct patterns of EDRF release exist to different agonists in both cultured and *in situ* EC, and that EDRF and PGI_2 do not necessarily follow the same time course of release. Furthermore, sustained release of EDRF does not require the constant infusion of the precursor, L-arginine, whereas sustained release of PGI_2 only occurs when AA, the precursor of PGI_2, is present in the extracellular medium.

Keywords: Bradykinin; adenosine diphosphate; arachidonic acid; L-arginine; D-arginine; alkaline buffer; acetylcholine; substance P

Introduction

Prostacyclin (PGI_2; Moncada *et al.*, 1976) and endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980) are both released from endothelial cells (EC) by a variety of agonists including acetylcholine (ACh), adenosine diphosphate (ADP), bradykinin (BK) and substance P (see Furchgott *et al.*, 1984; Gryglewski *et al.*, 1988). This receptor-mediated release of EDRF and PGI_2 is coupled most probably at the level of phospholipase C (de Nucci *et al.*, 1988a). In contrast the free base (FB) of arginine can change the pH of Krebs buffer by about 1.0 pH unit and thereby selectively release EDRF with very little accompanying PGI_2, through a mechanism which is receptor-independent (Mitchell *et al.*, 1991a). Thus, alkaline buffers appear to induce the release of EDRF by a mechanism that bypasses the coupled release stage, thereby provoking a release of EDRF independent from that of PGI_2.

Although EDRF and PGI_2 are co-released by several agonists their chemical nature, biosynthetic pathway and mode of action differ. PGI_2 is a metabolite derived from the 20 carbon fatty acid, arachidonic acid (AA; Moncada *et al.*, 1976) whereas EDRF is NO or a related molecule (Palmer *et al.*, 1987; Myers *et al.*, 1990) formed from L-arginine (Palmer *et al.*, 1988; Schmidt *et al.*, 1988). The biosynthetic pathways of EDRF and PGI_2 also differ with regard to substrate availability. The level of free AA available for PGI_2 synthesis is tightly controlled (see Irvine, 1982) whereas intracellular levels of L-arginine are relatively high (approximately 0.1–1 mM), and remain stable during prolonged EDRF release (Mitchell *et al.*, 1990a) due to constant generation from intracellular stores (Mitchell *et al.*, 1990b). On the other hand there are similarities between the two pathways for both EDRF (Förstermann *et al.*, 1991; Mitchell *et al.*, 1991b) and PGI_2 (Moncada *et al.*, 1976) are synthesized by particulate enzymes (EDRF synthase and cyclo-oxygenase/prostacyclin synthase respectively). In addition, both cyclo-oxygenase and the EDRF synthase-related enzyme, NO synthase in macrophages, utilize molecular oxygen (Ryhage & Samuelsson, 1965; Stuehr *et al.*, 1991).

Although the release of EDRF induced by several agonists has been extensively studied, the kinetics of release has not been fully explored. Moreover, the temporal relationship between EDRF and PGI_2 release from cultured EC has been investigated primarily with receptor-activating agents (White & Martin, 1989). The objective of this study was to determine the duration of release from cultured EC of EDRF and PGI_2 following infusions of their respective precursors, or stimulation of the EC by receptor-dependent or -independent agonists.

Some of these results were presented to the British Pharmacological Society (de Nucci *et al.*, 1988b).

Methods

Endothelial cell culture

Endothelial cells were isolated by treatment of bovine aortae with 0.02% (w/v) collagenase. Cells were grown to confluence

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in plastic vessels, then removed by treatment with 0.05% (w/v) trypsin, and seeded onto Cytodex 3 microcarrier beads (Pharmacia), as previously described (de Nucci *et al.*, 1988a). The beads were packed into a jacketed column and perfused with Krebs solution (5 ml min^{-1} at 37°C) containing superoxide dismutase (SOD, 10 units ml^{-1}; Gryglewski *et al.*, 1986).

Preparation of the rabbit aorta

New Zealand White rabbits were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.v.) and exsanguinated via the common carotid artery. The thorax and abdomen were opened and the aorta exposed and carefully cleared of connective tissue *in situ*. The mesenteric and coeliac arteries were ligated close to their origins from the aorta and a segment of the aorta from the aortic arch to the left renal artery was carefully removed. A cannula was inserted into the cardiac end of the aorta, secured with thread, mounted in a heated tissue bath (37°C) and the aorta was perfused through the lumen at 5 ml min^{-1} with warmed (37°C) gassed (95% O_2:5% CO_2) Krebs solution containing SOD (10 units ml^{-1}) and indomethacin (5 μM) (Warner *et al.*, 1989).

EDRF detection by bioassay

The effluent from either the column or from the luminally-perfused rabbit aorta superfused in a cascade (Vane, 1964) four-spirally cut rabbit aortic strips (RbAs) that were denuded of endothelium. Effluent from the column or donor aorta reached the consecutive RbAs after 1, 4, 7, and 10 s. Drugs were infused either over the assay tissues (o.t.) as a control, or through the EC column (t.c.) or donor aorta (t.a.). The assay tissues were superfused first with Krebs solution containing U46619 (30 nM) until a stable contraction was obtained. Aortic strips prepared from 5–10% of rabbits were insensitive to U46619 (30–300 nM) and were consequently contracted with noradrenaline (300 nM). The assay tissues were then calibrated by the relaxant effects of glyceryl trinitrate (GTN), and the sensitivities on the recordings of the RbAs adjusted electronically to be roughly equal. The sensitivity of batches of EC to EDRF/PGI_2 releasing agonists varied; it was therefore necessary to test each batch with doses of a given agonist in order to choose an optimum concentration (causing EDRF release which relaxed the first RbA $\geqslant$ 20 pmol GTN and PGI_2 release $\geqslant$ 0.5 ng ml^{-1}). The relaxations of the assay tissues were recorded with auxotonic levers (Paton, 1957), attached to Harvard isotonic transducers and displayed on a six channel Watanabe Recorder (type WR3101). Indomethacin (5 μM) was infused o.t. throughout the experiments to prevent synthesis of cyclo-oxygenase products by the assay tissues. When AA was infused, the dual cyclo-oxygenase and lipoxygenase inhibitor BW755C (30 μM; Higgs *et al.*, 1979) was infused o.t. to prevent the formation of eicosanoids in the tissues in the cascade. In experiments where arginine FB or alkaline buffers were used the column of EC was perfused with Krebs solution without KH_2PO_4 to avoid precipitation of calcium. An appropriate infusion o.t. of KH_2PO_4 restored the phosphate level in the cascade. In experiments using the luminally-perfused rabbit aorta homatropine (1.5 μM), phenoxybenzamine (0.3 μM), mepyramine (1 μM) and methysergide (0.3 μM) were infused o.t. The time course of release of EDRF, as measured by activation of isolated soluble guanylate cyclase, correlates with relaxation of rabbit aortic strips used in bioassay (Kondo *et al.*, 1989). Thus bioassay tissues give a reliable assessment of the duration of EDRF release.

Measurement of PGI_2 by radioimmunoassay

The effluent from the column was collected after passing over the bioassay cascade tissues and analyzed by radioimmunoassay (RIA) for 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$; Salmon, 1978) as a measure of PGI_2 release from the EC. The detection limit on the RIA was approximately 0.5 ng ml^{-1}.

Measurement of pH

The pH was measured with a Corning pH 105 meter.

Materials

The Krebs solution (pH; 7.5–8.0) had the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.17, $CaCl_2 \cdot 6H_2O$ 2.5, $NaHCO_3$ 25 and glucose 5.6. Bradykinin acetate (BK), adenosine diphosphate di (monocyclohexylammonium) salt (ADP), arachidonic acid sodium salt (AA), superoxide dismutase (SOD) from bovine erythrocytes, free base (FB) of L-arginine or D-arginine, indomethacin, substance P acetate, acetylcholine chloride (ACh), homatropine hydrobromide, trizma and glycine buffers were supplied by Sigma (Poole, U.K.). Sodium carbonate buffer (Na_2CO_3; anhydrous, Analar) was freshly prepared each day (100 mg ml^{-1}) in distilled water. This was then diluted to produce a solution of the same buffering capacity as infusions of L- or D-arginine FB (1–3 mM). The Krebs solution salts, glucose and Na_2CO_3 were obtained from B.D.H. (Dagenham, U.K.). Methysergide bimaleate was obtained from Sandoz Prod. Ltd. (Leeds, U.K.), mepyramine maleate from May & Baker (Dagenham, U.K.), and phenoxybenzamine from S.K. & F. (Stevenage, U.K.). Collagenase (Cooper Biomedical) was obtained from Lorne Diagnostics (Suffolk, U.K.) and trypsin and Dulbecco's modified Eagle's medium were obtained from Flow Laboratories. [^{3}H]-6oxo-$PGF_{1\alpha}$ was purchased from New England Nuclear. The antiserum and 6-oxo-$PGF_{1\alpha}$ for the RIA and BW755C 3-amino-1-[*m*-(trifluoromethyl)-phenyl] -2 pyrazoline) were gifts from J.A. Salmon and G. Higgs (Wellcome Research Laboratories, Beckenham, U.K.) respectively. U46619 (9,11-dideoxy-9α-methano epoxyprostaglandin $F_{2\alpha}$) was a gift from J. Pike (Upjohn, Kalamazoo, U.S.A.).

Statistics

Results are shown as mean $\pm$ s.e.mean for n experiments. Student's unpaired t test was used to assess the difference between means and a P value of <0.05 was taken as significant.

Results

Cultured endothelial cells

Release of EDRF and PGI_2 by bradykinin or ADP Neither BK (10 or 30 nM) nor ADP (1.6 or 4 μM) affected the strips of RbAs when infused o.t. Infusions of BK t.c. provoked transient release of EDRF (declining after 2.5 $\pm$ 0.3 min, $n = 6$). However, the release of EDRF induced by ADP (1–4 μM) was sustained throughout the 10 min infusion period (Figure 1a, $n = 6$).

The release of PGI_2 induced by infusions of BK or ADP reached a maximum in the 2nd–4th minute and then returned rapidly to basal levels (Figure 1b).

The transient release of EDRF induced by BK and the sustained release induced by ADP occurred at all concentrations used.

Release of EDRF and PGI_2 by arachidonic acid The release of EDRF induced by AA (30 or 90 μM) was sustained ($n = 4$) and in 2 experiments outlasted the infusion period (Figure 2a). The release of PGI_2 from EC induced by infusions of AA (30 or 90 μM) was not dose-dependent and always outlasted the infusion period (Figure 2b, $n = 4$).

Release of EDRF and PGI_2 by the free bases (FB) of L-arginine or D-arginine The free bases (FB) of L- or D-arginine (0.5–3 mM) or Na_2CO_3, at concentrations that increased the pH of the Krebs solution by about 1 pH unit to 8.6–9.0, infused o.t.

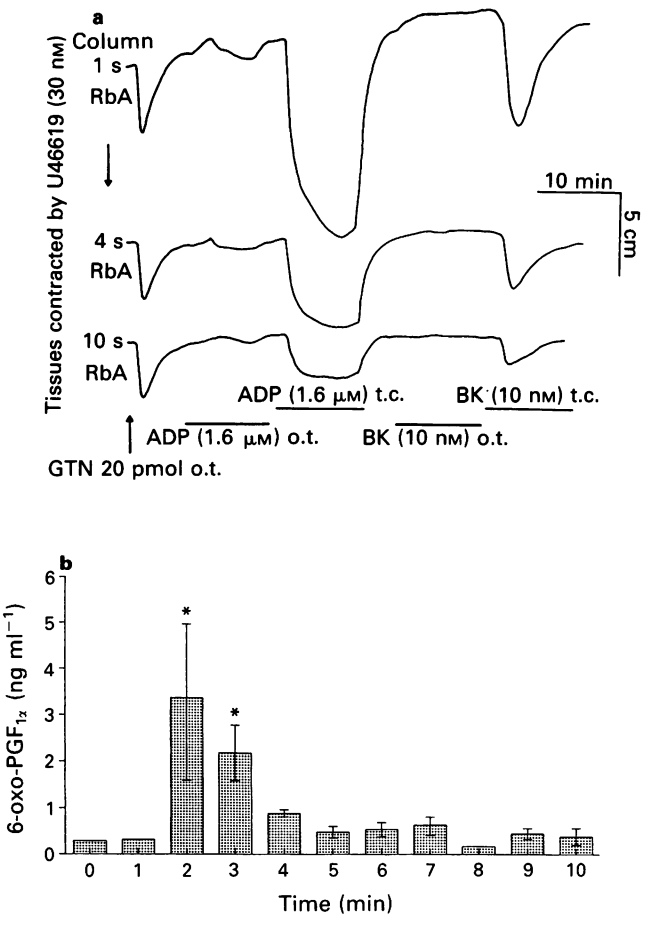

Figure 1 (a) Release of EDRF induced by 10 min infusions of ADP and bradykinin (BK) from bovine aortic cultured endothelial cells treated with superoxide dismutase (10 units ml^{-1}). The perfusate from the column of endothelial cells superfused a cascade of four rabbit aortic strips which were denuded of endothelium (RbAs; the first, second and fourth are shown). The RbAs were contracted by infusion of U46619 (10 nM). Control infusions of ADP (1.6 μM) or BK (10 nM) were given over the assay tissues (o.t.). Injections of glyceryl trinitrate (GTN) o.t. (40 pmol) caused relaxation of the RbAs. Infusions of ADP and BK through the column of endothelial cells (t.c.) caused release of EDRF. The release of EDRF induced by ADP was sustained throughout the infusion period and stopped abruptly on termination of the infusion. However the release of EDRF induced by BK was transient, the RbAs returning to their original baseline during the course of the infusion. This is a bioassay trace from a typical experiment. (b) Release of prostacyclin (PGI_2) induced by infusions of ADP. Effluent from a column of endothelial cells was collected at 1 min intervals and PGI_2 release measured by RIA for 6-oxo-$PGF_{1\alpha}$. Infusions of ADP (1.6 or 4 μM) through the column of endothelial cells (t.c.) for 10 min periods induced a transient release of PGI_2, that was maximal during the second and third minute, and subsequently returned to basal levels. Similar results were obtained with infusions of BK (10 or 30 nM). Data with different agonist concentrations were pooled due to variability of the endothelial cells (see methods). $^*P < 0.05$.

had small contractile effects on the RbAs. However, when infused t.c. they provoked a sustained release of EDRF similar to that seen with AA (Figure 3, $n = 4$). The accompanying release of PGI_2 was small and variable. There was no significant increase above basal in the case of L- or D-arginine free base ($n = 4$). However, there was a 3.5 ± 0.75 fold increase in PGI_2 during the 4th and 5th minutes of a 10 min infusion of Na_2CO_3 ($n = 4$). Infusions of L- or D-arginine FB increased the pH of the Krebs solution by 0.6–1.2 units when infused o.t. but the increase was less pronounced when the buffers were infused t.c. (Figure 3 lower panel). Trizma base or glycine buffer which changed the pH of the Krebs solution to a similar extent also resulted in the selective release of EDRF (data not shown).

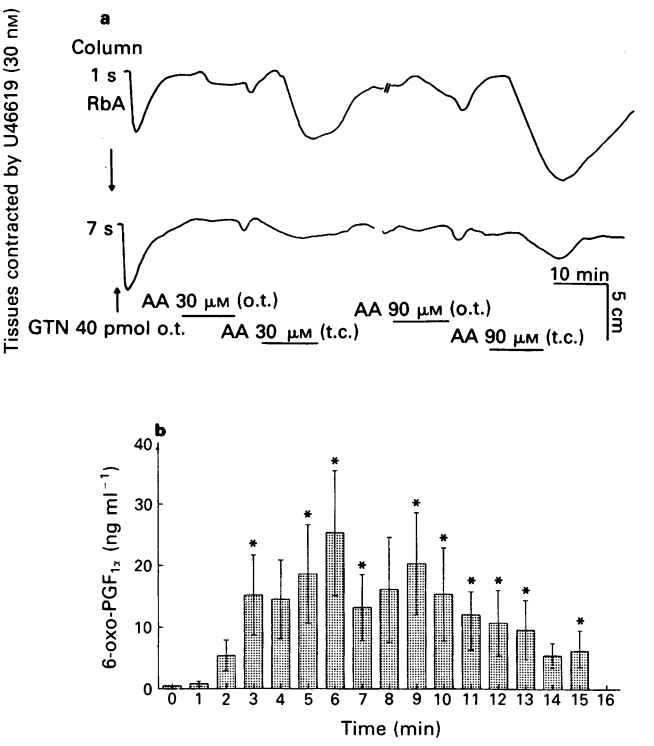

Figure 2 (a) Release of EDRF induced by infusions of arachidonic acid (AA) from columns of bovine aortic cultured endothelial cells. Experimental details are the same as for Figure 1. Infusions of AA (30 and 90 μM) over the RbAs (o.t.) induced small relaxations. When AA was infused for a period of 10 min through the column of endothelial cells (t.c.) it provoked a sustained and prolonged release of EDRF. Note that when the infusion of AA was stopped, the release of EDRF only gradually decreased. (b) Release of prostacyclin (PGI_2) induced by infusions of arachidonic acid (AA). The experimental procedures were the same as in Figure 1b. When AA (30–90 μM) was infused through the column of endothelial cells (t.c.) the level of PGI_2 released increased reaching a maximum by the third minute and remaining at this level for the duration of the infusion (10 min). When the infusion was removed the release of PGI_2 did not return immediately to basal but remained at an elevated level ($\geq$5 min, $^*P < 0.05$ are shown for values as compared to the basal level).

Luminally perfused rabbit aorta

Release of EDRF by ACh or ADP Acetylcholine (250 nM) had no effect on the assay tissues when given o.t. However, when infused for 10 min through the aorta (t.a.) of ACh provoked a sustained release of EDRF (Figure 4, $n = 4$). With the luminally perfused rabbit aorta higher concentrations of ADP (4 or 16 μM) were needed than with the EC columns to provoke the release of EDRF. ADP (4–16 μM) caused a sustained relaxation of the assay tissues when infused o.t. When ADP was infused t.a. the relaxation was greater ($n = 3$ out of 4; Figure 4) and this release of EDRF was sustained throughout the infusion period.

Release of EDRF by substance P Substance P (3.3 μM) had no effect when infused o.t. When infused t.a., substance P provoked a transient release of EDRF (Figure 4, $n = 4$).

Discussion

Here we have shown that different patterns of EDRF release occur from EC both in culture and *in situ* and the duration of PGI_2 release does not necessarily correlate with the duration of EDRF release induced by a given agonist.

Bradykinin infusions did not cause sustained release of either PGI_2 or EDRF. This desensitization to BK has been previously noted in guinea-pig isolated lungs, where infusions

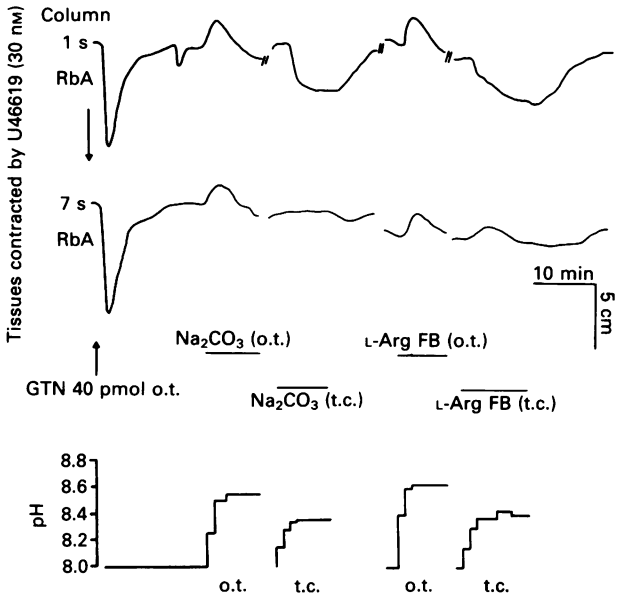

Figure 3 Release of EDRF induced by infusions of L-arginine free base (L-Arg, 3 mM) and Na_2CO_3 from bovine aortic cultured endothelial cells. Experimental details are the same as in Figure 1a. The first and second of 4 RbAs are shown. L-Arg and Na_2CO_3 infused over the RbAs (o.t.) had a contractile effect. However, when either agent was infused through the column of endothelial cells (t.c.) it induced a sustained and prolonged release of EDRF. Similar results were seen using the free base of D-arginine (3 mM; $n = 4$). The histogram at the bottom represents the change in the pH of the Krebs solution induced by L-Arg and Na_2CO_3 respectively.

of BK neither caused sustained release of thromboxane A_2 (Piper & Vane, 1969) nor of PGI_2 (Bakhle *et al.*, 1985), and in cultured EC where the release of PGI_2 induced by infusions of BK was transient (White & Martin, 1989). In our cells this short lived action of BK is unlikely to be due to inactivation of BK by angiotensin converting enzyme (ACE) for they have little or no ACE activity (de Nucci *et al.*, 1988c).

Substance P also induced a transient release of EDRF from the whole rabbit aorta, confirming previous observations on rabbit aortic strips with intact endothelium (Beny *et al.*, 1987). Thus this pattern of transient EDRF release is not peculiar to EC in culture. Whether this desensitization of the endothelium

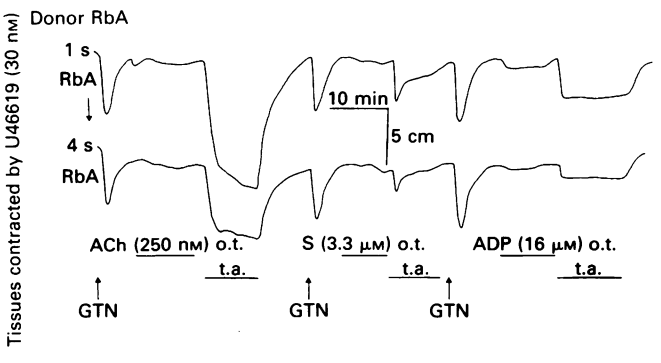

Figure 4 Release of EDRF induced by infusions of acetylcholine (ACh), adenosine diphosphate (ADP) and substance P (SP) from a luminally perfused rabbit aorta. Experimental details are the same as in Figure 1a with the exception of the source of EDRF generation. Infusions of ACh (250 nM; 10 min) or ADP (16 μM) through the rabbit aorta donor (t.a.) induced the release of EDRF which was sustained. The tissues returned to their original baseline immediately on removal of the infusion of ACh or ADP. In contrast, SP (3.3 μM; 10 min) infused t.a. induced a transient release of EDRF, the RbAs returning to their original baseline during the course of the infusion. o.t., over the RbAs.

to BK or substance P is due to rapid phosphorylation of G-proteins by protein kinase C and consequent uncoupling of the receptor complex remains to be clarified.

It is interesting that agents which increase the pH of the Krebs solution can cause a sustained release of EDRF. These observations support our previous findings that the free bases of L or D-arginine release EDRF to similar extents due to elevated pH and not by supplying substrate to NO synthase (Mitchell *et al.*, 1991a). Release by alkalinisation is mainly dependent on the presence of extracellular calcium, as opposed to receptor-mediated release where intracellular calcium mobilization may be important (Mitchell *et al.*, 1991a). This suggests that elevation of extracellular pH causes the entry of extracellular calcium, which would directly stimulate EDRF synthesis, for the enzyme responsible for EDRF formation (EDRF synthase; Förstermann *et al.*, 1991; Mitchell *et al.*, 1991b) by EC is calcium-dependent. However, an increase in the extracellular pH to 8.6 is unlikely to be a physiological stimulus for EDRF. Although EC respond to, and attempt to buffer, an elevated pH (possibly by exporting H^+ into the extracellular medium in exchange for Na^+), local changes in the blood will not produce this degree of alkalinisation.

The finding that ACh induces a sustained release of EDRF from the luminally perfused rabbit aorta, as suggested from experiments on isolated strips (Beny *et al.*, 1987) highlights the paradox in that ACh is a potent and prolonged releaser of EDRF, but is an unlikely endogenous agonist (see Kalsner, 1988). It has not been demonstrated that ACh diffuses from parasympathetic nerve terminals to activate the EC.

The sustained release of EDRF induced by ADP is common to columns of EC and to luminally perfused aortae and could indicate that ADP is an important physiological or pathophysiological agonist. One of the more likely pathophysiological sources of ADP may be activated platelets (Haslam, 1964), in which case it would be desirable for areas with an intact endothelium to release EDRF to decrease platelet adhesion, and possibly inhibit further platelet aggregation. As ADP increases intracellular calcium in EC initially by releasing calcium from intracellular stores, and subsequently by stimulating the entry of extracellular calcium (Pritton *et al.*, 1987; Luckhoff & Busse, 1986) the transient release of PGI_2 induced by ADP, which we describe here, is most probably correlated with the transient release of calcium from intracellular stores, as has been shown to be the case for porcine EC (Pearson *et al.*, 1983).

The rapid decrease in PGI_2 release following stimulation of the EC by agonists other than AA is unlikely to be due to diminished activity of cyclo-oxygenase, for infusions of exogenous AA produced a sustained release of PGI_2. However, it may possibly be explained by an increase in the reacylation of the released AA or the depletion of AA from the available pool of phospholipids. In contrast to PGI_2 the release of EDRF does not appear to be suppressed by the effective removal of L-arginine, the putative precursor. Indeed, as we show here, EC release EDRF for a prolonged period of time in the absence of extracellular L-arginine. This is explained by a high intracellular level of L-arginine (100 μM; Mitchell *et al.*, 1990a), some thirty times higher than the apparent K_m for purified endothelial NO synthase (Pollock *et al.*, 1991) and by the ability of EC to maintain intracellular arginine, despite continuous EDRF release, by generation from intracellular stores (Mitchell *et al.*, 1990b). Thus, it seems that the availability of substrate for EDRF and PGI_2 synthesis regulates the duration of release of these autocoids following stimulation of the endothelium.

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The actions of endothelins-1 and -3 on the vascular and capsular smooth muscle of the isolated blood perfused spleen of the dog

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1 Endothelin-1 (ET-1), endothelin-3 (ET-3) and noradrenaline (NA) were administered as intra-arterial bolus injections into the isolated, blood-perfused spleen of the dog to assess agonist properties and relative molar potencies on the vascular and capsular smooth muscle.

2 An initial small vasodilatation was observed occasionally at low doses (1.0–10 pmol) of ET-1.

3 ET-1, ET-3 and NA all caused graded increases in splenic arterial vascular resistance. The molar ED_{50} for the splenic vasoconstrictor response to ET-1 was significantly less ($P < 0.001$) than that to ET-3; both peptides were significantly more potent as vasoconstrictor agents than NA. The maximum increase in splenic arterial vascular resistance was not significantly different for either ET-1, ET-3 or NA.

4 The time course of the splenic vasoconstrictor response to ET-1 was significantly ($P < 0.01$) longer than that to equieffective doses of ET-3 or NA.

5 The splenic vasoconstrictor responses to ET-1 and ET-3 were accompanied by reductions in spleen volume. The rank order of molar potency in causing splenic capsular contraction was ET-1 > ET-3 > NA. The maximum reduction in splenic volume was significantly greater for NA than for either ET-1 or ET-3. The two peptides (ET-1, ET-3) were equiefficacious in contracting splenic capsular smooth muscle.

6 The high molar potency of ET-1 as a splenic arterial vasoconstrictor, over 1,700 times more potent than NA, suggests that it may play an important local role in the control of splenic haemodynamics.

Keywords: Endothelin-1 (ET-1); endothelin-3 (ET-3); splenic contraction; splenic vasoconstriction; splenic vasodilatation; spleen and ET-1; spleen and ET-3

Introduction

Endothelins-1 and -3 (ET-1, ET-3) are structurally related 21 amino acid residue peptides originally detected in the supernatant of cultured endothelial cells of porcine and rat origin respectively (Yanagisawa *et al.*, 1988a,b). Endothelial cells of all species studied can express ET-1 whereas ET-3 is associated with nervous tissue (Inoue *et al.*, 1989). The natural stimuli for their synthesis and release have not been elucidated.

Both ET-1 and ET-3 have potent and long lasting vasoconstrictor actions in many vascular beds (Walder *et al.*, 1989; Minkes & Kadowitz, 1989). In most vascular systems, ET-1 is more potent than ET-3. Contractile actions on extravascular smooth muscle have also been extensively documented (Eglen *et al.*, 1989).

Autoradiographical localization of [^{125}I]-ET-1 has revealed binding sites on intrasplenic vessels, especially the arterioles of the white pulp (Power *et al.*, 1989) and also on the trabecular network of extravascular (capsular) smooth muscle (Koseki *et al.*, 1989). Thus, ET-1 may contribute to the local control of both the conductive and capacitative aspects of splenic function. The splenic distribution of any ET-3 binding sites has not been established.

The present study assessed the actions of ET-1 and ET-3 on the smooth muscle of the splenic arterial system and the spleen capsule and compared their molar potencies at these sites with those of the principal transmitter, noradrenaline (NA). The results allowed a comparison to be made with the splenic actions of neuropeptide Y previously described in similar preparations (Corder *et al.*, 1987).

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Methods

The experiments were carried out on 6 dogs (mean weight 29.7 ± 0.8 kg; range 26.0–31.0 kg) anaesthetized with an intravenous injection of a mixture of chloralose and urethane (50 and 500 mg kg^{-1}, respectively) after induction with methohexitone sodium (6 mg kg^{-1}). The trachea was cannulated although respiration was always spontaneous. Systemic blood pressure was recorded from the cannulated left carotid artery with mean heart rate being derived electronically from this signal.

The procedures for isolation and perfusion of the spleen and recording of splenic arterial blood flow and changes in spleen volume were described previously (Corder *et al.*, 1987). Essentially, after vascular and nervous isolation from the donor, the spleen was placed in a perspex plethysmograph, covered with liquid paraffin and maintained at 37°C with an overhead heating lamp. The splenic arterial system was perfused with arterial blood derived from a cannulated femoral artery. At some stage during 2 experiments, the spleen was perfused with arterial blood at constant flow using a Watson Marlow Pump (Type MHRE) in order to dissociate the capsular volume changes from any concomitant changes in arterial blood flow. Splenic venous blood drained into the femoral vein. An electromagnetic flow probe and strain gauge transducer were incorporated into the splenic arterial circuit to measure splenic arterial mean blood flow (SABF) and splenic arterial mean perfusion pressure (SAPP) from which splenic arterial vascular resistance (SAVR) was calculated. The plethysmograph was filled with liquid paraffin and connected to a continuously weighed reservoir. Displacement of liquid paraffin to and from the reservoir, therefore, allowed a continuous monitoring of changes in spleen volume. A 'T' piece in the arterial inflow circuit permitted the close arterial administration of vasoactive substances without eliciting systemic effects and, therefore, changes in perfusion pressure.

Hourly arterial blood samples allowed the monitoring of arterial P_{CO_2}, P_{O_2} and pH and when necessary, correction to normal values was made by i.v. infusion of $NaHCO_3$. The flow probe was calibrated at the end of each experiment with whole blood at which stage the spleen was also weighed after clamping the artery and vein.

Drugs

ET-1, ET-3 or NA were administered as bolus injections directly into the splenic arterial perfusion line through the arterial 'T' piece and washed in with saline (0.9% w/v NaCl solution) to give a total volume of injection of 2.0 ml. ET-1 and ET-3 were obtained from The Peptide Institute, Osaka, Japan, and dilution was made with saline immediately before injection. Noradrenaline acid tartrate (Levophed; Winthrop) was diluted immediately before use with normal saline and the containers kept in ice.

Statistics

Results are expressed as means ± standard error of the mean (s.e.mean). Unless otherwise stated, tests for significance refer to Student's unpaired *t* test.

Results

Control values

The mean spleen weight was 493 ± 79 g representing 1.73 ± 0.3% of body weight. The mean splenic arterial blood flow was 118 ± 16.7 ml min^{-1} or 28.0 ± 6.0 ml min^{-1} 100 g^{-1}. Since the mean splenic arterial perfusion pressure was 134 ± 7.4 mmHg then the mean calculated splenic arterial vascular resistance was 1.27 ± 0.20 mmHg ml^{-1} min or 6.73 ± 1.94 mmHg ml^{-1} min 100 g. These values agree with those reported recently from this laboratory with similar preparations (Corder *et al.*, 1987; Corder & Withrington, 1988; Withrington, 1989).

Splenic smooth muscle responses to close arterial injections

Noradrenaline NA was injected on 31 occasions in 6 spleen perfusions in doses ranging from 5 to 300 nmol. Once the threshold dose was reached there was, usually, both a splenic capsular and vascular response. The splenic arterial vascular response was biphasic (Figure 1); an initial rapid reduction in flow being followed by a more prolonged increase in flow. These changes in flow reflect an increase and then a decrease in splenic arterial vascular resistance, respectively. The adrenoceptors responsible for this pattern of splenic arterial vasoconstriction and vasodilatation have been analysed previously (Davies *et al.*, 1969) and represent the successive activation of, and overlapping responses to, initially α-adrenoceptors followed by β-adrenoceptors. These splenic vascular responses were accompanied by a reduction in spleen volume which was rapid in onset but prolonged in duration well beyond the time-course of the vascular changes. This capsular contraction is due to α-adrenoceptor activation.

The maximum vascular response to NA in each experiment led to arrest of splenic arterial blood flow; the mean maximum reduction in spleen volume was 148.5 ± 14.5 ml ($n = 6$). The mean molar ED_{50}, i.e., the mean molar dose that causes 50% of the maximum response to NA in each experiment, was 59.6 ± 12.4 nmol for the vasoconstrictor response and 20.5 ± 3.4 nmol for the capsular contraction. These ED_{50} values are significantly different ($P < 0.01$) and confirm previous observations in similar preparations (Davies *et al.*, 1973) of differences in the sensitivities of the vascular and extravascular (capsular) smooth muscle, to the principal transmitter. The nearest experimental point to the ED_{50} for NA was 60 nmol; the calculated mean time to half recovery for this vasoconstrictor response was 0.80 ± 0.14 min. The mean time to half recovery for the capsular contraction was 3.46 ± 0.39 min.

Endothelin-1 In 5 of the perfused spleen preparations ET-1 was administered over the dose range of 1.0–200 pmol. The splenic vascular response was, in 32 out of 39 administrations, monophasic consisting of a reduction in blood flow of slow onset but very prolonged duration (Figure 1). At constant perfusion pressure, this fall in splenic arterial blood flow represents splenic arterial vasoconstriction. To 7 of the lower bolus doses of ET-1 (1.0–10 pmol) a small initial increase in blood flow was observed (mean increase in flow = 19.1 ± 3.5% of control flow). In any individual experiment (Figure 2), the molar dose-response curve relating the splenic vasoconstrictor responses showed ET-1 to be more potent than NA. The maximum effect of ET-1 was, like that of NA, to cause cessation of splenic arterial blood flow.

The mean ED_{50} for the splenic vasoconstrictor response to ET-1 was 52.2 ± 19.3 pmol; a value significantly less ($P < 0.001$) than the mean ED_{50} for NA (59.6 ± 12.4 nmol) in the same preparations. The mean molar potency ratio $(ED_{50})_{NA}/(ED_{50})_{ET-1}$ was 1,727. The nearest experimentally determined point to the ED_{50} for ET-1 was 50 pmol and the

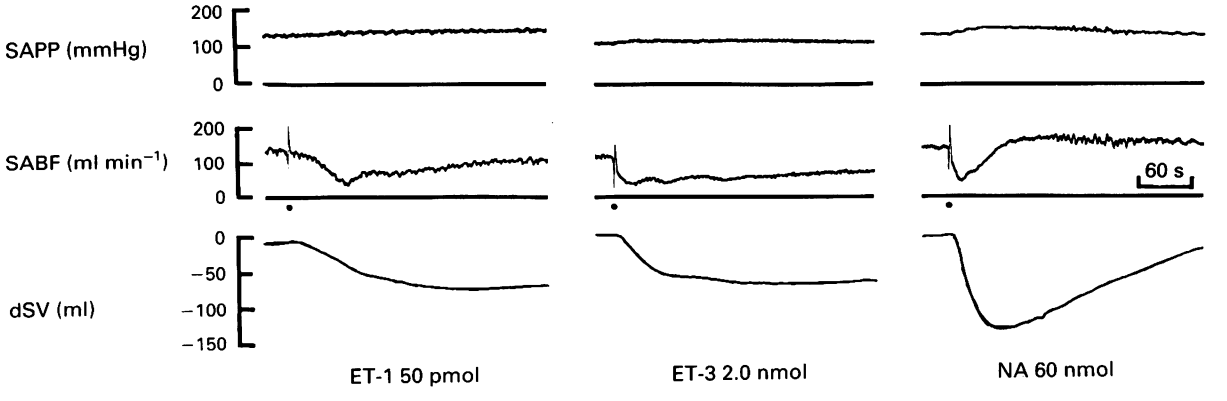

Figure 1 Isolated blood-perfused spleen (520 g) of the dog. Experimental records illustrating the changes (reductions) in splenic arterial blood flow (SABF) and spleen volume (dSV) in response to close arterial bolus injections of endothelin-1 (ET-1, 50 pmol); endothelin-3 (ET-3, 2.0 nmol) and noradrenaline (NA, 60 nmol). Records are (from the top) SAPP, splenic arterial mean perfusion pressure; SABF, splenic arterial mean blood flow; dSV, change in spleen volume. The change in splenic arterial blood flow to NA is biphasic but solely vasoconstrictor to both ET-1 and ET-3. The capsular and vascular responses to ET-1 and ET-3 are more prolonged that those to NA.

mean time to half recovery from the point of injection was 3.40 ± 0.53 min; a significantly longer interval ($P < 0.001$) than for the equieffective dose of NA (60 nmol).

Each vasoconstrictor response to ET-1 was accompanied by a reduction in spleen volume (Figure 1), the time course of which was prolonged both in onset and duration; the time to half recovery for 50 pmol ET-1 was 6.94 ± 1.04 min. These reductions in spleen volume to selected doses of ET-1 (20, 50, 100 pmol) were not significantly different ($P > 0.05$, paired t test; $n = 6$) when the spleen was perfused at either constant pressure or (see Methods) constant flow. They were, therefore, active capsular contractions and not passive reductions in volume arising from the falls in splenic arterial inflow. The mean molar ED_{50} for the capsular response was 39.5 ± 7.6 pmol; significantly less than the molar ED_{50} for NA ($P < 0.001$). However, in individual experiments the maximum reduction in spleen volume to ET-1 was always less than that to NA in the same experiment (Figure 2). The mean maximum reduction in spleen volume to ET-1 was 66.9 ± 14.9 ml; a value significantly less ($P < 0.01$) than the mean maximum reduction to NA (148.5 ml). The mean maximum reduction in volume to ET-1 was 55.3 ± 10.1% of the mean maximum to NA in the same experiments.

Endothelin-3 In 3 preparations, ET-3 was administered over the dose range 10 pmol to 2.0 nmol to construct 5 complete dose-response curves. The splenic arterial vascular response was, in all preparations and to all injections, monophasic. It consisted of a reduction in splenic arterial blood flow (Figure 1) of prolonged duration. No initial vasodilator response was observed to ET-3 similar to that seen in some preparations, at low doses of ET-1. In individual experiments (Figure 3) it was apparent that, in terms of molar potency, ET-3 was more effective on the splenic vascular and capsular smooth muscle than NA. However, whilst the maximum vascular responses to NA and ET-3 were the same, resulting in arrest of the splenic arterial inflow the maximum reduction in volume was much greater to NA than to ET-3. In one experiment, where complete dose-response curves to all three vasoactive substances

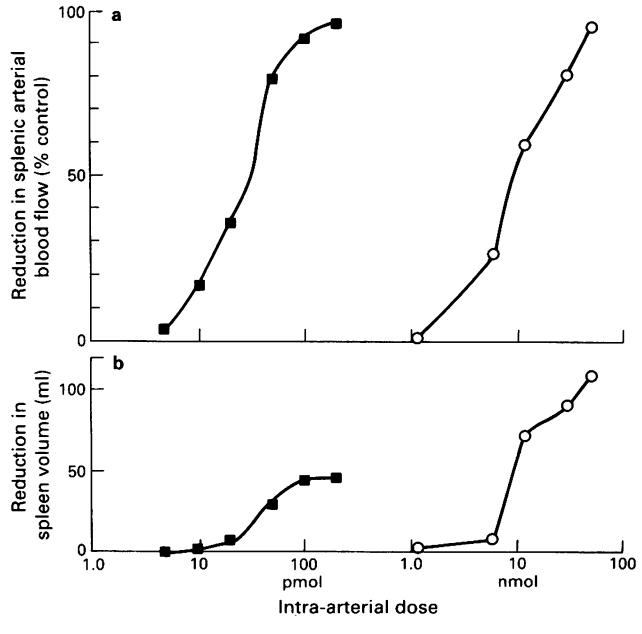

Figure 2 Relationship between the reduction in splenic arterial blood flow (splenic arterial vasoconstriction, a), reduction in spleen volume (b) and the intra-arterial bolus molar dose of endothelin-1 (ET-1, ■) and noradrenaline (NA, ○). All the points were obtained in a single experiment (spleen weight 374 g). The maximum splenic arterial vasoconstrictor response to ET-1 and NA are the same whilst the maximum capsular effect (reduction in spleen volume) is much greater to NA.

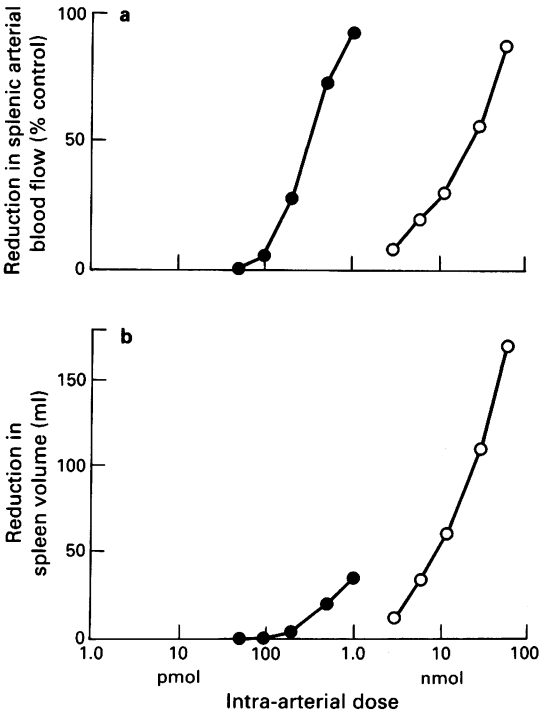

Figure 3 The relationship between the reduction in splenic arterial blood flow (splenic arterial vasoconstriction, a), reduction in spleen volume (b) and the intra-arterial bolus dose of endothelin-3 (ET-3, ●) and noradrenaline (NA, ○). Whilst the maximum vasoconstrictor effect to ET-3 and NA are the same the capsular response to NA is greater. All the points obtained in the same experiment (spleen weight 820 g).

(NA, ET-1, ET-3) were tested, then ET-3 occupied an intermediate position, in terms of molar potency, between ET-1 and NA for both the capsular and vascular responses.

The mean molar ED_{50} for the splenic vasoconstrictor response to ET-3 was 893 ± 157 pmol; a value significantly greater than for ET-1 (52.2 pmol, $P < 0.001$) but significantly less than that for NA (59.6 nmol; $P < 0.001$). The closest experimental point to the ED_{50} for ET-3 is 1.0 nmol and the mean time from injection point to half recovery for the vasoconstrictor response was 1.08 ± 0.15 min. This value is not significantly different from the half recovery time to NA ($P > 0.10$) but significantly shorter than that for ET-1 ($P < 0.001$). The mean time to half recovery for the capsular response to 1.0 nmol ET-3 was 2.72 ± 0.35 min. The mean splenic arterial vascular changes for ET-1, ET-3 and NA are illustrated in Figure 4.

Accompanying all the vascular responses to ET-3 were reduction in spleen volume. The mean molar ED_{50} for the capsular contraction to ET-3 was 530 ± 190 pmol; a value significantly greater than the molar ED_{50} to ET-1 (39.5 ± 7.6 pmol; $P < 0.05$) but significantly less than the molar ED_{50} for NA (20.5 ± 3.4 nmol; $P < 0.002$). However, it was evident from within experiment comparisons, that the absolute reductions in spleen volume to ET-3 were much less than those associated with splenic arterial vasoconstrictor responses to NA of a comparable magnitude. The mean maximum reduction in spleen volume to ET-3 was 39.5 ± 10.5 ml; not significantly different from the mean maximum to ET-1 but significantly less than that to NA ($P < 0.001$). The mean results (Figure 4) relating the splenic arterial vasoconstrictor response (increase in splenic arterial vascular resistance) and the concomitant reduction in spleen volume clearly indicate the relative molar potencies of NA, ET-1 and ET-3 on splenic vascular and capsular smooth muscle. They illustrate their intrinsic differences since doses of NA, equieffective in inducing the same maximum changes as

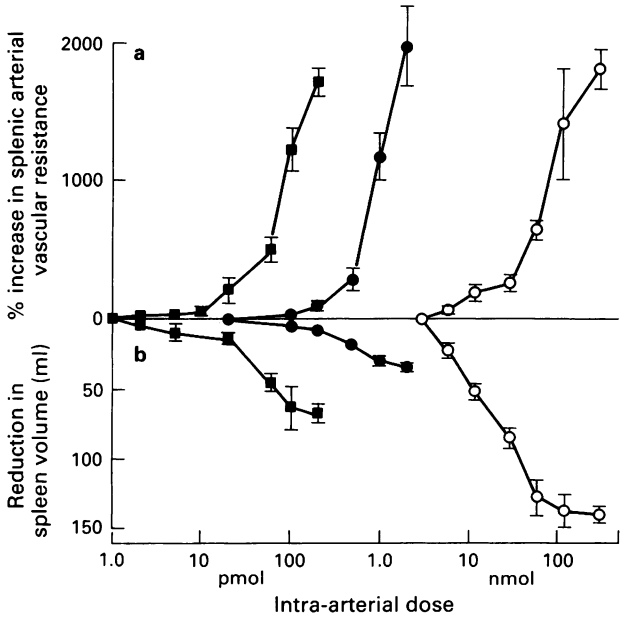

Figure 4 The mean results obtained in 6 spleen perfusion preparations illustrating the splenic vasoconstrictor responses (a, increase in splenic vascular resistance), and splenic capsular responses (b, reduction in spleen volume) in response to close-arterial bolus injections of endothelin-1 (ET-1, ■), endothelin-3 (ET-3, ●) and noradrenaline (NA, ○). The points represent the means and the vertical lines the standard errors of between 4 and 6 observations. It is apparent that the order of molar potency is ET-1 > ET-3 > NA on both splenic vascular and capsular smooth muscle. However whilst the maximum splenic vasoconstrictor effects to ET-1, ET-3 and NA are the same the maximum reductions in spleen volume are significantly less to either ET-1 or ET-3 compared with NA.

ET-1 or ET-3 in splenic arterial blood flow, are associated with much greater concomitant reductions in spleen volume.

Discussion

The earliest pharmacological property of ET-1 to be evaluated was the biphasic change in arterial blood pressure evoked by intravenous administration. This response consisted of a marked hypertensive phase of prolonged duration preceded by a brief hypotensive episode (Yanagisawa *et al.*, 1988a). The prolonged vasoconstrictor activity of ET-1 has been confirmed subsequently in many species and different vascular preparations. However, in some peripheral vascular territories such as the carotid and hindquarters of conscious Wistar and Long Evans rats (Gardiner *et al.*, 1989; 1990), and the isolated perfused mesentery of the rat (Warner *et al.*, 1989) the vasodilator component to ET-1 appeared to be the major phase. In the dog, the hepatic arterial vascular response to intra-arterial ET-1 was biphasic (Withrington *et al.*, 1989a) consisting of both a substantial initial vasodilatation succeeded by a prolonged vasoconstriction. In contrast, in the present experiments on the isolated blood perfused spleen of the dog, an initial vasodilator response to ET-1 was observed in only a few preparations and at just above threshold doses. The most evident splenic vascular response to ET-1 was a dose-related arterial vasoconstriction the duration of which was significantly longer than the response to equieffective doses of NA. However, it was apparent that the canine splenic arterial vasoconstrictor response was not so prolonged as found previously in the canine hepatic arterial circuit (see Figure 1, Withrington *et al.*, 1989a). Clearly there are substantial differences in the form of vascular responses to intra-arterial bolus doses of ET-1 between different vascular beds even in the same species, as previously demonstrated in the rat by Gardiner *et al.* (1990).

In the canine hepatic arterial vascular bed neither the initial vasodilator nor subsequent vasoconstrictor phases were significantly modified by the prior administration of indomethacin (Withrington *et al.*, 1989b). The release of vasoactive prostanoids by ET-1, demonstrated by de Nucci *et al.* (1988) and Rae *et al.* (1989) is unlikely, therefore, to contribute in a major way to the hepatic arterial vascular responses to ET-1. The release of endothelial derived relaxing factor (EDRF), now putatively identified as nitric oxide (NO), in response to ET-1 administration (de Nucci *et al.*, 1988; Warner *et al.*, 1989) presents an alternative origin for the initial vasodilator phase. Indeed, in the rat the vasodepressor responses to ET-1 were significantly inhibited (Whittle *et al.*, 1989) by N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of NO synthesis by endothelial cells. However, in the hindquarters of the conscious rat Gardiner *et al.* (1989) were unable to block the vasodilator phase to ET-1 by the prior administration of L-NMMA. This suggests that the cause of the vasodilator phase of ET-1 varies in different vascular territories and may be related to a different endothelin receptor spectrum between endothelial layers.

In the present experiments on splenic smooth muscle the molar potency of ET-3 was intermediate to that of ET-1 and NA in causing arterial vasoconstriction and capsular contraction. These effects of ET-3 may be through a partial agonist action on ET-1 receptors. Sakurai *et al.* (1990) have proposed, on the basis of receptor cloning and binding characterization, the existence of two major endothelin receptor types: a non-selective endothelin receptor (ET_B) that has equal affinity for ET-1, ET-2 and ET-3 whilst a more selective endothelin subtype (ET_A) is localized on vascular smooth muscle. It would appear that the splenic vasoconstrictor and capsular responses to both ET-1 and ET-3, described in the present paper, are probably the result of their differential activation of the ET_A receptor.

Binding sites for both ET-1 (Power *et al.*, 1989) and neuropeptide Y (NPY, Lundberg *et al.*, 1985) have been localized in vessels of the splenic pulp suggesting that both peptides may play a role in the regulation of the splenic microcirculation. On a molar basis ET-1 was over 1,700 times more potent than NA as a splenic arterial vasoconstrictor whilst previous results, in similar preparations (Corder *et al.*, 1987), showed that NPY was approximately 400 times more potent than NA. The present results therefore indicate that ET-1 is 4–5 times more potent, on a molar basis, as a splenic arterial vasoconstrictor than NPY, the co-transmitter in the sympathetic innervation (Lundberg *et al.*, 1989). In addition, the splenic vasoconstrictor response to ET-1 is significantly longer in duration of action than an equieffective dose of NA. A consequence inherent in the long time course of action of ET-1 is the cumulative effect which becomes apparent when ET-1 is continuously present at the receptor site during an infusion or during tonic local release.

In contrast to the splenic distribution of NPY, ET-1 binding sites have also been identified autoradiographically in the smooth muscle forming the capsule and trabecula network of the spleen (Koseki *et al.*, 1989); extravascular smooth muscle which plays an overwhelming role in the blood reservoir function of the spleen. In the present series, close arterial injection of ET-1 lead to reductions in spleen volume of prolonged duration. These capsular responses also occurred when the spleen was perfused at constant inflow and so were not simply passive consequences of the concomitant substantial and prolonged reduction in splenic arterial blood flow. On a molar basis ET-1 was more potent than NA in causing capsular contraction but the maximum reduction in spleen volume was significantly less than that to NA. These results for ET-1 contrast with those previously obtained for NPY since any capsular responses to that peptide were very small.

Within the spleen there are many peptides in addition to ET-1 and NPY with potent, yet differing, actions on vascular and extravascular smooth muscle. Calcitonin gene-related peptide (CGRP) and substance P (SP) are both co-located

within splenic sensory nerve terminals (Franco-Cereceda *et al.*, 1987): both peptides possess potent vasodilator properties (Lundberg *et al.*, 1985; Withrington, 1989). However, CGRP evokes an active capsular relaxation (Withrington, 1989) whilst SP causes a small capsular contraction (Lundberg *et al.*, 1985). Vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI) are 28-amino acid peptides co-synthesised and co-released from non-adrenergic splenic sympathetic nerves; they both cause splenic arterial vasodilatation but with little direct action on the capsular smooth muscle (Corder & Withrington, 1988). The situations in which these vasoactive peptides are released and the roles they play in different microcirculatory circumstances have yet to be elucidated.

The present study clearly demonstrates that ET-1 is the most potent splenic arterial vasoconstrictor yet examined. It has a prolonged action and in addition, may cause contraction of the splenic capsule. In view of the location of binding sites within the spleen and its intrinsic agonist potency on the vascular and capsular smooth muscle then ET-1 may have a functional role in the capacitative aspects of the splenic circulation in addition to a dominant role in modulating splenic arterial inflow resistance.

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Alterations in cellular cholesterol metabolism following administration of 6-hydroxydopamine to rabbits

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1 The role of adrenergic mechanisms in the regulation of cholesterol metabolism was investigated by studying the effects of 6-hydroxydopamine (6-OHDA) on serum cholesterol levels and on the activities of 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase, acyl coenzyme A : cholesterol-O-acyltransferase (ACAT) in the livers and intestines, and cholesterol 7α-hydroxylase in the livers of male New Zealand White rabbits.

2 Total serum cholesterol levels were significantly reduced ($P < 0.01$) in 6-OHDA-treated animals. This was reflected in the very low density lipoprotein, low density lipoprotein and high density lipoprotein fractions. The reduction in lipoprotein cholesterol levels reflected reduced cholesterol proportions in the lipoprotein fractions.

3 The 6-OHDA-treated animals also had significantly lower activities of intestinal ($P < 0.001$) and hepatic ($P < 0.01$) HMGCoA reductase. The specific activities of intestinal ACAT, hepatic ACAT and cholesterol 7α-hydroxylase were comparable in both groups.

4 In contrast to the results observed *in vivo*, 6-OHDA did not have any *in vitro* effect on cholesterol biosynthesis in cultured human leucocytes.

5 This latter finding suggests that the effects of 6-OHDA on cellular cholesterol biosynthesis *in vivo* are indirect, possibly resulting from the known toxic effect of this drug in sympathetic nerve terminals, and imply a potential role for the sympathetic nervous system in the regulation of cellular cholesterol biosynthesis *in vivo*.

Keywords: 6-Hydroxydopamine; lipoproteins; cholesterol metabolism

Introduction

The effects of adrenergic mechanisms on triacylglycerol metabolism have been well documented (Fain & Garcia-Sainz, 1983; Smith, 1983) but much less information is available on their role in the regulation of cholesterol metabolism. Studies in animals (Dury, 1957; Shafrir *et al.*, 1960; Barrett, 1966; Kunnihara & Oshima, 1983) have described increases in serum cholesterol following the administration of adrenaline, while an increased very low density lipoprotein (VLDL) cholesterol concentration has been observed following the administration of noradrenaline (O'Donnell *et al.*, 1988). *In vivo* (George & Ramasarma, 1977; Devery *et al.*, 1986) and *in vitro* (Edwards, 1975; Edwards *et al.*, 1979; Devery *et al.*, 1986) studies have also demonstrated an increased activity of hepatic-3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase (E.C.1.1.1.34), the rate-limiting enzyme of cholesterol synthesis, in response to noradrenaline and adrenaline. The large pharmacological doses of catecholamines used in these experiments contrast, however, with the low physiological levels present in serum. Consequently, the precise role of these hormones in the regulation of cholesterol metabolism merits further investigation.

This study evaluates the effects of adrenergic mechanisms on lipoprotein metabolism by studying the effects of 6-hydroxydopamine (6-OHDA), an analogue of noradrenaline, on cholesterol in rabbits. It has been demonstrated that 6-OHDA causes widespread destruction of the terminal endings of sympathetic neurones with a resultant decrease in the noradrenaline content of all sympathetically innervated end organs (Thoenen & Tranzer, 1968). The role of the sympathetic nervous system in the regulation of cholesterol metabolism is further studied by examining in rabbits the effects of 6-hydroxydopamine administration on key enzymes regulating cholesterol metabolism. These key enzymes include HMGCoA reductase, acyl coenzyme A : cholesterol-O-acyltransferase (ACAT) (E.C.2.3.1.26), the enzyme regulating cholesterol esterification and cholesterol 7α-hydroxylase (E.C.1.14.13.7), the key enzyme involved in the catabolism of cholesterol to bile acids.

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Methods

Animals

Male New Zealand White rabbits weighing 3–4 kg received purina rabbit chow *ad libitum* for two weeks before the start of the study. 6-Hydroxydopamine in 0.15 M saline was then injected intraperitoneally into 6 animals at a single dosage of 10 mg kg^{-1} body weight. The route of administration and the dose chosen were effective as judged by the observation of a marked sympathomimetic response in the 6-OHDA-treated animals within 5 min of injection; 6 control animals were injected intraperitoneally with 0.15 M saline alone. Throughout the study period the animals were housed in individual metabolic cages and food intake was monitored. No significant difference in food intake (g/day) was observed between the two groups. The mean weights of the control and 6-OHDA-treated rabbits at the time they were killed were comparable (3.32 ± 0.09 kg c.v. 3.68 ± 0.13 kg respectively). Blood was drawn from both rabbit groups for lipoprotein analysis after a 16 h overnight fast, on the 19th day after the administration of 6-OHDA. The animals were killed on day 21, and the livers and intestines were removed.

Experimental

The livers were chilled, minced and then homogenized in 4 vol. ice-cold 0.1 M potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 30 mM nicotinamide. The homogenate was centrifuged at 800 *g* for 10 min and the resulting supernatant was further centrifuged at 15,000 *g* for 20 min. This supernatant was then centrifuged at 104,000 *g* for 60 min

Table 1 Effect of 6-hydroxydopamine (6-OHDA) administration on serum lipoprotein levels in rabbits

	Untreated animals ($n = 6$)	*6-OHDA-treated animals* ($n = 6$)
Cholesterol (mmol l^{-1})		
Total	0.97 ± 0.17	0.54 ± 0.04*
VLDL	0.10 ± 0.01	0.02 ± 0.002*
LDL	0.29 ± 0.09	0.09 ± 0.01*
HDL_2	0.19 ± 0.05	0.14 ± 0.02
HDL_3	0.36 ± 0.03	0.27 ± 0.02*
Triglyceride (mmol l^{-1})		
Total	0.44 ± 0.06	0.41 ± 0.05
VLDL	0.14 ± 0.04	0.12 ± 0.02
LDL	0.07 ± 0.004	0.06 ± 0.01
HDL_2	0.04 ± 0.004	0.04 ± 0.01
HDL_3	0.16 ± 0.01	0.14 ± 0.02

Results are expressed as means ± s.e.mean.
VLDL: very low density lipoprotein; LDL: low density lipoprotein; HDL_2 and HDL_3: high density lipoprotein—see text for details.
* $P < 0.01$ different from untreated animals.

to obtain the microsomal pellet. The pellet was then suspended in 0.25 vol. 5 mM imidazole/HCl buffer, pH 7.4 containing 0.15 mM NaCl and 5 mM dithiothreitol (DTT).

Mucosal cell fractions were obtained from rabbit intestine by differential scraping of the mucosa as described by Dietschy & Siperstein (1965). Cells were homogenized in 0.1 M potassium phosphate buffer, pH 7.4, containing 1 M EDTA and 30 mM nicotinamide. Due to the relative difficulty in subfractionating intestinal cells compared to liver cells, the activities of HMGCoA reductase and ACAT were measured in the whole homogenate of intestinal cells. The protein content of the liver and intestinal cell preparations was determined by the method of Lowry *et al.* (1951).

Enzyme assays

HMGCoA reductase was determined as described by Mitropoulos & Balasubramaniam (1976) in liver microsomal and intestinal whole homogenate fractions of the rabbit. The incubation mixture contained 0.1 M potassium phosphate buffer pH 7.4, 2.5 mM NADP, 25 mM glucose 6-phosphate, 50 mM glutathione, 30 mM nicotinamide, 5 mM $MgCl_2$ and 5 units glucose 6-phosphate dehydrogenase in a total volume of 0.39 ml to which 0.05 ml of the various enzyme preparations were added. Following a preincubation of 10 min at 37°C, the reaction was initiated by the addition of 90 μM DL-hydroxymethyl [3-^{14}C]-glutaryl CoA (specific activity 6 Ci mol^{-1}). After incubation for various periods of time (30 min for liver microsomes and 60 min for intestinal cell homogenates, the reaction was terminated by the addition of 0.1 ml 12 N H_2SO_4, 0.5 ml mevalonolactone (10 mg ml^{-1}) and 10 μl DL-[2-^{3}H]-mevalonic acid (50,000 d.p.m./assay) was added as internal standard. Extraction, separation and quantitation of the product was as described previously (Mitropoulos & Balasubramaniam, 1976).

ACAT activity was determined as outlined by Balasubramaniam *et al.* (1978) in hepatic microsomes and intestinal cell whole homogenates. The incubation mixture contained 0.1 M potassium phosphate buffer, pH 7.4, 2 mM ATP, 4 mM $MgCl_2$, 0.2 mM coenzyme A and human albumin (1.2 mg/assay) in a total volume of 0.16 ml to which 0.02 ml of respective enzyme preparations were added. After a preincubation period of 5 min at 37°C, the reaction was started by the addition of 0.01 mM (1-^{14}C]-oleic acid. After 6 min, the reaction was stopped by the addition of two successive 2.0 ml aliquots of chloroform:methanol (2:1 v/v) and 0.8 ml water. [^{3}H]-cholesteryl-oleate 10 μl (20–30,000 d.p.m./assay) was added as internal standard. Quantitation of the product was as described previously (Balasubramaniam *et al.*, 1978).

The activity of cholesterol 7α-hydroxylase was measured in acetone-treated powder preparations of rabbit liver microsomal fractions as described by Shefer *et al.* (1981). The incubation mixture contained 0.1 M potassium phosphate buffer, pH 7.4, 10 mM NADPH and 5 mM $MgCl_2$, in a total volume of 0.5 ml to which 0.05 ml of the respective enzyme preparations were added. After a preincubation time of 5 min, 1.0 mM [4-^{14}C]-cholesterol (1.3 nCi $nmol^{-1}$) was added. The reaction was terminated after 20 min by the addition of 14 vol dichloromethane:ethanol (5:1 v/v).

Plasma lipoproteins were separated by a one step density gradient ultracentrifugation technique (Demacker *et al.*, 1983). The very low density lipoprotein (VLDL), low density lipoprotine (LDL), high density lipoprotein (HDL_2 and HDL_3) fractions correspond to the density intervals < 1.006 g ml^{-1}, 1.006–1.063 g ml^{-1}, 1.063–1.100 g ml^{-1} and 1.100–1.210 g ml^{-1} respectively. Total plasma cholesterol and the cholesterol concentrations in each lipoprotein fraction were assayed by an enzymatic colorimetric technique with Boehringer Mannheim GmbH diagnostic kits (Roschlau *et al.*, 1974) and triglycerides were measured with Biomerieux (Charbonnieres les Bains, France) kits (Takayama *et al.*, 1977).

Lymphocyte-enriched peripheral blood mononuclear cells were isolated from healthy laboratory volunteers' blood by centrifugation on Ficoll/Hypaque gradients as described by Mistry *et al.* (1981). *De novo* cholesterol synthesis was estimated by measurement of [^{14}C]-acetate incorporation into cholesterol in these cells after exposure to the mitogen, phytohaemagglutinin (PHA), as described by Owens *et al.* (1990). The effects of 6-OHDA on this process were assessed by culturing the cells in the presence of the drug over the 5 h exposure to [^{14}C]-acetate. The drug, dissolved in phosphate-buffered saline, was added (10 μl) to the cultures at two final concentrations, 4×10^{-5} M and 4×10^{-4} M. Phosphate-buffered saline (10 μl) alone was added to control cultures. Assays were performed in triplicate and results are expressed as nmol acetate incorporated into cholesterol per milligram cell protein.

Materials

3-Hydroxy-3-methyl [3-^{14}C]-glutaryl CoA (56.6 mCi $mmol^{-1}$), DL-[2-^{3}H]-mevalonic acid lactone (629 mCi $mmol^{-1}$), [1-^{14}C]-oleic acid (57.3 mCi $mmol^{-1}$), [1α, 2α(n)^{3}H]-cholesterol (40 Ci $mmol^{-1}$) and [4-^{14}C]-cholesterol (50–60 mCi $mmol^{-1}$) were obtained from the Radiochemical Centre (Amersham, U.K.). β-Nicotinamide adenine dinucleotide phosphate (NADP), β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), glucose 6-phosphate, glucose 6-phosphate dehydrogenase, dithiothreitol (DTT), glutathione, human serum albumin (fatty acid-free), oleoyl chloride, nicotinamide, mevalonic acid lactone, coenzyme A, cholesterol and 6-hydroxydopamine (6-OHDA) hydrochloride were all obtained from Sigma-London Chemical Co. Ltd. (London, U.K.). Phytohaemagglutinin (PHA) was obtained from Wellcome Diagnostics (Oxford, U.K.). Kieselgel 60 was obtained from Merck (Darmstadt, Germany).

Statistics

Statistical analysis of changes in the lipoprotein concentrations between the two groups of animals was performed using the Wilcoxon test (two-tailed) as these data were not normally distributed. The Student's *t* test was used to analyse differences in the activities of HMGCoA reductase, ACAT and cholesterol 7α-hydroxylase between the 6-OHDA-treated and untreated animals.

Results

Table 1 outlines the effect of 6-OHDA administration on serum triglyceride and cholesterol levels and their distribution throughout the lipoprotein fractions in the animals. The treated animals had a significantly lower total cholesterol

Table 2 Elemental composition of the lipoprotein fractions of the 6-hydroxydopamine (6-OHDA)-treated and control animals

	Control animals ($n = 6$)	*6-OHDA-treated animals* ($n = 6$)
VLDL (%)		
Cholesterol	16.1 ± 3.3	6.1 ± 0.6*
Triglyceride	40.4 ± 4.4	56.0 ± 5.0
Phospholipid	18.0 ± 1.6	11.8 ± 1.2
Protein	25·6 ± 1.6	26.2 ± 5.1
LDL (%)		
Cholesterol	21.2 ± 3.3	14.2 ± 1.4
Triglyceride	13.5 ± 1.7	14.0 ± 2.4
Phospholipid	26.2 ± 1.2	25.1 ± 1.5
Protein	39.1 ± 3.2	46.8 ± 1.6
HDL_2 (%)		
Cholesterol	15.0 ± 1.5	11.8 ± 0.5
Triglyceride	7.7 ± 1.1	8.0 ± 0.9
Phospholipid	34.4 ± 3.0	34.1 ± 1.2
Protein	42.9 ± 2.1	46.1 ± 1.3
HDL_3 (%)		
Cholesterol	8.7 ± 0.7	8.0 ± 0.3
Triglyceride	8.6 ± 0.9	9.3 ± 1.0
Phospholipid	18.4 ± 3.9	21.8 ± 0.5
Protein	64.4 ± 3.3	60.9 ± 1.2

Results expressed as means ± s.e.mean. For abbreviations, see Table 1.
* $P < 0.01$ different from control animals.

($P < 0.01$), VLDL cholesterol ($P < 0.01$), LDL cholesterol ($P < 0.01$) and HDL_3 cholesterol ($P < 0.01$). HDL_2 cholesterol levels were similar in both groups. No significant differences were observed in total and lipoprotein triglyceride levels between the two groups.

The elemental composition of the lipoprotein fractions of the treated and control rabbits is demonstrated in Table 2. A significant reduction in the proportions of cholesterol was observed in the VLDL particles of the treated group ($P < 0.01$). Although the percentage cholesterol in the LDL, HDL_2 and HDL_3 particles was also lower in the treated group, the differences were not significant.

The effect of 6-OHDA on the specific activities of the hepatic and intestinal cholesterol metabolising enzymes in the two animal groups is outlined in Table 3. The specific activities of HMGCoA reductase were significantly lower in the livers ($P < 0.01$) and intestines ($P < 0.001$) of the 6-OHDA-treated animals. Intestinal ACAT, hepatic ACAT and cholesterol 7α-hydroxylase activities were similar in both groups.

In an attempt to ascertain whether 6-OHDA might have a direct influence on cholesterol biosynthesis, the influence of 6-OHDA at two different concentrations on the incorporation of [^{14}C]-acetate into cholesterol by cultured human leucocytes is shown in Table 4. There was no significant difference between the amount of [^{14}C]-acetate incorporated by cells exposed to either drug concentration and that incorporated by control cells.

Table 3 The effect of 6-hydroxydopamine (6-OHDA) administration on cholesterol metabolizing enzymes in rabbit liver and intestine

	Control animals ($n = 6$)	*6-OHDA-treated animals* ($n = 6$)
HMGCoA reductase (nmol min^{-1} mg^{-1} protein)		
Hepatic	0.036 ± 0.005	0.016 ± 0.005*
Intestinal	0.024 ± 0.003	0.007 ± 0.002**
ACAT (nmol min^{-1} mg^{-1} protein)		
Hepatic	2.19 ± 0.19	2.03 ± 0.57
Intestinal	1.62 ± 0.26	1.95 ± 0.54
Cholesterol 7α-hydroxylase (pmol min^{-1} mg^{-1} protein)	14.29 ± 1.88	16.92 ± 1.2

Results expressed as mean ± s.e.mean.
HMGCoA reductase: 3-hydroxy-3-methylglutaryl coenzyme A, ACAT: cholesterol-O-acyltransferase.
* $P < 0.01$ different from control animals.
** $P < 0.001$ different from control animals.

Table 4 Effect of 6-hydroxydopamine (6-OHDA) on leucocyte cholesterol synthesis

	Control cells	*6-OHDA treated cells*	
6-OHDA concentration	0	4×10^{-5} M	4×10^{-4} M
[^{14}C]-acetate incorporated (nmol mg^{-1} protein)	70.7 ± 2.1	72.9 ± 2.6	68.4 ± 2.0

Results are expressed as mean ± s.e.mean.

Discussion

Given the potential of 6-OHDA administration to bring about a chemical sympathectomy in animals (Thoenen & Tranzer, 1968), it is likely that the 6-OHDA-treated rabbits in this study were noradrenaline-depleted and the metabolic effects observed may be a reflection of lower noradrenaline concentrations in these animals. The observation of a brisk sympathomimetic response (manifested by a profound tachycardia) following the administration of 6-OHDA suggests that the dose chosen was sufficient to cause destruction of sympathetic nerve endings (Stone *et al.*, 1964). Since the time interval necessary for the regeneration of sympathetic neurones following 6-OHDA is much longer than the duration of the present study (Goldman & Jacobowitz, 1971; Kostrzewa & Jacobowitz, 1974), it can be assumed that the 6-OHDA-treated rabbits were at least partially sympathectomized at the time of lipoprotein sampling and at the time they were killed. Thus, the alteration in HMGCoA reductase in the 6-OHDA-treated animals in this study may well be a result of the toxic effect of this drug on the sympathetic nerve fibre rather than due to a direct effect of the drug on hepatic and intestinal cholesterogenesis. This postulate is supported by the lack of any direct effect of 6-OHDA *per se* on cellular cholesterogenesis as measured by [^{14}C]-acetate uptake in cultured human leucocytes (Table 4). Mitogen-stimulated cultured human leucocytes have been used previously in this laboratory as a model system for studying cellular cholesterogenesis, using [^{14}C]-acetate as precursor (Owens *et al.*, 1990; 1991). The accessibility of these cells, their robustness relative to isolated hepatocytes and their capacity to amplify cholesterogenesis through mitogen stimulation makes them a suitable model system. The sensitivity of [^{14}C]-acetate uptake process to the specific inhibitor of HMGCoA reductase, mevinolin, validates the use of this procedure to assess indirectly the enzyme's activity in cells. Other workers (Cuthbert & Lipsky 1980: Cuthbert *et al.*, 1986) have used this tissue as a model for human cell sterol metabolism and for the assessment of LDL receptor status in familial hypercholesterolaemia. Given the marked *in vivo* effect of 6-OHDA administration on both hepatic and intestinal HMGCoA (Table 3), the rate-limiting enzyme in cholesterol biosynthesis from acetate in cells, a chemical sympathectomy-mediated depletion of noradrenaline would seem plausible.

The 6-OHDA-treated rabbits in this study had lower serum cholesterol levels compared with their untreated counterparts. These data are consistent with both the reduced cholesterol levels described in patients receiving the selective α-adrenoceptor antagonist, prazosin (Leren *et al.*, 1981; Rouffy & Jaillard, 1984) and the increased VLDL cholesterol levels observed in New Zealand White rabbits following the administration of noradrenaline (O'Donnell *et al.*, 1988). The results

suggest reduced availability of cholesterol for lipoprotein formation in these 6-OHDA-treated animals. The lipoprotein data confirm this, as the reduced lipoprotein cholesterol levels in the treated rabbits (Table 1) are reflected in reduced cholesterol proportions in virtually all the lipoprotein fractions (Table 2). A reduced availability of cholesterol within hepatocytes might reflect decreased cholesterol catabolism to bile acids. The unchanged activity of cholesterol 7α-hydroxylase in the 6-OHDA-treated animals suggests that cholesterol catabolism was not altered. However, the reduced activity of hepatic HMGCoA reductase in the 6-OHDA-treated animals implies suppressed endogenous cholesterogenesis in the liver, thereby reducing the amount of cholesterol available within the hepatocyte for VLDL formation. A similar reduction in intestinal HMGCoA reductase activity in the 6-OHDA-treated animals may also reduce the amount of cholesterol available for lipoprotein formation in this tissue.

The dramatic fall in hepatic and/or intestinal HMGCoA reductase in the 6-OHDA-treated animals raises the possibility that the effects of 6-OHDA might be mediated by a non-specific destruction of hepatocytes and/or intestinal cells. In such an eventuality, we would also have expected a dramatic reduction in hepatic ACAT, cholesterol 7α-hydroxylase and intestinal ACAT activities. However, the specific activities of these 3 enzymes were not altered in the 6-OHDA-treated animals. Furthermore, the doses of 6-OHDA used in these studies were modest and there is considerable evidence accumulated from many animal species to suggest that under such circumstances 6-OHDA destroys catecholaminergic neurones with a high degree of selectivity (Kostrzewa & Jacobowitz, 1974).

The altered hepatic HMGCoA reductase activity in the 6-OHDA-treated model is consistent with the *in vivo* (George & Ramasarma, 1977; Devery *et al.*, 1986) and *in vitro* (Edwards, 1975; Edwards *et al.*, 1979; Devery & Tomkin, 1986) evidence demonstrating enhanced HMGCoA reductase activity in response to noradrenaline. Since this influence of supraphysiological doses of noradrenaline on hepatic HMGCoA reductase activity in rat isolated hepatocytes is not mediated through the adenylate cyclase system (Edwards, 1975), it is unlikely to represent an α_2-adrenoceptor-mediated effect. It is possible that the increased HMGCoA reductase activity in response to noradrenaline is mediated through activation of α_1-adrenoceptors or it could represent a direct effect of noradrenaline on the enzyme (George & Ramasarma, 1977). In support of the former possibility, it has been shown that noradrenaline induces DNA synthesis in rat hepatocytes via α_1-adrenoceptor stimulation (Cruise *et al.*, 1985). The lack of effect of 6-OHDA administration on ACAT and cholesterol 7α-hydroxylase activity is also consistent with the lack of change in the activity of these enzymes following noradrenaline administration (Devery *et al.*, 1986). Thus, it appears that the sympathetic nervous system regulates cholesterol metabolism within cells by promoting cholesterol synthesis. By contrast, the increased serum cholesterol concentration and increased activity of hepatic HMGCoA reductase observed following vagotomy (Scott & Tomkin, 1985) suggest that the parasympathetic nervous system regulates intracellular cholesterol metabolism by suppressing cholesterol synthesis. However, in addition to regulating the activity of HMGCoA reductase, the parasympathetic nervous system also regulates the activity of hepatic ACAT and cholesterol 7α-hydroxylase (Scott & Tomkin, 1985), whereas the sympathetic nervous system does not appear to have any effect on these enzymes.

In this study, 6-OHDA did not have any effect on serum triglyceride levels. Previous studies in male New Zealand White rabbits have demonstrated that pharmacological doses of noradrenaline increased VLDL triglyceride concentrations (O'Donnell *et al.*, 1988), and there is experimental evidence (Taggart & Carruthers, 1971) suggesting that the rise in free fatty acid and triglyceride concentrations during periods of acute stress in motor racing drivers may be due to the extremely high levels of catecholamines in their serum (levels which are often supraphysiological). Despite the extensive evidence in the literature implicating the importance of adrenergic mechanisms in the regulation of triglyceride metabolism (Fain & Garcia Sainz, 1983), the data in the present study raise the possibility that adrenergic mechanisms do not have a significant influence on triglyceride metabolism under basal conditions and the effects only become manifest when the levels of catecholamines are very high. In support of this view, it has previously been demonstrated by use of graded infusions of noradrenaline, that plasma levels of greater than 1800 pg ml^{-1} (or nearly 10 times the basal level) are required to produce measurable metabolic changes (Silverberg *et al.*, 1978). By contrast, haemodynamic effects are seen with increments in plasma noradrenaline which are as low as 100 pg ml^{-1} (Cryer *et al.*, 1976). Thus, while noradrenaline can function as a neurotransmitter at relatively low mean concentrations (presumably due to high local synaptic levels), it only functions as a hormone in those rare instances where supraphysiological concentrations are attained (Cryer, 1980).

In summary, this study demonstrates reduced serum cholesterol levels in rabbits that were treated with 6-OHDA. This was associated with a reduction in cellular cholesterol biosynthesis. Similar effects on cholesterol synthesis were not observed *in vitro*, suggesting that the alteration in cholesterol metabolism *in vivo* is not a direct effect of this pharmacological agent. Instead, 6-OHDA may mediate its effect via an indirect mechanism, possibly through its well described effect on sympathetic nerve endings; such a hypothesis implicates the sympathetic nervous system in the regulation of cholesterol synthesis *in vivo*. The clinical significance, if any, of this latter observation remains to be determined.

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Agonist interactions with 5-HT_3 receptor recognition sites in the rat entorhinal cortex labelled by structurally diverse radioligands

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1 The pharmacological properties of 5-HT_3 receptor recognition sites labelled with [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330 in membranes prepared from the rat entorhinal cortex were investigated to assess the presence of cooperativity within the 5-HT_3 receptor complex.

2 In rat entorhinal cortex homogenates, [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330 labelled homogeneous densities of recognition sites (defined by granisetron, 10 μM) with high affinity ($B_{max} = 75 \pm 5$, 53 ± 5, 92 ± 6 and 79 ± 6 fmol mg^{-1} protein, respectively; p$Kd = 9.41 \pm 0.04$, 8.69 ± 0.14, 8.81 ± 0.06 and 10.14 ± 0.04 for [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330, respectively, $n = 3$–8).

3 Quipazine and granisetron competed for the binding of each of the radioligands in the rat entorhinal cortex preparation at low nanomolar concentrations (pIC_{50}; quipazine 9.38–8.51, granisetron 8.62–8.03), whilst the agonists, 5-hydroxytryptamine (5-HT), phenylbiguanide (PBG) and 2-methyl-5-HT competed at sub-micromolar concentrations (pIC_{50}; 5-HT 7.16–6.42, PBG 7.52–6.40, 2-methyl-5-HT 7.38–6.09).

4 Competition curves generated with increasing concentrations of quipazine, PBG, 5-HT and 2-methyl-5-HT displayed Hill coefficients greater than unity when the 5-HT_3 receptor recognition sites in the entorhinal cortex preparation were labelled with [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330. These competing compounds displayed Hill coefficients of around unity when the sites were labelled with [^{3}H]-(S)-zacopride. Competition for the binding of [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330 by granisetron generated Hill coefficients around unity.

5 The nature of the interaction of competing compounds (quipazine, granisetron, PBG, 5-HT, 2-methyl-5-HT) for the [^{3}H]-(S)-zacopride binding site in the rat entorhinal cortex preparation was not altered by the removal of the Krebs ions or the addition of the monoamine oxidase inhibitor, pargyline, to the HEPES/Krebs buffer.

6 In conclusion, the present studies provide further evidence towards the presence of cooperativity within the 5-HT_3 receptor macromolecule and indicate that either [^{3}H]-(S)-zacopride labels a different site on the receptor complex from [^{3}H]-LY278,584, [^{3}H]-granisetron or [^{3}H]-GR67330, or it binds in such a manner as to prevent the conformatory change in the receptor protein responsible for the cooperative binding of agonists (and quipazine).

Keywords: 5-HT_3 receptor recognition sites; 5-HT_3 receptor agonists; quipazine; radioligand binding; rat entorhinal cortex

Introduction

Antagonists for a type of 5-hydroxytryptamine (5-HT) receptor, the 5-HT_3 receptor, have been proposed for the therapeutic management of emesis, anxiety, psychosis, withdrawal from drugs of abuse and cognitive impairment (for reviews see Costall *et al.*, 1988; Barnes *et al.*, 1992). Radioligand binding sites, pharmacologically indistinguishable from functional 5-HT_3 receptors, have been identified in cell lines (e.g. Neijt *et al.*, 1988) and both peripheral and central tissues (e.g. Kilpatrick *et al.*, 1987; 1991; Peroutka & Hamik, 1988; Waeber *et al.*, 1988; Watling *et al.*, 1988; Barnes *et al.*, 1988; 1989; Nelson & Thomas, 1989; Pinkus *et al.*, 1989; Wong *et al.*, 1989; Bolanos *et al.*, 1990; Robertson *et al.*, 1990; Sharif *et al.*, 1991). Highest densities within the brain are associated with the dorsal vagal complex (comprising area postrema, nucleus tractus solitarius and dorsal motor nucleus of the vagus nerve; for review see Pratt *et al.*, 1990), limbic (e.g., amygdala, hippocampus) and cortical areas (e.g. primary olfactory cortex, entorhinal cortex, frontal cortex; e.g. Barnes *et al.*, 1990). It is plausible that the action of 5-HT_3 receptor antagonists in these brain regions is responsible for their ability to inhibit emesis and modify behaviour.

In common with functional studies with the 5-HT_3 receptor, where agonists display steep response curves (for review see Peters & Lambert, 1989), the inhibition of the binding of some selective radioligands (e.g. [^{3}H]-GR65630, [^{3}H]-GR67330, [^{3}H]-ICS205-930; Hoyer & Neijt, 1988; Kilpatrick *et al.*, 1987; 1990) by agonists (and the purported antagonist quipazine; Ireland & Tyers, 1987) generate Hill coefficients greater than unity, suggesting the occurrence of cooperativity within the 5-HT_3 receptor complex. This phenomenon, however, is not apparent for all the 5-HT_3 receptor recognition site radioligands (e.g. [^{3}H]-Q ICS 205-930, [^{3}H]-quipazine, [^{3}H]-(R/S)-zacopride, [^{3}H]-(S)-zacopride; Barnes *et al.*, 1988; 1990; Hamon *et al.*, 1989; Milburn & Peroutka, 1989; Bolanos *et al.*, 1990; Stanton *et al.*, 1990; Sharif *et al.*, 1991). In addition, it is noteworthy that inter-laboratory differences have been reported with respect to the presence of cooperativity associated with the competition by various compounds for a radioligand. For instance quipazine, 5-HT and 2-methyl-5-HT have been reported to compete for the binding of [^{3}H]-GR65630 to membranes from the rat cortex with Hill coefficients both greater than unity (Kilpatrick *et al.*, 1987) and not significantly different from unity (Sharif *et al.*, 1991). In addition, the nature of the competition by agonists has not been

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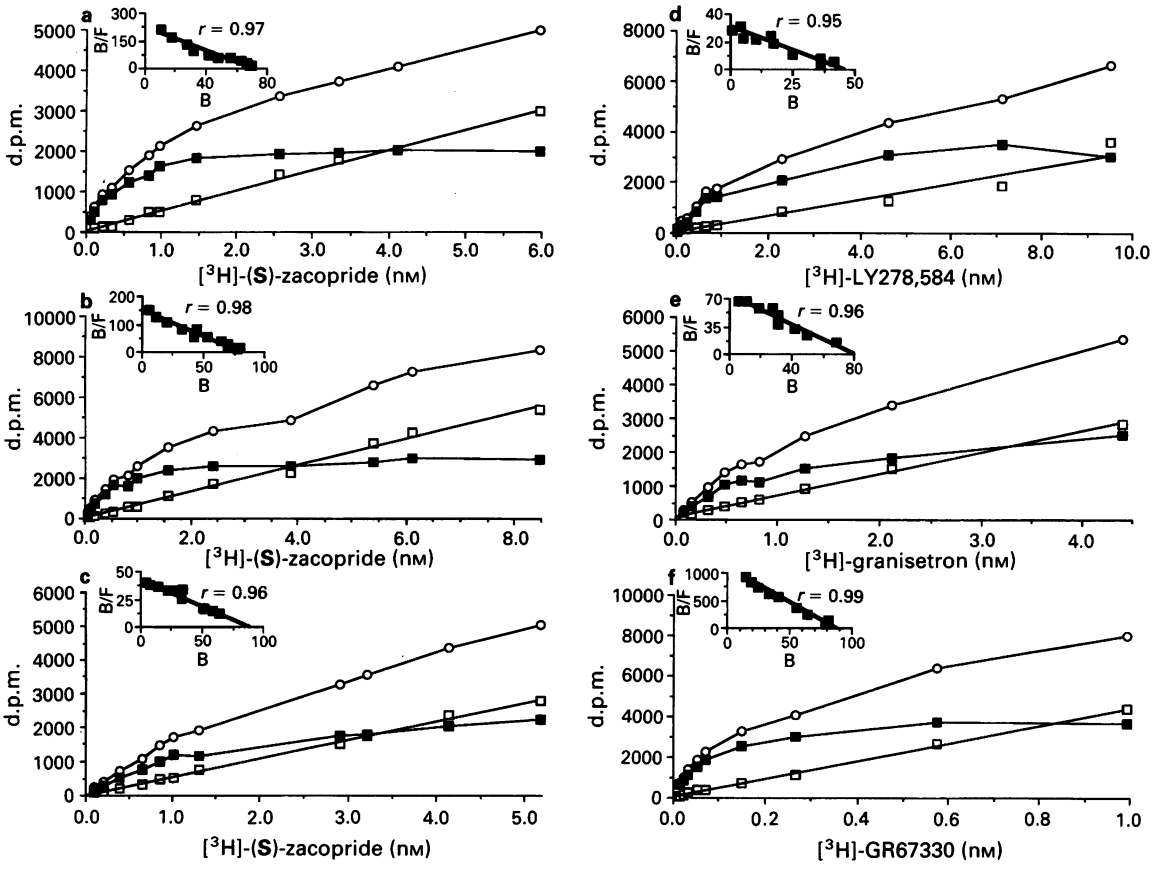

Figure 1 Equilibrium saturation studies of [^{3}H]-(S)-zacopride in HEPES/Krebs buffer (a), HEPES/Krebs/pargyline buffer (b) or HEPES buffer (c) and [^{3}H]-LY278,584 in HEPES/Krebs buffer (d), [^{3}H]-granisetron in HEPES/Krebs buffer (e) and [^{3}H]-GR67330 in HEPES buffer (f) binding to homogenates prepared from the entorhinal cortex of the rat. Typical results are presented from a single experiment where the total binding (○) and the non-specific binding (□, defined by the presence of granisetron, 10 μM) were determined in triplicate. Inset: Scatchard analysis of the resulting specific binding (■). B, bound radioligand fmol mg^{-1} protein; B/F, bound radioligand/free radioligand fmol mg^{-1} protein nM^{-1}; r, linear correlation coefficient.

reported for all the selective 5-HT$_3$ receptor recognition site radioligands (e.g. [^{3}H]-granisetron, [^{3}H]-LY278,584; Nelson & Thomas, 1989; Robertson *et al.*, 1990).

In the present studies, we assess the interaction of various 5-HT receptor agonists (and quipazine) for the binding site in the rat entorhinal cortex labelled by [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330.

Methods

Preparation of binding homogenate

Female hooded-Lister rats (200–300 g) were killed by cervical dislocation and the entorhinal cortex was rapidly dissected and placed in approximately 20 volumes of ice-cold buffer (either HEPES (50 mM, pH 7.4), HEPES/Krebs (mM: HEPES 50.0, NaCl 118.0, KCl 4.75, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, glucose 11.0, pH 7.4) or HEPES/Krebs/pargyline (HEPES/Krebs plus 10 μM pargyline, pH 7.4)). The tissue was homogenized in a Polytron blender (full power, 10 s) and the homogenate centrifuged (48 000 g for 10 min at 4°C). The resultant pellet was gently resuspended in the appropriate buffer and recentrifuged. The final pellet was gently resuspended in the appropriate buffer to form the binding homogenate at a concentration of 0.5–1.0 mg protein ml^{-1}. Protein content was assayed by the Bio-Rad Coomassie Brilliant Blue method (Bradford, 1976), with bovine serum albumin used as the standard.

Radioligand binding assay

Assay tubes (in triplicate) contained 650 μl of competing drug or vehicle (HEPES, HEPES/Krebs or HEPES/Krebs/pargyline buffer) and 100 μl radioligand ([^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron or [^{3}H]-GR67330; 0.3–0.4, 0.9–1.1, 0.5–0.9, 0.1–0.2 nM, respectively, for competition studies or at a range of concentrations between 0.02 and 9.5 nM for saturation studies, in the appropriate buffer). The assay tubes were preincubated for 2 min at 37°C before the addition of 250 μl brain homogenate to initiate binding which was allowed to proceed at 37°C for 30 min before termination by rapid filtration under vacuum through pre-wet (0.01% v/v polyethyleneimine in the appropriate buffer) Whatman GF/B filters followed by washing with ice-cold buffer (HEPES, HEPES/Krebs or HEPES/Krebs/pargyline; wash time 2 × 8 s). Radioactivity remaining on the filters was assayed in 10 ml Ultima-Gold (Packard) by liquid scintillation spectroscopy at an efficiency of approximately 47%.

Data analysis

Saturation and competition data were analysed with the computer programmes EBDA and LIGAND (Munson & Rodbard, 1980).

Drugs

Granisetron (HCl, SmithKline Beecham), 5-HT (bimaleate, Sigma), 2-methyl-5-HT (maleate, Research Biochemicals Incorporated), phenylbiguanide (PBG, Aldrich), quipazine (dimaleate, Research Biochemicals Incorporated) were dissolved in a minimum quantity of distilled water and diluted with the appropriate buffer. [^{3}H]-(S)-zacopride (83 Ci mmol^{-1}, Amersham), [^{3}H]-LY278,584 (1-methyl-N-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-1H-indazole-3-carboxamide, 84 Ci mmol^{-1}, Amersham), [^{3}H]-granisetron

Table 1 Saturation parameters generated with [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330 binding to membranes prepared from the rat entorhinal cortex (non-specific binding defined by granisetron, 10 μM)

Radioligand/buffer	pKd	B_{max} (fmol mg^{-1} protein)
[^{3}H]-(S)-zacopride (HEPES/Krebs)	9.41 ± 0.04	75 ± 5
[^{3}H]-(S)-zacopride (HEPES/Krebs/pargyline)	9.31 ± 0.05	84 ± 8
[^{3}H]-(S)-zacopride (HEPES)	8.57 ± 0.14	76 ± 9
[^{3}H]-LY278,584 (HEPES/Krebs)	8.69 ± 0.14	53 ± 5
[^{3}H]-granisetron (HEPES/Krebs)	8.81 ± 0.06	92 ± 6
[^{3}H]-GR67330 (HEPES)	10.14 ± 0.04	79 ± 6

Data represent the mean ± s.e.mean, n = 3–8.

(61 Ci mmol^{-1}, SmithKline Beecham) and [^{3}H]-GR67330 (1,2,3,9-tetrahydro-9-methyl-3[(5-methyl-1H-imidazol-4-yl) methyl]- 4H-carbazol-4-one, 85 Ci mmol^{-1}, Glaxo Group Research) were supplied in ethanol and diluted in the appropriate buffer.

Results

Saturation studies

[^{3}H]-(S)-zacopride (in HEPES, HEPES/Krebs or HEPES/Krebs/pargyline buffer), [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330 bound to a saturable population of binding sites (defined by the presence of granisetron, 10 μM) in the rat entorhinal cortex homogenate (Figure 1). Scatchard analysis of the specific binding indicated that each of the radioligands bound with high affinity to a homogeneous population of binding sites (Table 1, Figure 1).

Competition studies

Quipazine, PBG, 5-HT, 2-methyl-5-HT and granisetron competed for the binding of each radioligand with differing affinities (Table 2, Figure 2). The potent 5-HT$_3$ receptor antagonists, quipazine and granisetron, inhibited the binding of each of the radioligands at low nanomolar concentrations (Table 2, Figure 2). The natural and synthetic agonists, 5-HT, PBG and 2-methyl-5-HT also inhibited the binding of each of the radioligands but at sub-micromolar concentrations (Table 2, Figure 2).

Analysis of the competition curves for [^{3}H]-(S)-zacopride (in the presence of HEPES, HEPES/Krebs or HEPES/Krebs/pargyline buffer) indicated that quipazine, PBG, 5-HT, 2-methyl-5-HT and granisetron displayed Hill coefficients around unity (Table 2), whilst quipazine, PBG, 5-HT and 2-methyl-5-HT displayed Hill coefficients greater than unity when competing for the binding sites labelled by [^{3}H]-LY278,584, [^{3}H]-granisetron or [^{3}H]-GR67330 (Table 2). Granisetron generated Hill coefficients around unity when competing for these latter three radioligands (Table 2).

Discussion

The present studies have demonstrated that the radioligands, [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330 label, with nanomolar affinity, homogeneous populations of binding sites (defined by the inclusion of the potent and selective 5-HT$_3$ receptor antagonist, granisetron; Van Wijngaarden *et al.*, 1990) in membranes prepared from the rat entorhinal cortex. The densities of the binding sites labelled by the different radioligands ranged from 53 ([^{3}H]-LY278,584) to 92 ([^{3}H]-granisetron) fmol mg^{-1} protein. These differences, however, are more likely to be due to animal batch variation rather than the labelling of different recognition site populations since saturation studies with [^{3}H]-LY278,584 and [^{3}H]-granisetron utilising the same cortical homogenate indicated that they label a near identical density of specific sites (defined by the inclusion of granisetron (10 μM); Barnes, unpublished observations). The binding characteristics of all the radioligands employed in the present studies have previously been the subject of detailed pharmacological analysis which showed that they selectively label 5-HT$_3$ receptor recognition sites (Nelson & Thomas, 1989; Barnes *et al.*, 1990; Kilpatrick *et al.*, 1990; Robertson *et al.*, 1990), this being consistent with the pharmacological data generated in the present studies.

The competition for the recognition sites labelled by [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330 by 5-HT$_3$ receptor agonists (5-HT, 2-methyl-5-HT, PBG) and the 'antagonist', quipazine (Ireland & Tyers, 1987), generated steep displacement curves with Hill coefficients greater than unity. Similar findings have previously been reported for [^{3}H]-GR67330 (Kilpatrick *et al.*, 1990), whereas the nature with which these compounds compete for [^{3}H]-LY278,584 and [^{3}H]-granisetron has not been previously described (although quipazine has been reported to compete for [^{3}H]-granisetron binding with a Hill coefficient close to unity; Nelson & Thomas, 1989). The results from the present study, therefore add further evidence to the presence of cooperativity within the 5-HT$_3$ receptor complex and raise questions as to

Table 2 Affinities and Hill coefficients with which various 5-HT$_3$ receptor ligands compete for the binding sites in rat entorhinal cortex membranes labelled by the radioligands [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330; pIC_{50}, $-\log_{10}$ molar concentration of the competing compound to reduce the specific binding by 50%

	pIC_{50} (Hill coefficient)				
Radioligand/buffer	*Quipazine*	*PBG*	*5-HT*	*2-Methyl-5-HT*	*Granisetron*
[^{3}H]-(S)-zacopride (HEPES/Krebs)	8.65 ± 0.19 (1.03 ± 0.13)	7.07 ± 0.14 (0.89 ± 0.11)	6.42 ± 0.10 (1.07 ± 0.14)	6.29 ± 0.08 (1.14 ± 0.24)	8.26 ± 0.07 (1.08 ± 0.10)
[^{3}H]-(S)-zacopride (HEPES/Krebs/pargyline)	8.51 ± 0.12 (1.09 ± 0.13)	6.40 ± 0.07 (0.90 ± 0.13)	6.69 ± 0.12 (1.04 ± 0.13)	6.09 ± 0.09 (1.19 ± 0.10)	8.03 ± 0.29 (0.89 ± 0.07)
[^{3}H]-(S)-zacopride (HEPES)	8.68 ± 0.10 (0.94 ± 0.09)	6.93 ± 0.08 (1.16 ± 0.05)	6.51 ± 0.08 (0.94 ± 0.07)	6.63 ± 0.08 (1.01 ± 0.03)	8.58 ± 0.15 (1.09 ± 0.13)
[^{3}H]-LY278,584 (HEPES/Krebs)	8.84 ± 0.04 (1.43 ± 0.15)	7.24 ± 0.04 (1.53 ± 0.18)	6.60 ± 0.06 (1.52 ± 0.15)	6.65 ± 0.11 (1.65 ± 0.14)	8.36 ± 0.07 (1.04 ± 0.02)
[^{3}H]-granisetron (HEPES/Krebs)	9.38 ± 0.10 (2.03 ± 0.31)	7.52 ± 0.10 (1.59 ± 0.33)	7.16 ± 0.06 (1.94 ± 0.35)	7.38 ± 0.12 (1.67 ± 0.34)	8.62 ± 0.11 (0.99 ± 0.12)
[^{3}H]-GR67330 (HEPES)	8.86 ± 0.12 (1.86 ± 0.22)	6.77 ± 0.04 (2.26 ± 0.37)	6.52 ± 0.07 (1.53 ± 0.31)	6.68 ± 0.08 (1.97 ± 0.32)	8.50 ± 0.10 (1.11 ± 0.06)

Data represent the mean ± s.e.mean, n = 3–6.

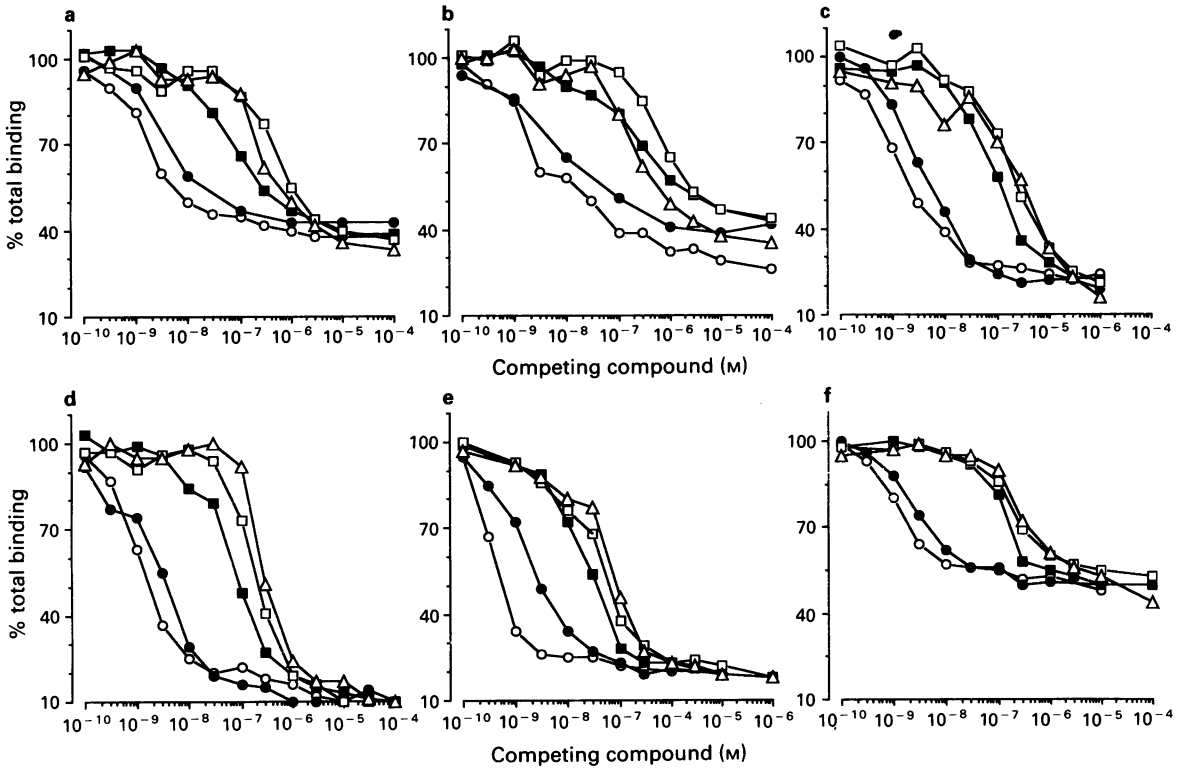

Figure 2 Ability of the agonists, phenylbiguanide (PBG), 5-hydroxytryptamine (5-HT) and 2-methyl-5-HT, and the antagonists, quipazine and granisetron, to compete for the binding of [^{3}H]-(S)-zacopride in HEPES/Krebs buffer (a), HEPES/Krebs/pargyline buffer (b) or HEPES buffer (c) and [^{3}H]-LY278,584 in HEPES/Krebs buffer (d), [^{3}H]-granisetron in HEPES/Krebs buffer (e) and [^{3}H]-GR67330 in HEPES buffer (f) in homogenates prepared from the entorhinal cortex of the rat. Values represent the mean calculated from 3–6 separate experiments. Standard errors were in the range of 1–22% of the mean value. PBG (■), 5-HT (△), 2-methyl-5-HT (□), quipazine (○), granisetron (●).

whether quipazine displays agonist activity at the 5-HT$_3$ receptor.

In contrast to the structurally dissimilar 5-HT$_3$ receptor recognition site radioligands, competition studies with [^{3}H]-(S)-zacopride failed to yield high Hill coefficients for the agonists 5-HT, 2-methyl-5-HT, PBG and the antagonist quipazine. This confirms earlier studies (e.g. Barnes *et al.*, 1990), and in the present studies, we investigated further the competition by various compounds for the [^{3}H]-(S)-zacopride binding site by altering the ionic composition of the incubation medium and also by inhibiting monoamine oxidase in an attempt to prevent the enzymatic degradation of the natural ligand 5-HT. In agreement with former results (Barnes *et al.*, 1988; Barnes, unpublished observations), we found that the removal of Krebs ions from the binding medium decreased the affinity of [^{3}H]-(S)-zacopride for its recognition site; however, their removal did not alter the nature of the competition by 5-HT, 2-methyl-5-HT, PBG or quipazine. A related observation by Stanton and colleagues (1990) was the detection of an increase in the affinity of antagonists (e.g. L-686,470 (exo 5-(azabicyclo[2.2.1]heptan-3-yl)-3-(1-methyl-1H-indol-3-yl)-1,2,4-oxadiazole oxalate), zacopride) for the 5-HT$_3$ receptor following the addition of Ca^{2+} and Mg^{2+} ions to the incubation buffer. It should be noted, however, that a previous study (Bolanos *et al.*, 1990) was unable to detect an increase in the specific binding of [^{3}H]-zacopride at a single, sub-equilibrium dissociation constant, concentration (and hence an increase in the affinity of the radioligand) with the addition of various ions (e.g. Na^+, K^+, Cs^+, Ba^{2+}, Ca^{2+}, Co^{2+} Mg^{2+}, Mn^{2+}, Zn^{2+}). The reasons for this apparent inconsistency are unclear, but may relate to the individual addition of these ions to the incubation buffer or the use of a lower concentration of a different buffer (25 mM Tris HCl) since these authors found that the amount of specific [^{3}H]-zacopride binding was dependent on the molarity of this buffer. In addition to the alteration in the ionic constituents of the incubation buffer, in the present studies, the inhibition of monoamine oxidase by pargyline (A19120; Taylor *et al.*, 1960), which might be expected to 'steepen' the competition curve of 5-HT since it would proportionally increase the concentration of 5-HT at higher concentrations, also failed to alter the nature of the competition of 5-HT with [^{3}H]-(S)-zacopride. Although it would be anticipated that the monoamine oxidase activity in the homogenate preparations for the different radioligands would have been comparable, this latter finding implies that metabolism of 5-HT does not account for the discord in the nature of the competition of 5-HT for the binding sites labelled by the different radioligands.

The available evidence suggests that the radioligands, [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330, label the same receptor macromolecule in the rat entorhinal cortex preparation. However, a potential explanation for the disparity between the radioligands with respect to the nature of the interaction of agonists (and quipazine) is that [^{3}H]-(S)-zacopride labels a different recognition site on the receptor or interacts in such a manner as to prevent the conformatory changes in the receptor complex which manifest the cooperative binding of agonists (and quipazine).

In conclusion, [^{3}H]-(S)-zacopride, [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330 labelled a similar density of pharmacologically comparable binding sites within a membrane preparation from the rat entorhinal cortex. Analysis of the competition curves demonstrated that quipazine, PBG, 5-HT and 2-methyl-5-HT generated Hill coefficients greater than unity when competing for the sites labelled by [^{3}H]-LY278,584, [^{3}H]-granisetron or [^{3}H]-GR67330, whilst the same competing compounds generated Hill coefficients of around unity when competing for the sites labelled by [^{3}H]-(S)-zacopride. Such findings provide further evidence of the

presence of cooperativity within the 5-HT_3 receptor and suggest that [^{3}H]-(S)-zacopride interacts in a different manner with the 5-HT_3 receptor from [^{3}H]-LY278,584, [^{3}H]-granisetron and [^{3}H]-GR67330.

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