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Angiotensin converting enzyme activity is present in the endothelium-denuded aorta

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Angiotensin converting enzyme activity was measured in intact aortic rings utilizing the synthetic tripeptide [^{3}H]-benzoyl-Phe-Ala-Pro as the substrate. Intact aortic rings possessed angiotensin converting enzyme activity which was blocked by captopril. Enzyme activity was reduced by approximately 30% after removal of the endothelium by chemical or mechanical methods. The remaining activity was also captopril-sensitive. These results suggest that in addition to endothelium, angiotensin converting enzyme activity is present in other vascular cells and may contribute to the metabolism of angiotensin I generated within the vessel wall.

Introduction Angiotensin converting enzyme (ACE) catalyzes the release of histidyl-leucine from the —COOH terminus of angiotensin I (AI) to yield the potent vasoactive octapeptide, angiotensin II. The ectoenzyme is primarily located on the luminal membrane of vascular endothelium cells where it hydrolyzes the circulating (blood-borne) hormones (Ryan *et al.*, 1976). Although it is not found within the endothelial cytoplasm (Catravas & Watkins, 1985) indirect evidence suggests that it may be present elsewhere within the vascular wall (Saye *et al.*, 1984; Dzau, 1986). In order to address this question directly, ACE activity was measured in intact and endothelium-denuded aortic rings *in vitro*. The results of the present study clearly indicate that significant ACE activity remains after removal of the endothelium and confirm a postulated role for this enzyme in the metabolism of locally (intra-wall) produced angiotensin I.

Methods Male Wistar-Kyoto (WKY) rats were anaesthetized with pentothal (50 mg kg^{-1}, weight). The thoracic aorta was removed and placed in oxygenated McEwen's solution of the following composition (mM): NaCl 130, $NaHCO_3$ 25, KCl 5.6, $CaCl_2$ 2.2, NaH_2PO_4 1.2, glucose 11 and sucrose 13. It was then cleaned carefully and cut into rings (2–3 mm wide). The rings were either set up for tension experiments or examined for ACE activity. The endothelium was removed either by infusing 3 ml sodium deoxycholate *in situ* (1 mg ml^{-1} for about 30 s) Byfield *et al.*, 1986) or by gently scraping the endothelium away from the intima. To test for the successful removal of endothelium, samples for light microscopy were prepared by the method of Abrol *et al.* (1984) for silver staining.

ACE activity was determined by using [^{3}H]-benzoyl-Phe-Ala-Pro ([^{3}H]-BPAP) as the substrate (Catravas & Watkins, 1985). The enzyme removes Ala-Pro (in much the same way as it removes histidyl-leucine from angiotensin I). The product ([^{3}H]-benzoyl-Phe) is quantitatively extracted from the acidified sample into toluene. In our system, 2 rings per sample were incubated in 1 ml HEPES buffer (0.1 M, pH 7.4, 37°C) containing 50 nCi [^{3}H]-BPAP, for 15 min. A total of 15 samples was used for experiments with intact endothelium and a total of 10 samples for the experiments where the endothelium was removed. The aortic rings for these samples were taken from 6 rats. The reaction was stopped with the addition of captopril (200 μg ml^{-1} final concentration) at 4°C and acidified by adding 0.12 N HCl. Product ([^{3}H]-B-Phe) was extracted into Ventrex No. 2 (a toluene-containing scintillation mixture) by incubating in the dark for 72 h and radioactivity was measured in a scintillation spectrometer. Results are expressed as units of ACE activity per mg of wet weight of tissue. One unit is determined as the amount of enzyme converting 1% of the substrate in 1 min under first order reaction conditions. Appropriate controls included captopril to ensure the specificity of the reaction. Statistical analysis was done by Student's *t* test.

Results The successful removal of the endothelium was tested pharmacologically by examining the effect of acetylcholine (ACh 10^{-5} M) on aortic rings precontracted with noradrenaline (10^{-9} M). In the presence of endothelium ACh caused a substantial fall in tension whereas in the absence of endothlium it was totally ineffective. In addition histological examination (silver nitrate stain) of opened aortic rings showed that both gentle scraping ($n = 4$) and

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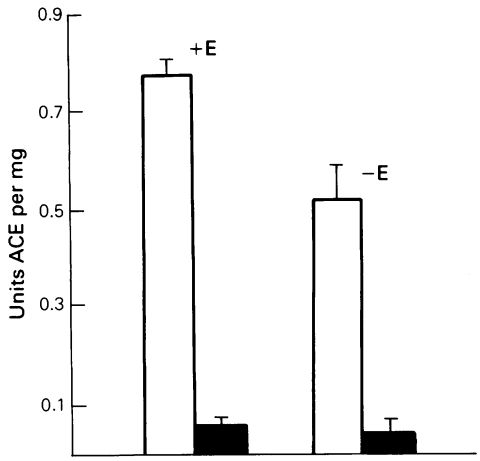


Figure 1 Angiotensin converting enzyme activity in the presence (+E) and absence (−E) of endothelium and with (solid column) and without (open column) captopril (1 mg ml^{-1}); $n = 15$ for +E; $n = 10$ for −E.

sodium deoxycholate ($n = 4$) infusion resulted in the successful removal of the endothelium.

In the presence of endothelium, ACE activity was found to be 0.76 ± 0.04 units per mg tissue wet weight ($n = 15$) (Figure 1). In endothelium-denuded vessels this activity was reduced partially but significantly ($P < 0.05$) to 0.52 ± 0.07 units per mg tissue wet weight ($n = 10$) (Figure 1). In both cases ACE activity was almost completely blocked by captopril (4×10^{-3} M during the 15 min incubation). Thus a substantial amount of ACE activity remained in the medial and adventitial layer of the aorta. These findings were similar whether sodium deoxycholate infusion or gentle scraping techniques were used to remove the endothlium.

Discussion The substantial ACE activity remaining after endothelium removal suggests that this activity may not be limited to the vascular endothelium. This nonendothelial enzyme appears to be similar to the one found in endothelium in so far as it is blocked by the classical ACE inhibitor, captopril. It bears, therefore, no relation to the captopril-insensitive, chymostotin-sensitive angiotensin II generating enzyme which was recently located in the adventitia of dog, monkey and human aorta (Okunishi *et al.*, 1987).

These findings are in agreement with those of Peach and colleagues (Saye *et al.*, 1984) who recently reported that endothelium removal only attenuated, but did not abolish angiotensin-I-elicited contractile responses in rabbit aortic rings. It might be argued that under the present experimental conditions, the endothelium of the vasa vasorum is unmasked and this may be an important source of ACE activity. However, removal of endothelium from the aorta does not unmask any measurable endothelium-derived relaxing factor (EDRF) activity, the main source of which is the endothelial cell, as reflected in the total inhibition of the relaxing responses to ACh in endothelium-denuded vessels. In addition it is known that other endothelial cell markers, such as the surface blood group antigens, are absent from the underlying structures of the vessel wall (Szulman, 1960). It therefore appears probable that the endothelium of the vasa vasorum makes a negligible contribution to the overall endothelium-associated metabolic activities.

The locus of the ACE activity remaining after endothelium removal (approximately 70% of total) is still unclear. Interstitial fibroblasts might be a likely candidate, since investigators have reported significant levels of ACE activity as well as immunofluorescence localization of the enzyme in cultured fibroblasts from various species (Rubin *et al.*, 1982; Weinberg *et al.*, 1982). Of course, it should be emphasized that given the minimal fraction of total vessel weight associated with endothelium (<1%) the presently measured ACE activity still represents specific activity which is vastly higher than in any other cell type. There is considerable experimental evidence today suggesting that angiotensin II can be formed outside the vascular endothelium (Dzau, 1986; Swales & Heagerty, 1987; Story & Ziogas, 1987). Furthermore, all the necessary components of the renin-angiotensin system are believed to be located within the vascular wall where angiotensin II can be formed and act *in situ* (Desjardins-Giasson *et al.*, 1981). The physiological function of vascular wall angiotensin II is believed to be the facilitation of NA release from nerve endings as ACE inhibitors have been found to reduce its release from the rat mesentery (Kawasaki *et al.*, 1984). Circulating angiotensin I and bradykinin are believed to be hydrolysed by endothelial ACE (Ryan *et al.*, 1970).

In conclusion, removal of the endothelium from rat aortic segments causes a partial reduction in total ACE activity directly assayed in this tissue. These results indicate that ACE activity is not limited to the vascular endothelium and suggest a role for intrawall ACE in the hydrolysis of locally produced angiotensin I.

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The effect of M&B 22948 on methacholine- and histamine-induced contraction and inositol 1,4,5-trisphosphate levels in guinea-pig tracheal tissue

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The effect of a cyclic GMP phosphodiesterase inhibitor, M&B 22948, on methacholine- and histamine-induced contraction and inositol 1,4,5-trisphosphate (IP_3) elevation was studied in guinea-pig tracheal rings. After addition of methacholine or histamine the rise in IP_3 content was rapid and transient reaching a maximum after 5–15 s, which coincided with the maximum rate of tension development. Cyclic GMP levels of the tissue were elevated by M&B 22948 before agonist stimulation and further elevated by addition of methacholine or histamine. Cyclic AMP levels were not altered by any of these agents. M&B 22948 abolished IP_3 generation induced by methacholine or histamine, but did not alter the rate or magnitude of tension development. Thus, IP_3 generation does not appear to be responsible for the contractions induced by methacholine or histamine in this tissue.

Introduction Contractions of airway smooth muscle induced by many putative mediators of airways hyper-reactivity are believed to involve increased inositol phosphate generation (Hashimoto *et al.*, 1985; Duncan *et al.*, 1987; Hall & Hill, 1988; Langlands *et al.*, 1988). There is substantial evidence demonstrating the ability of inositol 1,4,5-trisphosphate (IP_3) to release Ca^{2+} from non-mitochondrial calcium stores such as the sarcoplasmic reticulum (Hashimoto *et al.*, 1985). For this reason, it is believed that IP_3 may be of fundamental importance in the regulation of cytosolic Ca^{2+} and the contractility of airway smooth muscle.

Recent studies have proposed that agents elevating tissue adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels may inhibit agonist induced inositol phospholipid metabolism (Hall & Hill, 1988; 1989) and tension development (Langlands *et al.*, 1989) in airway smooth muscle.

In the present study, we have used a protein binding assay system to determine levels of IP_3 during contraction of guinea-pig tracheal rings induced by methacholine and histamine. In addition, the study examines the effects of a cyclic GMP phosphodiesterase inhibitor, M&B 22948 (Kukovetz *et al.*, 1979), on agonist-induced IP_3 formation and tension development in this tissue.

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Methods Tracheal rings from male Hartley guinea-pigs (300–400 g) were suspended under an initial 20 mN resting tension in Krebs-Henseleit solution (KHS) of the following composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and D-glucose 11.7. Changes in isometric tension were recorded on an ink-writing curvilinear polygraph (Grass, Model 7C, Quincy, Mass). The bathing medium was maintained at 37°C and aerated with 5% CO_2 in O_2. Flurbiprofen (1 μM), a cyclo-oxygenase inhibitor, was present in the bathing medium throughout the experiment (Bryson & Rodger, 1987).

After an initial 45 min equilibration period, tissues were freeze-clamped, using tongs pre-cooled in liquid nitrogen, at pre-determined times (0, 5, 10, 15, 30, 60 s) after addition of the agonist. Where appropriate, tissues were pretreated with atropine (1 μM), mepyramine (1 μM) or M&B 22948 (10 μM) 20 min before addition of agonist. Cyclic AMP and cylic GMP levels were determined before addition of M&B 22948, before agonist addition but in the presence of M&B 22948 and after 60 s agonist stimulation.

Inositol 1,4,5-trisphosphate levels were determined by a protein binding assay system (TRK 1000; Amersham). This assay measures levels of the 1,4,5-isomer of IP_3 without interference from other inositol phosphates. Cyclic AMP and cyclic GMP levels were determined by protein-binding and radioimmunoassay as described by Bryson & Rodger (1987).

The following drugs were used: acetyl-β-methylcholine (methacholine) and histamine acid phosphate (Sigma), atropine sulphate (Koch-Light), sodium

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flurbiprofen (Boots PLC), mepyramine sulphate (Rhone-Poulenc). All other chemicals were analar grade obtained from BDH. Cyclic AMP assay kit (TRK 432), cyclic GMP assay kit (TRK 500), guanosine 3',5'-cyclic phosphoric acid 2'-0-succinyl-3-[^{125}I]-iodotyrosine methyl ester (IM 107) and IP_3 assay kit were obtained from Amersham. M&B 22948 (2-*o*-propoxyphenyl-8-azapurin-6-one) was a gift from Rhone-Poulenc.

Results are expressed as mean ± s.e. mean ($n = 5$ in all experiments) and were analysed by the Mann-Whitney U test. Significance was accepted when $P < 0.05$.

Results Methacholine (EC_{90}; 10 μM) induced a rapid rise in the IP_3 level of the guinea-pig tracheal tissue after 10 s. Levels remained elevated at 15 s before returning to basal after 30 s (Figure 1a). Similarly, histamine (EC_{90}; 30 μM) induced a rapid rise in the IP_3 level after 5 s, which continued to rise until 10 s, before rapidly returning to basal level at 15 s (Figure 1b). The transient elevations in IP_3 content of the tissues induced by both methacholine and histamine occurred during the period of rapid tension development (Figure 1a and b) and could be abolished, together with the contractile responses, by pretreatment of the tissues with atropine (1 μM) and mepyramine (1 μM) respectively (data not shown).

After pretreatment of the tissues with M&B 22948 (10 μM), no alteration in IP_3 content was observed during stimulation with either methacholine or histamine. However, in the presence of M&B 22948 the rate and magnitude of tension development induced by both methacholine and histamine were not significantly different from control responses (Figure 1).

M&B 22948 (10 μM) produced a significant rise in the basal cyclic GMP content of the tissue after 20 min incubation (from 14.5 ± 3.2 to 34.3 ± 4.4 fmol cyclic GMP mg^{-1} tissue).

Cyclic GMP levels were further increased to 69.2 ± 17.4 and 52.3 ± 13.8 fmol cyclic GMP mg^{-1} tissue 60 s after addition of methacholine (10 μM) and histamine (30 μM) respectively. The cyclic AMP levels were not altered significantly from basal (0.35 ± 0.02 pmol cyclic AMP mg^{-1} tissue) after 20 min incubation with M&B 22948 (0.40 ± 0.04 pmol cyclic AMP mg^{-1} tissue) or after 60 s stimulation with either methacholine (0.38 ± 0.06 pmol cyclic AMP mg^{-1} tissue) or histamine (0.41 ± 0.05 pmol cyclic AMP mg^{-1} tissue).

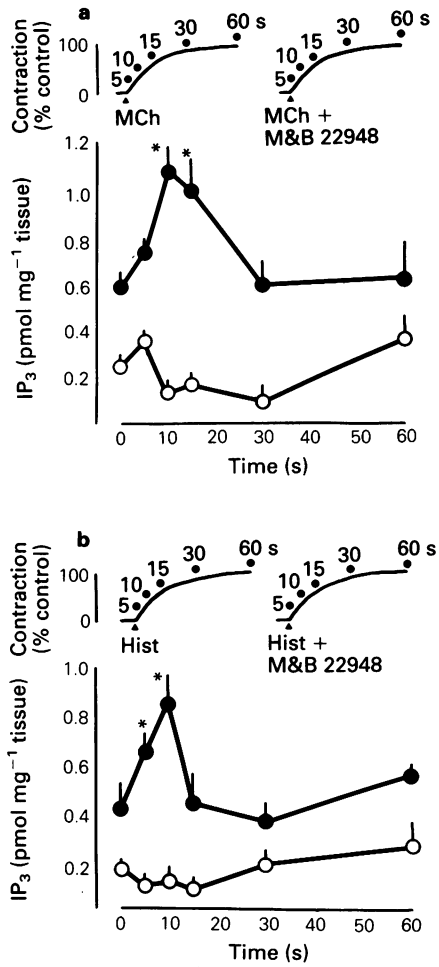


Figure 1 The effect of (a) methacholine 10 μM and (b) histamine 30 μM alone (●) and in the presence of M&B 22948 10 μM (○) on inositol 1,4,5-triphosphate (IP_3) levels and tension development in guinea-pig tracheal rings. Tissues were freeze-clamped at 0, 5, 10, 15, 30 and 60 s after addition of the agonist, and the IP_3 levels were determined by a protein binding assay. The contractile response is expressed as a percentage of the maximum response observed in the absence of M&B 22948. *Indicates significant difference from basal level of IP_3 (in the absence or presence of M&B 22948) $P < 0.05$, Mann-Whitney.

Discussion In the present study, we have demonstrated the ability of methacholine and histamine, acting via specific receptors, to induce rapid transient elevations in IP_3 content during contraction of the guinea-pig trachea. In addition, the level of IP_3 becomes elevated rapidly enough with both agonists to suggest that it may play an important role in the initiation of tension development by these agonists. Since only a transient elevation in IP_3 content is observed, it is likely that the formation of IP_3 is only

responsible for the initiation of temsion development. The significance of the transient nature of the response is still unclear.

Due to the variety of cell types within the tissue under study, it is possible that non-smooth muscle cells contribute to the changes in IP_3 content observed during contraction. However, preliminary studies (data not shown) have demonstrated that levels of IP_3 which may be present in the cartilage are below the level of detection for this assay system. In addition, autoradiographic studies have demonstrated localization of muscarinic receptors on airway smooth muscle cells with a much poorer distribution of the receptors in epithelial and vascular smooth muscle cells (Barnes *et al.*, 1983). Thus, non-smooth muscle cells probably contribute only minimally to the levels of IP_3 determined during tissue contractions.

M&B 22948 abolished the rapid transient elevation in IP_3 which was observed during the first 60 s of stimulation with methacholine and histamine. A previous report has demonstrated inhibition of PI hydrolysis in vascular smooth muscle by agents elevating cyclic GMP content (Rapoport, 1986). The ability of M&B 22948 to inhibit IP_3 formation induced by methacholine and histamine is consistent with this observation since basal levels of cyclic GMP were elevated by M&B 22948. Cyclic AMP was unchanged and therefore does not appear to play a role in the M&B 22948-induced inhibition of IP_3 generation. Quite surprisingly, the contractile responses to methacholine and histamine were unaltered in the presence of M&B 22948. If generation of IP_3 is necessary for release of intracellular Ca^{2+} and the initiation of airway smooth muscle contraction, we would expect the contractile response to be greatly reduced. Clearly this is not the case and it would appear that some other, as yet unknown, mechanism, independent of IP_3 generation, is responsible for the development of tension induced by both methacholine and histamine.

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Dual effects of 5-hydroxytryptamine on the release of γ-aminobutyric acid from myenteric neurones of the guinea-pig ileum

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The effects of 5-hydroxytryptamine (5-HT) on the release of γ-aminobutyric acid (GABA) were examined in the longitudinal muscle-myenteric plexus (LM-MP) preparation of guinea-pig ileum. 5-HT increased the spontaneous release and inhibited the electrically-evoked release of [^{3}H]-GABA. The 5-HT-evoked release was Ca^{2+}-dependent and tetrodotoxin-sensitive, and was antagonized by (3α-tropanyl)-1H-indole-3-carboxylic acid ester (ICS 205-930), but not by methysergide and ketanserin. The inhibitory effect of 5-HT was antagonized by methysergide, but not by ketanserin and ICS 205-930. 8-Hydroxy-2-(di-*n*-propylamino)tetralin mimicked the inhibitory effect of 5-HT. Thus, 5-HT may exert an excitatory effect on the enteric GABAergic neurone via the 5-HT_3 receptor and an inhibitory effect via the 5-HT_{1A} receptor.

Introduction 5-Hydroxytryptamine (5-HT) has been shown to exert stimulatory and inhibitory effects on acetylcholine (ACh) release from the guinea-pig ileum (Kilbinger & Pfeuffer-Friederich, 1985). γ-Aminobutyric acid (GABA) has similar dual effects on ACh release (Kleinrok & Kilbinger, 1983; Erdö, 1985; Tanaka, 1985; Jessen *et al.*, 1987). We proposed that excitatory peptides present in the ileum, including substance P, neurotensin and cholecystokinin may modulate the enteric GABAergic neurone (Tanaka & Taniyama, 1987). To assess 5-HT as a possible modulator of GABAergic activity in the guinea-pig ileum, we have now examined the effect of 5-HT on the release of GABA from longitudinal muscle-myenteric plexus preparations.

Methods Adult guinea-pigs of either sex, weighing between 300 and 350 g, were killed by cervical dislocation. Strips of ileum were excised 10 cm proximal to the ileocaecal sphincter, and longitudinal muscle-myenteric plexus (LM-MP) preparations were dissected. The methods of incubation and perfusion were as described by Hashimoto *et al.* (1986). Strips were incubated at 35°C for 60 min with [^{3}H]-GABA at a final concentration of 5×10^{-8} M in Krebs solution of the following composition (in mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.19, $NaHCO_3$ 25.0, KH_2PO_4 1.18 and glucose 11. After washing in fresh Krebs solution for 30 min, the strips were mounted between two parallel platinum electrodes and superfused at a rate of 1.2 ml min^{-1} with Krebs solution. The Krebs solution used throughout contained 10^{-5} M aminooxyacetic acid (AOAA) to prevent the metabolism of [^{3}H]-GABA and 10^{-3} M β-alanine to prevent the glial uptake of [^{3}H]-GABA, and was gassed with 95% O_2-5% CO_2. The superfusate was collected every 1 min, and the radioactivity was measured with a liquid scintillation spectrometer. The proportion of unchanged GABA in the tritium efflux was determined as described by Hashimoto *et al.* (1986). The release of GABA was represented as the fractional rate in which the amount of GABA in the superfusate was divided by the respective GABA contents in the tissue at the start of the respective period. The amount of GABA contained in the tissue at the start of the respective period was calculated by adding cumulatively the amounts of GABA released during the collection period to the amount of GABA in the tissue at the end of the experiment. Data were analyzed by Students' *t* test and a $P \leqslant 0.05$ was considered statistically significant. All chemicals and drugs were obtained commercially.

Results The experiment was started 60 min after the superfusion, during which time the spontaneous release of [^{3}H]-GABA declined in a multiphasic exponential course and approached a slow phase with a fractional rate of 0.00411 ± 0.00035 min^{-1} ($n = 30$) 60 min after superfusion. The effect of 5-HT on the GABA release was examined in the presence of 10^{-6} M imipramine. Superfusion with 5-HT at 10^{-9} to 3×10^{-5} M for 1 min led to an increase in the release of [^{3}H]-GABA from the LM-MP preparations (Figure 1a), which then returned to the basal release levels within 2 min during the continued presence of 5-HT. The EC_{50} value of 5-HT was 6.0×10^{-8} M ($n = 6$). The 5-HT (10^{-6} M)-evoked release of [^{3}H]-GABA was prevented by pretreatment with tetrodotoxin (TTX) at 10^{-6} M for 15 min

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and in Ca^{2+}-free medium containing 1 mM EGTA, thereby indicating that the released [^{3}H]-GABA is of neuronal origin. (3α-Tropanyl)-1H-indole-3-carboxylic acid ester (ICS 205–930, 3×10^{-7} M), a 5-HT_3 receptor antagonist, but not methysergide (10^{-6} M) and ketanserin (10^{-5} M) antagonized the facilitatory effect of 5-HT (Figure 1a). Hexamethonium at 3×10^{-4} M, atropine at 10^{-6} M and [D-Pro^2, D-Trp^{7,9}]substance P at 3×10^{-6} M, a substance P antagonist, did not affect the 5-HT (10^{-6} M)-evoked release of [^{3}H]-GABA.

Electrical field stimulation (EFS) with pulses of 0.5 ms duration, 15 V intensity, and 5 Hz frequency for 30 s stimulated [^{3}H]-GABA release, which was prevented by superfusion with 10^{-6} M TTX or with Ca^{2+}-free medium containing 1 mM EGTA. Imipramine (10^{-6} M) did not affect the EFS-evoked release of [^{3}H]-GABA. Pretreatment with 5-HT for 3 min, but not 1 or 2 min, inhibited the release of [^{3}H]-GABA evoked by EFS (in the presence of ICS 205-930 (10^{-6} M) to prevent a transient increase in the release of [^{3}H]-GABA), in a concentration-dependent manner (Figure 1b). The IC_{50} value for 5-HT was 1.46×10^{-7} M ($n = 6$). 8-Hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT) at 10^{-7} M, a 5-HT_{1A} receptor agonist, also inhibited the EFS-evoked release of [^{3}H]-GABA to 62.1 ± 5.3% ($n = 5$) of the release in the absence of 8-OH-DPAT. The inhibitory effects of 5-HT and 8-OH-DPAT on the EFS-evoked release of [^{3}H]-GABA were antagonized by methysergide (3×10^{-7} M) (Figure 1b), but not by ketanserin (10^{-5} M) and ICS 205-930 (10^{-6} M). Prazosin (10^{-6} M), an α_1-adrenoceptor antagonist and yohimbine (10^{-7} M), an α_2-adrenoceptor antagonist did not affect the inhibition by 5-HT of the EFS-evoked release of [^{3}H]-GABA.

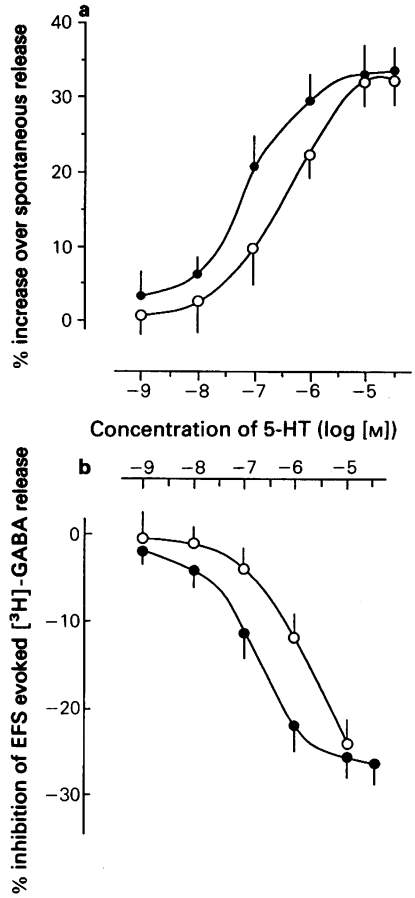


Figure 1. Concentration-response curve for the effect of 5-hydroxytryptamine (5-HT) on the release of [^{3}H]-γ-aminobutyric acid ([^{3}H]-GABA). (a) 5-HT-evoked release of [^{3}H]-GABA in the absence (●) and presence (○) of ICS 205-930. ICS 205-930 (3×10^{-7} M) was applied 10 min before and during stimulation with 5-HT for 1 min, in the presence of 10^{-6} M imipramine. (b) Inhibition by 5-HT of electrical field stimulation (EFS, 5 Hz, 15 V, 0.5 ms for 30 s)-evoked release of [^{3}H]-GABA in the absence (●) and presence of methysergide (○). Pretreatment with 5-HT was for 3 min. Methysergide (3×10^{-7} M) was applied 10 min before and during stimulation with EFS.

The effect of 5-HT at concentrations of 10^{-9}, 10^{-7} and 10^{-5} M was examined in one preparation and that at concentrations of 10^{-8}, 10^{-6} and 3×10^{-5} M in another preparation from the same animal. Each point represents the mean from 5 or 7 animals with s.e. shown by vertical lines.

Discussion The present study revealed that in guinea-pig ileum, 5-HT exerts a dual effect on the release of GABA: a transient increase in a spontaneous release of GABA and an inhibition of EFS-evoked release of GABA. This finding is similar to certain effects of 5-HT on the release of ACh (Kilbinger & Pfeuffer-Friederich, 1985). The excitatory 5-HT receptor on the GABAergic neurone may be the 5-HT_3 receptor, because the 5-HT-evoked release of GABA was antagonized by ICS 205-930, a 5-HT_3 antagonist, but not by methysergide and ketanserin. However, ICS 205-930 has also been reported to antagonize responses evoked by 5-HT acting at a receptor other than 5-HT_1, 5-HT_2 or 5-HT_3 (Craig & Clarke, 1989; Dumuis *et al.*, 1989) and it remains a possibility that the 5-HT-evoked release of GABA may be mediated by the stimulation of a non-classical 5-HT receptor. It has been reported that 5-HT induces the release of sub-

stance P (Buchheit *et al.*, 1985), and that substance P induces GABA release (Tanaka & Taniyama, 1987). However the lack of effect of [D-Pro^2, D-Trp^{7,9}]substance P on the 5-HT-evoked release of GABA does not support an involvement of substance P in the excitatory effect of 5-HT on the GABAergic neurone.

5-HT and 8-OH-DPAT (5-HT$_{1A}$ agonist) inhibited the EFS-evoked release of GABA. These inhibitory effects were antagonized by methysergide, but not by ketanserin or ICS 205-930. The inhibitory effect of 5-HT on the GABA release may, therefore, occur via the 5-HT$_{1A}$ receptor.

The affinity of 5-HT for the putative 5-HT$_3$ receptor was only twice as high as that for 5-HT$_{1A}$ receptor, calculating from the EC_{50} value. However, the time course of these receptor-mediated effects of 5-HT differed. The 5-HT$_3$ receptor-mediated effect occurred immediately after treatment with 5-HT and returned to the basal level within 2 min, even in the presence of 5-HT. The 5-HT$_{1A}$ receptor-mediated effect occurred 3 min after treatment with 5-HT. It remains to be determined whether the 5-HT$_{1A}$ and 5-HT$_3$ receptors are present in the same or distinct GABAergic neurones.

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The effects of alterations in electrogenic Na^+/K^+-pumping in guinea-pig isolated trachealis: their modulation by the epithelium

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1 An examination was made of the effect of epithelium removal on mechanical responses of guinea-pig isolated tracheal strips after inhibition or activation of electrogenic Na^+/K^+-pumping.

2 The Na^+/K^+-pump inhibitor ouabain (0.1–10 μM) evoked concentration-dependent contractions which were potentiated by epithelium removal.

3 K^+-free solution, which inhibits Na^+/K^+-pumping, produced a slow, sustained relaxation in intact preparations. In epithelium-free preparations the relaxation was transient and of lesser magnitude.

4 The addition of K^+ (10 or 30 mM), which activates Na^+/K^+-pumping, to preparations bathed in K^+-free solution caused a relaxation of preparations under spontaneous tone or contracted with methacholine; the magnitude and duration of relaxation was greater in the epithelium-free preparations. Ouabain (0.1 μM) attenuated the relaxation to K^+ in intact preparations and converted the response of epithelium-free preparations to a contraction. In the presence of a higher concentration of ouabain (1 μM), intact preparations contracted in response to K^+.

5 In normal K^+ solution, ouabain (0.1 μM) increased the sensitivity of intact preparations to methacholine but reduced their sensitivity to K^+. Ouabain was without these effects in epithelium-free preparations.

6 Thus, responses of intact preparations to perturbations which affect electrogenic Na^+/K^+-pumping in trachealis are influenced by an epithelium-derived factor. The production of the factor may be linked to an epithelial Na^+/K^+-pump, or the factor may modulate the activity of an electrogenic Na^+/K^+-pump in the muscle.

Introduction

An electrogenic Na^+/K^+-pump is present in airway smooth muscle (Souhrada *et al.*, 1981; Souhrada & Souhrada, 1981; 1983; Gunst & Stropp, 1988). Inhibition of the electrogenic Na^+/K^+-pump with ouabain or by reducing the extracellular K^+ concentration induces depolarization of airway smooth muscle (Souhrada *et al.*, 1981). Activation of the Na^+/K^+-pump by the addition of K^+ to K^+-free solution leads to membrane hyperpolarization (Souhrada *et al.*, 1981). In response to pump inhibition the smooth muscle may contract, or the response to other contractile agents may be augmented (Fleming, 1980; Souhrada & Souhrada, 1981). In contrast, the addition of K^+ to K^+-free medium results in hyperpolarization and relaxation which may be inhibited by ouabain (Fleming, 1980; Souhrada & Souhrada, 1981). The activity of the Na^+/K^+-pump is increased in ovalbumin-sensitized guinea-pigs. The resting membrane potential of the trachealis cells assumes a more negative value and the tissue exhibits enhanced reactivity to bronchoconstrictor agents such as histamine (Souhrada & Souhrada, 1984).

Cardiac glycosides cause contraction of canine airway smooth muscle *in vivo* (Marco *et al.*, 1968) and potentiate bronchoconstrictors acting on guinea-pig airway smooth muscle *in vitro* (Kolbeck *et al.*, 1981) and *in vivo* (Agrawal & Hyatt, 1986).

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Ouabain can evoke bronchoconstriction in asthmatics (Agrawal *et al.*, 1986).

In several mammalian species the airway epithelium modulates the reactivity of the underlying airway smooth muscle (see reviews by Vanhoutte, 1987; Fedan *et al.*, 1988) by releasing an epithelium-derived relaxing factor (EpDRF) (Flavahan *et al.*, 1985; Hay *et al.*, 1986; 1987; Ilhan & Sahin, 1986; Tschirhart & Landry, 1986). The relevance of this modulation to bronchial asthma lies in the fact that epithelial cell loss or damage occurs in association with airway hyperreactivity (Laitinen *et al.*, 1985).

Epithelium removal increases the reactivity of airway smooth muscle preparations to some agents but not to others (see Fedan *et al.*, 1988 for examples). This is a situation analogous to that which occurs in postjunctional supersensitivity, where increases in reactivity are sometimes associated with a reduction in electrogenic Na^+/K^+-pumping (Fleming, 1980). Procedures which inhibit the Na^+/K^+-pump prevent the endothelium-dependent relaxation of arterial smooth muscle to acetylcholine (De Mey & Vanhoutte, 1980; Rapoport *et al.*, 1985).

In view of the relationship between electrogenic Na^+/K^+-pumping, resting potential and reactivity in airway smooth muscle, we reasoned that the Na^+/K^+-pump could be involved in the regulation of smooth muscle reactivity by the epithelium. We examined the effects of epithelium removal on responses of airway smooth muscle after alterations in the activity of the Na^+/K^+-pump. A preliminary account of this study has been given (Raeburn *et al.*, 1987).

Methods

Preparation of tracheal strips

Male English short-hair guinea-pigs (340–500 g); Camm Research Institute, Inc.; Wayne, NJ) were killed by stunning and bleeding. The trachea was removed, placed in a modified Kreb-Henseleit (MKH) solution and cleaned. The trachea was opened by cutting in the longitudinal axis of the organ, diametrically opposite the trachealis muscle. Strips consisting of two adjacent cartilage rings were prepared. The strips were set up in organ baths containing MKH at 37°C for the isometric measurement of tension changes. The preparations were equilibrated for 1 h under an optimum resting load of 1 g, and washed every 15 min before the start of each experiment. The MKH contained (mM): NaCl 113, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 5.7. The solution was gassed with 95% O_2–5% CO_2 to give a pH of 7.4. K^+-free MKH was prepared by omitting KCl from the MKH and replacing KH_2PO_4 with NaH_2PO_4.

The epithelium was removed by gently rubbing the luminal surface with a cotton-tipped applicator. This procedure has been demonstrated histologically to remove the epithelium (Hay *et al.*, 1986). To minimize possible regional differences in reactivity, comparisons were made between adjacent strips from the same area of trachea, i.e. one intact and one rubbed. This permitted paired analysis between intact and epithelium-free preparations. The region of trachea used was randomized with respect to the particular protocol being studied.

Concentration-response curves

Cumulative concentration-response curves for ouabain, K^+ or methacholine were constructed by use of 1.6 fold (K^+), 3.2 fold (methacholine) or 10 fold (ouabain) concentration increments. The effect of ouabain on methacholine and K^+ concentration-response curves was assessed after 5 min incubation with ouabain.

K^+-free MKH and addition of K^+ to K^+-free MKH

After equilibration of the tracheal strips with MKH, the bathing solution was changed to K^+-free MKH. K^+-free MKH solution itself induced relaxant responses, which were monitored for up to 60 min. As no further changes in force occurred after 40 min, this time was chosen as the period of exposure to K^+-free MKH solution. The preparations were then either left under spontaneous tone, or tone was increased with methacholine. EC_{60} concentrations of methacholine (as observed in MKH) were used to raise the tone of intact or epithelium-free preparations, i.e. 2 μM and 1 μM, respectively. K^+ (10 or 30 mM) was then added. In some experiments, ouabain (0.1 or 1 μM) was added 5 min before the addition of K^+.

Effects of a mixture of inhibitors and indomethacin

Where indicated, the effects of a mixture of the following inhibitors were evaluated 15 min after it was added to the organ chambers: diphenhydramine (0.1 μM), atropine (0.1 μM), propranolol (1 μM) and tetrodotoxin (1 μM). These inhibitors were used to ascertain whether the effects of ouabain, K^+-free MKH, or the addition of K^+ to K^+-free MKH, were direct or involved the release of histamine, acetylcholine or catecholamines present in the preparations. The mixture of inhibitors had no effect on tone.

In some experiments, indomethacin (1 μM) was added to the baths, as indicated, to inhibit cyclo-oxygenase activity.

Vehicle controls (drug solvent only) were run in parallel; no vehicle effects were observed.

Statistical analysis

The results were expressed as % maximum response, force of contraction or relaxation (mg), or as the duration of the response(s) and are given as mean ± s.e.mean; n is the number of separate experiments. Geometric mean EC_{50} values were determined from linear regression analysis of probit-transformed data. The data were evaluated for differences by use of Student's t test for paired samples; $P < 0.05$ was considered significant. In the figures the s.e.mean is shown by the vertical bars, unless enclosed within the symbol.

Drugs

The following drugs (Sigma Chemical Co., St. Louis, MO, U.S.A.) were used: atropine sulphate, diphenhydramine hydrochloride, indomethacin, methacholine chloride, ouabain, (±)-propranolol and tetrodotoxin. All were dissolved in distilled water, with the exception of indomethacin (in 95% ethanol) and tetrodotoxin (in 0.9% NaCl). Drug solutions were prepared freshly each day.

Results

Ouabain concentration-response curves

Ouabain (0.1–10 μM) evoked contraction of intact and epithelium-free strips (Figure 1). The maximum responses of both preparations occurred at 10 μM; larger concentrations evoked no further contraction. EC_{50} values were obtained from probit analysis by use of two data points (as there is no probit value for 100%), and are, therefore, approximations. Epithelium removal increased significantly the sensitivity to ouabain ($-\log EC_{50}$ (M) values were 5.66 ± 0.18 in intact, and 6.30 ± 0.88 in denuded, preparations). The magnitude of responses, expressed both as % maximum response and force developed, also were significantly increased (maximum responses were 273 ± 89 mg in intact, and 560 ± 180 mg in denuded, preparations).

Similar results were obtained in the presence of the mixture of inhibitors. In denuded preparations the sensitivity to ouabain increased significantly, with $-\log EC_{50}$ (M) values decreasing from the control value of 5.77 ± 0.10 to 6.10 ± 0.30 in the absence of the epithelium. The maximum force of contraction again was increased after epithelium removal (from 356 ± 53 mg for intact preparations to 432 ± 108 mg for epithelium-free preparations), but the difference in the maximum force of contraction just failed to achieve significance.

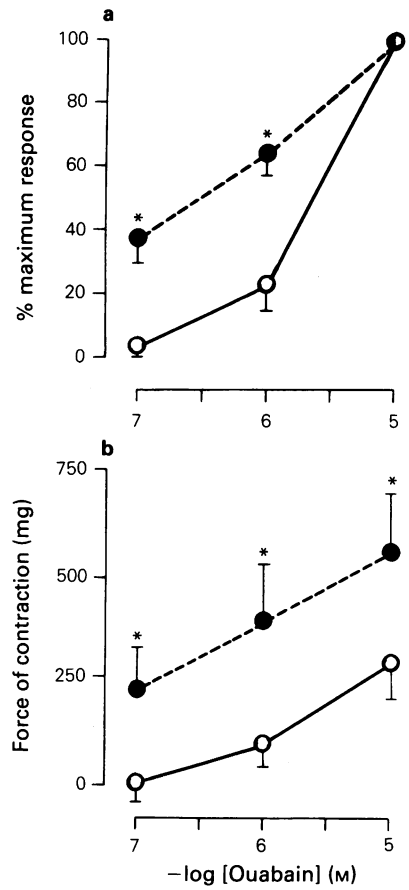


Figure 1 Guinea-pig isolated tracheal strips: effect of epithelium removal on the ouabain concentration-response curve. (○) Intact preparations; (●) epithelium-free preparations. In (a) the contractile responses to ouabain are given as % of the respective maximum responses; in (b) the responses to ouabain are expressed as the force of contraction. *Significantly different from epithelium-containing, paired preparations. $n = 5$.

The mixture of inhibitors did not significantly affect sensitivity to ouabain either in intact or denuded preparations.

Effect of K^+-free MKH

Upon exposure to K^+-free MKH, intact preparations relaxed, reaching a maximum relaxation of 533 ± 60 mg at approximately 40 min (Figures 2 and 3). Epithelium-free strips also relaxed during the first 10 min but then gained some tone. The peak tone achieved was less than the initial level, was well-maintained between 20 and 30 min after exposure to the K^+-free MKH but thereafter began to decline.

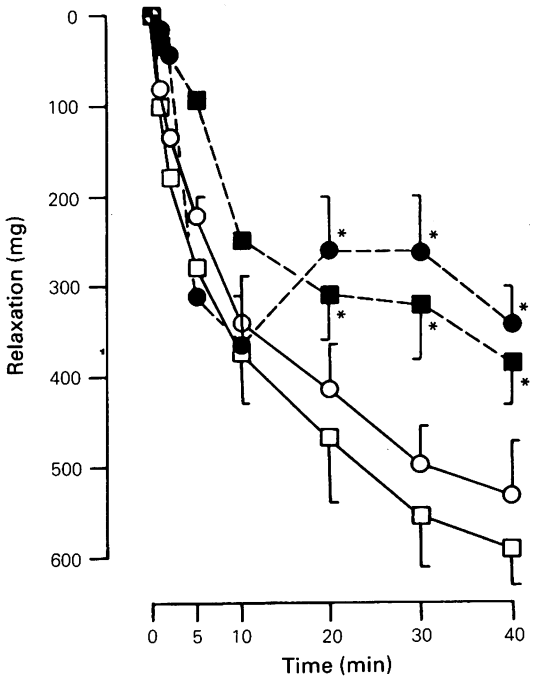


Figure 2 Guinea-pig isolated tracheal strips: time-course of the effect of K^+-free modified Krebs-Henseleit solution (MKH) on tone of intact and epithelium-free preparations in the absence or presence of the mixture of inhibitors. (□, ○) Intact preparations; (■, ●) epithelium-free preparations. (○, ●) Mixture of inhibitors absent; (□, ■) mixture of inhibitors present. Ordinate scale: the magnitude of relaxation from the level of force present in MKH solution before the solution was changed to K^+-free MKH. * Significantly different from epithelium-containing, paired preparations. $n = 9$, mixture of inhibitors absent or present.

Neither the time-course nor the magnitude of relaxation at 40 min (593 ± 56 mg) was affected in the presence of the mixture of inhibitors. In the absence or presence of the mixture of inhibitors, the relaxation at 40 min by epithelium-free strips was significantly less than that seen in intact preparations. No relaxation was observed during this time period when intact or epithelium-free strips were incubated in K^+-containing MKH.

Effects of K^+ added to preparations incubated in K^+-free MKH

K^+ (10 or 30 mM), added to preparations equilibrated with K^+-free MKH and in the absence or presence of methacholine, caused an initial relaxation (Figure 3; Table 1); this was followed by an increase in tone to a level greater than the pre-K^+ level. Relaxations produced by 10 mM K^+ (the EC_{25} for contraction in normal MKH) were larger and were of longer duration than relaxations to 30 mM K^+ (the EC_{50} for contraction in normal MKH). Relaxations to 30 mM K^+ in methacholine-contracted strips were larger than those obtained in the absence of methacholine. With one exception (not shown), the magnitude and duration of the K^+-induced relaxations were always greater in the epithelium-free preparations. When the mixture of inhibitors was present and the K^+ concentration was 30 mM, the relaxation to K^+ was attenuated and there was no difference in the magnitude of the relaxations between intact and epithelium-free preparations. However, the duration of relaxation was prolonged in denuded preparations.

In intact and rubbed preparations indomethacin (1 μM) alone was without effect on the response to the re-addition of K^+, i.e. results similar to those shown in Figure 3 were obtained ($n = 4$).

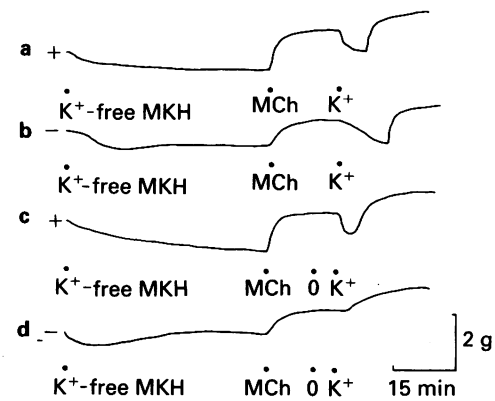


Figure 3 Guinea-pig isolated tracheal strips equilibrated with K^+-free modified Krebs-Henseleit solution (MKH) and methacholine: effects of epithelium removal and ouabain (0; 0.1 μM) on relaxant responses evoked by 30 mM K^+. (a and b) K^+-induced relaxation of intact (a) and epithelium-denuded (b) preparations in the absence of ouabain; (c and d) K^+-induced relaxation of intact (c) and epithelium-denuded (d) preparations in the presence of ouabain. The concentrations (EC_{60}) of methacholine (MCh) were 1 μM in denuded preparations and 2 μM in intact preparations. (+) and (–) refer to intact and rubbed preparations, respectively. After their addition (dots), methacholine, ouabain and K^+ remained present for the duration of the experiment. Note that in (a, b, c) the relaxation to K^+ was followed by an abrupt (a, b), gain in tone in the continued presence of K^+. The tracings shown are representative of identical results obtained in six separate experiments. Note that ouabain itself had no effect on methacholine-induced tone.

Table 1 Effect of K^+ (10 or 30 mM) on intact (+Epi) and epithelium-free (−Epi) tracheal preparations incubated for 40 min in K^+-free Krebs-Henseleit solution

Treatment	n	*Relaxation* (mg) +*Epi*	*Relaxation* (mg) −*Epi*	*Duration of relaxation* (s) +*Epi*	*Duration of relaxation* (s) −*Epi*
Spontaneous tone					
10 mM K^+	4	825 ± 87*	1038 ± 80*‡	550 ± 79	1150 ± 120‡
30 mM K^+	6	180 ± 77*	345 ± 89*‡	323 ± 6	413 ± 26‡
Methacholine-contracted					
30 mM K^+	6	718 ± 138*	1125 ± 193*‡	405 ± 12	615 ± 43‡
30 mM K^+, mixture of inhibitors present	6	388 ± 53*	344 ± 91*	324 ± 53	442 ± 72‡
10 mM K^+, 0.1 μM ouabain present	4	210 ± 56*	†	130 ± 18	ND
30 mM K^+, 0.1 μM ouabain present	6	375 ± 102*	†	142 ± 19	ND

* Transient relaxation followed by sustained contraction (see Figure 3).
† Only contractions were obtained.
‡ Significantly different from +Epi.
ND, not determined; response stable > 2 h.

Effect of ouabain on K^+-induced relaxation responses

The effect of ouabain (0.1 μM) on the relaxation response of methacholine-contracted strips to 30 mM K^+ depended on whether the epithelium was present or not (Figure 3; Table 1). In intact preparations, the K^+-induced relaxation was still evident in the presence of ouabain (Figure 3c); the relaxation, which had an altered profile, was followed by a sustained contraction. In the absence of the epithelium, however, ouabain (0.1 μM) inhibited the K^+-induced relaxation (Figure 3c); the response was converted to a contraction. Similar results were obtained with 10 mM K^+, and in tissues under 'basal' tone (not shown). In intact and rubbed control preparations incubated only in normal MKH and pre-contracted with methacholine, K^+ (10 and 30 mM) elicited only contractile responses (not shown; $n = 3$). The K^+-induced relaxations were therefore obtained only in preparations which had been incubated in K^+-free MKH. Identical results were obtained in the presence of the mixture of inhibitors ('basal' tone preparations). At a higher concentration of ouabain (1 μM), the relaxation of intact preparations was fully inhibited, and K^+ elicited a contraction as it did in the epithelium-free preparations, i.e. results comparable to Figure 3d were obtained ($n = 5$).

Effect of ouabain on methacholine and K^+ concentration-response curves

In intact preparations ouabain (0.1 μM) produced a significant (ca. 4 fold) leftward shift in the methacholine concentration-response curve, and a significant (ca. 1.6 fold) rightward shift in the K^+ concentration-response curve (Figure 4; Table 2). In the absence of the epithelium, ouabain had no effect on the sensitivities of the preparations to meth-

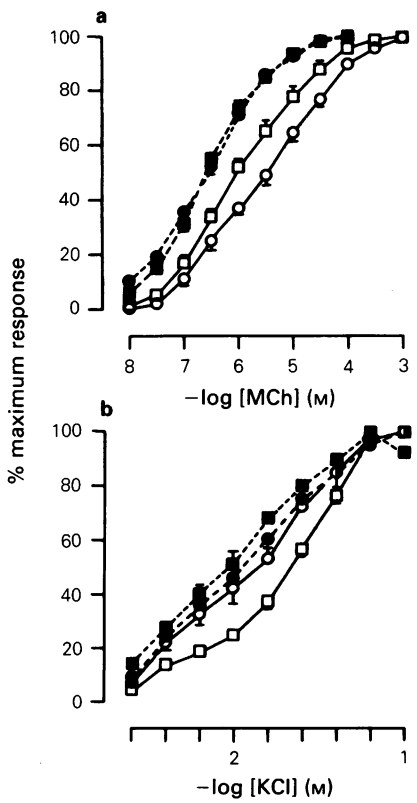


Figure 4 The effect of ouabain (0.1 μM) on cumulative concentration-response curves for methacholine (a; $n = 6$) and K^+ (b; $n = 5$) in guinea-pig isolated tracheal strips. (□, ○) Intact preparations; (■, ●) epithelium-free preparations. (○, ●) Ouabain absent (control); (□, ■) ouabain present. Responses are plotted as a % of the preparations' respective maximum responses.

Table 2 Effect of ouabain (0.1 μM) on the sensitivities and maximum responses to methacholine and K^+ of intact (+Epi) and epithelium-free (−Epi) tracheal strips

		+Epi		−Epi	
Agent	n	$-log\ EC_{50}$ (M)	*Maximum* (mg)	$-log\ EC_{50}$ (M)	*Maximum* (mg)
Methacholine					
Control	6	5.32 ± 0.14	743 ± 108	6.70 ± 0.01*	590 ± 53
Ouabain	6	5.89 ± 0.12†	600 ± 95	6.70 ± 0.01*	643 ± 141
K^+					
Control	5	1.87 ± 0.10	1285 ± 330	1.92 ± 0.08	1120 ± 181
Ouabain	5	1.67 ± 0.01†	920 ± 133	1.99 ± 0.10*	910 ± 180

* Significantly different from intact preparations.
† Significantly different from control.

acholine or K^+ (Figure 4; Table 2). Ouabain (0.1 μM) did not affect the maximum responses to methacholine or K^+ in strips containing or denuded of epithelium (Table 2).

Discussion

This study indicates that the epithelium influences the response of airway smooth muscle to alterations in electrogenic Na^+/K^+-pumping activity caused by ouabain, K^+-free MKH and the addition of K^+ to preparations in K^+-free MKH. The results indicate a direct action of ouabain, K^+-free MKH and added K^+ on the muscle, and an indirect component involving the epithelium. Responses to these manipulations do not appear to involve non-epithelial, indirect components to any appreciable extent, since similar results were obtained in the presence of the mixture of inhibitors. Chideckel *et al.* (1987) also found no evidence for an indirect component in the responses of airway smooth muscles of several species to ouabain.

The reactivities of intact guinea-pig tracheal preparations to methacholine and K^+ were altered by ouabain in an epithelium-dependent manner. Ouabain decreased the reactivity to K^+ only in the presence of the epithelium, and had no effect in epithelium-free preparations. Ouabain increased the responsiveness of intact preparations to methacholine, and, as such, partially mimicked the effect of epithelium removal. However, ouabain had no effect on the reactivity to methacholine in denuded preparations. The effects of ouabain and epithelium removal were not additive, and could, therefore, involve the same mechanism, i.e. the electrogenic Na^+/K^+-pump.

Both intact and epithelium-free preparations incubated in K^+-free MKH responded to added K^+ with relaxation; this presumably occurs in association with membrane hyperpolarization due to activation of electrogenic Na^+/K^+-pumping (Souhrada *et al.*, 1981). In the presence of ouabain (0.1 μM) to block activation of the Na^+/K^+-pump, the addition of K^+ to epithelium-free preparations caused only a contraction. As the pump was inhibited, this response presumably reflected membrane depolarization. Our results obtained with denuded strips are identical to those obtained by Souhrada *et al.* (1981), who also removed the epithelium but used a higher concentration of ouabain (10 μM).

An explanation must be sought for our observation that the addition of K^+ to intact preparations, equilibrated with K^+-free MKH and with 0.1 μM ouabain, induced relaxation while contraction was observed under similar circumstances for the epithelium-denuded preparations. A satisfactory explanation should also take into account our observation that 1 μM ouabain abolished the relaxant responses to K^+ in both intact and epithelium-free preparations. Two possibilities, either alone or in combination, may account for our findings. The first is that the production and/or release of an inhibitory factor is linked to the activity of the epithelial Na^+/K^+-pump. The basolaterally-located Na^+/K^+-pump in epithelial cells (reviewed by Nadel *et al.*, 1985; Widdicombe *et al.*, 1987), which is involved in glandular secretory activity (Marin, 1986), should be affected, although not necessarily identically, by the perturbations which affect the corresponding pump in the trachealis, i.e. it would be inhibited in K^+-free MKH, and activated upon the addition of K^+. Our findings can be explained if the epithelial pump differs from the muscle pump in other aspects, such as sensitivity to ouabain and K^+.

The alternative hypothesis is that EpDRF stimulates the activity of the muscle electrogenic Na^+/K^+-pump. The K^+-induced relaxation of intact preparations in the presence of 0.1 μM ouabain might reflect the release of EpDRF from the epithelium, such as has been described by Munakata *et al.* (1988). However, the inhibitory effect of 1 μM ouabain on K^+-induced relaxation of intact preparations must also be explained, and it is convenient

to consider a muscle Na^+/K^+-pump site to explain why this higher concentration is effective. The effects of ouabain and of the presence of the epithelium are, in a sense, opposite. EpDRF possibly facilitates muscle pump turnover when K^+ is added, even when the pump is partially inhibited (by 0.1 μM ouabain), or EpDRF reduces the binding of 0.1 μM ouabain, allowing K^+ to promote pump cycling. However, the amount of EpDRF is insufficient to prevent the effect of 1 μM ouabain, and the response to K^+ is abolished.

Inhibition of electrogenic Na^+/K^+-pumping with ouabain or reduced extracellular K^+ results in a partial membrane depolarization and alters airway smooth muscle reactivity to several agents (Souhrada & Souhrada, 1981; Souhrada *et al.*, 1981; Gunst & Stropp, 1988). The loss of an epithelium-derived factor, which stimulates the muscle Na^+/K^+-pump and causes membrane hyperpolarization, could explain the increases in reactivity after epithelium removal. However, epithelium removal does not significantly change the resting membrane potential in canine bronchial smooth muscle, but nevertheless potentiates the contraction and depolarisation induced by acetylcholine (Gao & Vanhoutte, 1988). It must be admitted that these findings in canine preparations are difficult to reconcile with the hypothesis that a change in activity of the Na^+/K^+-pump in the smooth muscle rather than in the epithelium is involved in the effects observed in the present study.

In the absence of ouabain the relaxation of intact preparations to K^+ was faster in onset compared to the epithelium-free strips, but its duration was shorter (Figure 3). It is probable that a linkage of EpDRF production to the epithelial Na^+/K^+-pump, or a stimulating action of EpDRF on the smooth muscle pump, explains the difference in the relaxation profiles of intact and epithelium-free strips. It is unlikely that K^+-induced relaxation involved a direct action of EpDRF, inasmuch as it was produced by 10 mM K^+. The hyperosmotic challenge-induced release of the factor (Munakata *et al.*, 1988) at 10 mM K^+ would have led to very small relaxation responses compared to the large ones seen in the present study.

Why the preparations relaxed when exposed to K^+-free MKH is unclear. This effect, not observed by Souhrada & Souhrada (1981), was seen by Chideckel *et al.* (1987). It is possible that, in our experiments, hyperpolarization in K^+-free MKH has a greater initial effect on membrane potential than depolarization resulting from inhibition of electrogenic pump current (Fleming, 1980). Indeed, Souhrada *et al.* (1981) observed hyperpolarization to K^+-free solution at times similar to those where we saw relaxation.

When placed in K^+-free solution, denuded preparations first lost and then gained some tone, whereas intact preparations only exhibited relaxation (Figures 2 and 3). The difference in the profiles of this response appears to be related to the presence of the epithelium, but the mechanism involved is unknown.

It is likewise difficult to explain the reduced sensitivity of intact strips to K^+ in the presence of ouabain under conditions that increased sensitivity to methacholine. This finding argues against the hypothesis that the potentiation of responses to methacholine by ouabain or by epithelium removal results from reduced electrogenic Na^+/K^+-pumping, since responses to K^+, being triggered electromechanically, should in theory have been potentiated (Fleming, 1980). Nevertheless, the findings that only ouabain reduced sensitivity to K^+, and that this effect occurred only in intact preparations, favours the general conclusion that the epithelium affects reactivity of the smooth muscle by a mechanism involving the Na^+/K^+-pump.

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The mode of action of caffeine on catecholamine release from perfused adrenal glands of cat

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1 Adrenaline and noradrenaline secretion induced by caffeine was investigated in the perfused cat adrenal glands.

2 Caffeine (10–80 mM) caused a dose-dependent increase in both adrenaline and noradrenaline secretion when applied for 1 min and 10 min after replacing Ca^{2+} with 10^{-5} M EGTA in the perfusion solution. The ratio of adrenaline to noradrenaline was about 1:1. Mg^{2+} and/or Ca^{2+} inhibited the response to caffeine.

3 When caffeine (40 mM) was repeatedly applied in the absence of extracellular Ca^{2+}, the secretory response almost disappeared but only at the second challenge with caffeine. However, the response was partially restored after readmission of Ca^{2+} (2.2 mM) and was augmented after the readmission of Ca^{2+} with ouabain (10^{-5} M).

4 Caffeine-induced secretion of adrenaline and noradrenaline increased with the increase in the preloaded concentration of Ca^{2+} and attained a maximum at 16 mM Ca^{2+}.

5 During perfusion with Ca^{2+}-free Locke solution containing hexamethonium (10^{-3} M), acetylcholine (10^{-4} M) caused increases in both adrenaline and noradrenaline secretions with a ratio of about 1:2. The secretory responses were partially inhibited by preceding stimulation with exposure to caffeine (80 mM).

6 These results suggest that caffeine mobilizes Ca^{2+} from an intracellular storage site that may not be entirely the same as that linked to muscarinic receptors, and causes an increase in both adrenaline and noradrenaline secretion from cat adrenal chromaffin cells.

Introduction

Xanthine derivatives, caffeine and theophylline are known to increase catecholamine secretion from perfused adrenal glands of the cat (Peach, 1972) and ox (Poisner, 1973a), isolated and halved adrenal glands of the rat (Cohen & Gutman, 1979) and cultured bovine adrenal chromaffin cells (Morita *et al.*, 1987a,b). In these papers, the stimulant action of xanthine derivatives has been interpreted as associated with an accumulation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) resulting from the inhibition of cyclic nucleotide phosphodiesterase, despite the lack of any direct supporting evidence.

According to Poisner (1973b), stimulation by caffeine is as effective in increasing catecholamine secretion in the absence of extracellular Ca^{2+} as in its presence, and the response to caffeine in Ca^{2+}-free medium is increased by the loading of Ca^{2+} stores in perfused bovine adrenal glands. Recently, we found that caffeine was much more effective in increasing catecholamine secretion in the absence of extracellular Ca^{2+} and Mg^{2+} than in their presence, and that the responses to caffeine and muscarinic receptor activation were reversibly inhibited by an intracellular Ca^{2+} antagonist, TMB-8, in perfused cat adrenal glands (Yamada *et al.*, 1988). These findings support the view that caffeine (Poisner, 1973b), as well as muscarinic agonists (Nakazato *et al.*, 1984; 1988), mobilize intracellular Ca^{2+} from some storage sites to stimulate catecholamine secretion from adrenal chromaffin cells.

The present experiments were designed to gain an insight into the properties of the intracellular Ca^{2+} storage site responsible for this agonist-induced catecholamine secretion. This paper describes the effects

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of divalent cations, ouabain and Ca^{2+} loading on caffeine-induced catecholamine secretion in perfused adrenal glands of the cat.

Methods

Isolation and perfusion of cat adrenal glands

Cats (1.5–4.0 kg) of either sex were anaesthetized with sodium pentobarbitone (40 mg kg^{-1}, i.p.). Under artificial respiration, both adrenal glands were perfused through a cannula inserted into the lower aorta below the renal artery at a flow rate of about 0.7 ml min^{-1} at room temperature (approximately 25°C). The adrenal effluent was drained through a cannula inserted into the vena cava at the same level as the arterial cannula. After this procedure was completed, the perfused adrenal glands were isolated and placed on a moistened cork board. The adrenal effluents were collected at 5 min intervals and acidified with 8 M perchloric acid, giving a final concentration of 0.4 M, and stored on ice until assayed. Drugs were administered 40–60 min after the start of perfusion. Secretagogues were administered for 1 min prior to the following 5 min sample-collection period, because of the 1 min dead time of the arterial cannula.

Perfusion media

The standard perfusion medium was modified Locke solution of the following composition (mM): NaCl 154, KCl 5.6, $CaCl_2$ 2.2, Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0) 3 and glucose 10. In Ca^{2+}-free solutions, $CaCl_2$ was replaced with glycoletherdiaminetetraacetic acid (EGTA) (10^{-5} M or 10^{-4} M). The perfusion medium was continuously aerated with O_2.

Catecholamine assay

Catecholamines (adrenaline and noradrenaline) were separated by high performance liquid chromatography (h.p.l.c.) (Jasco) and assayed by electrochemical detector (LC-4B, BAS). The treatment of samples for h.p.l.c. was carried out according to the method described by Salzman & Sellers (1982). Total catecholamine levels were also assayed by a conventional fluorometric method (Anton & Sayre, 1962).

Materials

The following drugs were used: acetylcholine chloride (Ovisort, Daiichi), caffeine monohydrate (Wako Pure Chem.), hexamethonium chloride dihydrate (Wako Pure Chem.), glycoletherdiaminetetraacetic acid (EGTA, Wako Pure Chem.) and g-strophanthin (ouabain, Tokyo Kasei). All compounds were dissolved in Locke solution.

Results

Dose-dependent increase in catecholamine secretion induced by caffeine

Various concentrations of caffeine were applied to the adrenal glands for 1 min, 10 to 15 min after the replacement of Ca^{2+} with 10^{-5} M EGTA in the perfusion solution. Adrenaline and noradrenaline secretions in response to caffeine started to increase at 10 mM and increased with the increase in the concentration of caffeine until they attained a maximum at 40 mM. At concentrations over 40 mM, the secretory responses declined. As shown in Figure 1, the ratio between adrenaline and noradrenaline in the

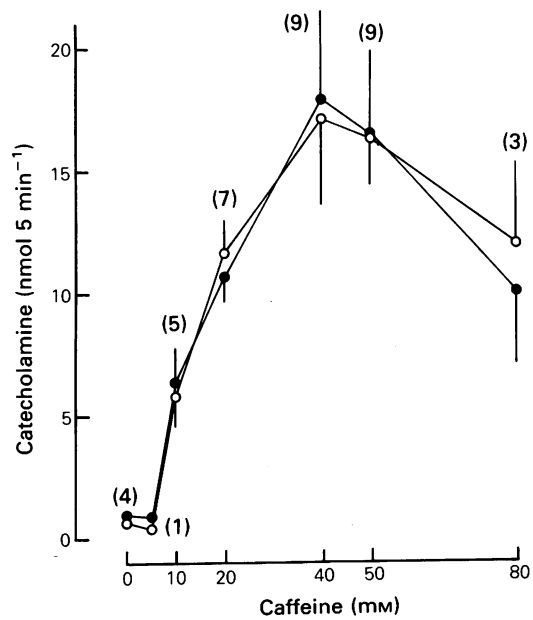


Figure 1 Dose-response curve for caffeine-induced catecholamine secretion in the absence of extracellular Ca^{2+}. A single dose of caffeine ranging from 5 to 80 mM was applied for 1 min to single preparations of perfused adrenal glands 10 to 15 min after replacing Ca^{2+} with 10^{-5} M EGTA in the standard Locke solution. Means, with s.e.means indicated by vertical lines that exceed the size of the symbol, of adrenaline (○) and noradrenaline (●) secretions are shown. The numbers in parentheses represent the number of experiments. The ordinate scale represents the amounts of catecholamines released and the abscissa scale the concentration of caffeine.

response to caffeine was approximately 1:1 with all concentrations of caffeine used. The maximum amounts of adrenaline and noradrenaline released were 17.2 ± 3.5 and 18.0 ± 3.6 nmol 5 min^{-1} respectively.

Effects of Mg^{2+} and Ca^{2+} on caffeine-induced catecholamine secretion

The adrenal glands were sequentially stimulated with caffeine (40 mM) in the presence of various concentrations of Mg^{2+} during perfusion with Ca^{2+}-free Locke solution containing 10^{-5} M EGTA. The concentration of Mg^{2+} was progressively decreased from 1.2 mM to 0.4 mM and 0.1 mM. Before each stimulation with caffeine, the adrenal glands were loaded with Ca^{2+} by exposure to the standard Locke solution for 20 min. To prevent Ca^{2+} from evoking the secretory response by itself when it was added after exposure to Ca^{2+}-free medium, 1.2 mM Mg^{2+} was always included in the standard Locke solution perfusate for Ca^{2+} loading for the first 5 min, after which it was removed. As shown in Figure 2a, the secretory responses of adrenaline and noradrenaline to caffeine started to appear when Mg^{2+} was reduced to 0.1 mM. When Mg^{2+} was totally removed, both adrenaline and noradrenaline secretions were further increased to the same degree.

In a similar fashion, the effect of reducing the concentration of Ca^{2+} on caffeine-induced catecholamine secretion was studied (Figure 2b). Until the concentration of Ca^{2+} was reduced to 0.3 mM, there appeared to be no, or only a trace of, secretory response to caffeine. However, adrenaline and noradrenaline secretions were suddenly increased by caffeine when Ca^{2+} was replaced with 10^{-5} M EGTA.

Dependence of caffeine-induced catecholamine secretion on the concentration of Ca^{2+} preloaded

We examined whether or not the caffeine-induced catecholamine secretion was dependent on the preloaded concentration of Ca^{2+} within the range of 2.2 mM to 32 mM. First, caffeine (40 mM) was applied for 1 min, 10 min after the replacement of Ca^{2+} with EGTA (10^{-4} M) in the standard perfusion solution. Then the adrenal glands were loaded with 8 mM Ca^{2+} for 25 min. After the end of this Ca^{2+} loading, the glands were again stimulated with a second dose of caffeine (40 mM). In a similar fashion, the effects of loading 16 mM and 32 mM Ca^{2+} on the responses to the third and fourth doses of caffeine, respectively, were observed. As shown in Figure 3, caffeine-induced adrenaline and noradrenaline secretions were increased with the increase in the concentration of Ca^{2+} loading until they attained a maximum at 16 mM. The rates of increase were almost the same

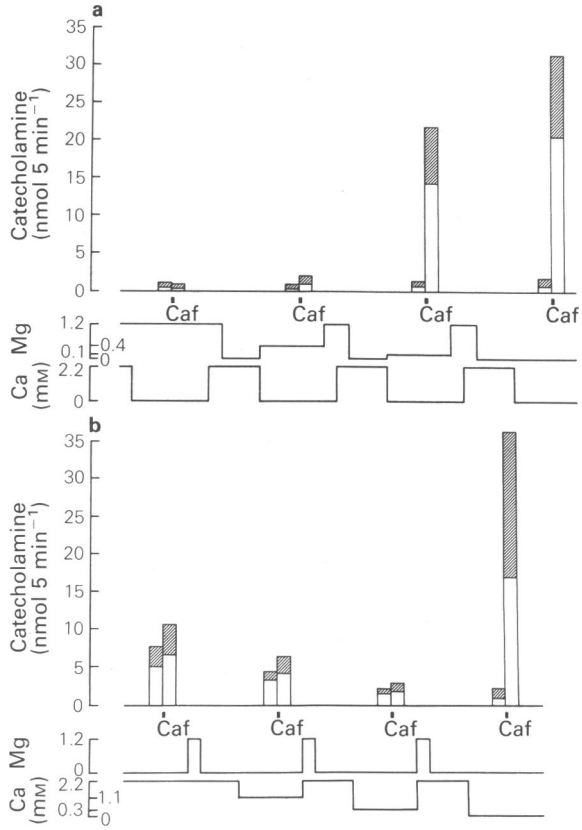


Figure 2 Effects of varying the concentrations of Mg^{2+} and Ca^{2+} on caffeine-induced catecholamine secretion. Caffeine (40 mM) was sequentially applied for 1 min in the presence of 1.2, 0.4 and 0.1 mM Mg^{2+} and in the absence of Mg^{2+} during perfusion with Ca^{2+}-free Locke solution containing 10^{-5} M EGTA in (a), and in the presence of 2.2, 1.1 and 0.3 mM Ca^{2+} and 10^{-5} M EGTA instead of Ca^{2+} during perfusion with Mg^{2+}-free solution in (b). Preceding each challenge with caffeine, the adrenal glands were loaded with Ca^{2+} by exposure to the standard Locke solution for 20 min. To prevent Ca^{2+} from evoking the secretory response by itself, 1.2 mM Mg^{2+} was added during the first 5 min of the Ca^{2+} loading period. Caffeine was applied 15 min after the end of Ca^{2+} loading. Columns represent the amounts of adrenaline (open columns) and noradrenaline (hatched columns). A representative experiment with each is illustrated.

for adrenaline and noradrenaline. Qualitatively similar results were obtained in the three other experiments.

Effects of ouabain on caffeine-induced catecholamine secretion

Previously, we suggested that ouabain increases either the entry of extracellular Ca^{2+} or the concen-

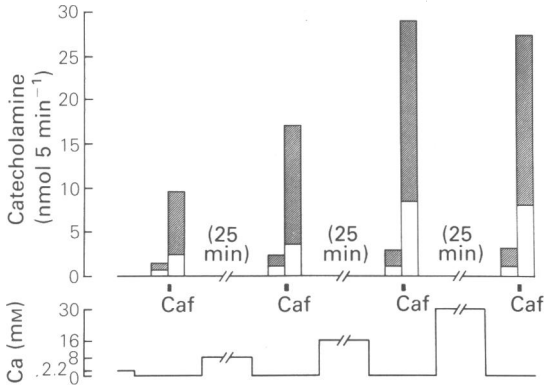


Figure 3 Effect of varying the concentration of preloaded Ca^{2+} on caffeine-induced catecholamine secretion. Caffeine (Caf, 40 mM) was applied for 1 min, 10 min after the removal of Ca^{2+} from the standard Locke solution in the presence of 10^{-4} M EGTA. Then the adrenal glands were loaded with 8 mM Ca^{2+} in the presence of 1.2 mM Mg^{2+} for 25 min and, 10 min after this ended, Ca^{2+} loading took place, after which caffeine was again applied in the presence of 10^{-4} M EGTA instead of both Ca^{2+} and Mg^{2+}. In a similar fashion, the effects of loading 16 and 32 mM Ca^{2+} on caffeine-induced response were observed in a subsequent period. Columns represent the amounts of adrenaline (open columns) and noradrenaline (hatched columns). A representative experiment is illustrated.

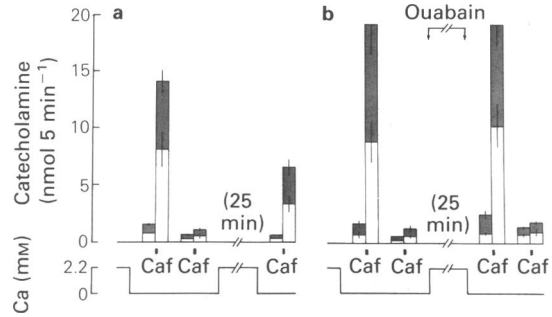


Figure 4 Effect of ouabain on deteriorated caffeine-induced catecholamine secretion. Caffeine (Caf, 40 mM) was applied for 1 min during perfusion with Ca^{2+}-free Locke solution containing 10^{-5} M EGTA before and after readmission of Ca^{2+} without (a) and with (b) ouabain (10^{-5} M). Columns represent the means (and, if vertical lines are present, s.e.) of adrenaline (open columns) and noradrenaline (hatched columns) obtained from 4 experiments with each.

tration of intracellularly stored Ca^{2+} responsible for acetylcholine-receptor mediated catecholamine secretion (Nakazato *et al.*, 1986; Yamada *et al.*, 1989). It was, therefore, of interest to study how ouabain affected caffeine-induced catecholamine secretion.

First, the reproducibility of caffeine-induced catecholamine secretion was determined by repeatedly applying caffeine (40 mM) for 1 min at 15 min intervals in the absence of extracellular Ca^{2+} throughout the course of the experiment. It was found that both adrenaline and noradrenaline secretions induced by caffeine disappeared only on the second challenge with the drug. However, the secretory responses of adrenaline and noradrenaline were restored to about 40 and 50% of the control level after the reintroduction of Ca^{2+} (Figure 4a). In a similar fashion, the glands were challenged with two doses of caffeine (40 mM) at 15 min intervals in the absence of extracellular Ca^{2+}. Then the perfusion solution was changed to the standard Locke solution containing ouabain (10^{-5} M) for 25 min, and the glands were again stimulated with caffeine (40 mM). Caffeine applied after exposure to ouabain caused releases of adrenaline and noradrenaline that were almost the same as or greater in magnitude than those induced by the first challenge with caffeine (Figure 4b).

Effect of caffeine on the subsequent acetylcholine-induced catecholamine secretion

Previously, we assumed that the activation of muscarinic receptors mobilized Ca^{2+} from some intracellular Ca^{2+} pools to release catecholamine in adrenal chromaffin cells (Nakazato *et al.*, 1984; 1988; Yamada *et al.*, 1988; 1989). Thus, it was of interest to examine whether caffeine and muscarinic agonists utilized a common hypothetical intracellular Ca^{2+} pool to release catecholamine.

In the absence of extracellular Ca^{2+} and Mg^{2+}, three doses of acetylcholine (10^{-4} M) were sequentially applied for 1 min at 55 min intervals in the presence of hexamethonium (10^{-3} M) throughout the course of the experiment. Prior to each challenge with acetylcholine, the adrenal glands were loaded with 2.2 mM Ca^{2+} for 25 min. The response to acetylcholine was gradually decreased on repetition of stimulation (Figure 5a). As previously observed (Yamada *et al.*, 1989), acetylcholine caused a preferential release of noradrenaline (about 70% of the total catecholamine released) in the absence of extracellular Ca^{2+}. To determine how caffeine influenced acetylcholine-induced catecholamine secretions, caffeine (80 mM) was applied for 1 min before the second challenge with acetylcholine. As shown in Figure 5b, acetylcholine applied after caffeine still caused increases in both adrenaline and noradrenaline secretions, though the amounts released were partially inhibited. The responses were restored at the third challenge with acetylcholine. The ratio of

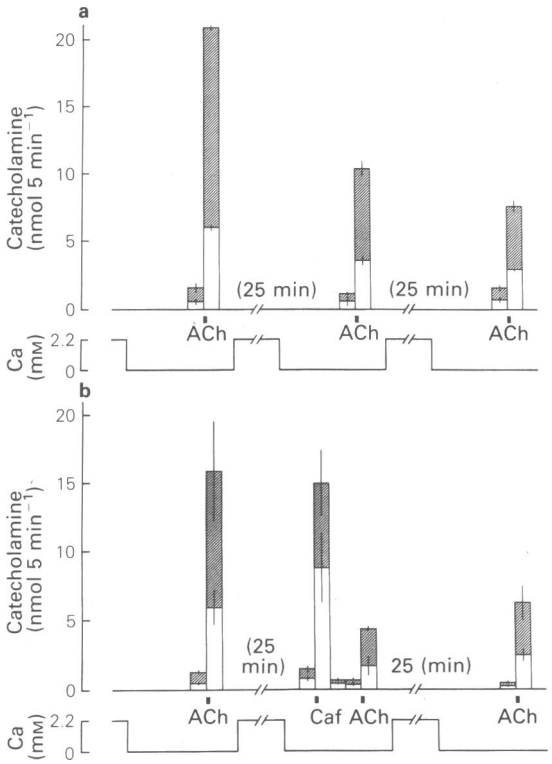


Figure 5 Effect of caffeine on acetylcholine-induced catecholamine secretion. Acetylcholine (ACh, 10^{-4} M) was applied for 1 min, 25 min after the removal of Ca^{2+} from the standard Locke solution in the presence of 10^{-5} M EGTA. Then the adrenal glands were loaded with Ca^{2+} for 25 min by perfusing the Locke solution containing 1.2 mM Mg^{2+} before each challenge with ACh in the following period. The control experiment for ACh-induced responses is shown in (a). In (b), caffeine (Caf, 80 mM) was applied 10 min before the second challenge with ACh. Columns represent the means (and, if vertical lines are present, s.e.) of adrenaline (open columns) and noradrenaline (hatched columns) obtained from 3 experiments.

adrenaline to noradrenaline in the responses to acetylcholine was fairly constant at about 1:2 throughout the experiment.

Discussion

As we previously reported, caffeine was much more effective in releasing catecholamine in the absence of extracellular Ca^{2+} than in its presence (Yamada *et al.*, 1988). In fact, Ca^{2+}, even in lower than normal concentrations, inhibited caffeine from initiating the secretory response. On the other hand, Ca^{2+} loading was indispensible for caffeine to be effective in releasing catecholamine in the subsequent Ca^{2+}-free period. Therefore, it seems likely that extracellular Ca^{2+} plays a dual role in the pharmacological action of caffeine; it both inhibits caffeine-induced catecholamine secretion when it is present outside the chromaffin cells and maintains or augments the action of caffeine when it enters the cells. Mg^{2+} has only an inhibitory action. The normal concentration of Ca^{2+} is known to cause a large increase in catecholamine secretion when added after exposure to Ca^{2+}- and Mg^{2+}-free medium in perfused cat adrenal glands (Douglas & Rubin, 1961). A low concentration of Mg^{2+} such as 2 mM completely blocked this Ca^{2+}-induced catecholamine secretion (Douglas & Rubin, 1963). The most probable action of this Ca^{2+} deprivation was assumed to be an increase in cell membrane permeability that allowed extracellular Ca^{2+} to penetrate into the medullary cells. The reason for this may be that both Ca^{2+} and Mg^{2+} have a stabilizing action on the plasma membrane (Frankenhaeuser & Hodgkin, 1957). Therefore, divalent cations (Ca^{2+} and Mg^{2+}) may decrease the caffeine-induced secretory response through a stabilizing effect on the plasma membrane. In addition, Mg^{2+} may block the catecholamine secretion induced by caffeine by competing with Ca^{2+} for intracellular sites (Aguirre *et al.*, 1977).

As with muscarinic receptor-mediated catecholamine secretion (Nakazato *et al.*, 1984; 1988), the response that deteriorated as a result of repeated application of caffeine in the absence of extracellular Ca^{2+} was partially restored after readmission of Ca^{2+}, depending on its concentration, and was augmented after the readmission of Ca^{2+} with ouabain. In addition, we previously found that an intracellular Ca^{2+} antagonist, TBM-8, reversibly blocked the catecholamine secretions induced both by caffeine and by acetylcholine in the absence of extracellular Ca^{2+} (Yamada *et al.*, 1988). These data fit well with the view that caffeine, as well as muscarinic agonists, possesses an intracellular Ca^{2+} storage site from which the agonists release Ca^{2+} into the cytosol to cause catecholamine secretion (Poisner, 1973b; Nakazato *et al.*, 1984; 1988). However, the process of caffeine-induced catecholamine secretion seems to differ from that of muscarinic agonists, because muscarinic agonists are able to increase catecholamine secretion both in the presence and in the absence of extracellular Ca^{2+} and Mg^{2+} (Yamada *et al.*, 1988; 1989). Recently, it was reported that Ca^{2+} stores in smooth muscle consisted of two classes; one possessing both caffeine (Ca^{2+}-induced Ca^{2+} release) and inositol 1,4,5-triphosphate (IP_3)-induced Ca^{2+} release mechanisms, and the other only an IP_3-induced Ca^{2+} release mechanism (Iino *et al.*, 1988). In our experiments, it was also suggested that Ca^{2+} stores linked to caffeine and muscarinic

agonists do not entirely overlap, because acetylcholine was still effective in releasing catecholamine after a substantial secretory response to caffeine during perfusion with Ca^{2+}-free Locke solution containing hexamethonium (10^{-3} M). The identification of these hypothetical Ca^{2+} storage sites and the mechanism releasing Ca^{2+} from them will certainly be an important subject for further investigation.

Unlike muscarinic agonists, which caused a preferential release of noradrenaline in the absence of extracellular Ca^{2+} (Yamada *et al.*, 1989), caffeine increased adrenaline and noradrenaline secretions in approximately equal ratios throughout the concentrations used. Furthermore, in contrast ouabain enhanced noradrenaline secretion much more than adrenaline secretion in response to muscarinic receptor activation (Yamada *et al.*, 1989), it caused an equal enhancement of the two catecholamines in caffeine-induced secretory response. It appears, therefore, that the population or the capacity of Ca^{2+} storage sites sensitive to caffeine and muscarinic agonists is different between adrenaline and noradrenaline cells. This may partly account for the differential release of adrenaline and noradrenaline by different secretagogues in cat adrenal glands.

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Effect of bile acid on electrophysiological properties of rabbit sino-atrial node *in vitro*

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1 In order to examine the action of bile acid on cardiac pacemaker activity, the effect of sodium taurocholate (NTC) was studied on the membrane potential and current of rabbit sino-atrial node preparations by means of a double microelectrode voltage clamp technique.

2 In spontaneously beating sino-atrial node preparations, NTC (above 30 μM) decreased the maximum rate of rise of the action potential. Above 100 μM, the compound also exerted a bradycardiac effect and decreased the rate of diastolic depolarization significantly.

3 On the current systems, NTC produced dose-related decreases in the slow inward Ca^{2+} current and the time-dependent outward K^+ current.

4 It is concluded that NTC depresses the spontaneous discharge of the sino-atrial node through decreases in both inward and outward current systems.

Introduction

Bile acids have been shown to exert a direct depressant effect on cardiac function (Wakim *et al.*, 1940; Joubert, 1978; Bogin *et al.*, 1983; Green *et al.*, 1984). Furthermore, Song *et al.* (1983) demonstrated that sinus bradycardia is induced by obstructive jaundice. A recent *in vitro* study (Binah *et al.*, 1987) showed that bile acids exert a negative inotropic effect mainly by a depression of Ca^{2+} influx of the cell membrane. Since Ca^{2+} conductance is predominantly responsible for the depolarization in sino-atrial node (Irisawa, 1978), a decrease in Ca^{2+} influx would alter the pacemaker spontaneous discharge. However, it is not obvious whether bile acids have a direct chronotropic action on the cardiac pacemaker cells. Hence, in the present study, we examined the effects of a bile acid (sodium taurocholate) on the membrane potential and the membrane current of rabbit sino-atrial node by means of a double microelectrode voltage clamp method.

Methods

Rabbits, weighing 1.5–2.0 kg, were stunned by a blow behind the neck, and the heart was quickly removed. The right atrium, including the sino-atrial node region, was dissected in Tyrode solution. A small sino-atrial node preparation of about 0.3 × 0.3 mm was prepared according to the method of Noma & Irisawa (1976). The preparation was usually smaller than one half of the length constant of the rabbit sino-atrial node preparation described by Bonke (1973) and Seyama (1976). In the experiments, we used the central area of the node, termed the compact area, as previously described by Bleeker *et al.* (1980) and Brown (1982).

The two-microelectrode voltage clamp method, one electrode to measure the membrane potential and the other to supply current intracellularly, was used. Glass microelectrodes were filled with 3 M KCl and the resistance was 10–30 MΩ. Membrane potential and current were monitored on an oscilloscope (Kikusui Denshi, 5516 ST) and were recorded with a penrecticorder (Nihon Kohden, RJG-4100). The feedback circuit was the same as that described by Noma and colleagues (1980), and the ground amplifier was essentially the same as that used by New & Trautwein (1972). When both microelectrodes recorded identical action potentials the preparation was clamped to −40 mV (holding potential), where the holding current was nearly zero (Noma *et al.*, 1979).

The composition of the normal Tyrode solution was (in mM): NaCl 137.0, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, Na_2HPO_4 0.6 and the pH was adjusted to 7.4 by adding Na_2HPO_4. Sodium taurocholate (NTC) was of highest purity and was obtained from Sigma Chemical Co. The temperature of the solution was maintained at 36–37°C throughout the experiments.

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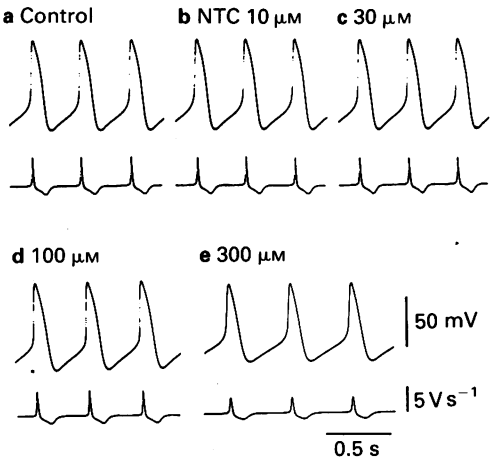


Figure 1 Effect of sodium taurocholate (NTC) (b–e) on spontaneous action potentials in rabbit sino-atrial node. In each panel, upper trace shows action potentials and lower shows their first derivative.

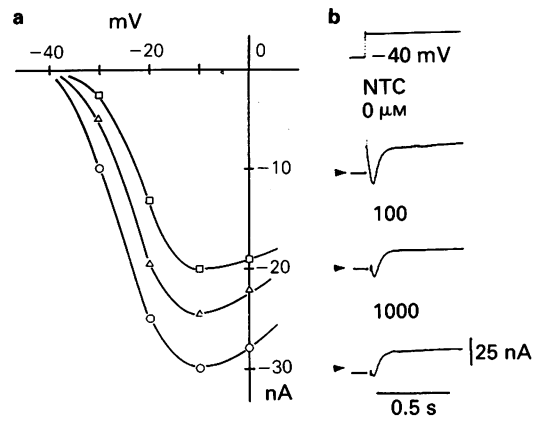


Figure 2 Voltage-dependence of sodium taurocholate (NTC)-induced decrease in the slow inward current (I_{si}). (a) Current-voltage relationship for I_{si}. (b) Voltage clamp recordings. In (b) (▶) shows the holding current before exposure to NTC. I_{si} was measured before (○) and after an application of 100 μM (△) and 1000 μM (□) NTC.

Electrophysiological measurements were made 15 min after changing to a new solution.

For the various parameters of the action potentials and membrane currents, the values are presented as mean ± s.d. Statistical analysis was performed by use of Student's t test, and P values less than 0.05 were considered significant.

Results

Effects on the transmembrane action potentials

In seven spontaneously beating sino-atrial node preparations, the effect of sodium taurocholate (NTC), a bile acid, on membrane potentials was examined by means of a conventional microelectrode method.

Figure 1 shows the action potential traces during increasing concentrations of NTC. Although 10 μM NTC did not produce any significant changes in the action potential, the compound reduced the maximum rate of depolarization ($\dot{V}_{max}$) at above 30 μM. After an application of a NTC concentration higher than 100 μM, the sinus rate and the rate of diastolic depolarization (RDD) were also significantly decreased. These changes in the action potential parameters are summarized in Table 1. The action potential amplitude (APA) tended to be decreased by the drug, but this change was not statistically significant. NTC 1000 μM induced sinus arrest in 3 out of 7 sino-atrial node preparations; this is not shown in the figure. These results indicate that the NTC induced a negative chronotropic effect associated with the reduction of $\dot{V}_{max}$ and RDD, which may reflect a decrease in Ca^{2+} influx through the cell membrane.

Table 1 Effect of sodium taurocholate (NTC) on various characteristics of sino-atrial node action potentials

	SCL (ms)	$\dot{V}_{max}$ ($V s^{-1}$)	*APA* (mV)	APD_{50} (ms)	*RDD* ($mV s^{-1}$)
Control	397.9 ± 56.7	6.6 ± 1.1	93.4 ± 6.5	85.6 ± 7.8	105.3 ± 10.2
NTC					
10 μM	411.6 ± 61.0	6.1 ± 1.3	92.6 ± 6.7	86.1 ± 8.4	102.5 ± 14.0
30 μM	401.1 ± 74.0	5.9 ± 1.1*	93.5 ± 5.9	86.7 ± 8.7	101.5 ± 12.6
100 μM	440.9 ± 70.0*	5.1 ± 1.2**	92.7 ± 5.8	86.7 ± 8.5	92.7 ± 16.4*
300 μM	459.0 ± 79.7*	4.2 ± 1.4**	88.1 ± 8.9	86.4 ± 10.3	86.7 ± 20.4*

Data show means ± s.d., $n = 7$. SCL: spontaneous cycle length; $\dot{V}_{max}$: maximum rate of rise; APA: action potential amplitude; APD_{50}: action potential duration at half-amplitude; RDD: rate of diastolic depolarization. * $P < 0.05$, ** $P < 0.01$ with respect to control values.

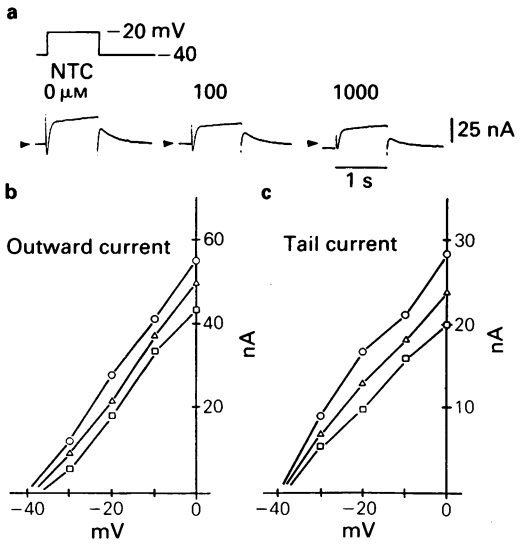


Figure 3 Voltage-dependence of sodium-taurocholate (NTC)-induced decrease in the outward K^+ current (I_K). (a) Voltage clamp recordings. Current-voltage relationships for outward current measured at 1 s after the test pulse (b) and for tail current (c). Outward current amplitude was measured before (○) and after exposure to 100 μM (△) and 1000 μM (□) NTC. In (a) (▶) shows the holding current before NTC treatment.

Effects on the membrane current systems

To investigate the ionic mechanism of the NTC-induced bradycardia, voltage clamp experiments were performed.

Figure 2 shows the effect of NTC on the slow inward current (I_{si}) in sino-atrial node preparations. The membrane potential was held at −40 mV, and depolarizing test pulses were applied with 10 mV increments. These pulses were usually applied at 0.1 Hz. The amplitude of I_{si} was measured as the difference between the peak inward current and the current at 100 ms after the onset of the clamp pulse (McDonald & Trautwein, 1978; Satoh & Hashimoto, 1986), and a current-voltage relationship for I_{si} was obtained before and after exposure to NTC (Figure 2a). NTC obviously depressed I_{si} in a dose-dependent fashion. At a concentration of 100 μM, I_{si} was decreased by a factor of 0.79 ± 0.09 ($P < 0.01$) during depolarization to −10 mV ($n = 4$).

In Figure 3, the effect of NTC on the time-dependent outward K^+ current (I_K) was examined. I_K in sino-atrial node cell is equivalent to I_x in Purkinje fibre (Irisawa, 1978). A depolarizing test pulse elicited a slowly developing outward current following the inactivation of I_{si}, while a gradually decaying tail current was observed after repolarization (Figure 3a). The former shows activation of I_K and the latter shows deactivation of I_K. Current-voltage relationships for the outward current amplitude during depolarization and for the tail current amplitude were plotted in the absence and presence of NTC (Figure 3b,c). NTC clearly decreased both current amplitudes dose-dependently, implying a depression of I_K. NTC (100 μM) decreased the outward current amplitude measured at 1 s after the onset of the depolarizing test pulse to −10 mV by a factor of 0.81 ± 0.14 ($P < 0.01$, $n = 4$).

Discussion

The present study has demonstrated that sodium taurocholate (NTC), a bile acid, decreased the sinus rate associated with decreases in $\dot{V}_{max}$ and RDD. Under the voltage clamp experiments, NTC depressed both I_{si} and I_K in a dose-related manner.

Above 100 μM, NTC produced a slight but significant decrease in the rate of phase 4 depolarization (RDD), wihch mainly contributed to the reduced heart rate. There are several hypotheses for the pacemaker current of sino-atrial node cells (Noble, 1984), but no clear-cut mechanism has at yet emerged. In the present experiments, a decrease in I_{si} seems to be the most important factor for a reduction of RDD and a negative chronotropic effect induced by NTC. In fact, we have already shown that drugs which reduce Ca^{2+} influx through the cell membrane decrease the rate of phase 4 depolarization (Kotake *et al.*, 1986; 1987; 1988). Furthermore, decreases in I_K would reduce the maximum diastolic potential, which might also inactivate pacemaker currents.

On the other hand, the action potential duration at half-amplitude (APD_{50}) was unaffected after an application of NTC. Changes in APD of the sino-atrial node are generally due to changes in I_{si} and I_K (Senami & Irisawa, 1981). A decrease in I_K would prolong APD, whereas a depression of I_{si} would shorten it. As a result of such a combined effect, APD_{50} was not significantly affected after exposure to NTC.

The circulating plasma concentration of all the bile acids is usually less than 1 μg ml^{-1}, which is equivalent to about 2 μM. However, obstruction of bile duct results in higher circulating concentrations of bile acids, as high as 1000 μg ml^{-1} (Makino *et al.*, 1969; Pennington *et al.*, 1977; Song *et al.*, 1983). In rats whose bile ducts have been ligated, the total bile acid concentration rises from 0.2 μg ml^{-1} to about 200 μg ml^{-1} (Bogin *et al.*, 1983; Hardison *et al.*, 1983). A previous electrophysiological study by Binah *et al.* (1987) demonstrated that NTC significantly reduced the developed tension of rat isolated papillary muscle at concentrations between 0.001

and 1.0 μM, which are almost within the physiological range. They also showed that 1 μM NTC depressed I_{si} of the rat ventricular myocyte. In our study, NTC failed to modify the electrical activity of rabbit sino-atrial node at the physiological concentration range, but it exerted significantly depressant membrane actions at concentrations above 100 μM. However, such a high concentration of bile acid is found to occur in obstructive jaundice, as mentioned above. Although the threshold concentration of NTC for inducing a negative chronotropic action is much higher than that for inducing a negative inotropism, sinus bradycardia could occur in severe jaundice through its direct membrane action. These findings might also support the observation by Song *et al.* (1983) in which obstructive jaundice could cause sinus bradycardia. It is difficult to relate clinical plasma concentrations to those of the compound perfusing isolated cardiac tissues and there may be species differences in pacemaker sensitivity to NTC. Nevertheless, our experimental results indicate that high concentrations of bile acid induce an inhibitory effect on the electrophysiological properties of sino-atrial node pacemaker cells and that severe jaundice might modify the spontaneous discharge of the heart.

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Endothelin-induced contractions of tracheal smooth muscle and identification of specific endothelin binding sites in the trachea of the rat

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1 The presence of specific binding sites and the contractile activity of the novel peptide, endothelin have been investigated in rat trachea.

2 Endothelin (10^{-8}–10^{-5} M) induced long-lasting contraction of rat tracheal rings superfused with Krebs solution (EC_{50} 5.4×10^{-6} M). Contractions of the tissue to 10^{-6} M endothelin were attenuated in Ca^{2+}-free medium containing 0.1 mM EGTA but unaffected by nicardipine (10^{-7} M).

3 After equilibration in Ca^{2+}-free medium (without EGTA) a return to normal Ca^{2+} concentrations (2.5 mM), 30 min or 60 min following endothelin (10^{-6} M), produced a sustained contraction of the tissue.

4 Specific binding sites for endothelin were identified on rat tracheal smooth muscle (K_D 1.34×10^{-10} M, maximal binding 1.2 fmol mm^{-2}). Specific binding sites were also identified on nerve trunks. Endothelin binding was unaffected by co-incubation with nicardipine (10^{-7} M) or verapamil (10^{-7} M).

5 The discrepancy between the apparent K_D for endothelin binding and the EC_{50} for endothelin-induced contraction suggests that the endothelin binding sites identified in this study may not be associated with the receptors mediating contraction.

6 These results indicate that endothelin binding sites are present on tracheal smooth muscle. The mechanism of endothelin-induced contraction, whilst being dependent on extracellular calcium, does not appear to involve binding to the dihydropyridine- or verapamil-sensitive sites on the voltage-dependent Ca^{2+} channel. Its long duration of action may be associated with a sustained increase in Ca^{2+} permeability.

Introduction

Yanagisawa *et al.* (1988) have recently described a potent vasoconstrictor peptide, endothelin, isolated from cultured endothelial cells that elicits sustained increases in arterial pressure. Endothelin is a 21 amino acid peptide that has a regional homology with some neurotoxins which act on membrane ion channels. The action of endothelin on isolated vasculature has been shown to be dependant on extracellular Ca^{2+}, unaffected by antagonists of other vasoconstrictors and to be attenuated by nicardipine (Yanagisawa *et al.*, 1988), which lead to the hypothesis that this compound might be an endogenous ligand of dihydropyridine-sensitive, voltage-dependent Ca^{2+} channels. Since the calcium agonist Bay K 8644 elicits contractions of airway smooth muscle (Advenier *et al.*, 1986), we have examined the possibility that endothelin may also be an endogenous agonist of Ca^{2+} channels in airway smooth muscle and elicit sustained increases in airway tone.

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Methods

Effects on smooth muscle

Male Wistar rats (150–350 g) were stunned by a blow to the head and killed by cervical dislocation. The

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trachea was removed, placed in Krebs solution and adherent fat and connective tissue removed. Rings consisting of 3 adjacent cartilage plates were suspended under a resting tension of 1 g and superfused with Krebs solution (composition in mM: NaCl 113, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 5.7) at 2 ml min^{-1}. The solution was pre-gassed with 95% O_2, 5% CO_2 and superfused the tissues at 37°C. Two tissues were used from each animal and were pre-equilibrated for 60 min. The resting tension at the end of this period was 0.9 ± 0.03 g. Agonists were added in the superfusing buffer and were in contact with the tissue for 30 s (60 s in the case of KCl).

In all tissues contractile responses to methacholine were established before examination of endothelin responses. Responses to methacholine or KCl were determined to single additions of the drug and complete recovery to resting tensions was allowed between concentrations. Cumulative concentration-response curves to endothelin were obtained by incremental increasing concentrations at the plateau of the previous response. Contractions of tracheal smooth muscle by endothelin were compared to those of rings of rat aorta under the conditions described for the tracheal rings. In parallel experiments, where the effects of nicardipine (10^{-7} M) on endothelin responses were investigated, nicardipine was included in the superfusate and the tissues were equilibrated with the antagonist for 30 min before endothelin was introduced.

In another series of experiments, following the initial equilibration, responses to methacholine 10^{-6} M and 50 mM KCl were determined. The tissues were then incubated in Ca^{2+}-free Krebs containing 0.1 mM EGTA for 15 min. Responses to methacholine and KCl were repeated and the contractile responses to endothelin 10^{-6} M established. The results of these experiments were compared to those of time-matched controls superfused with normal Krebs solution. In another study following a 60 min preincubation in nominally Ca^{2+}-free medium (i.e. without EGTA), the effect of adding 2.5 mM Ca^{2+} back to the superfusing medium 30 or 60 min after addition of endothelin was determined.

In vitro *autoradiography*

Adult male Wistar rats ($n = 15$) were killed by decapitation, the trachea was rapidly removed, tissue blocks prepared by snap freezing in melting Arcton (dichlorodifluoromethane) and stored under liquid nitrogen until required. From each animal consecutive cryostat sections were cut at a thickness of 10 μm and mounted onto acid alcohol washed chrome alum scrubbed slides. All of the sections were pre-incubated in 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl, 5 mM $MgCl_2$, 40 mg ml^{-1} Bacitracin and 0.1% bovine serum albumin (w/v) for 15 min at room temperature. Following this some sections ($n = 4$) were incubated, alone, in the same buffer with [^{125}I]-endothelin (25 pM–2.5 nM; specific activity 1145 Ci $mmol^{-1}$) for 15 min at room temperature. To assess non-specific binding, additional serial sections from each animal were co-incubated with radiolabelled peptide (200 pM) and an excess of unlabelled endothelin (10^{-6} M). For competition experiments serial sections from each animal were co-incubated with radiolabelled peptide (200 pM) and other vasoactive but unrelated peptides (CGRP, VIP, ANP, gastrin all 10^{-6} M), the calcium antagonists nicardipine (10^{-7} M) and verapamil (10^{-7} M), and the K^+ channel blocking agents 4-aminopyridine (5 mM) and tetraethylammonium (30 mM) for 30 min at room temperature. The incubation was terminated by washing (2 × 5 min) in cold buffer (4°C) followed by one rinse in ice-cold distilled water, after which all sections were dried rapidly under a stream of cold air. Autoradiographs were generated by exposing labelled tissue sections and the polymer based ^{125}I standards (10 μm thick) to Hyperfilm-^{3}H for 4 days at 4°C. From the subsequent autoradiograms, binding sites on smooth muscle were analysed by IBAS 2000 computer assisted image analysis. Grey values of the radioactive standards were measured and a standard curve produced and stored on disc. The grey values of the tissue images were then measured by means of this standard curve and converted to the amount of ^{125}I-bound per unit surface area of tissue component measured. Smooth muscle and nerve trunks were identified in serial sections counterstained with haemotoxylin and eosin.

Drugs

Endothelin (Scientific Marketing, London, U.K.); acetyl-β-methylcholine bromide, 4-aminopyridine, bovine serum albumin (BSA), ethylene glycol-tetraacetic acid (EGTA), nicardipine hydrochloride, tetraethylammonium chloride, verapamil hydrochloride, (Sigma, Poole, Dorset, U.K.); calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), atrial naturetic peptide (ANP), gastrin (Peninsula Laboratories, St Helens, U.K.); Hyperfilm-^{3}H (Amersham International, Aylesbury, U.K.).

Statistics

Results are expressed as mean ± s.e.mean. Values were compared by means of a two-tailed Mann-Whitney U test for unpaired observations. Log EC_{50}

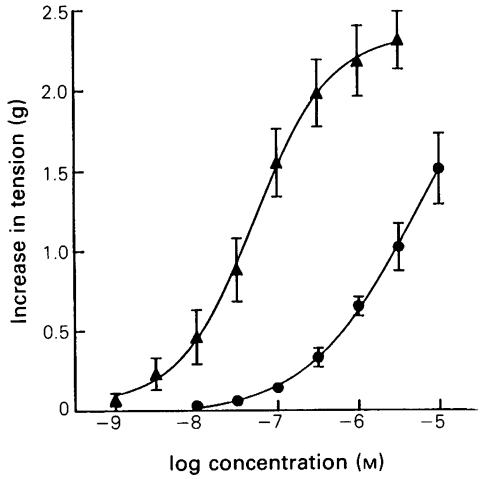


Figure 1 Concentration-response curves for endothelin on rat aorta (▲) or trachea (●) superfused with Krebs solution at $2\,ml\,min^{-1}$ and under a resting tension of $0.9 \pm 0.03\,g$. Results are expressed as the increase in tension (g) over resting and are a minimum of 6 observations. Vertical lines show s.e.mean.

values were calculated from the mean data following fitting to a sigmoid curve; K_D and maximal binding capacity were calculated by Scatchard analysis of the binding data. (GraphPad, ISI Software).

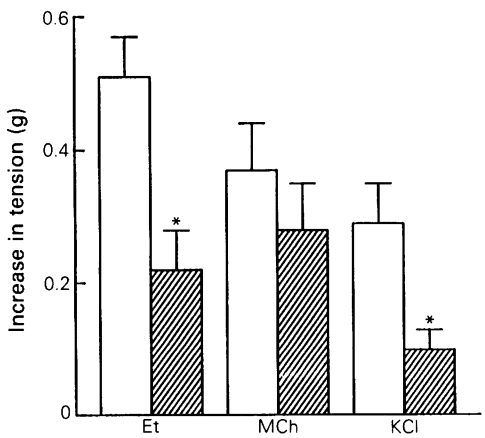


Figure 2 The response of rat trachea to 10^{-6} M endothelin (Et) after 15 min incubation in calcium free medium containing 0.1 mM EGTA compared to time matched controls. Also shown are the responses to methacholine (MCh 10^{-6} M) and KCl (50 mM) in normal medium and following 15 min incubation in calcium-free medium containing 0.1 mM EGTA. Open columns: responses in normal medium and hatched columns: responses in calcium-free medium containing 0.1 mM EGTA. $^*P < 0.05$ $n = 5$. Vertical bars show s.e.mean.

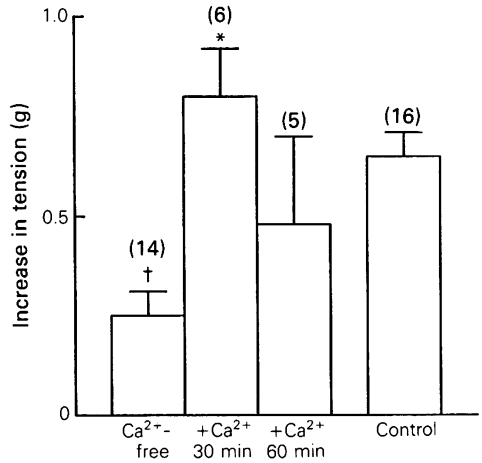


Figure 3 Endothelin (10^{-6} M) induced increases in tension of rat tracheal rings after 1 h incubation in calcium-free medium and the subsequent increases in tracheal tension following return to 2.5 mM calcium 30 or 60 min post endothelin. $^*P < 0.05$; ($\dagger P < 0.05$ compared to responses to endothelin in normal Krebs solution) (Mann-Whitney U test). Values in parentheses are the number of observations.

Results

In Krebs solution with normal Ca^{2+} concentrations, endothelin (10^{-8}–10^{-5} M) produced a concentration-dependent contraction of rat trachea and had a similar potency to methacholine (log EC_{50} − 5.27 and −5.89 respectively, $n \geqslant 5$). Concentrations of endothelin sufficient to produce a maximum contractile response were not achieved due to limited availability of the peptide. However, the maximal effect of endothelin 10^{-5} M (1.51 ± 0.22 g, $n = 6$) was not different from the maximum contraction to methacholine 3×10^{-5} M (1.24 ± 0.24, $n = 6$). Under the same conditions endothelin (10^{-9}–10^{-6} M) was 100 times more potent in producing contraction of rat aortic rings (log EC_{50} − 7.27 ($n \geqslant 6$) compared to −5.27 in trachea) (Figure 1). Endothelin-induced contraction of rat trachea was long lasting, responses to 10^{-6} M taking 5.3 ± 1.4 min to achieve a plateau and 57.0 ± 7.9 min to return to resting tensions ($n = 8$).

In Ca^{2+}-free solutions containing 0.1 mM EGTA responses to endothelin 10^{-6} M were reduced by 67% ($P < 0.03$) and those to 50 mM KCl by 76% ($P < 0.05$), the response to methacholine 10^{-6} M was not significantly affected ($n = 5$; Figure 2). Under these conditions the duration of action of endothelin was also reduced to 17 ± 6 min; at equivalent times,

contractions of tissues in Krebs with normal calcium concentrations were 75.6 ± 14% of their maximum levels ($n = 5$).

Thirty minutes after addition of endothelin (10^{-6} M) to tissues superfused with nominally Ca^{2+}-free medium for 60 min, changing the superfusate to Krebs containing 2.5 mM Ca^{2+} produced a sustained contraction of the tissue which was the same as the response to endothelin in normal medium but which was significantly greater than that following 60 min pre-incubation in Ca^{2+}-free medium, ($P < 0.05$, $n = 6$) (Figure 3). Return to normal Ca^{2+} concentrations 60 min after addition of endothelin (10^{-6} M) also elicited contraction of the tissues ($n = 5$) but the effect was smaller than those seen at 30 min post endothelin. In tissues that had not been exposed to endothelin return to normal Ca^{2+} concentrations had no effect on their resting tone. The contractile effects of endothelin (10^{-7}–3×10^{-6} M) were unaffected by nicardipine 10^{-7} M compared to time matched controls ($n = 4$; Figure 4).

In vitro *autoradiography*

Specific high affinity binding sites were identified in the smooth muscle of the trachea and on nerve trunks in the adventitia (Figure 5). These structures were identified in labelled tissue sections which were post-fixed in 10% formalin and counterstained with haematoxylin and eosin. Binding was rapid and reached equilibrium within 15 min ($n = 4$). The binding was specific; almost 100% of the total binding was displaced by coincubation with unlabelled endothelin (10^{-6} M). Scatchard analysis of the binding data suggested that there is a single class of binding site to smooth muscle (dissociation constant (K_D) 1.34×10^{-10} M, maximum binding 1.2 fmol mm^{-2}). However, in view of the scatter ($r = 0.89$) a second lower affinity site may be present. Endothelin binding was unaffected by co-incubation with CGRP, VIP, ANP or gastrin, the K^+ channel blocking agents tetraethylammonium and 4-aminopyridine or the Ca^{2+} antagonists nicardipine and verapamil. Non-displaceable binding to the respiratory epithelium was seen.

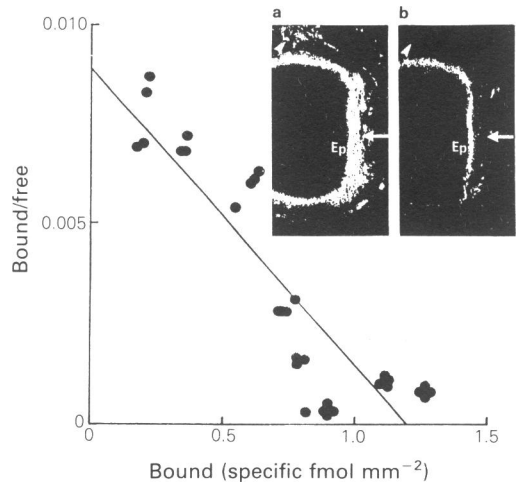


Figure 5 [^{125}I]-endothelin binding in the absence (a) and presence (b) of a 1000 fold excess of unlabelled endothelin. Specific binding is seen on the smooth muscle (arrow) and nerve trunks (arrowheads). Non-specific binding to the epithelium (Ep) was also seen. The scatchard plot of [^{125}I-endothelin (25–2500 pM) binding to 10 μm sections of rat trachea suggested a specific endothelin binding site with an apparent K_D of 1.34×10^{-10} M. Each point is the mean of 3 observations taken from serial sections from a total of 4 animals.

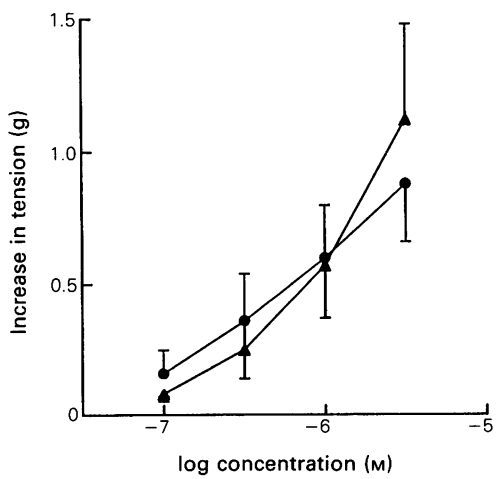


Figure 4 Effect of 10^{-7} M nicardipine (▲) on the increase in tension of rat tracheal rings elicited by endothelin. Nicardipine was present in the superfusate and the tissues were pre-incubated for 30 min before the addition of endothelin. (●) control responses to endothelin ($n = 4$). Vertical lines show s.e.mean.

Discussion

Calcium ions are essential for contraction of smooth muscle, calcium in the extracellular medium

gaining entry to the cell following the opening of specific ion channels. Of the two types of channel that have been proposed, voltage-dependent calcium channels have a calcium permeability that is linked to the transmembrane potential difference (Rodger, 1985; 1988; Daniel, 1988), and are specifically inhibited by dihydropyridine derivatives such as nifedipine and nicardipine (Advenier *et al.*, 1984). Endothelin, a potent vasoconstrictor (Yanagisawa *et al.*, 1988; Tomobe *et al.*, 1988; Hirata *et al.*, 1988) has recently been suggested as a putative endogenous ligand for the dihydropyridine-sensitive calcium channels in vascular smooth muscle (Yanagisawa *et al.*, 1988).

Yanagisawa *et al.* (1988) have shown that preproendothelin mRNA is not expressed in porcine lung, suggesting that little endothelin is produced by the pulmonary microvasculature. We, however, have demonstrated that there are specific binding sites for this ligand in the rat trachea and that these are associated predominantly with the smooth muscle and nerve trunks. Furthermore, endothelin elicits contractions of tracheal smooth muscle, with a potency similar to that of substance P (on guinea-pig trachea), but 1–2 orders of magnitude less potent than neurokinins A or B (Devillier *et al.*, 1988). Contraction of the tissue to endothelin, however, is long lasting, persisting for up to 60 min at 10^{-6} M, even though the tissue was constantly washed by superfusion, which suggests that bound endothelin might be resistant to dissociation from its binding sites.

Scatchard analysis of the binding data gave an apparent dissociation constant in the order of 10^{-10} M. In contrast our observed EC_{50} for contraction of tracheal smooth muscle is 5.4×10^{-6} M. This disparity between the K_D for binding and the EC_{50} may in part be related to loss of the peptide during the superfusion, although in 3 experiments we have determined that losses of ^{125}I endothelin through the superfusion apparatus are negligible. Hirata *et al.* (1988), have, similarly demonstrated a discrepancy between the K_D for endothelin binding to rat aortic smooth muscle ($2–4 \times 10^{-10}$ M) and concentrations of the peptide that increase intracellular calcium levels ($10^{-9}–10^{-7}$ M). Although in their study the concentration of endothelin eliciting a maximum response was not obtained, this concentration range is consistent with our observed EC_{50} for contraction of rat aorta (5.4×10^{-8} M). Whilst the nature of the superfusion may not result in equilibrium conditions, these observations do suggest that in the rat trachea 'specific' endothelin binding over the concentrations studied ($25 \times 10^{-12}–25 \times 10^{-10}$ M) may not be related to the receptors mediating the contractile response.

It has been suggested that the mechanism of action of contractile agonists such as acetylcholine, histamine and leukotriene D_4 may be independent of Ca^{2+} entry through either voltage-dependent or receptor coupled channels, but is related to the release of activator Ca^{2+} from intracellular sites, whilst those to KCl are dependent on extracellular calcium (Farley & Miles 1978; Rodger 1988). In the present study endothelin-induced contractions were attenuated in Ca^{2+}-free medium containing 0.1 mM EGTA and its duration of action was shortened. In the same tissues the contactile response to KCl was also reduced but that to methacholine was not significantly affected. These observations, therefore, are consistent with a requirement for extracellular calcium in the expression of the contractile effects of endothelin.

Although endothelin-induced contraction of the trachea was attenuated in Ca^{2+}-free medium (residual contractile activity perhaps being due to the availability of Ca^{2+} bound to cartilage; Raeburn & Rodger, 1984) addition of Ca^{2+} to the medium superfusing the tissues elicited a contraction of the tissue, which was dependent or prior exposure to endothelin. The sustained contractions of the tissue, therefore, may be related to the tight association of endothelin to its binding site resulting in prolonged Ca^{2+} channel opening. Furthermore, since a contractile response to Ca^{2+} addition could still be achieved 60 min post-endothelin, the mechanism of recovery to resting tensions (60 min at 10^{-6} M) in the presence of a continued increase in Ca^{2+} permeability remains uncertain.

Under identical experimental conditions, endothelin was 100 times more potent in eliciting contractions of rat aorta. This greater affinity of endothelin on vascular smooth muscle may indicate that the endothelin binding sites in the rat trachea are different from those in the rat aorta. Furthermore, unlike porcine coronary artery (Yanagisawa *et al.*, (1988) or guinea-pig trachea (Uchida *et al.*, 1988), contraction of the rat trachea was unaffected by the calcium antagonist nicardipine. Whilst, Hirata *et al.* (1988) have recently shown that endothelin binding to rat aorta is not affected by calcium channel blockers, in their studies the endothelin-induced increase in intracellular calcium was. We have shown in the rat trachea that the binding of endothelin to airway structures is unaffected by either nicardipine or verapamil and this, coupled with the absence of effect of nicardipine on the contractile activity of endothelin, suggests that, at least in the rat airway, endothelin is unlikely to be interacting with the dihydropyridine binding site.

Finally potassium channel blocking agents such as tetraethylammonium elicit contractions of smooth muscle through opening of voltage-dependent calcium channels (Allen *et al.*, 1986; Daniel, 1988). Our binding studies have indicated that endothelin

binding to tracheal smooth muscle is unaffected by either tetraethylammonium or 4-aminopyridine, thus specific endothelin binding is unlikely to be related to an interaction with K^+ channels.

In conclusion, specific endothelin binding sites have been identified in the rat airway and endothelin has been shown to elicit long-lasting contractions of tracheal smooth muscle, albeit less potent than as an agonist on vascular smooth muscle. However, its molecular site of action does not appear to be at the dihydropyridine binding site or at the K^+ channel. Its long duration of action may be associated with a sustained increase in Ca^{2+} permeability.

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Purinoceptors in the pulmonary circulation of the rat and their role in hypoxic vasoconstriction

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1 P_2-purinoceptors have been characterized in the systemic circulation of a variety of species but little is known about their nature in the pulmonary vasculature.

2 In the isolated, blood perfused and ventilated lung of the rat the P_{2x} selective analogues α,β-methylene ATP(α,β-meATP) (25 μg) and β,γ-methylene ATP (400 μg) caused a rise in pulmonary artery pressure (127 $\pm$ 32% and 110 $\pm$ 23% increase respectively, $n = 6$), demonstrating the existence of vasoconstrictor P_2 receptors in the pulmonary circulation.

3 Repeated boluses of α,β-meATP resulted in complete desensitization of the constrictor response to β,γ-meATP, but a small vasodilator response remained indicating unopposed stimulation of P_{2y} receptors. The desensitization by α,β-meATP had no effect on angiotensin II-induced vasoconstriction.

4 After desensitization of the vasoconstrictor P_2 receptors with α,β-meATP, hypoxic pulmonary vasoconstriction remained intact, indicating that these receptors are not involved in this response.

Introduction

Purinoceptors have been classified into two types, P_1 and P_2 which are selective for adenosine and adenosine triphosphate (ATP) respectively (Burnstock, 1978). For more than 30 years it has been known that ATP is more potent than adenosine in causing vasodilatation (Winbury *et al.*, 1953; Wolff & Berne, 1956), but it has only recently been proposed that the P_2 receptor may be subclassified into two subtypes, P_{2x} and P_{2y}, on the basis of the potency of a series of ATP analogues. At the P_{2x} receptor the potency order of agonists is α,β-methylene ATP (α,β-meATP), β,γ-methylene ATP (β-γ-meATP) > ATP = 2-methylthio ATP (2-meSATP). At the P_{2y} receptor the order of potency is 2-meSATP $\gg$ ATP > α,β-meATP = β,γ-meATP (Burnstock & Kennedy, 1985a). In the systemic vasculature the physiological consequences of stimulation of each of these receptor subtypes has been documented. It has been found that P_{2x} receptors, which are located on vascular smooth muscle, mediate contraction whereas P_{2y} receptors, located on endothelial cells and occasionally on vascular smooth muscle (Kennedy & Burnstock, 1985; Mathieson & Burnstock, 1985), mediate relaxation (White *et al.*, 1985; Kennedy *et al.*, 1985; Kennedy & Burnstock, 1985). Furthermore, the P_{2x} receptor may be selectively desensitized by α,β-meATP, both *in vitro* (Burnstock & Kennedy, 1985a) and *in vivo* (Hedlund *et al.*, 1983; Bulloch & McGrath, 1988), making it possible to elucidate the role of this receptor in physiological responses.

Although the phenomenon of hypoxic pulmonary vasoconstriction (HPV) has been recognized for over 40 years (von Euler & Liljestrand, 1946), its mechanism remains unknown. Since purines may be released in hypoxia it is possible that purinoceptors might influence HPV. Burnstock has suggested that if an adenyl compound is involved in the pulmonary vasoconstrictor response to hypoxia, the compound is likely to be ATP stimulating a vasoconstrictor P_{2x} receptor (Burnstock, 1980; Burnstock & Kennedy, 1985b). We therefore used an isolated, perfused and ventilated lung model in the rat to characterize the purinoceptors in the pulmonary vascular bed and to

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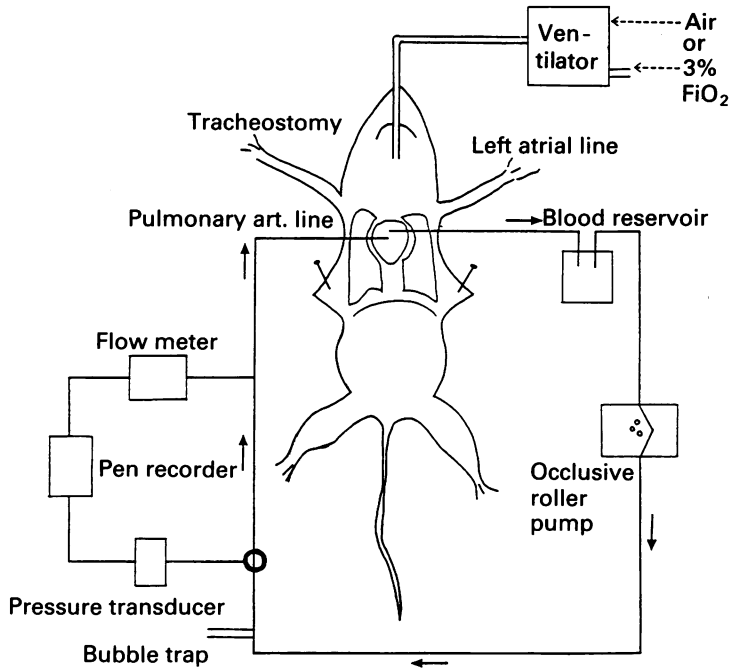


Figure 1 Isolated perfused and ventilated lung of the rat. The fraction of inspired oxygen concentration (FiO_2) could be altered such that the animal was ventilated with either 21% O_2/74% N_2/5% CO_2 (normoxia) or 3% O_2/92% N_2/5% CO_2 (hypoxia). Drugs were injected into the circuit immediately proximal to the pulmonary artery line.

test the hypothesis that stimulation of purinoceptors on pulmonary blood vessels may be involved in HPV.

Methods

Tissue preparation

We used an isolated, perfused and ventilated *in situ* model of the rat lung as previously described (Emery *et al.*, 1981), which is shown schematically in Figure 1. Male Wistar rats weighing 270–360 g were anaesthetized with fentanyl and diazepam. Before each experiment one rat was exsanguinated and the blood placed in a reservoir, maintained at 40°C by a surrounding water bath and used to 'prime' the perfusion circuit. This consisted, in order, of a left atrial cannula, reservoir, roller pump (Watson Marlow MHRE 200, Falmouth), connecting tubing, bubble trap, in-line flow probe connected to flow meter (SEM 275, S.E. medic, Feltham), side arm pressure transducer (Bio Medical Systems Ltd., Strathclyde) and a pulmonary artery cannula. The flow meter and pressure transducer were connected to a recorder (Multitrace 2, Ormed, Hertfordshire) to permit constant monitoring of these parameters. The total blood volume of the circuit, including the reservoir, was 15–20 ml.

The experimental animal was anaesthetized and ventilated at a tidal volume of 4 ml and a frequency of 15 breaths per min, via a tracheostomy. The lungs were exposed through a median sternotomy and the animal was heparinised and exsanguinated via the abdominal aorta. A purse string suture was placed around the left atrium to secure a catheter inserted through an atriotomy, which was allowed to drain freely into a blood reservoir, thereby ensuring that 'left atrial pressure' was maintained at zero. A cannula was inserted into the main pulmonary artery via a right ventriculotomy and tied securely in place. A constant flow of 18 ml min^{-1} resulted in a mean initial pulmonary artery pressure (Ppa) of 16 ± 1 mmHg ($n = 12$). Blood gas tensions were measured at the beginning and during each experiment by collecting blood anaerobically from the left atrial cannula, and analysing it with a Corning blood gas analyzer (178 pH/blood gas analyzer, Corning, Essex). Deviations in pH from normal were corrected with small amounts of $NaHCO_3$ (1.0 N) added to the reservoir.

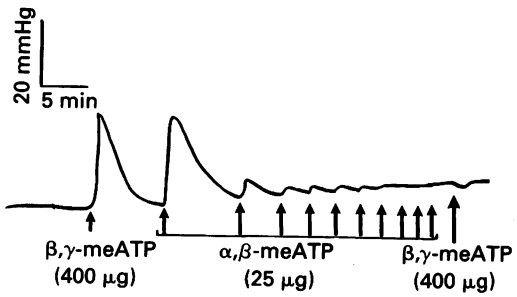


Figure 2 Pulmonary artery pressure (Ppa) trace from a rat after administration of 400 μg β,γ-meATP before and after desensitization of the P_2 receptor with repeated (×10) administrations of 25 μg boluses of α,β-meATP. Upward deflection represents an increase in Ppa.

Experimental protocol

Vasoconstrictor P_2 receptor desensitization Six animals were continuously ventilated with gases of composition 21% O_2, 5% CO_2 and 74% N_2 and after a stable baseline had been achieved, β,γ-meATP (400 μg) was injected as a 130 μl bolus into the circuit immediately proximal to the pulmonary artery. A rapid increase in Ppa followed which had returned to baseline levels within 10 min. Ten bolus injections of α,β-meATP (25 μg, 8 μl) were then given at 2–4 min intervals which caused transient, but successively smaller increases in Ppa which was allowed to return to baseline between administrations. Two minutes after the last injection of α,β-meATP, a further injection of β,γ-meATP (400 μg) was given and the effect on Ppa measured.

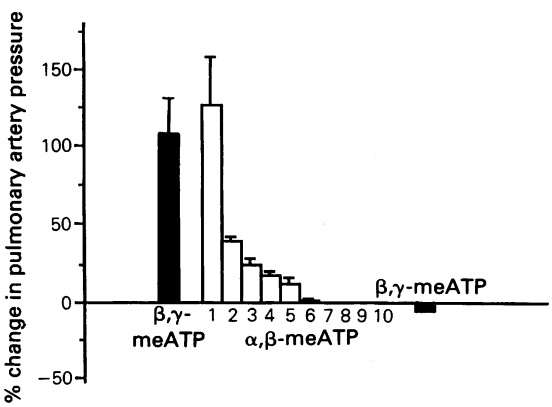


Figure 3 Effect of β,γ-meATP (400 μg) on isolated perfused lung of rat before and after desensitization by α,β-meATP (injections 1–10) as described in Figure 2. Mean values, with s.e.mean shown by vertical bars ($n = 6$).

In another group of animals ($n = 5$), to demonstrate that the desensitization by α,β-meATP was selective for vasoconstrictor P_2 receptors, angiotensin II (1 μg) was injected into the circuit and the rise in Ppa noted. After the Ppa had returned to baseline, the vasoconstrictor P_2 receptor was desensitized as described above with repeated 25 μg injections of α,β-meATP. When the desensitization was complete a second injection of angiotensin II (1 μg) was administered and the rise in Ppa recorded.

Vasoconstrictor P_2 receptor desensitization and HPV Six animals were initially ventilated with gas composition 21% O_2, 5% CO_2 and 74% N_2. After baseline Ppa was stable the inspired gas mixture was changed to a 'hypoxic' mixture composed of 3% O_2, 5% CO_2 and 92% N_2 until maximum hypoxic pulmonary vasoconstriction (HPV) was obtained. Animals were then re-ventilated with the normoxic mixture of 21% O_2, 5% CO_2 and 74% N_2 and Ppa permitted to return to baseline. Ventilation with the hypoxic gas mixture was repeated to obtain a second (control) HPV response. Ten bolus injections of α,β-meATP (25 μg) were then given at 2–4 min intervals, allowing the Ppa to return to baseline between administrations. Two minutes after the last injection of α,β-meATP the animal was again ventilated with the hypoxic gas mixture and the rise in Ppa measured. The animal was then returned to ventilation with 21% O_2, 5% CO_2 and 74% N_2 and after a stable Ppa had been obtained an i.v. injection of 400 μg of β,γ-meATP was administered to confirm desensitization of the vasoconstrictor P_2 receptors.

Drugs

β,γ-methylene 5′ adenosine triphosphate, α,β-methylene 5′ adenosine triphosphate and angiotensin II were obtained from Sigma (Poole, UK) and dissolved in aqueous solution.

Data analysis

Changes in Ppa as a result of β,γ-meATP, angiotensin II and hypoxia before and after desensitization of the vasoconstrictor P_2 receptors were compared by the paired Student's t test. $P < 0.05$ was considered significant.

Results

Figure 2 is a sample trace showing changes in Ppa from an animal given β,γ-meATP before and after desensitization of the vasoconstrictor P_2 receptor with α,β-meATP. Mean values of six experiments are shown in Figure 3. Initially, 400 μg of β,γ-meATP

caused a mean increase in Ppa of 110 ± 23% ($n = 6$) above baseline. The Ppa returned to baseline within 10 min in all animals. The initial 25 μg bolus of α,β-meATP resulted in a large increase in Ppa (127 ± 32% above baseline), but subsequent injections of the same dose over a 30 min period caused progressively smaller increases in Ppa such that after 6 injections α,β-meATP administration had no discernible effect. To show that the vasoconstrictor P_2 receptor was desensitized, a second injection of β,γ-meATP (400 μg) was given which resulted in a mean decrease in Ppa of 5 ± 1%.

Figure 4 demonstrates that the effect of α,β-meATP was not due to non-specific desensitization of the pulmonary vasculature. In five animals the first injection of angiotensin II resulted in an increase of Ppa of 16 ± 2 mmHg (99 ± 17% above baseline). The vasoconstrictor P_2 receptor was desensitized and then a second injection of angiotensin II resulted in an increase in Ppa of 18 ± 2 mmHg (82 ± 9% above baseline), not different from the first angiotensin response.

Figure 5 shows the mean values from animals ($n = 6$) exposed to two episodes of hypoxic ventilation prior to 10 consecutive 25 μg doses of α,β-meATP to desensitize the vasoconstrictor P_2 receptor, followed by a further period of ventilation with hypoxic gas mixture. The mean increases in Ppa during the initial hypoxic episodes were 72 ± 16 and 79 ± 11% respectively. The first injection of α,β-meATP (25 μg) caused a rise in Ppa of 153 ± 13%,

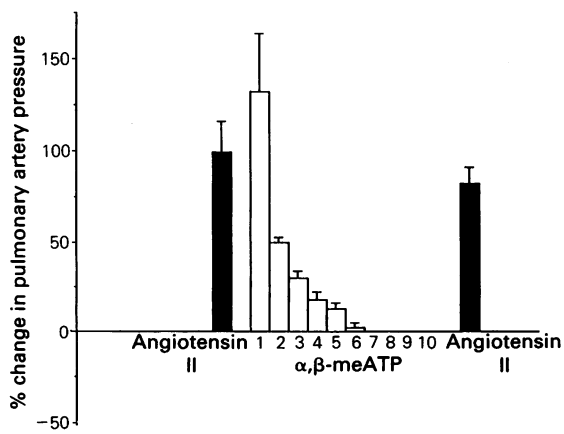


Figure 4 Increase in Ppa caused by angiotensin II (1 μg) in isolated perfused lung of rat before and after desensitization of the P_2 receptor with repeated administrations of 25 μg of α,β-meATP (injections 1–10). Mean values, with s.e.mean shown by vertical bars ($n = 5$).

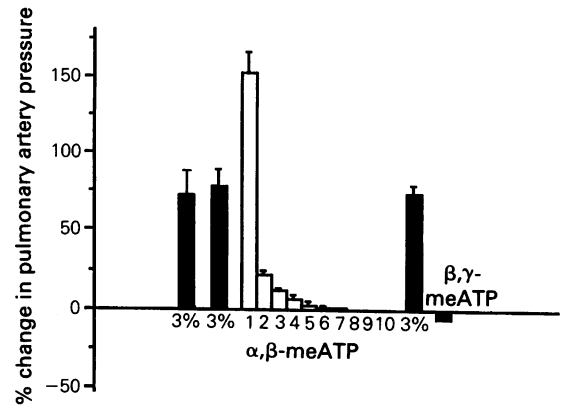


Figure 5 Increases in pulmonary artery pressure (Ppa) from isolated lung preparations ($n = 6$) ventilated with (3% oxygen (hypoxia)) before and after desensitization of the P_2 receptor with repeated boluses of α,β-meATP. Desensitization of the P_2 receptor did not diminish HPV. β,γ-meATP (400 μg) was injected at the end of the experiment to confirm blockade of the P_2 receptor. Mean values, with s.e.mean shown by vertical bars.

but subsequent injections resulted in progressively smaller increases, with no discernible effect after 7 doses, indicating desensitization of the vasoconstrictor P_2 receptor. The animal was then again ventilated with hypoxic gas mixture with a consequent rise in Ppa of 74 ± 6%. This was not significantly different from control values. Lastly, to confirm the continuing blockade of the vasoconstrictor P_2 receptor, β,γ-meATP (400 μg i.v.) was given and the Ppa was found to decrease by 5 ± 1%.

Discussion

Our data show the presence of vasoconstrictor P_2 receptors in the pulmonary artery of the rat. Vasoconstrictor P_2 receptors have not previously been described in the pulmonary circulation. However, contrary to the suggestion of Burnstock (1980), ATP does not seem to play a direct role in mediating HPV, which was not attenuated by complete desensitization of the vasoconstrictor P_2 receptor.

P_{2x} receptors are defined according to the potency order of a series of ATP analogues. Although we have not documented for certain that the vasoconstrictor P_2 receptor that we have studied on the pulmonary circulation is a P_{2x} receptor there is sufficient evidence to suggest that this is the case. Firstly, α,β-meATP, the ATP analogue that we used in this study has been shown to stimulate potently the P_{2x} receptor with little agonist activity at the P_{2y}

receptor. Secondly, α,β-meATP has been shown to desensitize selectively the P_{2x} receptor (Burnstock & Kennedy 1985a). Thirdly, the only P_2 receptor reported to mediate constriction in blood vessels has been the P_{2x} receptor.

We used α,β-meATP to desensitize the vasoconstrictor (P_{2x}) receptor in the pulmonary vasculature in a manner similar to that reported by others (Hedlund *et al.*, 1983; Bulloch & McGrath, 1988). This compound can selectively desensitize the P_{2x} receptor whilst the smooth muscle responses to other stimuli remain unaffected (Kasakov & Burnstock, 1983; Hedlund *et al.*, 1983). Selectivity of α,β-meATP for the P_{2x} receptor was confirmed in our study by demonstrating that the pressor response to angiotensin II was not affected by the desensitization by α,β-meATP. In all our preparations, desensitization of the vasoconstrictor P_2 receptor was confirmed by the administration of β,γ-meATP, a compound which has agonist activity at the P_{2x} and to a lesser extent P_{2y} receptors (Kennedy & Burnstock, 1985). Administration of β,γ-meATP prior to desensitization of the vasoconstrictor P_2 receptor resulted in a large increase in Ppa (110% above baseline) indicating stimulation of a vasoconstrictor receptor. Following desensitization, β,γ-meATP administration caused a small but significant decrease in Ppa (5% below baseline), which was presumably due to unopposed stimulation of the P_{2y} receptor.

For the desensitization of the vasoconstrictor P_2 receptor we used repeated doses of 25 μg of α,β-meATP. The first dose of this agonist produced an increase in Ppa approximately twice the magnitude of the increase due to HPV. After 10 injections of 25 μg of α,β-meATP the pressor response to this P_{2x} agonist was abolished, however, the pressor response to HPV remained intact. It is possible that after desensitization of the vasoconstrictor P_2 receptor, doses of α,β-meATP larger than 25 μg would have resulted in some contraction, implying that there was not complete desensitization of this receptor. However, this is probably irrelevant to the study of HPV as we have demonstrated that the vasoconstrictor P_2 receptor is desensitized to a stimulation resulting in a contraction larger than that caused by HPV.

The mechanism underlying HPV has been the subject of much investigation in the past 20 years. A great deal of the interest has focused on potential mediators including histamine (Bergofsky, 1979), angiotensin II (Berkov, 1974), 5-hydroxytryptamine (Keith & Will, 1981) and more recently leukotrienes (Ahmed & Oliver, 1983). However, there have been inconsistencies in all of these proposed theories and to date the mechanism of HPV has remained elusive. It has been suggested that venoarterial or alveolar-vascular reflexes confined to the lungs may be important in HPV (Fishman, 1976). Studies of HPV investigating the role of the autonomic nervous system have commonly used conventional antagonists of the parasympathetic and sympathetic components (Silove & Grover, 1968; Lewis, 1976). Burnstock (1972a) has proposed the 'purinergic hypothesis' that a purine nucleotide, probably ATP, is the principal transmitter released from non-adrenergic, noncholinergic 'purinergic' nerves. Recently Inoue & Kannan (1988) have presented evidence for purinergic (non-adrenergic) excitatory nerves of rat intrapulmonary arteries. With the recent discovery of agonists and antagonists of the purinoceptor subtypes (P_{2x} and P_{2y}) (Burnstock & Kennedy, 1985b) it is now possible to evaluate the role of the purine nucleotides in the control of pulmonary vascular tone.

It has been reported previously that ATP infused intravenously into human subjects with pulmonary hypertension, causes a fall in pulmonary artery pressure (Gaba *et al.*, 1986), suggesting activation of P_{2y} receptors located on the vascular endothelium. Furthermore, ATP relaxes isolated human pulmonary arteries by an endothelium-dependent mechanism (Greenberg *et al.*, 1986). Adenosine also causes vasodilatation in human pulmonary vessels which is independent of endothelium via an A_2-adenosine receptor (McCormack *et al.*, 1989). The excitatory (vasoconstrictor) P_{2x} receptors are located on the vascular smooth muscle (White *et al.*, 1985), such that ATP given intravenously does not cause vasoconstriction but preferentially stimulates receptors on the endothelial surface (P_{2y} receptors) leading to vasodilatation.

Although our findings do not suggest a role for vasoconstrictor P_2 purinoceptors in HPV, their physiological role remains to be determined. The hypothesis that some nerves release more than one transmitter was proposed by Burnstock (1972b) and purinergic cotransmission with noradrenaline has been claimed for a number of different blood vessels. The presence and function of purinoceptors in the human pulmonary vasculature requires characterization as this may have important future therapeutic consequences.

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Pharmacological properties of a C-fibre response evoked by saphenous nerve stimulation in an isolated spinal cord-nerve preparation of the newborn rat

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1 An isolated spinal cord-peripheral nerve preparation of the newborn rat was developed. In this preparation it is possible to record spinal reflexes from a lumbar ventral root in response to stimulation of the ipsilateral saphenous or obturator nerve.

2 Single shock, weak intensity stimulation of the saphenous nerve induced a fast conducted compound action potential in the L3 dorsal root and a fast depolarizing response in the ipsilateral L3 ventral root. As a stronger stimulus was applied to the saphenous nerve, a slowly conducted compound action potential appeared in the dorsal root and a slow depolarizing ventral root potential (v.r.p.) in the L3 ventral root.

3 Single shock stimulation of the obturator nerve induced a rapidly conducted compound action potential in the L3 dorsal root and monosynaptic and polysynaptic reflexes, with a fast time course, in the ipsilateral L3 ventral root.

4 The slow v.r.p. evoked by saphenous nerve stimulation was depressed by the tachykinin antagonist, [D-Arg^1, D-Trp^{7,9}, Leu^{11}] substance P (spantide), 4–16 μM. The response recovered its original shape and size 30–60 min after the removal of this antagonist.

5 The saphenous nerve-evoked slow v.r.p. was depressed by [Met^5] enkephalin (0.1–1 μM), dynorphin (1–13)(0.2 μM) and morphine (1–2 μM), and these effects were reversed by naloxone (1 μM).

6 Two endogenous peptides, galanin (1–2 μM) and somatostatin (1–2.5 μM), inhibited the slow v.r.p. evoked by saphenous nerve stimulation, whereas another endogenous peptide, calcitonin gene-related peptide (0.1–0.5 μM), potentiated the slow v.r.p. The slow v.r.p. was also inhibited by γ-aminobutyric acid (GABA, 20 μM) and muscimol (0.2 μM), and their effects were antagonized by bicuculline (1 μM).

7 The present results suggest that substance P and neurokinin A are involved in the saphenous nerve-evoked C-fibre response in the spinal cord of the newborn rat.

Introduction

The tachykinin antagonist, [D-Arg^1, D-Trp^{7,9}, Leu^{11}] substance P (spantide) (Rosell, 1986), depresses the nociceptive reflex induced by application of capsaicin to the tail of an isolated spinal cord-tail preparation of the newborn rat (Otsuka & Yanagisawa, 1988). Based on this finding, as well as many other lines of evidence, we suggested that substance P (SP) and neurokinin A (NKA), which are present in a subpopulation of primary afferent C fibres, are involved as neurotransmitters in this nociceptive reflex. We assumed that the nociceptive reflex was induced by stimulation of primary afferent C fibres, basing our assumption entirely on the pharmacological specificity of capsaicin (Kenins, 1982; Heyman & Rang, 1985; Marsh, 1985). Indeed, we were not able to prove, by recording from sensory nerves, that the application of capsaicin resulted in an afferent C-fibre volley which induced the nociceptive reflex. In the present study we used a newly developed isolated spinal cord-peripheral nerve preparation of the newborn rat in which a purely cutaneous nerve, i.e. the saphenous nerve, and a typical muscle nerve, i.e. the obturator nerve, can be separately stimulated. Electrical stimulation of saphenous nerve at C-fibre strength evoked a slow ventral root depolarization similar to that evoked by

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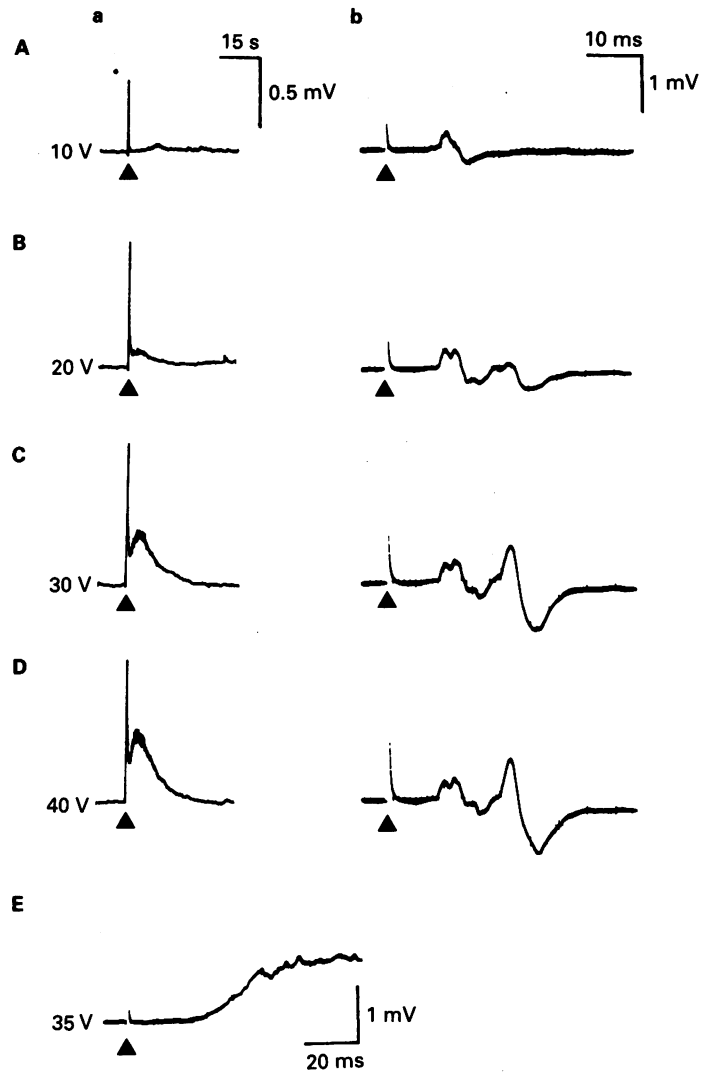


Figure 1 Effects of stimulation of the saphenous nerve. Isolated spinal cord-peripheral nerve preparation of a 3 day-old rat. The nerve was stimulated with single shocks of 200 μs duration and increasing intensities at ▲. The intensities are indicated on the left side of the records. At first the potentials from the ipsilateral L3 ventral root were monitored on a pen-recorder (Aa–Da). In (E) part of the response of the ventral root is displayed with a fast sweep speed on an oscilloscope. Then the L3 dorsal root was cut at the point of entry into the spinal cord and the potentials were recorded on an oscilloscope from the cut distal end of the dorsal root (b). Calibrations in (A) apply to (A–D).

the application of capsaicin to the tail (Otsuka & Yanagisawa, 1988). We therefore used this preparation to test the effects of spantide and other drugs on a response with a slow time course evoked by electrical stimulation of C fibres. Some of the present results have been published in a preliminary form (Otsuka & Yanagisawa, 1987b).

Methods

Neonatal Wistar rats aged 1–4 days were anaesthetised with ether, decapitated and placed in a dissecting dish perfused with oxygenated artificial cerebrospinal fluid (CSF; for composition see Akagi *et al.*, 1985) at room temperature. After the removal

of visceral organs, a ventral laminectomy was performed and the dural sac opened in order to expose the spinal cord to O_2 and the nutrients contained in the artificial CSF. The inguinal ligament was then resected exposing the distal end of the psoas muscle with the femoral nerve lying laterally to it. Resection of the psoas muscle exposed the thick nervous trunk which gives rise to the femoral and obturator nerves. The obturator nerve was sectioned at the level of the obturator foramen and dissected free. The saphenous nerve was sectioned where it runs superficially over the medial aspect of the knee joint, and was dissected free up to its origin from the femoral nerve (for anatomical data, see Greene, 1968).

The spinal cord, without hemisection below the lower thoracic level, together with the lumbar ventral and dorsal roots, dorsal root ganglia (L3–4), and the attached femoral, saphenous and obturator nerves on one side, was dissected out, placed in a recording chamber of about 0.4 ml volume and perfused with artificial CSF saturated with 95% O_2/5% CO_2 at a rate of about 6 ml min^{-1}. The temperature was kept at 27°C.

Extracellular recordings were made from the L3 ventral root or from the distal end of the cut dorsal root with a tightly fitting suction electrode on a pen-recorder or an oscilloscope through a preamplifier. Details of the experimental procedures and the sources of drugs were as described previously (Otsuka & Yanagisawa, 1988).

Results

Effects of stimulation of the saphenous and obturator nerves

In the experiment illustrated in Figure 1, the saphenous nerve was stimulated with single shocks of 200 μs duration and increasing intensity, and the responses were recorded from the ipsilateral L3 ventral root. At a stimulus intensity of 10 V, a depolarizing response lasting about 200 ms was induced (Figure 1Aa). When the stimulus intensity was increased to 30 V, a slow depolarizing response lasting 20–30 s was elicited (Figure 1Ca). After these observations, the L3 dorsal root was severed at the entrance to the spinal cord and the incoming afferent volley was recorded from the peripheral stump of the dorsal root with a suction electrode, while the position of the saphenous nerve in the stimulating suction electrode was left unchanged. When the saphenous nerve was stimulated with a stimulus intensity of 10 V, a rapidly conducted compound action potential was recorded from the dorsal root (Figure 1Ab), which corresponded to the depolarizing response with a fast time course recorded from the ventral root. At a stimulus intensity of 30 V, a slowly conducted wave was recorded (Figure 1Cb), which corresponded to the response with a slow time course recorded from the ventral root. We estimated the conduction velocity of the rapidly conducted wave to be about 1 ms^{-1}, a speed which would be expected in A fibres. The slowly conducting wave had an estimated conduction velocity of 0.5 ms^{-1}, a speed that would be expected in C fibres (Fitzgerald & Gibson, 1984; Fitzgerald, 1985).

Stimulation of the obturator nerve with increasing intensity, elicited a response in the ipsilateral ventral root when the intensity of the stimuli attained a value of 15 V (Figure 2Ba). When the potential was recorded from the peripheral stump of the severed L3 dorsal root, a compound action potential with a conduction velocity of about 1.2 ms^{-1} was observed at a stimulus intensity of 15 V (Figure 2Bb). With stimuli of varying intensities a close correlation between the amplitude of the compound action potential in the dorsal root and the size of the evoked response in the ventral root was observed (Figure 2A–D). The short delay suggests that the initial response in the ventral root is a monosynaptic reflex. There is evidence that monosynaptic reflexes evoked by Ia muscle afferents are already developed at the time of birth of rats (Saito, 1979; Konishi, 1982; Kudo & Yamada, 1985). Saphenous nerve stimulation, by contrast, did not evoke a monosynaptic reflex in the L3 ventral root as shown in Figure 1E with a fast sweep speed.

Recordings from ventral roots with a slow time base showed that stimulation of the obturator nerve elicited a depolarizing response with a relatively fast time course, which, even with intense stimulation, was followed by a slow depolarization of small amplitude (Figure 2E). In this paper, therefore, we present data about effects of drugs on the slow response evoked by stimulation of a cutaneous nerve only. Single or double shocks at 20–50 Hz were used for stimulation. Stable responses could be recorded from the ventral root for several hours.

Effects of spantide on the saphenous nerve-evoked slow response

Since the above experiments suggested that the slow depolarizing response of the ventral root upon intense stimulation of the saphenous nerve was evoked by primary afferent C fibres, it was of interest to examine the possible involvement in this reflex of SP and NKA which are contained in certain primary afferent C fibres (Nagy *et al.*, 1981; Leah *et al.*, 1985; Hua *et al.*, 1985). We therefore tested the effects of spantide, which has been shown to be a specific and

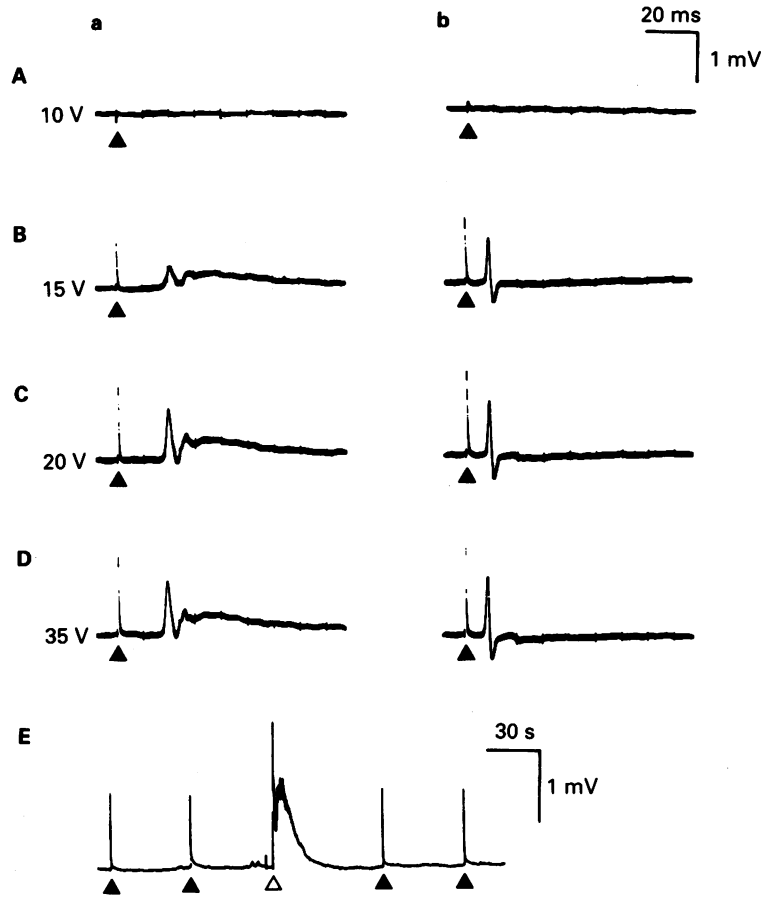


Figure 2 Effects of stimulation of the obturator nerve. The same preparation as in Figure 1 was used except in (E). The nerve was stimulated at ▲ with single square pulses of 200 μs duration and increasing intensities, which are indicated on the left side of the records. Other details of the experimental procedures are the same as for Figure 1. (a) Records from the L3 ventral root. (b) Records from the L3 dorsal root. Time and voltage scales in (Ab) apply to both (a) and (b) except (E). In (E) the responses of the ventral root to supramaximal stimulation of obturator (▲) and saphenous (△) nerves are monitored on a pen-recorder with a slow time scale.

effective tachykinin antagonist (Otsuka & Yanagisawa, 1988), on the saphenous nerve-evoked slow ventral root potential (v.r.p.). Perfusion of the preparation with spantide at a concentration of 16 μM markedly depressed the slow v.r.p. and recovery occurred 30–60 min after removal of the antagonist (Figure 3). Similar depressant effects of spantide (4–16 μM) were observed in 4 other preparations.

Surprenant *et al.* (1987) showed that [D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}]substance P, a tachykinin antagonist with a structure closely related to spantide, causes release of noradrenaline in guinea-pig submucosal plexus. It was conceivable, therefore, that the effect of spantide as illustrated in Figure 3, might be due to a release of some inhibitory transmitters. However, the depressant effect of spantide on the saphenous nerve-evoked C-fibre response was as clearly observed in the presence of naloxone (0.5 μM), bicuculline (0.5 μM) or prazosin (1 μM) as in the absence of these antagonists.

Post *et al.* (1985) found that two substance P analogues with tachykinin antagonist activity have a potent local anaesthetic action. It might therefore be argued that spantide inhibited the slow v.r.p. through its depressant action on the afferent input in the present preparation. However, upon stimulation of the saphenous and obturator nerves the compound action potentials recorded from the cut dorsal root were not at all affected by 16 μM spantide. Furthermore, both the monosynaptic reflex evoked by

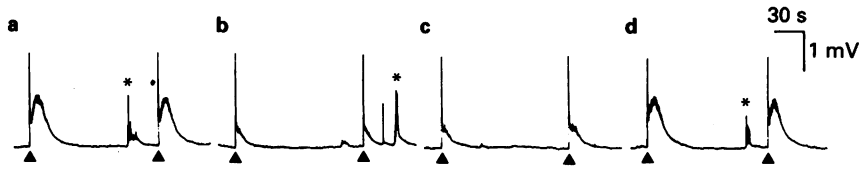


Figure 3 Effect of spantide on the reflex responses evoked by saphenous nerve stimulation. Isolated spinal cord–peripheral nerve preparation of a 1 day-old rat. Potentials were recorded from an L3 ventral root and the ipsilateral saphenous nerve was stimulated every 2 min at ▲ with double square pulses of 100 μs duration and supramaximal intensity at 20 Hz. (a) Control responses, (b) 3 min after adding 16 μM spantide, (c) and (d) are 10 min and 42 min, respectively, after removal of spantide. In each record two consecutive responses are shown. Asterisks indicate spontaneous activity.

stimulation of obturator nerve and the saphenous nerve-evoked depolarizing v.r.p. of fast time course were little affected by spantide 16 μM (Figure 3).

Effects of endogenous peptides, γ-aminobutyric acid and related drugs

We have further studied the effects of several endogenous peptides, γ-aminobutyric acid (GABA) and related drugs to examine their possible physiological roles in the spinal cord. In the experiment shown in Figure 4, the effects of opioids were examined. The saphenous nerve-evoked slow v.r.p. was markedly depressed by [Met^5]enkephalin (0.1–1 μM; number of preparations (n) = 3), dynorphin (1–13) (0.2 μM; n = 3) and morphine (1–2 μM; n = 2). The effect of [Met^5] enkephalin disappeared after the peptide was washed out, which led to a transient enhancement of the magnitude of the reflex (Figure 4Ac). The effects of dynorphin (1–13) and morphine, by contrast, were sustained for more than 30 min after removal of the drugs. These effects of opioids were reversed by naloxone (1 μM) (Figure 4Bc). Application of naloxone (0.5 μM) alone to fresh preparations which had not received any opioids previously, markedly potentiated the saphenous nerve-evoked slow v.r.p. (cf. Yanagisawa *et al.*, 1984).

As shown in Figure 5, two other endogenous neuropeptides, galanin (1–2 μM; n = 2) and somatostatin (1–2.5 μM; n = 3), also depressed markedly and reversibly the saphenous nerve-evoked slow v.r.p. By contrast, calcitonin gene-related peptide (CGRP; 0.1–0.5 μM; n = 5) reversibly potentiated the saphenous nerve-evoked slow v.r.p. (Figure 6).

Figure 7 shows the effects of GABA (20 μM; n = 4) and the $GABA_A$-receptor agonist, muscimol (0.2 μM; n = 2) on the saphenous nerve-evoked slow v.r.p. which was depressed by these drugs. These effects were reversed by either washing out the drugs or by

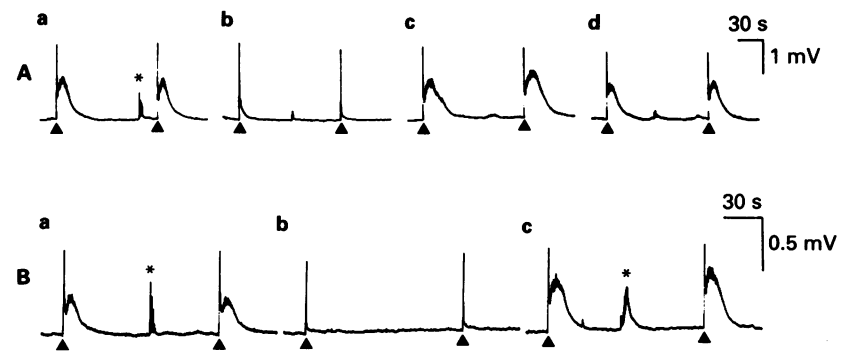


Figure 4 Effects of opioids and naloxone on the reflex responses evoked by saphenous nerve stimulation. Isolated spinal cord-peripheral nerve preparations of 1 day-old (A) and 2 day-old (B) rats. (A) Effect of [Met^5]enkephalin. Potentials were recorded extracellularly from an L3 ventral root upon stimulation of the ipsilateral saphenous nerve every 2 min at ▲ with double square pulses of 100 μs duration and supramaximal intensity at 50 Hz. (a) Control, (b) 1 min after adding 1 μM [Met^5]enkephalin, (c) and (d) are 1 min and 5 min, respectively, after removal of [Met^5] enkephalin. (B) Effects of morphine and naloxone. Experimental procedures are the same as for (A). (a) Control, (b) 5 min after adding 2 μM morphine, (c) 3 min after further addition of 1 μM naloxone in the continuous presence of 2 μM morphine. In each record 2 consecutive responses are shown. Asterisks indicate spontaneous activity.

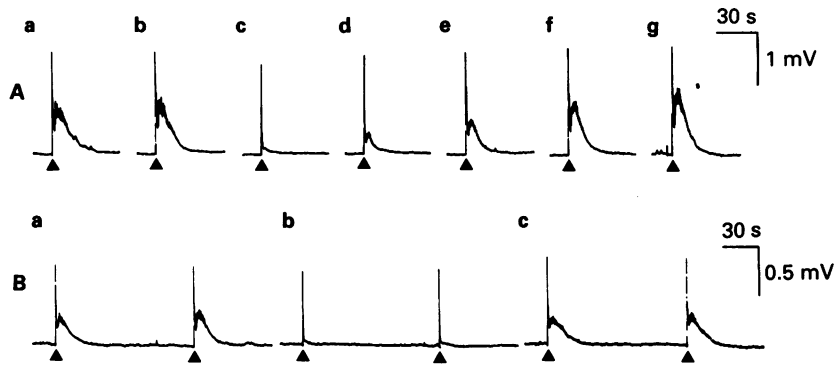


Figure 5 Effects of galanin and somatostatin on the reflex responses evoked by saphenous nerve stimulation. Isolated spinal cord-peripheral nerve preparations of 2 day-old rats. Potentials were recorded extracellularly from L3 ventral roots and the ipsilateral saphenous nerve was stimulated every 4 min (A) or 2 min (B) at ▲ with double square pulses of 100 μs duration and supramaximal intensity at 50 Hz. (A) Effect of galanin. (a) and (b) Controls, (c) 2 min after adding 2 μM galanin, (d), (e), (f) and (g) are 3, 7, 15, and 23 min, respectively, after removal of galanin. (B) Effect of somatostatin. (a) Conrol; (b) 2 min after adding 2.5 μM somatostatin, (c) 65 min after removal of the peptide. In (B) each record shows two consecutive responses.

bicuculline (1 μM). In addition, GABA exerted a slight depolarizing action on the ventral root.

The saphenous nerve-evoked fast v.r.p. was not affected by calcitonin gene-related peptide (CGRP, 1 μM), but slightly depressed by [Met^5]enkephalin (1 μM), dynorphin (1–13) (0.2 μM), morphine (2 μM),

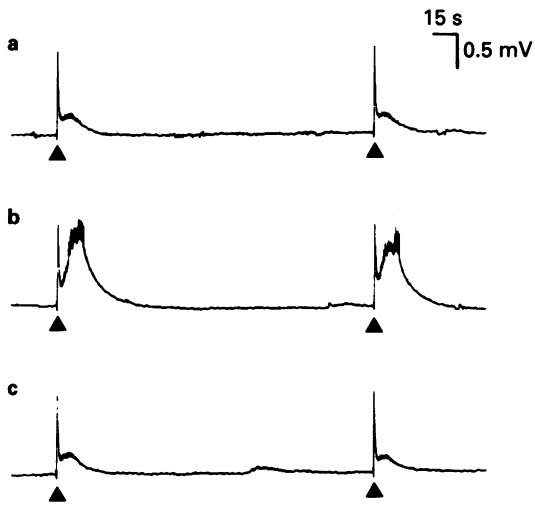


Figure 6 Effects of calcitonin gene-related peptide (CGRP) on the reflex responses evoked by saphenous nerve stimulation. Isolated spinal cord-peripheral nerve preparation of a 2 day-old rat. Recordings are as in Figures 3–5. Ipsilateral saphenous nerve was stimulated every 3 min at ▲ with double square pulses of 150 μs duration and supramaximal intensity at 50 Hz. (a) Control, (b) 4 min after adding 0.2 μM CGRP, (c) 30 min after removal of the peptide.

galanin (2 μM), somatostatin (2.5 μM), GABA (20 μM) and muscimol (0.2 μM). The obturator nerve-evoked monosynaptic reflex was little affected by these drugs at the above concentrations.

Discussion

Intense stimulation of a cutaneous, i.e. saphenous, nerve which contains more than 80% of unmyelinated fibres (Jancsó *et al.*, 1985), induces a slowly conducted afferent volley in the L3 dorsal root which is presumably attributable to C fibres (Fitzgerald, 1985), and at the same time a slow depolarizing response in the ipsilateral ventral root. The functional significance of the saphenous nerve-evoked slow v.r.p. is at present unknown, but may be related to various C fibre-evoked activities of spinal neurones. In rats, flexor-withdrawal reflexes can be evoked by cutaneous C-fibre stimulation at birth (Fitzgerald & Gibson, 1984). These reflexes are exaggerated and of long duration in the neonatal period (Ekholm, 1967; Fitzgerald & Gibson, 1984). Our preliminary experiments with the present preparations showed that the saphenous stimulation at C-fibre strength induced spike discharges in pectineus nerve during the 2 to 4 s period after the stimulus. The latter observation suggests that the saphenous nerve-evoked slow v.r.p. contains, at least in part, components of flexor reflexes.

A tachykinin antagonist, spantide (Rosell, 1986), depressed the saphenous nerve-evoked slow v.r.p., suggesting that SP and NKA may be involved as neurotransmitters in this slow response. A likely possibility is that SP and NKA are released from

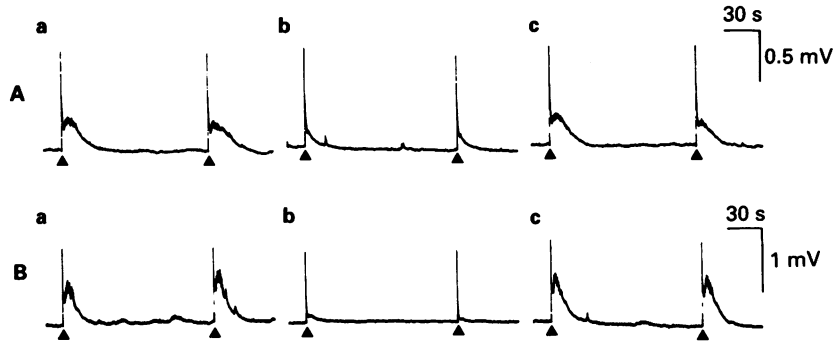


Figure 7 Effects of γ-aminobutyric acid (GABA) and muscimol on the reflex responses evoked by saphenous nerve stimulation. Isolated spinal cord-peripheral nerve preparation of 2 day-old (A) and 3 day-old (B) rats. Recordings and stimulation are as in Figure 4. (Aa) Control, (Ab) 3 min after adding 20 μM GABA, (Ac) 15 min after washing out GABA. (Ba) Control, (Bb) 1 min after adding 0.2 μM muscimol, (Bc) 40 min after washing out muscimol.

certain primary afferent C fibres upon stimulation of the saphenous nerve and produce slow excitatory postsynaptic potentials (e.p.s.ps) in dorsal horn neurones which, via synapses in the spinal cord, elicit a sustained depolarization of motoneurones (Otsuka & Yanagisawa, 1988). In this connection, Cook *et al.* (1987) observed in rat spinal cord that brief inputs from peripheral afferent C fibres can greatly increase the size of cutaneous receptive fields of dorsal horn neurones for prolonged periods, and suggested that this may be due to slow subthreshold excitation of dorsal horn neurones.

In the present study we used spantide at 4–16 μM. Our previous study (Otsuka & Yanagisawa, 1988) showed that spantide 16 μM displays a considerable degree of specificity as a tachykinin antagonist, although it also antagonized bombesin (cf. Yachnis *et al.*, 1984). However, the role of bombesin in the C-fibre response in the present preparation is probably small because the amount of bombesin in the spinal cord was shown to be about one fiftieth of that of SP (McGregor *et al.*, 1984). The pA_2 values of spantide were estimated against various tachykinin agonists in various tissues. The estimated pA_2 values varied between 5.2 and 6.9, which suggests the existence of more than one class of spantide-susceptible tachykinin receptor (Chahl, 1985; Greiner, 1985; Featherstone *et al.*, 1986; Buck & Shatzer, 1988). In the newborn rat spinal cord we observed that spantide, 2–16 μM, depressed the SP- and NKA-induced depolarization of the ventral root, and estimated the pA_2 value of spantide against SP to be 5.4 (Otsuka & Yanagisawa, 1988). This value probably represents mainly the pA_2 values of tachykinin receptors of possibly more than one class located on spinal interneurones. On the other hand, Wienrich & Harting (1985) found that the SP-induced depolarization of ventral roots was unaffected or potentiated by spantide 10 μM in the newborn rat spinal cord treated with tetrodotoxin, which may reflect different properties of tachykinin receptors located on motoneurones.

Immunohistochemical, autoradiographic and neurochemical studies show that there is a dense concentration of GABA terminals (Miyata & Otsuka, 1975; Barber *et al.*, 1978; Hunt *et al.*, 1981), GABA receptors (Price *et al.*, 1984; Young & Kuhar, 1980), enkephalin-immunoreactive terminals (Hökfelt *et al.*, 1977; Hunt *et al.*, 1981) and opiate receptors (Atweh & Kuhar, 1977) in superficial layers of the dorsal horn. Probably both GABA and enkephalins act as inhibitory neurotransmitters of spinal interneurones in this region. It is conceivable that an incoming primary afferent volley activates not only secondary sensory neurones but inhibitory interneurones containing GABA or enkephalins, and that these transmitters modulate the nociceptive input by presynaptic and/or postsynaptic inhibition. In support of this, the tail-pinch potential, i.e. the nociceptive reflex induced by tail-pinch and recorded from a lumbar ventral root of the isolated spinal cord-tail preparation, was potentiated by naloxone (Yanagisawa *et al.*, 1984) and by bicuculline (Otsuka & Yanagisawa, 1987a), suggesting that endogenous enkephalinergic and GABA-mediated mechanisms are involved. It should be noted, however, that the inhibitory mechanisms operated by GABA and enkephalins might be either tonic, acting as a background inhibition, or phasic, being activated by primary afferent stimulation.

The saphenous nerve-evoked slow v.r.p. was depressed by opioids, galanin, somatostatin, GABA and muscimol. The pharmacological properties of the slow v.r.p. are therefore similar to those of the capsaicin-induced nociceptive reflex observed in the isolated spinal cord-tail preparation of the newborn

rat (Yanagisawa & Otsuka, 1985; Yanagisawa *et al.*, 1986a,b; Otsuka & Yanagisawa, 1988), which is consistent with the assumption that both responses are evoked by C-fibre stimulation. The effects of opioids may be relevant to the fact that C-fibre-mediated nociception is particularly sensitive to morphine (Le Bars *et al.*, 1976). Galanin, somatostatin and CGRP are known to occur in subpopulations of sensory C fibres (Hökfelt *et al.*, 1976; Nagy *et al.*, 1981; Ch'ng *et al.*, 1985; Gibson *et al.*, 1984; Gibbins *et al.*, 1985). Based on the present findings of the peptide actions, it is conceivable that galanin, somatostatin and CGRP are released from sensory C fibres as neurotransmitters or co-transmitters and modulate nociceptive inputs in the substantia gelatinosa. The potentiating effect of CGRP on the saphenous nerve-evoked slow v.r.p. is consistent with the observation of Woolf & Wiesenfeld-Hallin (1986) that CGRP increased the excitability of the nociceptive flexion reflex. A possible mechanism of action of CGRP is the increase of SP release from C fibres (Oku *et al.*, 1987), because the intrathecal application of CGRP did not affect the response to SP applied to the spinal cord in mice (Gamse & Saria, 1986).

The present spinal cord-peripheral nerve preparation appears to offer many possibilities for further study of the spinal reflexes involving C fibres. For example, it should now be possible to examine pharmacological aspects of the effects of concomitant stimulation of A fibres on the C-fibre response. According to the gate control theory (Melzack & Wall, 1965), stimulation of some A fibres would be expected to suppress the C-fibre reflex induced by saphenous nerve stimulation. It may also be possible to examine the effect of descending inhibition on C-fibre reflexes by stimulating appropriate nuclei in the brainstem (García-Arrarás *et al.*, 1986). Furthermore, it will be possible to elaborate the present preparation to include various afferent and efferent pathways in the peripheral nervous system and study reflexes which are physiologically meaningful, such as autonomic reflexes.

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The effect of a single dose of oestradiol on tamoxifen-induced uterine hyperaemia and growth in the rat

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1 The effect of oestradiol on tamoxifen – (single doses of each agent) induced responses in the uterus of the mature ovariectomized rat was investigated. The parameters examined were uterine blood flow (measured by the microsphere technique), weight and oestrogen receptor concentrations.

2 Initially the addition of oestradiol to the tamoxifen regimen produced a reduction in uterine blood flow and dry weight which was reflected by a reduction in nuclear oestrogen receptor. However, as the tamoxifen response became more established from 48 h onwards the addition of oestradiol produced an additive effect. These changes were not mirrored by increases in nuclear oestrogen receptor.

3 The results suggest that tamoxifen and oestradiol may, in part, act via different mechanisms; the role of possible mediatory systems such as histamine release and eosinophil migration is discussed. Heterogeneous control systems may also be involved in the early and late uterine response.

Introduction

Tamoxifen, although generally classified as a non-steroidal antioestrogen, can, under certain conditions, act as a partial agonist. This dualism depends upon the target organ and species investigated; endocrine status may also be relevant in breast cancer patients. In the rat, tamoxifen behaves as a partial agonist (Harper & Walpole, 1967; Emmens, 1970; Jordan *et al.*, 1978). It is possible that tamoxifen exerts its effect via the oestrogen receptor system as it has been shown that the triphenylethylene anti-oestrogens can compete and bind to oestrogen receptor sites (Sutherland & Murphy, 1982).

After formation of the oestrogen receptor/ligand complex, a process known as transformation occurs which increases the affinity of the complex for the chromatin. Recent evidence suggests that the ligand associated with the receptor is important with respect to conformational changes of the receptor molecules (Giambiagi & Pasqualini, 1988); the end result being the specific genomic responses associated with hormone stimulation.

A previous study in this laboratory has shown that a single dose of tamoxifen has a persistent effect on uterine blood flow and weight (Marshall & Senior, 1987). As a result of this work, we suggested that the classical genomic oestrogen receptor interaction may not fully account for the uterotrophic events seen after tamoxifen administration. In the current work, oestradiol was given at various times after single dose tamoxifen administration in order to investigate the possibility of compromising or altering the established tamoxifen uterotrophic response, thus further elucidating the relationship between the uterine response and oestrogen receptor dynamics.

Methods

Mature virgin female rats of a CD-derived Sprague-Dawley random-bred strain from the animal house, University of Bradford, were used throughout and were housed in light (07 h 00 min–19 h 00 min) and temperature (18°C) controlled rooms. Food and water were available *ad libitum*. All the animals used in this study were bilaterally ovariectomized at least 21 days before further experimentation and were randomly assigned to groups.

Measurement of blood flow

The microsphere technique (previously described by Phaily & Senior, 1978) was used to determine blood flow in the rat anaesthetized with sodium pentobarbitone (60 mg kg^{-1}). This dose was selected to ensure anaesthetic control throughout the experimental procedure and minimal cardiovascular changes. The microspheres used were 15 μm in diameter (NEN-Trac, New England Nuclear, Boston, MA, U.S.A.), uniformly labelled with ^{46}Sc and suspended in 10% (w/v) dextran containing 0.1% (w/v) Tween 80.

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Uterine blood flows were calculated using wet weight of tissue. In all cases blood flow between the adrenals and kidneys was balanced, indicating a uniform distribution of microspheres. Cardiac output was also unaffected by the treatments. When dry weights are quoted the tissue was dried until the weight remained constant.

Measurement of cytoplasmic and nuclear oestrogen receptors by [^3H]-oestradiol exchange

Homogenization, cytosol and nuclear pellet preparation and the determination of oestrogen receptors therein was carried out by the method of Marshall & Senior (1987). Briefly, animals were killed by cervical dislocation and the uteri were rapidly removed and homogenized in PE buffer (10 mM phosphate buffer, pH 7.4, containing 1.5 mM EDTA and 2 mM mercaptoethanol). All procedures were performed at 4°C unless otherwise stated. Homogenates were centrifuged at 100,000 g for 1 h. Aliquots of cytosol were incubated at 30°C for 1 h with 1–20 nM of [^{3}H]-oestradiol ([2, 4, 6, 7,-^{3}H]-oestradiol, 84–104 Ci mmol^{-1}) (Radiochemical Centre, Amersham, Bucks) plus or minus 100 fold excess of oestradiol. Separation of bound from free ligand was achieved by use of dextran-coated charcoal (0.5% dextran (BDH, Poole), 5% Norit A acid washed charcoal w/v in phosphate buffer). The radioactivity in the supernatant was determined by liquid scintillation counting. The assay of nuclear oestrogen receptors involved the preparation of a low speed nuclear pellet (prepared by centrifugation at 800 g of homogenate). Aliquots of the nuclear suspension were treated in the same way as in the cytosolic assay. Separation was achieved with a 60% hydroxylapatite suspension in phosphate buffer. After ethanolic extraction of the pellet, radioactivity was determined by liquid scintillation counting (44% efficiency).

Drug administration

Oestradiol (oestra-1,3,5-(10)-triene-3,17β-diol; BDH, Poole, UK) was dissolved in a 10% (v/v) solution of propylene glycol and was administered intravenously (i.v.) at a dose level of 0.5 μg kg^{-1} into the tail vein of the conscious rat 3 h before measurements were made. This dose of oestradiol was chosen as it gives maximal uterine blood flow 3 h after administration and is the minimum dose to produce this effect. Tamoxifen (ICI, Macclesfield, Cheshire) was dissolved in ethanol and dispersed in arachis oil before subcutaneous (s.c.) injection at a dose level of 1 mg kg^{-1}. The solvents used did not have any effect on the parameters measured and these results have been grouped as controls.

Statistical analysis

The results (expressed as mean $\pm$ s.e.mean) were compared by analysis of variance and Student's t test (two tailed) (Snedecor & Cochran, 1979).

Results

The exchange conditions employed were found to be optimal with respect to maximal exchange with minimal loss of binding sites.

In this work the term cytoplasmic receptor is used: this refers to the receptor form that separates into the cytoplasmic cell fraction after homogenization. It represents the unoccupied inactive form of the receptor thought to be located in the nucleus *in vivo*. The nuclear or activated form of the receptor remains in the nuclear compartment after fractionation (Jordan, 1984). Although receptor localization is important, in terms of this study the receptor measurements made are used in a qualitative manner to assess the effect of drug treatments on the receptor population as a whole, rather than as an attempt to compartmentalise the oestrogen receptor.

Effect of oestradiol on tamoxifen-stimulated blood flow (Table 1)

When oestradiol was included in the treatment regimen 24 h after tamoxifen administration and uterine blood flow was measured 3 h later, the presence of oestradiol caused significantly reduced blood flow when compared to the result with tamoxifen alone at 27 h. However this reduction was no longer apparent at 48 h after the tamoxifen treatment (oestradiol was given at 45 h); 72 h after tamoxifen administration the addition of oestradiol had caused significant elevation in uterine blood flow.

Blood flow to the adrenals, kidneys, spleen and stomach was not significantly altered by the treatments, except for splenic flow which showed an increase ($P < 0.05$) 72 h after tamoxifen injection.

Effect of oestradiol on tamoxifen-stimulated uterine weight and water content (Table 1)

In terms of uterine wet weight oestradiol caused the most significant alteration in this parameter when administered 45 h after the tamoxifen dose (measurement made 3 h after the oestradiol injection), when the addition of oestradiol caused a further elevation of wet weight to a uterus already enlarged by tamoxifen. This increase was due mainly to water imbibition in the uterus. Similarly, at this time (48 h) the inclusion of oestradiol also enhanced tamoxifen-stimulated uterine dry weight. This dry weight increase was still evident 72 h after tamoxifen administration.

Table 1 The effect of oestradiol on tamoxifen (1 mg kg^{-1} s.c., single dose)-stimulated uterine blood flow and weight in rats: measurements were made at 27 h, 48 h and 72 h after administration of tamoxifen; where the combination was used oestradiol (0.5 μg kg^{-1}, i.v.) was injected 3 h before flood flow determinations

Pretreatment	*No. of rats*	*Body weight* (g)	*Cardiac output* (ml min^{-1})	*Uterine blood flow* ml min^{-1}	*Uterine blood flow* ml min^{-1} 100 g^{-1}	*Uterine weight* *Wet*	*Uterine weight* *Dry*	*Water content of uterus* (mg)
None (Control)	9	304 ± 7	93 ± 6	0.065 ± 0.01	66 ± 12	107 ± 8	23 ± 3	85
Oestradiol 3 h	8	321 ± 7	104 ± 11	0.94 ± 0.10	735 ± 66	128 ± 7	35 ± 2	93
Tamoxifen 27 h	6	312 ± 14	106 ± 12	0.88 ± 0.20	649 ± 117	129 ± 9	29 ± 2	100
Tamoxifen + oestradiol (24 h + 3 h)	7	314 ± 10	82 ± 17	0.53 ± 0.11*	376 ± 66**†	139 ± 6	26 ± 2**	113
Tamoxifen 48 h	6	338 ± 15	99 ± 8	0.80 ± 0.19	489 ± 123	167 ± 8**	28 ± 1*	139
Tamoxifen + oestradiol (45 + 3 h)	6	345 ± 12	88 ± 4	1.23 ± 0.42	633 ± 181	190 ± 15***	37 ± 4†	153
Tamoxifen 72 h	6	360 ± 11	124 ± 12	1.58 ± 0.09	988 ± 56*	160 ± 7**	34 ± 1	126
Tamoxifen + oestradiol (69 + 3 h)	6	346 ± 9	98 ± 9	2.13 ± 0.23**†	1328 ± 147**†	160 ± 5**	38 ± 2	121

Values are mean ± s.e.mean; *$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$ values are significantly different from those of oestradiol alone group. †$P < 0.05$ values are significantly different from those of the equivalent tamoxifen alone group.

Effect of oestradiol in the presence of tamoxifen on oestrogen receptor levels (Figure 1)

Three hours after treatment with oestradiol alone, cytosolic receptor concentration had decreased when compared to control values with a concomitant increase in nuclear receptor concentration. When tamoxifen was used alone, cytosolic receptor concentration was lowest at 27 h but increased with time after injection, whilst nuclear receptor levels were highest at 27 h after injection and were significantly lower at 48 h and 72 h.

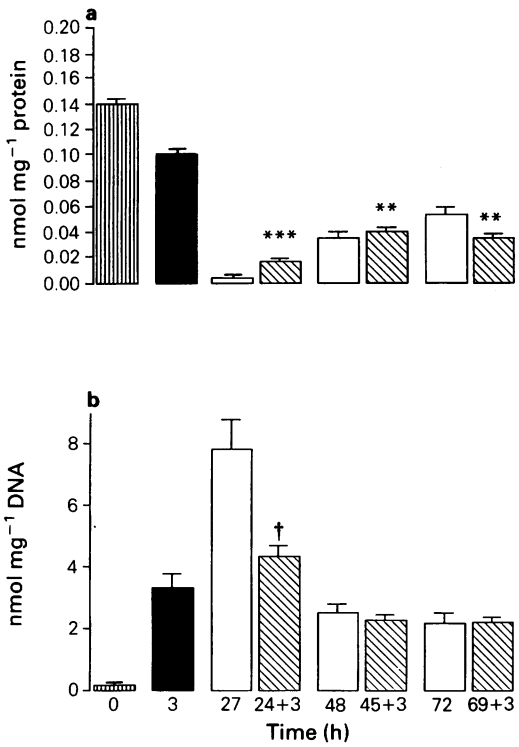


Figure 1 The effect of single injections of oestradiol ($0.5\,\mu g\,kg^{-1}$, i.v. administered 3 h before measurements were made) and tamoxifen ($1\,mg\,kg^{-1}$, s.c. single dose) used singly and together on cytosolic (a) and nuclear (b) oestrogen receptor distribution in mature ovariectomized rats. In (a) and (b) untreated control, vertically lined column; oestradiol, solid column; tamoxifen, open column; oestradiol plus tamoxifen, hatched column. The results are shown at various times after tamoxifen injection. Each column represents mean of 3–6 determinations, with 6 uteri per determination; vertical bars show s.e.mean. $^{**}P < 0.005$, $^{***}P < 0.001$, values are significantly different from those of oestradiol alone group. $\dagger P < 0.05$, values are significantly different from those of equivalent tamoxifen alone group.

When oestradiol was included in the tamoxifen regimen it had the most marked effect when given 24 h after tamoxifen. When the measurements were made 27 h after tamoxifen administration, oestradiol caused a significant reduction in nuclear receptor content compared to treatment with tamoxifen alone; at the same time there was also a small increase in the concentration of receptor in the cytosolic compartment.

Discussion

When oestradiol is used alone it causes a shift in oestrogen receptor from the cytosolic to the nuclear compartment. A similar, although more marked movement of receptor is seen when tamoxifen is used alone at 27 h. As the time after tamoxifen administration increases, nuclear receptor concentration begins to decline but the concentration of cytosolic receptor remains depressed, compared to untreated control values. This pronounced depletion of cytosolic receptor has been well documented (Katzenellenbogen *et al.*, 1983; Kirchoff *et al.*, 1983) and may be attributable to resynthesis/recycling abnormalities with respect to the antioestrogen-receptor complex. The presence of the so-called anti-oestrogen binding site may also be relevant in this context. As Sudo *et al.* (1983) have suggested, these high affinity and widely distributed sites may influence the action of antioestrogens in a passive manner by altering their biological potency and pharmacokinetics.

In the earlier stages of the tamoxifen response (a single $1\,mg\,kg^{-1}$ dose of tamoxifen exerts a uterotrophic effect for up to six weeks, Marshall & Senior, 1987) the addition of oestradiol to the regimen produces a reduction in uterine blood flow and dry weight. In terms of oestrogen receptor dynamics, this decline is reflected by a decrease in nuclear oestrogen levels. Therefore, in the presence of oestradiol it would seem that tamoxifen at this stage of the response is assuming the role of an antagonist. It may also be feasible that when oestradiol is included in the regimen, tamoxifen is forced to compete for oestrogen receptor sites and some of its binding capacity is switched to another site, possibly the antioestrogen/H_3 site described by Brandes & Bogdanovic (1986), so in effect the stimulatory effects of oestradiol are counterbalanced by a change in emphasis in tamoxifen binding.

Later in the tamoxifen response at 48 h, the effect of oestradiol on tamoxifen is manifested as an increase in both uterine wet weight (this increase is accompanied by increased uterine oedema) and dry weight. However, these differences do not appear to

be reflected in changes in oestrogen receptor levels. By 72 h after tamoxifen administration, oestradiol causes a small increase in the dry weight response. However, this additive effect is most marked in terms of the dramatic increase seen in uterine blood flow at this time, although again these changes are apparently independent of alterations in oestrogen receptor levels.

This additive effect may be attributed to the two agents acting via different mechanisms, for example the effect of oestradiol may involve histamine synthesis and release. Results from this laboratory suggest that histamine is involved in the early (3 h) response to oestradiol but that it does not appear to play a major role in the uterine response to tamoxifen (Marshall & Senior, 1986). Tamoxifen may be more dependent on, for example, the eosinophil-mediated mechanism proposed by the Tchernitchin group (Galand *et al.*, 1985; Tchernitchin, 1979; Tchernitchin, 1983) as they have shown that nafoxidine, also a triphenylethylene derivative, can induce eosinophil migration (Galand *et al.*, 1984). The reversal of the combined blood flow response at 27 h and 72 h may also indicate a different mediatory system.

The results of this work would seem to indicate that changes in the uterotrophic parameters examined in this study do not always correspond with alterations in oestrogen receptor levels. It is difficult to correlate exactly the uterine response with oestrogen receptor dynamics. Even though receptor concentrations are measurable, it is not possible to assess the quality of the binding taking place. A study by Giambiagi & Pasqualini (1988) has shown that oestradiol, tamoxifen and its major metabolite 4-hydroxytamoxifen induce different conformational modifications of the oestrogen receptor molecule and the way it interacts with the genome. The changes in direction seen when oestradiol was added at the various stages of the tamoxifen response may be suggestive of different regulatory systems coming into play with respect to the early and later uterotrophic events. The oestrogen receptors present on the endothelial cell membrane in uterine arteries (Pietras & Szego, 1977) may be of importance in this respect as postulated by Arvidson & Terenius (1982).

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Possible therapeutic effect of naftidrofuryl oxalate on brain energy metabolism after microsphere-induced cerebral embolism

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1 The present study was designed to determine whether naftidrofuryl oxalate exerts a possible therapeutic effect on brain energy metabolism impaired by microsphere-induced cerebral embolism *in vitro*.

2 Injection of microspheres into the right carotid canal resulted in a decrease in tissue high-energy phosphates both in the right and left hemispheres, and an increase in tissue lactate in the right hemisphere, on the 3rd and the 5th day after the embolism. The embolism also induced a marked reduction in mitochondrial oxidative phosphorylation ability and succinate dehydrogenase activity. The results suggest that severe ischaemia was induced in the brain by the microsphere administration.

3 Treatment of microsphere-injected rats with naftidrofuryl oxalate ($15\,mg\,kg^{-1}$) for 3 or 5 days elicited a significant recovery of tissue high-energy phosphate and lactate levels. The recovery was associated with a significant restoration of mitochondrial succinate dehydrogenase activity on the both days and of mitochondrial oxidative phosphorylation rate on the 5th day.

4 The results suggest that naftidrofuryl oxalate is beneficial in the recovery of cerebral energy metabolism impaired by microsphere-induced cerebral ischaemia, presumably through a mechanism involving its direct effect on the cerebral mitochondrial enzyme activities.

Introduction

Cerebral ischaemia or oligemia primarily induces various degrees of disturbances in brain energy metabolism. After a certain period of interrupted blood flow, tissue high-energy phosphate levels markedly decrease and tissue lactate levels increase (Lowry *et al.*, 1964; Ljunggren *et al.*, 1974; Siesjö *et al.*, 1976; Welsh *et al.*, 1977). In such a way, lack of energy and accumulation of this metabolite are considered to produce disturbance in the transmembrane fluxes of ions (Crowe *et al.*, 1981) and cellular acidosis (Welsh *et al.*, 1987). Ischaemic insults have also been shown to induce mitochondrial impairment such as a decrease in calcium accumulating ability (Mela, 1979) and oxidative phosphorylation ability (Lazarewicz *et al.*, 1972; Mela, 1979). In a previous study (Takeo *et al.*, 1989), we have shown that the rat brain is ischaemic over a period of at least 5 days after microsphere-induced cerebral embolism. During these periods mitochondrial oxidative phosphorylation ability and succinate dehydrogenase activity of the microsphere-injected cerebral hemisphere remained markedly decreased. Under these experimental conditions, naftidrofuryl oxalate, 2-(diethylamino)ethyltetrahydro-alpha-(1-naphthyl-methyl)-2-furanpropionate ester oxalate (naftidrofuryl), improved the decreased mitochondrial activities of microsphere-injected rats *in vitro*. The results suggested a direct and beneficial effect of naftidrofuryl on brain mitochondrial activity impaired by ischaemia. Furthermore, since naftidrofuryl has been shown to exert other pharmacological actions such as an improvement in the brain blood flow under normal or pathological conditions (Hagiwara *et al.*, 1973; Kobayashi *et al.*, 1984; Yamada, 1984; Hiramatsu *et al.*, 1988), therapeutic effects of the agent on impaired brain energy metabolism could be expected, even when it is administered after the onset of ischaemia-induced

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derangements. The present study was designed to elucidate a possible therapeutic action of naftidrofuryl on the ischaemic brain energy metabolism *in vivo*.

Methods

Introduction of microsphere-induced cerebral embolism in rats

Male Wistar rats, weighing 210–290 g (CRJ; Japan Charles River Co.), were used in the present study. Microsphere-induced cerebral embolism was performed by methods described previously (Takeo *et al.*, 1989). Briefly, rats were anaesthetized by intraperitoneal administration of 30 mg kg^{-1} sodium pentobarbitone, and fixed in the supine position to an operation plate. After cervical incision, the right common carotid artery was isolated. The right external carotid and the right pterygopalatine arteries were ligated with strings. A polyethylene catheter (2 FR, Atom Co., Tokyo) was inserted into the common carotid artery. Six hundred and eighty microspheres (47.5 ± 0.5 μm in diameter), suspended in 150 μl of 20% dextran solution, were injected into the right carotid canal through this cannula. In the present study we have used slightly more microspheres (about 15% more than those used in the previous study). This could induce more definite changes in the cerebral energy metabolism and the behaviour of the animals operated on. After the injection, the right common carotid artery was ligated and the wound was closed by sutures. The rats which underwent sham operation were injected with the same volume of 20% dextran solution without microspheres. Fifteen hours after the surgery, the behaviour of operated rats was monitored. The behaviour of microsphere-injected rats was scored on the basis of paucity of movement, truncal curvature and forced circling towards the right during locomotion, which are considered to be typical symptoms of stroke (Furlow & Bass, 1976; McGraw, 1977). The score for each item was ranked from 0 to 2. The rats which had 5 to 6 points of the score were considered to be A type, 3 to 4 to be B type and less than 2 to be C type. In the present biochemical study, the A type rats were used.

Treatment with naftidrofuryl

After inspection of the symptoms of the animals on the first day after surgery, the rats which showed typical symptoms (A type) were treated twice daily with naftidrofuryl (15 mg kg^{-1}, i.p., 10 h 00 min and 18 h 00 min). The agent was dissolved in distilled water at a concentration of 45 mg ml^{-1}. The vehicle was similarly administered to the control group. The rats killed on the 3rd day received 5 injections, and those on the 5th day, 9. The dose employed in the present study has been shown to be effective in preventing ischaemia-induced damage to brain energy metabolism of bilateral carotid artery-ligated mice (Takeo *et al.*, 1988).

Determination of tissue high-energy phosphates, lactate and pyruvate

Adenosine triphosphate (ATP), creatine phosphate (CP), lactate and pyruvate in the brain hemispheres were determined by methods described previously (Takeo *et al.*, 1987; 1988; 1989). Briefly, the rats were decapitated in an appropriate experimental sequence and the heads were immersed in liquid nitrogen within 0.5 s after decapitation. Both hemispheres were isolated and their frozen weights were determined. Each hemisphere was pulverized separately in a stainless centrifuge tube with a stainless steel plunger under liquid nitrogen-cooling. The brain tissue powder was extracted with 0.3 M $HClO_4$ + 0.25 mM EDTA. After centrifugation of the extract at 1,000 *g*, the supernatant fluid was neutralized with 2.5 M K_2CO_3 and sampled for determination of tissue high-energy phosphates, lactate and pyruvate. The ATP content was determined enzymatically (Bücher, 1947) and CP, according to the methods of Lowry & Passonneau (1972). The tissue lactate and pyruvate were determined by the methods of Gutman & Wahlefeld (1974) and Czok & Lamprecht (1974), respectively. The content of tissue metabolites was expressed as μmol g^{-1} dry tissue. The ratio of dry tissue weight to the frozen tissue weight was estimated in a preliminary study and found to be 0.200 ± 0.002 (n = 10).

Isolation of cerebral mitochondria

Isolation of cerebral mitochondria was carried out according to the methods of Sordahl *et al.* (1971). The brain was removed within 15 s after decapitation, and immersed in cold 0.3 M mannitol + 0.1 mM EDTA solution, pH 7.4 (ME buffer). The cerebral hemispheres were separated in the cold ME buffer and employed for isolation of the mitochondria from both sides. After determining the wet weight, the brain was homogenized with a loose fitting glass teflon homogenizer. The homogenate was centrifuged at 600 *g* for 8 min at 0–4°C. The supernatant fluid was centrifuged at 10,000 *g* for 10 min at 0–4°C. The pellet was rehomogenized in ME buffer, then centrifuged at 5,000 *g* for 10 min. After decantation of the supernatant fluid, the pellet was washed with 20 ml of ME buffer. The washing solution and the supernatant fluid described above

were combined, and the resulting suspension was centrifuged at 5,000 *g* for 10 min. The collected pellet and the pellet described above were suspended in ME buffer or 30 mM potassium phosphate – 0.1% bovine serum albumin and sampled for determination of oxidative phosphorylation ability or succinate dehydrogenase activity of the cerebral mitochondria, respectively. Mitochondrial protein concentrations were determined by the methods of Lowry *et al.* (1951). The protein yields of the mitochondria from the right and the left cerebral hemisphere ranged from 0.55 ± 0.01 to 0.60 ± 0.01 in each experimental group. There were no significant differences in the protein yield of the isolated mitochondria irrespective of the operation performed or the hemisphere to which microspheres were administered.

Measurements of the oxidative phosphorylation ability

The oxidative phosphorylation ability of the isolated cerebral mitochondria was measured according to the method of Sordahl *et al.* (1971). The oxygen consumption of the mitochondria was continuously monitored at 25°C on an oximeter with Clark type DO/O_2 sensor (Oxygraph-8, Central Sciences Co., Tokyo). The reaction mixture (Total volume: 1 ml) contained 0.3 M mannitol, 10 mM KCl, 10 mM Tris/HCl, 5 mM K_2HPO_4, 0.2 mM EDTA and about 1.0 mg of mitochondrial protein. Either 10 mM potassium glutamate or 10 mM potassium succinate was used as the substrate for the phosphorylation. State 3 respiration was initiated by the addition of 250 nmol ADP. As indicators for the oxidative phosphorylation ability of the mitochondria, the following parameters were measured: respiratory control index (RCI; ratio of oxygen consumption at state 3 to that at state 4), ADP/O (nmol ADP natom^{-1} oxygen consumption at state 3), rate of oxygen consumption at state 3 (QO_3; natoms oxygen min^{-1} mg^{-1} protein), rate of oxygen consumption at state 4 (QO_4; natoms oxygen min^{-1} mg^{-1} protein) and oxidative phosphorylation rate (OPR; nmol ATP produced during state 3 min^{-1} mg^{-1} protein).

Measurements of succinate dehydrogenase activity

The mitochondrial succinate dehydrogenase activity was measured according to the methods of Slator & Bonner (1952). In a 1 ml cuvette, 300 μl of 0.3 M potassium phosphate buffer (pH 7.6), 30 μl of 30 mM EDTA, 90 μl of 0.4 M potassium succinate (pH 7.6), 30 μl of 3% bovine serum albumin, 30 μl of 75 mM $K_3Fe(CN)_6$, 360 μl of distilled water and 25 μl of mitochondrial protein (about 500 μg) were mixed and the absorbance of the mixture was measured at 455 nm for 2 min at 25°C. The initial rate of reduction of absorbance was taken as the succinate dehydrogenase (SDH) activity. The absorbance of the mixture without succinate served as the blank value.

Statistics

Values in the text represent the mean ± s.e.mean. The observed values of activities of rats without operation were used as standard (normal value). As statistical tests, Student's *t* test was used for comparison of the activities of the mitochondria and the amounts of metabolites measured. Differences with a probability of 5% or less were considered to be statistically significant ($P < 0.05$).

Results

Microsphere-induced cerebral embolism

Microsphere-induced embolism was evoked in 310 rats. Numbers of animals with A type symptoms were 168 (54%). Eighty-three rats (27%) died before inspection of the behaviour or determination of metabolites. Thirty-seven rats showed slightly milder symptoms than A type (B type; 12%). There were twenty-two rats which showed very light symptoms of stroke (C type; 7%). Twenty-nine rats with A type symptoms were used for a preliminary study and morphological examination. The rest of the A type animals, 115 rats, were used for biochemical examination. Sham operations were performed with another 42 rats. None of these animals died during the experiment. An additional 19 rats were used for measurement of tissue metabolites and mitochondrial SDH activity as standard values.

The characteristic behaviour of rats with cerebral embolism on the 3rd and 5th day after the operation was appreciably milder than that observed on the 1st day. The behaviour of microsphere-injected rats on both days tended to be improved by treatment with naftidrofuryl. For example, the score of the rats after treatment with naftidrofuryl ranged from 1 to 3, whereas that without the treatment was 1 to 5. However, the effect could not be clearly defined merely by studying animal behaviour.

Tissue high-energy phosphates

Tissue high-energy phosphate levels of the rat hemispheres isolated on the 3rd day and the 5th day after the onset of microsphere-induced cerebral embolism are shown in Table 1. The tissue ATP and CP levels of the brain hemispheres from the sham-operated rats were slightly lower than the values for non-

Table 1 Tissue high-energy phosphate content of rat hemispheres isolated on the 3rd day and the 5th day after microsphere-induced cerebral embolism

	No. of experiments	*Right hemisphere*		*Left hemisphere*	
		ATP	*CP*	*ATP*	*CP*
No operation	12	12.70 ± 0.11	14.10 ± 0.17	12.61 ± 0.16	14.23 ± 0.13
3rd day					
Sham-operation	7	11.70 ± 0.13	12.26 ± 0.33	12.65 ± 0.10	13.51 ± 0.12
Microsphere-embolism	8	6.43 ± 0.28*	5.53 ± 0.20*	9.26 ± 0.30*	9.16 ± 0.36*
Microsphere-embolism + naftidrofuryl	8	7.62 ± 0.09†	6.96 ± 0.15†	10.67 ± 0.19†	11.13 ± 0.36†
5th day					
Sham-operation	7	11.23 ± 0.27	12.16 ± 0.43	12.16 ± 0.21	13.28 ± 0.16
Microsphere-embolism	8	7.80 ± 0.24*	7.56 ± 0.43*	10.22 ± 0.18*	10.19 ± 0.24*
Microsphere-embolism + naftidrofuryl	8	9.55 ± 0.28†	9.66 ± 0.34†	11.45 ± 0.14†	12.25 ± 0.28†

Values are expressed as $\mu mol\ g^{-1}$ dry wt tissue. Each value represents the mean ± s.e.mean.
* Significantly different from the value for sham-operated rats ($P < 0.05$).
† Significantly different from the value for microsphere-injected rats ($P < 0.05$). CP, creatine phosphate.

operated rats. Microsphere-induced cerebral embolism caused reductions in ATP and CP of about 50 to 40 and 60 to 46% in the right hemisphere on both days monitored, respectively. Similarly, but to a lesser degree, the tissue ATP and CP contents of the left hemisphere were significantly reduced after embolism. Treatment of the rats with naftidrofuryl restored the tissue ATP and CP levels in both hemispheres isolated on the 3rd day after the surgery to a significant degree. Similar results were obtained in the brain hemispheres isolated from rats on the 5th day as shown in Table 1.

Tissue lactate and pyruvate contents

The lactate and pyruvate contents of the rat brain hemispheres isolated on the 3rd and the 5th days after the onset of microsphere-induced cerebral embolism are shown in Figures 1 and 2, respectively. The tissue lactate content of the right cerebral hemisphere of the normal rat was 22.09 ± 1.34 $\mu mol\ g^{-1}$ dry tissue ($n = 12$), whereas the tissue pyruvate content was 1.36 ± 0.16 $\mu mol\ g^{-1}$ dry tissue ($n = 12$). The tissue lactate contents of the right hemisphere of the rat brain on the 3rd and the 5th day (37.97 and 33.96 $\mu mol\ g^{-1}$ dry tissue, respectively; mean value of 8 experiments) were significantly higher than those of the sham-operated rat. The pyruvate content of the right hemisphere of the microsphere-injected rat was also higher than that of the sham-operated rats. When the microsphere-injected rats were treated with naftidrofuryl for 3 or 5 consecutive days, the tissue lactate and pyruvate levels of the right hemisphere returned toward the standard levels to a significant extent (31.81 and 24.20 or 1.56 and 1.11 $\mu mol\ g^{-1}$ dry tissue, respectively; mean value of 8 experiments). Tissue lactate and pyruvate levels of

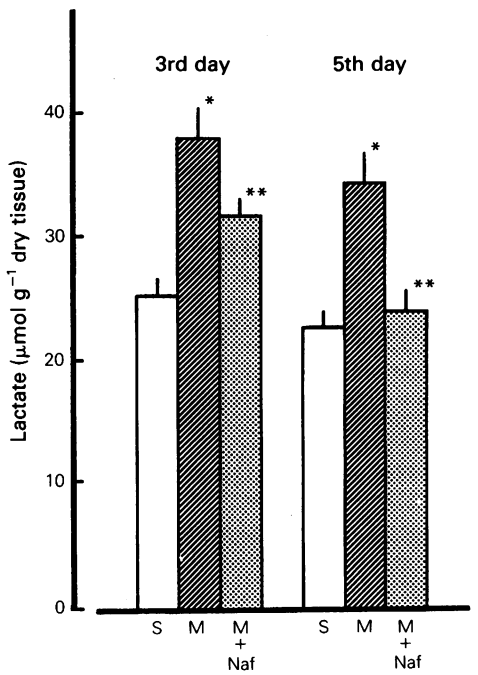


Figure 1 Tissue lactate levels of the right cerebral hemispheres isolated on the 3rd and 5th day after microsphere-induced cerebral embolism in rats. S (open columns): sham-operated group ($n = 7$). M (hatched columns): microsphere-injected group ($n = 8$). M + Naf (stippled columns): microsphere-injected group with naftidrofuryl-treatment ($n = 8$). * Significantly different from the values for the sham-operated group ($P < 0.05$). ** Significantly different from the values for the microsphere-injected group ($P < 0.05$). Vertical bars show s.e.mean.

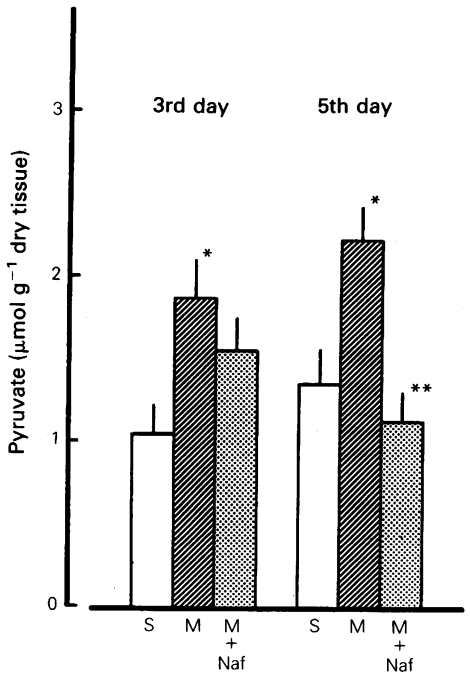


Figure 2 Tissue pyruvate levels of the right cerebral hemispheres isolated on the 3rd and 5th day after microsphere-induced cerebral embolism in rats. S (open columns): sham-operated group ($n = 7$). M (hatched columns): microsphere-injected group ($n = 8$). M + Naf (stippled columns): microsphere-injected group with naftidrofuryl-treatment ($n = 8$). * Significantly different from the values for the sham-operated group ($P < 0.05$). ** Significantly different from the values for the microsphere-injected group ($P < 0.05$). Vertical bars show s.e.mean.

the left hemisphere on both days were not significantly altered by either microsphere-embolism or treatment with naftidrofuryl (data not shown).

Mitochondrial oxidative phosphorylation activity

Standard values of the mitochondrial oxidative phosphorylation activity were determined previously (Takeo *et al.*, 1989). All the values in sham-operated rats were similar to those of standard rats except for a slightly higher oxidative phosphorylation rate (OPR) of the right hemisphere in the presence of glutamate and a slight lower OPR in the right hemisphere in the presence of succinate. The OPR of the right hemisphere of the microsphere-injected rats tended to be higher on the 3rd and 5th days after the embolism in the presence of glutamate as a substrate (15 and 25% increases, respectively), whereas the OPR was significantly lower when measured in the presence of succinate as a substrate (30 and 24% decreases, respectively). Trends similar to those mentioned above were seen in the changes of other activities such as RCI, ADP/O and QO_3.

The mitochondrial oxidative phosphorylation activity of microsphere-injected rats on the 5th day was essentially similar to that on the 3rd day, and no significant effects of naftidrofuryl on the mitochondrial oxidative phosphorylation activity were observed in rats on the 3rd day. The results of the rat mitochondria on the 5th day are shown in Table 2. A significant recovery of the OPR was seen only in the right hemisphere isolated on the 5th day from the microsphere-injected rats treated with naftidrofuryl, when the activity was measured in the presence of succinate. This was accompanied by a slight improvement of ADP/O in the right hemisphere.

Table 2 Effects of naftidrofuryl (Naf) on the oxidative phosphorylation activity of the mitochondria isolated from rat brain on the 5th day after microsphere-induced embolism *in vivo*

	Glutamate			*Succinate*		
	Sham	*Microsphere*	*Microsphere + Naf*	*Sham*	*Microsphere*	*Microsphere + Naf*
OPR	83.6 ± 3.5	104.2 ± 5.5*	113.6 ± 4.8	207.6 ± 5.0	158.7 ± 6.1*	182.9 ± 8.7†
RCI	3.10 ± 0.05	3.80 ± 0.21*	3.93 ± 0.13	2.81 ± 0.08	2.44 ± 0.08*	2.55 ± 0.11
ADP/O	2.35 ± 0.05	2.57 ± 0.05*	2.69 ± 0.04	1.92 ± 0.02	1.87 ± 0.04	2.01 ± 0.03†
QO_3	35.6 ± 0.9	40.4 ± 1.7*	42.2 ± 1.7	108.4 ± 3.1	84.9 ± 2.7*	91.0 ± 3.7
QO_4	11.5 ± 0.4	10.8 ± 0.4	10.8 ± 0.3	38.2 ± 1.0	34.9 ± 1.0*	35.9 ± 0.9

* Units: RCI (respiratory control index) = ratio of QO_3/QO_4. ADP/O (ADP consumed during state 3) = nmol ADP natoms^{-1} oxygen. QO_3 (oxygen uptake at state 3) = natoms oxygen mg^{-1} protein min^{-1}. QO_4 (oxygen uptake at state 4) = natoms oxygen mg^{-1} protein min^{-1}. OPR (oxidative phosphorylation rate) = nmol ATP produced during state 3 mg^{-1} protein min^{-1}. Each value represents the mean ± s.e.mean of 7 (sham), 11 (microsphere) and 11 (microsphere plus naftidrofuryl). All parameters seen in the mitochondria isolated from the left cerebral hemisphere were not significantly altered in the present experimental conditions.

* Significantly different from the values for the sham-operated group ($P < 0.05$).

† Significantly different from the values for the microsphere-injected group ($P < 0.05$).

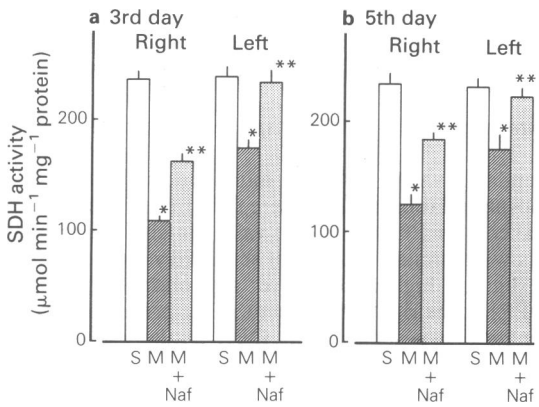


Figure 3 The succinate dehydrogenase activity of the cerebral mitochondria isolated on the 3rd (a) and 5th (b) day after microsphere-induced cerebral embolism in rats. The activity is expressed as μmol NADPH min^{-1} mg^{-1} protein. S (open columns): sham-operated group ($n = 7$). M (hatched columns): microsphere-injected group ($n = 8$). M + Naf (stippled columns): microsphere-injected group with naftidrofuryl-treatment ($n = 8$). * Significantly different from the values of the sham-operated group ($P < 0.05$). ** Significantly different from the values for the microsphere-injected group ($P < 0.05$).

The mitochondrial oxidative phosphorylation activities of the left hemisphere on both days were not affected by either microsphere-embolism or treatment with naftidrofuryl (data not shown).

Mitochondrial succinate dehydrogenase activity

The mitochondrial SDH activity on the 3rd and the 5th days is shown in Figures 3a,b. The mitochondrial SDH activity of the right and the left hemispheres of the normal rat (without operation) was 266.6 ± 5.2 and 272.4 ± 4.1 μmol NADPH min^{-1} mg^{-1} protein respectively ($n = 7$). Microsphere-induced cerebral embolism markedly reduced the mitochondrial SDH activity in the right and left hemispheres regardless of the site of microsphere-administration (125.6 and 177.1 μmol NADPH min^{-1} mg^{-1} protein on the 3rd day, and 110.2 and 175.2 μmol NADPH min^{-1} mg^{-1} protein on the 5th day, respectively; mean value of 8 experiments). Treatment of the microsphere-injected rats with naftidrofuryl markedly restored the decreased SDH activity of both hemispheres on the 3rd and the 5th day after embolism (183.3 and 222.9 μmol NADPH min^{-1} mg^{-1} protein on the 3rd day and 164.3 and 236.5 μmol NADPH min^{-1} mg^{-1} protein on the 5th day, respectively; mean value of 8 experiments). The SDH activity in the left hemisphere recovered almost completely as a result of naftidrofuryl treatment.

Discussion

In a previous study we observed that symptoms of stroke in rats on the 3rd day after the embolism were more severe than in rats on the 5th day, and that the tissue ATP and CP contents were most markedly reduced on the 3rd day after the embolism and, on the 5th day, these levels tended to be slightly restored toward the normal levels (Takeo *et al.*, 1989). These findings allowed us to study extensively the cerebral energy metabolism of microsphere-injected rats, on the 3rd and the 5th days after the embolism as severely injured and recovering stages of impaired brain function and metabolism, respectively.

The high-energy phosphate levels of the cerebral hemispheres were markedly reduced both on the 3rd and the 5th days after microsphere-induced cerebral embolism. The tissue lactate of the right hemisphere also showed higher levels than control on both days. An increase in tissue lactate and a decrease in tissue high-energy phosphate are well accepted to be markers of an ischaemic state in the tissue, as discussed in the Introduction. Thus, the results confirmed that microsphere-induced cerebral embolism in the present study induces cerebral ischaemia, which is sustained for at least 5 days after the surgery. The reduction in tissue high-energy phosphate levels was associated with a significant decrease in oxidative phosphorylation activity of the cerebral mitochondria, the ATP producing ability, and in SDH activity, an activity which plays an important role in the tricarboxylic acid cycle of glucose metabolism in cerebral mitochondria. The changes in these parameters in the right hemisphere are more severe than those in the left hemisphere because the latter site might indirectly receive microspheres through the circle of Willis. It should be mentioned that histological examination of the brain cortex after haematoxylin-eosin staining indicated a spread of a haemorrhage to the forebrain, which was accompanied by necrosis and oedema around the haemorrhagic area. In addition, yellow-brown round substances were seen in the microvessels of the nectrotic area, which can be identified as microspheres.

Under these ischaemic conditions, treatment of the rats which showed typical symptoms of cerebral impairment with naftidrofuryl appreciably influenced these metabolic parameters. That is, treatment with naftidrofuryl significantly restored the tissue high-energy phosphate levels in both hemispheres and suppressed the increase in tissue lactate levels in the right cerebral hemisphere on the 3rd and the 5th days. Welsh and co-workers (1980, 1987) have

emphasized the crucial role of lactic acidosis in the genesis of ischaemic damage to cerebral function and metabolism. Thus, the reduction in cerebral lactate levels by treatment with naftidrofuryl, observed in the present study, would be beneficial to brain function and metabolism after ischaemia with respect to the prevention of brain damage from lactic acidosis.

On the 5th day, the recovery of tissue high-energy phosphate levels was accompanied by a slight but significant recovery of the OPR, assessed in the presence of succinate. As mentioned in the Results section, the OPR after the cerebral embolism was increased when measured in the presence of glutamate, whereas it was decreased when measured in the presence of succinate. It should be noted that embolism-induced increases in the mitochondrial oxidative phosphorylation activities, such as RCI, ADP/O, QO_3 and OPR, in the presence of glutamate still persisted after treatment with naftidrofuryl, whereas the embolism-induced decrease in the mitochondrial oxidative phosphorylation activities in the presence of succinate was significantly restored by the treatment. These findings positively suggest that naftidrofuryl facilitates the cerebral mitochondrial activity and so improves mild or reversible ischaemic damage to brain energy metabolism, particularly during the recovery stage of metabolic function (on the 5th day). Presumably this may be exerted through a direct effect of the agent on mitochondrial oxidative phosphorylation, or through activation of the tricarboxylic acid cycle, because we found, in a previous study, significant improvements in oxidative phosphorylation ability and succinate dehydrogenase activity of cerebral mitochondria of microsphere-injected rats under *in vitro* conditions (Takeo *et al.*, 1989).

A decrease in mitochondrial SDH activity is thought to represent damage to the mitochondrial membrane or an impairment of the tricarboxylic acid cycle. It has been shown that acute hypoxia also induces a significant decrease in SDH activity of rat brain homogenate (Purshottam & Ghosh, 1975). In the present study, mitochondrial SDH activity was greatly diminished on both the 3rd and 5th days to a similar extent, suggesting a sustained impairment of mitochondrial membrane function due to microsphere-induced cerebral embolism. It is obviously beneficial that treatment with naftidrofuryl significantly restored the decreased SDH activity of the rat brain on both days, suggesting a restoration of mitochondrial membrane function by the agent.

In a previous study from our laboratory, we have shown, *in vitro*, that naftidrofuryl restores mitochondrial SDH activity of the cerebral hemispheres from microsphere-injected rats (Takeo *et al.*, 1989). This is comparable with the findings of the current *in vivo* study. It should be noted that treatment of sham-operated rats with naftidrofuryl (in two experiments) did not produce any appreciable changes in tissue high-energy phosphate and lactate levels or in mitochondrial SDH and oxidative phosphorylation activity, suggesting no effect of the agent *per se* on cerebral function and metabolism. The extent of the recovery of SDH activity was more profound and more consistent than that of the oxidative phosphorylation ability in the *in vitro* as well as in the *in vivo* study. Furthermore, the recovery was associated with a slight, but significant restoration of microsphere-induced decreases in brain high-energy phosphate levels. These results alllow us to conclude that restoration of mitochondrial SDH activity or activation of the tricarboxylic acid cycle in the mitochondria may substantially contribute to the therapeutic effect of naftidrofuryl on ischaemic brain energy metabolism.

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A comparison of basal and agonist-stimulated release of endothelium-derived relaxing factor from different arteries

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1 The release of endothelium-derived relaxing factor (EDRF) from rabbit aorta and pig coronary artery vessels in response to acetylcholine (ACh), substance P (SP) and the calcium ionophore A23187 has been studied by means of a bioassay cascade system.

2 A technique is described which allows the quantification of EDRF release rates from vessels of different sizes, perfused at different flow rates and with different donor-recipient transient times.

3 Rabbit aorta and pig coronary arteries, perfused at flow rates which equalise endothelial shear stress, released EDRF at a similar basal rate.

4 In response to ACh, rabbit aortic endothelium released EDRF at a significantly greater maximum rate than pig coronary artery endothelium.

5 In response to SP, both endothelium types released EDRF; SP was a significantly more potent agonist in pig coronary artery than in rabbit aorta, but maximum SP-induced EDRF release from rabbit aorta was twice that of pig coronary artery.

6 These data indicate that different endothelium types can release EDRF at widely different rates, according to the agonist used, and that the previously obtained lack of relaxant response to ACh in pig coronary artery was due to a lack of EDRF release rather than concomitant smooth muscle constriction.

Introduction

Endothelium-derived relaxing factor (EDRF) activity varies greatly in different arteries. For example, Griffith and colleagues (1984a) have shown large differences in basal EDRF activity between rabbit aorta and rabbit coronary preparations, with activity being greatest in coronary vessels. Similarly Collins and co workers (1986) have demonstrated that agonist-induced Ca^{2+} influx is inhibited more by basal EDRF activity in dog isolated coronary artery preparations than in rabbit aorta.

Receptor-activated EDRF responses also vary according to vessel type. Relaxant responses to substance P (SP) and noradrenaline in isolated preparations of dog large arteries vary in the order coronary > carotid > femoral $\gg$ renal = mesenteric (Angus *et al.*, 1986), and EDRF responses in perfused rat renal and mesenteric preparations differ widely according to the agonist used to release EDRF (Bhardwaj & Moore, 1988). These differences could be due to variations in the amount of EDRF released by different arteries, or a variation in the response of the smooth muscle to EDRF. We have previously demonstrated that differences in EDRF activity between pig coronary artery and rabbit aorta can be explained in part by differences in their smooth muscle response to EDRF, with pig coronary artery being more responsive than rabbit aorta (Christie & Lewis, 1988).

Substance P is a potent endothelium-dependent relaxant in both pig coronary artery (Cocks & Angus, 1983) and rabbit aorta (Zawadzki *et al.*, 1981), and acetylcholine (ACh) is the standard EDRF-releasing agent in both endothelium-intact rings of rabbit aorta (Furchgott & Zawadzki, 1980) and in bioassay preparations (Griffith *et al.*, 1984b). In pig coronary artery preparations, however, endothelium-dependent relaxation cannot be elicited by ACh (Kalsner, 1985), and dose-response curves to the constrictor effects of ACh in this preparation are unaffected by the presence of an intact endothelium

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(Graser et al., 1986). This lack of ACh-mediated EDRF activity in pig coronary artery may be due to either a lack of EDRF release, or masking of the relaxant response to EDRF by increased activation of smooth muscle muscarinic receptors mediating constriction.

In this study the amount of EDRF released from each endothelial type both basally and in response to the endothelium-dependent relaxants SP and ACh has been quantified by use of cascade bioassay techniques. Direct comparisons between EDRF release from the pig coronary artery and rabbit aorta are complicated by differences in diameter (d) and flow rate ($\dot{Q}$) as shear stress, which is proportional to $\dot{Q}/d^3$, enhances EDRF release in bioassay systems (Pohl et al., 1986; Rubanyi et al., 1986). To compensate for this effect, in the present study, pig coronary artery and rabbit aorta donors were perfused at flow rates which result in equivalent endothelial shear stresses. As differences in perfusion rate will influence both the dilution of EDRF released into the lumen of each donor, and the transit time (during which EDRF decays exponentially) between donor and recipient vessels, these factors were also taken into account before EDRF release rates from the two endothelium types were calculated and compared.

The results demonstrated that, although basal EDRF release was similar in the two vessel types, rabbit aortic endothelium releases EDRF at a higher rate in response to both SP and ACh. The relevance of these findings to results obtained from endothelium-intact vascular preparations is discussed.

Methods

Vessel preparations

Rabbit aorta Male New Zealand white rabbits (2–2.5 kg) were killed by a blow to the neck. The descending thoracic aorta was removed and transferred to pre-gassed (95% O_2 : 5% CO_2) Holman's solution of the following composition (mM): NaCl 120, KCl 5, NaH_2PO_4 1.3, $NaHCO_3$ 25, $CaCl_2$ 2.5, D-glucose 11, sucrose 10, containing indomethacin (10^{-5} M) as a cyclo-oxygenase inhibitor to prevent prostanoid synthesis. A 3 cm length of rabbit aorta was carefully cleaned of connective tissue and mounted in the bioassay system as an EDRF donor.

Pig coronary artery Pig circumflex coronary arteries were collected from a local abbatoir, cut into 2–3 mm wide rings, denuded of endothelium and mounted as recipients in the bioassay system as previously described (Christie & Lewis, 1988). Recipient rings were perfused initially at 2 ml min^{-1} with gassed Holman's solution at 37°C, and tension responses recorded on a devices chart recorder. Rings were equilibriated for 60 min at a resting tension of 5 g before being tested for the successful removal of endothelium by preconstriction with 5-hydroxytryptamine (5-HT) 10^{-5} M, and brief (1 min) exposure to A23187, 10^{-7} M. Any rings showing a relaxant response to A23187 were considered to have some functional endothelium and discarded. Pig coronary artery rings were then allowed to return to a resting tension of 5 g, and the tension was adjusted if basal tone was altered by the initial exposure to 5-HT.

A 3 cm length of pig coronary artery was prepared and mounted as an EDRF donor in the bioassay system in a manner similar to rabbit aorta.

Experimental protocol

Recipient pig coronary artery rings were preconstricted with 5-HT, (3×10^{-6} M) infused post-donor (i.e. into the perfusate between donor and recipient) by means of an LKB (type 3132) peristaltic pump. Atropine (3×10^{-6} M) was also infused post-donor in all experiments to prevent stimulation of pig coronary artery muscarinic receptors. After constrictor responses had stabilised, the donor vessel (either rabbit aorta or pig coronary artery) was perfused with Holman's solution for 3 min before being allowed to perfuse the recipient rings. Basal EDRF release, and EDRF release in response to either cumulative doses of ACh (10^{-7}–10^{-5} M) or 60 s pulses (to avoid tachyphylaxis) of SP (10^{-11}–3×10^{-8} M) was measured. At the end of each experiment a supramaximal (10^{-6} M) dose of A23187 was added to cause maximal EDRF release. This ensured that recipient relaxation in response to EDRF was submaximal throughout the dose-response curve and served as a test for the presence of a functional endothelium on donor vessels. At the concentrations used, ACh, SP and A23187 were without direct effect on the pig coronary artery rings used in this study.

Calculation of donor dimensions and endothelial shear stress

At the end of each experiment the donor vessel was removed from the bioassay, and its central portion sectioned into four 3–4 mm wide rings with a fixed-blade ring cutter. Each ring was cut longitudinally and the resulting strip laid on graduated (1 mm square) paper. The length of each strip could then be estimated to the nearest 0.5 mm, and used to calculate the mean circumference (c) of the intact donor. The diameter (d) of each donor could then be calculated (as $d = c/\pi$). For all donors the tips of the infu-

sion cannulae were 20 mm apart, making the effective length (L) of each donor identical. The endothelial shear stress of each donor at a fixed flow rate $\dot{Q}$ was derived from the formula $32\mu\dot{Q}/\pi d^3$ dyne/cm^2, where the coefficient of viscosity, μ, for Holman's solution at 37°C was taken as 0.7 centipoise.

In a preliminary series of experiments the dimensions of a representative sample of pig coronary artery and rabbit aorta vessels were measured and used to calculate the flow rates that produced similar levels of endothelial shear stress in each donor/vessel. These were 1 ml min^{-1} (0.017 cm^3 s^{-1}) and 7 ml min^{-1} (0.117 cm^3 s^{-1}) in pig coronary artery and rabbit aorta respectively, and were used in all experiments. The higher flow rate was obtained by fitting wider bore tubing in the Watson-Marlow perfusion pump, so that the frequency of flow pulsation was the same for each donor vessel (1.2–1.7 Hz).

Quantification of relaxant responses to EDRF

Sodium nitroprusside (NaNP) has been shown to exert its relaxant action by spontaneous release of nitric oxide (NO) (Feelisch & Noake, 1987), and EDRF is either NO or a closely related species (Palmer *et al.*, 1987). EDRF responses were therefore quantified by reference to the linear part of a standard NaNP concentration-response curve, constructed by mounting endothelium-denuded pig coronary artery rings as recipients in the bioassay, preconstricting them with 5-HT (3×10^{-6} M), and exposing them to increasing cumulative concentrations of NaNP (3×10^{-9}–3×10^{-7} M). Relaxant responses induced by EDRF were then expressed as units of EDRF activity, where 3×10^{-6} units EDRF causes the same percentage relaxation of pig, 5-HT-preconstricted coronary artery rings as 3×10^{-6} M NaNP.

Calculation of rate of EDRF decay post-donor

The short half-life of EDRF must be taken into account at different flow rates. In this bioassay system, the transit time of EDRF between donor and recipient was 6.5 s at a flow rate of 1 ml min^{-1}, and 1.5 s at a flow rate of 7 ml min^{-1}. EDRF activity decreases exponentially as a function of time during transit (Griffith *et al.*, 1984b) so that its decay can be described by the equation:-

$$C_R = C_D \cdot e^{-\lambda t} \qquad (1)$$

Where C_R = concentration of EDRF at recipient (units), C_D = concentration of EDRF leaving donor (units), λ = decay constant for EDRF (units s^{-1}) and t = transit time between donor and recipient (s).

The decay constant for EDRF was calculated by increasing transit time of EDRF between donor and recipient as described previously (Griffith *et al.*, 1984b).

Calculation of rate of EDRF release into, and decay within donor

The concentration of EDRF at the distal end of a donor vessel is a combination of the rate of release of EDRF into the luminal perfusate, and the rate of decay of EDRF in the perfusate as it travels through the donor. The rate of EDRF release from endothelium in a donor vessel can be calculated from the equation:-

$$f = \frac{\lambda d C_D}{4 \cdot \left[1 - e\left(\frac{-\lambda v}{\dot{Q}}\right)\right]}$$

(2) (see Appendix)

Where f = rate of EDRF release per unit area of endothelium (units cm^{-2} s^{-1}), C_D = concentration of EDRF leaving donor (units), λ = decay constant for EDRF (units s^{-1}), d = diameter of donor (cm), V = volume of donor (cm^3) and $\dot{Q}$ = flow rate (cm^3 s^{-1}).

The equation combines the rate of decay and rate of production of EDRF in each donor type, the resultant of which is the concentration of EDRF in the perfusate leaving the donor.

Each pig coronary artery relaxant response induced by EDRF was first expressed as a percentage of the initial 5-HT-induced constriction. This response was then converted to EDRF units by comparing it with the NaNP calibration curve to give a value for C_R (total concentration of EDRF at recipient). This value for C_R was inserted into equation 1 to give a value for C_D which was then inserted into equation 2 to give a value of f for each response. These f values are directly comparable for pig coronary artery and rabbit aorta donors, as they describe the rate of EDRF release from a unit area of endothelium at the same shear stress, and correct for differences in vessel dimensions, flow rates and donor recipient transit times.

Statistics

Percentage relaxation and absolute EDRF release concentration-response curve slopes, and EC_{50} values for SP and ACh, were calculated from a line fitted to the linear part of the concentration-response curve by a least squares minimisation procedure. Absolute EDRF values, and decay constants for EDRF were also calculated from a line fitted by least

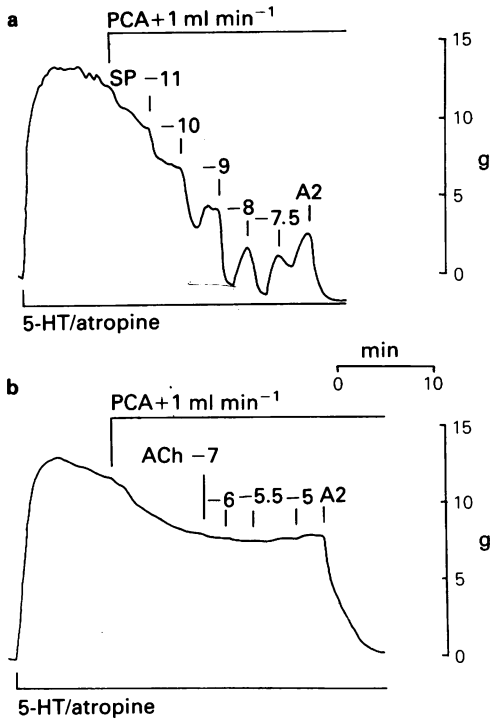


Figure 1 Typical responses of pig coronary artery rings to endothelium-derived relaxing factor (EDRF) released from pig coronary artery donors (PCA+) by 60 s pulses of substance P (SP, a), or cumulative concentrations of acetylcholine (ACh, b) followed by a supramaximal concentration of A23187 (10^{-6} M). Points indicate a change in the concentration of the EDRF-releasing agonist (log M). Rings are preconstricted with 5-hydroxytryptamine (3×10^{-6} M) in the presence of atropine (3×10^{-6} M). Note that pig coronary artery releases EDRF in response to SP but not ACh, and that A23187 induces supramaximal release of EDRF.

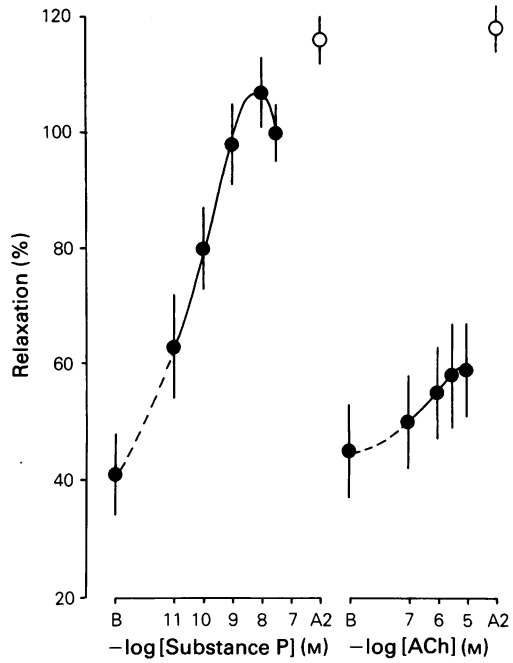


Figure 2 Mean percentage relaxation of endothelium denuded, 5-hydroxytryptamine-preconstricted coronary artery rings of the pig in response to endothelium-derived relaxing factor (EDRF) released from endothelium-intact coronary artery donors of the pig basally (B), and in response to 60 s pulses of substance P ($n = 5$) or cumulative concentrations of acetylcholine (ACh) ($n = 5$) (●), followed by a supramaximal concentration (10^{-6} M) of A23187 (○). Vertical lines show s.e.mean.

squares minimisation to the percentage relaxation vs $\log_{10}$ [NaNP], and EDRF units vs transit time data, respectively.

Results are presented as mean ± s.e.mean, compared by use of Student's t test for unpaired data, and considered significant when $P < 0.05$.

Drugs used

5-Hydroxytryptamine (creatinine sulphate complex), acetylcholine chloride, substance P (acetate salt), A23187 (calcium, magnesium mixed salts), sodium nitroprusside and indomethacin were purchased from the Sigma Chemical Co Ltd, Poole, UK. Atropine sulphate was purchased from BDH Ltd, Poole, UK. Stock solutions of 5-HT, ACh and NaNP (10^{-2} M) were made up in distilled water. Indomethacin (10^{-2} M) and A23187 (5×10^{-3} M) stock solutions were made up in 99% ethanol and DMSO, respectively. Substance P was diluted in 0.1 M HCl immediately before use. The final concentrations of ethanol, DMSO and HCl used did not affect tissue responses in this system.

Results

Relaxant responses to EDRF released from donor vessels

Pig coronary artery Figure 1 shows typical tension recordings obtained from atropine-treated, endothelium-denuded, coronary artery rings of the pig exposed to EDRF released from a pig coronary

artery donor. Both rings were perfused at a flow rate of 1 ml min^{-1} and preconstricted with 5-HT (3×10^{-6} M) infused post-donor. Atropine (3×10^{-6} M) was also infused post-donor. (a) Shows EDRF released basally, that in response to 60 s pulses of SP ($10^{-4} - 3 \times 10^{-8}$ M) or in response to a supramaximal concentration of A23187 (10^{-6} M). (b) Shows EDRF release basally, in response to cumulative concentrations of ACh (10^{-7}–10^{-5} M) and in response to A23187 (10^{-6} M). Pig coronary artery endothelium therefore releases EDRF basally and in response to SP, but not to ACh, although in both experiments A23187 induces maximal EDRF release.

Figure 2 shows the mean relaxant responses obtained in rubbed coronary rings of the pig preconstricted with 5-HT, to EDRF released from pig coronary artery donors. The pig coronary artery rings used to detect SP- and ACh-induced EDRF release were preconstricted to 7.6 ± 1.2 g and 8.4 ± 1.1 g tension by 5-HT and relaxed by 116 ± 4% and 118 ± 4%, respectively, in response to maximal EDRF release induced by A23187. The pig coronary artery donors used for the SP experiments were perfused at a flow rate of 1 ml min^{-1}, which produced a mean shear stress of 0.16 ± 0.02 dyne cm^{-1}, and basal EDRF release corresponding to 41 ± 7% relaxation. The concentration-response curve to SP had a slope of 14.1 ± 2.8, an EC_{50} value of $7.4 \pm 2.7 \times 10^{-11}$ M, and an asymptote corresponding to 107 ± 6% relaxation. Pig coronary artery donors used for the ACh experiments experienced a mean shear stress of 0.17 ± 0.02 dyne cm^{-2}, which produced basal EDRF release corresponding to 45 ± 8% relaxation. The concentration-response curve to ACh had a slope of 4.6 ± 1.3, an EC_{50} value of $3.1 \pm 0.8 \times 10^{-7}$ M, and an asymptote corresponding to 59 ± 8%.

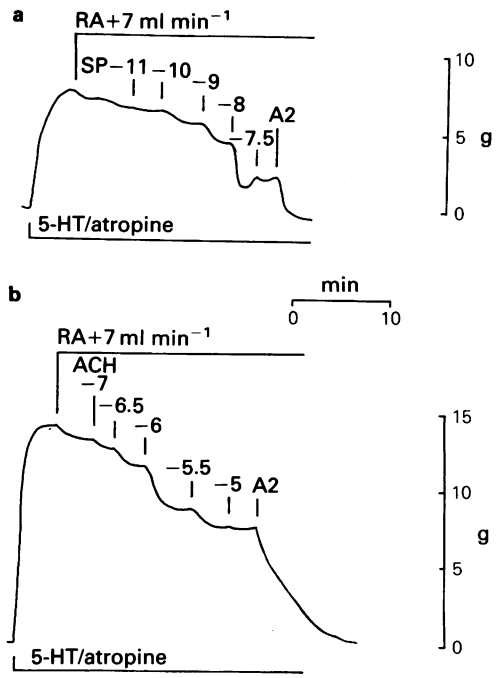


Figure 3 Typical responses of pig coronary artery rings to endothelium-derived relaxing factor (EDRF) released from rabbit aorta donors (RA+) by 60 s pulses of substance P (SP, a) or cumulative concentrations of acetylcholine (ACh, b), followed by A23187 (10^{-6} M). Points indicate a change in the concentration of the EDRF-releasing agonist (log M). Rings are preconstricted with 5-hydroxytryptamine (3×10^{-6} M) in the presence of atropine (3×10^{-6} M). Note that rabbit aorta releases EDRF in response to both SP and ACh, and that A23187-induced release is supramaximal.

Rabbit aorta Figure 3 shows typical tension recordings obtained from atropine-treated, endothelium-denuded coronary artery rings of the pig exposed to EDRF released from a rabbit aorta donor. Both rings were perfused at 7 ml min^{-1} and preconstricted with 5-HT infused post-donor. Atropine was also infused post-donor. The upper trace shows EDRF released basally and in response to 60 s pulses of SP, and in response to a supramaximal concentration of A23187. The lower trace shows EDRF released basally and in response to cumulative concentrations of ACh, and in response to A23187. Note that, unlike pig coronary artery, both SP and ACh released EDRF from rabbit aortic endothelium.

Figure 4 shows the mean relaxant responses of rubbed coronary artery rings of the pig, preconstricted with 5-HT, to EDRF released from rabbit aorta donors. The pig coronary artery rings used to detect SP- and ACh-induced EDRF release were preconstricted to 7.8 ± 0.7 g and 9.8 ± 0.7 g tension by 5-HT, and relaxed by 126 ± 8% and 99 ± 5% respectively, in response to maximal EDRF release induced by A23187. The rabbit aorta donors used for the SP experiments were perfused at a flow rate of 7 ml min^{-1}, which produced a mean shear stress of 0.22 ± 0.03 dyne cm^{-2}, and basal EDRF release corresponding to 28 ± 6% relaxation. The concentration-response curve to SP had a slope of 18.5 ± 3.8, an EC_{50} value of $3.2 \pm 0.9 \times 10^{-10}$ M, and an asymptote corresponding to 96 ± 13% relaxation. The rabbit aorta donors used for the ACh experiments experienced a mean shear stress of 0.19 ± 0.02 dyne cm^{-2}, which produced basal EDRF release corresponding to 27 ± 8% relaxation. The concentration-response curve to ACh had a slope of 29.9 ± 5.6, an EC_{50} value of $3.2 \pm 0.8 \times 10^{-7}$ M, and an asymptote corresponding to 85 ± 7% relaxation.

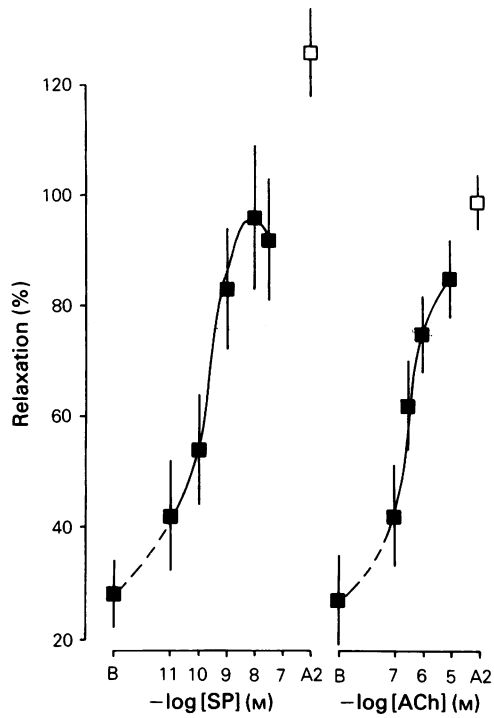


Figure 4 Mean percentage relaxation of endothelium-denuded, 5-hydroxytryptamine-preconstricted coronary artery rings of the pig in response to endothelium-derived relaxing factor (EDRF) released from endothelium-intact rabbit aorta donors basally (B) and in response to 60 s pulses of substance P (SP, $n = 5$) or cumulative doses of acetylcholine (ACh, $n = 6$) (■) followed by a supramaximal concentration (10^{-6} M) of A23187 (□). Vertical lines show s.e.mean.

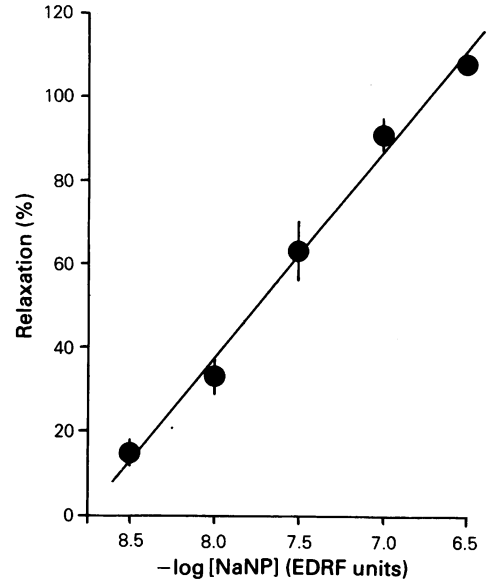


Figure 5 The calibration curve used to convert percentage relaxations of pig coronary artery rings in response to endothelium-derived relaxing factor (EDRF), to units of EDRF activity. It is a standard concentration-response curve of endothelium-denuded, 5-HT (3×10^{-6} M) preconstricted coronary artery rings of the pig, to increasing cumulative concentrations of NaNP. A regression line fitted to the pooled data ($r = 0.96$) is also shown ($n = 12$).

Mean shear stress values for pig coronary artery and rabbit aorta donors were not significantly different.

Calibration curves

Figure 5 shows the standard curve used to convert EDRF-induced pig coronary artery relaxant responses to total EDRF units released from each donor (C_R). The standard curve was constructed by use of the technique described in the Methods section. A regression line fitted to the data had a slope of 49.2 ($r = 0.96$, $P < 0.001$).

In order to calculate a value for the EDRF decay constant, λ, endothelium-intact coronary artery of the pig and rabbit aorta vessels were mounted as donors in the bioassay, perfused at 2 ml min^{-1}, and EDRF released by A23178 (3×10^{-7} M) measured. Pig coronary artery rings, used to detect EDRF at various donor-recipient transit times from pig coronary artery and rabbit aorta donors, were preconstricted with 5-HT (3×10^{-6} M) to 5.7 ± 0.6 g and 8.5 ± 0.4 g respectively. Results were then expressed as EDRF units by reference to the NaNP standard curve. An exponential curve fitted to the pig coronary artery EDRF data gave a decay constant $\lambda = -0.1072$ ($r = 0.646$, $P < 0.001$). An exponential curve fitted to the rabbit aorta EDRF data gave a decay constant $\lambda = -0.0975$ ($r = 0.690$, $P < 0.001$). These values for λ which were not significantly different were used in all calculations.

EDRF release from pig coronary artery and rabbit aorta endothelium in response to SP

Figure 6 shows EDRF release rates (normalised for vessel dimensions, flow rates and transit times) from pig coronary artery and rabbit aorta endothelium in response to 60 s pulses of increasing concentrations of SP. Basal EDRF release from pig coronary artery and rabbit aorta endothelium was 0.73 ± 0.25 and

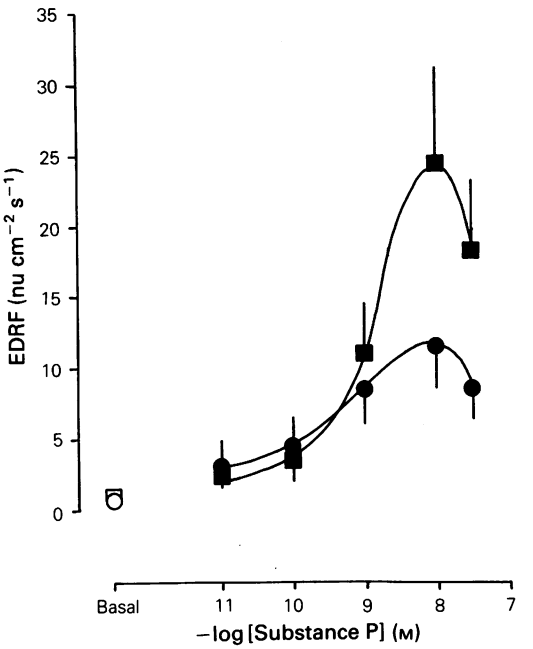


Figure 6 The mean rate of endothelium-derived relaxing factor (EDRF) release from pig coronary artery (●, $n = 5$) and rabbit aorta (■, $n = 5$) endothelium in response to 60 s pulses of substance P (closed symbols). Basal release (open symbols). Note that although basal EDRF release rates are similar, pig coronary artery endothelium has significantly lower EC_{50} value ($P < 0.01$) and rabbit aorta endothelium is capable of releasing EDRF at twice the maximum rate of that of pig coronary artery. Vertical lines show s.e.mean.

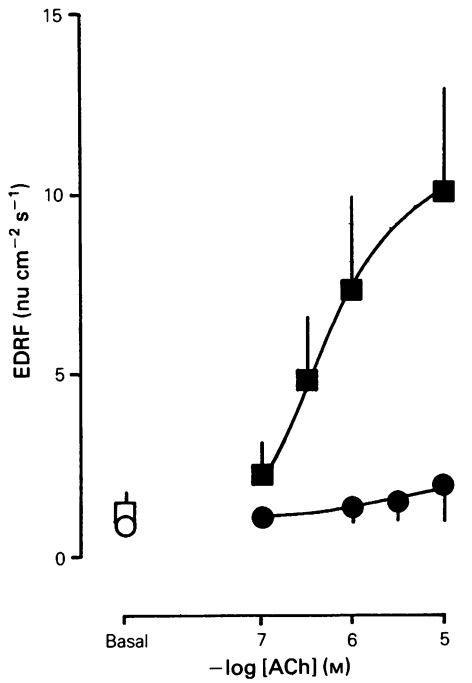


Figure 7 The mean rate of endothelium-derived relaxing factor (EDRF) release from pig coronary artery (●, $n = 5$) and rabbit aorta (■, $n = 6$) endothelium in response to cumulative concentrations of acetylcholine (ACh, closed symbols). Basal release (open symbols). Note that although basal EDRF release rates are similar, the concentration-response curve for rabbit aorta endothelium has a significantly steeper slope ($P < 0.05$) and a significantly higher asymptote ($P < 0.05$). Vertical lines show s.e.mean.

0.90 ± 0.33 nu cm^{-2} s^{-1}, respectively. The pig coronary artery concentration-response curve had a slope of 3.4 ± 1.0, EC_{50} value of $3.3 \pm 0.6 \times 10^{-10}$ M, and an asymptote of 11.40 ± 2.90 nu cm^{-2} s^{-1}. The rabbit aorta concentration-response curve had a slope of 7.4 ± 2.2, EC_{50} value of $8.6 \pm 1.4 \times 10^{-10}$ M and asymptote of 24.60 ± 6.85 nu cm^{-2} s^{-1}. The EC_{50} value for pig coronary artery endothelium was significantly less than that for rabbit aorta endothelium ($P < 0.01$).

EDRF release from pig coronary artery and rabbit aorta endothelium in response to ACh

Figure 7 shows normalised EDRF release rates from pig coronary artery and rabbit aorta endothelium in response to cumulative doses of ACh. Basal EDRF release from pig coronary artery and rabbit aorta endothelium was 0.85 ± 0.25 and 1.18 ± 0.55 nu cm^{-2} s^{-1}, respectively. The pig coronary artery concentration-response curve had a slope of 0.3 ± 0.1, an EC_{50} value of $8.1 \pm 4.4 \times 10^{-7}$ M, and an asymptote of 2.00 ± 0.48 nu cm^{-2} s^{-1}. The concentration-response curve in the rabbit aorta had a slope of 5.9 ± 1.8, an EC_{50} value of $7.2 \pm 1.8 \times 10^{-7}$ M, and an asymptote of 10.23 ± 2.83 nu cm^{-2} s^{-1}. The slope and asymptote of the rabbit aortic endothelium concentration-response curve was significantly greater than that of the pig coronary artery ($P < 0.05$).

Discussion

The results demonstrate that the intraluminal release of EDRF from pig coronary artery donors occurs in

response to SP, but not ACh. Rabbit aortic endothelium however, released EDRF in response to both SP and ACh. Basal EDRF release from both pig coronary artery and rabbit aorta donors, at comparable levels of endothelial shear stress, was quantitatively similar.

The value of λ, the decay constant for EDRF, provided an indirect measure of the chemical half-life for EDRF and was not significantly different for EDRF released from the pig coronary artery and rabbit aorta. Previous estimates of EDRF half-lives are based on relaxation of recipient vessels with no reference to a relaxation standard e.g. NaNP. The calculation of such 'biological' half-lives for EDRF could thus be dependent on the sensitivity of the recipient tissue to EDRF, more sensitive recipients giving an apparently longer half-life. Differences in the experimental procedures used and the presence or absence of superoxide dismutase may also be relevant. One or more of these factors could account for the marked differences between the half-lives found by different workers (for review see Angus & Cocks 1989). The present data therefore support the view that the chemical structure of EDRF released from different vessels is similar, at least in pig and rabbit, and probably also in other vessels. The release of EDRF from pig coronary artery in response to pulses of SP confirms both the potency of this agent in this preparation (threshold dose < 10 pM), and the onset of desensitization at higher doses, as shown by the tendency of the concentration-response curve asymptote to fall at doses above 10 nM SP. Both these phenomena have previously been observed in endothelium-intact conduit arteries of the rabbit, dog, cat and pig (Furchgott *et al.*, 1983; Cocks & Angus, 1983) and also in resistance arteries of the rabbit (Griffith *et al.*, 1988).

The lack of EDRF release from pig coronary endothelium in response to ACh explains the previously observed lack of endothelium-dependent relaxation to this agent in this preparation (Kalsner, 1985; Graser *et al.*, 1986), despite the fact that ACh-induced constrictions in pig coronary arteries are inhibited by NaNP (Itoh *et al.*, 1981). The small rise in EDRF release demonstrated could have been secondary to increased endothelial shear, as a result of constriction of the smooth muscle of the donor wall in response to ACh. This potential artifact would be technically very difficult to prevent in this system, where diffusion of luminal ACh into the media is likely to occur, activating the (normally quiescent) smooth muscle. The lack of EDRF release from pig coronary endothelium in response to ACh could be due to either a lack of muscarinic receptors, or high levels of cholinesterase activity.

Gordon & Martin (1983a) have demonstrated endothelium-dependent relaxation of pig aortic rings in response to ACh, which they later showed to be independent of prostacyclin synthesis (Gordon & Martin 1983b) and likely, therefore, to be due to release of EDRF. This suggests that either regional variations in the endothelial response to ACh can occur, or that the vascular response of neonates (Gordon & Martin routinely used 10–14 day old piglets) are not representative of the adult animal. Endothelium-dependent relaxation of aortic strips in response to ACh has not been tested in the pigs used for the present experiments.

The release of EDRF from rabbit aorta donors by pulses of SP occurred at threshold concentrations of 10 pM, and showed desensitization at high doses, as demonstrated by Zawadzki *et al.* (1981) who used endothelium-intact rabbit aortic rings. Comparison of the absolute amount of EDRF released from pig coronary artery and rabbit aorta endothelium in response to maximal doses of SP (Figure 7), showed that rabbit aortic endothelium is capable of releasing EDRF at a rate of approximately twice that of pig coronary artery endothelium.

The results of the present investigation together with those of a previous study by Christie & Lewis (1988), showing greater vascular smooth muscle sensitivity to EDRF in the pig coronary artery than rabbit aorta, provide a possible explanation for the previous findings of Collins and co-workers (1986), in which it was shown that basal EDRF 'activity' was greater in the dog coronary artery than rabbit aorta. If, as the present study shows for pig coronary artery, basal EDRF release in the dog coronary artery is similar to rabbit aorta, then the findings of Collins and colleagues are likely to be due to a greater sensitivity of the dog coronary to EDRF rather than greater release.

The release of EDRF from rabbit aorta donors in response to ACh directly confirms the results obtained by other workers, who have used endothelium-intact rabbit aortae as donors for EDRF in bioassay systems that employ widely different detectors of EDRF activity. EDRF released from rabbit aorta donors in this system had an EC_{50} of between 0.01 and 0.1 μM ACh, as originally found in the intact ring studies of Furchgott & Zawadzki (1980). EDRF release in response to SP reached a higher maximal rate than release induced by ACh (98.4 and 40.9 nu $cm^{-2} s^{-1}$, respectively), which is probably the mechanism responsible for maximal SP-induced relaxations being greater than ACh-induced relaxations in intact rabbit aortic rings (Zawadzki *et al.*, 1981).

Release of EDRF from both pig coronary artery and rabbit aorta donors in response to 10^{-6} M A23187 occurred at a higher rate than receptor-stimulated release of EDRF in response to either ACh or SP. Ionophore-induced release also effec-

tively defines the upper limit of EDRF detection by a rubbed 5-HT-preconstricted coronary artery ring of the pig, as A23187 normally induces the release of EDRF from pig coronary artery and rabbit aorta donors at such high rates that the relaxant response of the recipient ring becomes saturated (i.e. greater than 100%). The concentration-response curve to NaNP for rubbed, 5-HT-preconstricted coronary artery rings of the pig begins to plateau at concentrations which induced greater than 100% relaxation i.e. about 3×10^{-6} M NaNP. This renders the calibration curve for EDRF non-linear at the high EDRF concentrations induced by A23187, so that absolute EDRF release rates in response to A23187 could not be accurately calculated. This ability of A23187 to stimulate supramaximally EDRF release was therefore used in all experiments simply as a test for the presence of a functional endothelium on donor vessels.

In conclusion, we have developed a technique for quantitation of EDRF release from different artery types in bioassay which is independent of vessel dimensions, flow rates and donor recipient transit times.

This work was supported by the British Heart Foundation.

Appendix

Consider an arterial segment of length L and cross-sectional area A acting as a source of EDRF in a bioassay experiment. Let c(x) be the equilibrium concentration of EDRF at a distance x from the proximal end of the segment. Let f be the rate of release of EDRF into the lumen of the vessel per unit length of artery, d be its diameter and $\dot{Q}$ the mean flow rate. Let the concentration of EDRF emerging from the donor segment be C_D (see Figure 8).

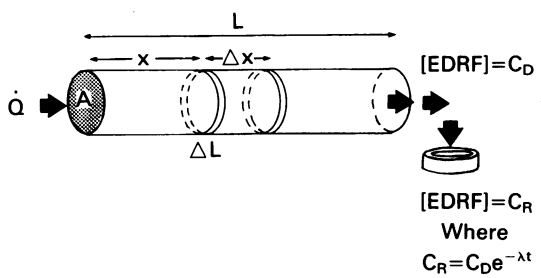


Figure 8

If a small volume of perfusate ($\Delta L \cdot A$) moves a distance Δx in time Δt it will acquire $f \cdot \Delta L \cdot \Delta t$ mol of EDRF and its concentration of EDRF will increase by

$$\frac{f \cdot \Delta L \cdot \Delta t}{\Delta L \cdot A} = \frac{f \cdot \Delta t}{A} = \frac{f}{A} \cdot \frac{A}{\dot{Q}} \Delta x = \frac{f}{\dot{Q}} \cdot \Delta x$$

since the velocity of the small volume

$$\frac{\Delta x}{\Delta t} = \frac{\dot{Q}}{A}$$

If EDRF is assumed to decay exponentially with a half-life $\frac{\log_e 2}{\lambda}$ then the small volume under consideration will lose $-\lambda c(x) \cdot \Delta t = -\lambda c(x) \cdot \frac{A}{\dot{Q}} \cdot \Delta x$ mol of EDRF in a time Δt.

Thus,

$$c(x + \Delta x) = c(x) + \frac{f}{\dot{Q}} \Delta x - \lambda c(x) \frac{A}{\dot{Q}} \Delta x$$

so that

$$\frac{dc(x)}{dx} = \frac{c(x + \Delta x) - c(x)}{\Delta x} = \frac{f}{\dot{Q}} - \frac{\lambda A}{\dot{Q}} c(x)$$

Thus

$$\frac{dc(x)}{f - \lambda A c(x)} = \frac{1}{\dot{Q}} \cdot dx$$

Integrating we obtain

$$\frac{-1}{\lambda A} [\log_e (f - \lambda A c(x))]_0^{c(L)} = \frac{1}{\dot{Q}} [x]_0^L$$

so that

$$\log_e \left[\frac{f - \lambda A C(L)}{f}\right] = \frac{-\lambda A L}{\dot{Q}} = \frac{-\lambda V}{\dot{Q}}$$

where V is the volume of the segment and

$$C_D = \frac{f}{\lambda A} [1 - e^{(-\lambda V/Q)}]$$

Thus the rate of intraluminal release of EDRF per unit surface area = $f/\pi d$

$$= \frac{\lambda dC_D}{4[1 - e^{(-\lambda V/Q)}]}$$

This equation is similar in form to a simpler expression derived independently by Kelm *et al.* (1988), to describe EDRF release rates from cultured endothelial cells mounted on microcarrier beads and perfused in columns.

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Prejunctional modulatory action of neuropeptide Y on peripheral terminals of capsaicin-sensitive sensory nerves

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1 We have determined the effect of neuropeptide Y (NPY) on motor responses produced by activation of capsaicin-sensitive primary afferents in the guinea-pig isolated left atria (reserpine-pretreatment, atropine in the bath) and bronchi (atropine and indomethacin in the bath) using capsaicin itself and electrical field stimulation as stimuli.

2 In both preparations, NPY inhibited or suppressed the response produced by electrical field stimulation while leaving the response to a submaximal concentration of capsaicin unaffected.

3 NPY had no effect on motor responses produced by a submaximal concentration of calcitonin gene-related peptide (atria) or neurokinin A (bronchi), the putative endogenous mediators of the responses produced by activation of the 'efferent' function of sensory fibres in these preparations.

4 We conclude that NPY exerts a prejunctional inhibitory action on transmitter release from peripheral endings of capsaicin-sensitive nerves. Failure of NPY to modulate responses activated by capsaicin provides further evidence for the existence of two independent modes of activation of the 'efferent' function of capsaicin-sensitive sensory nerves.

Introduction

Capsaicin-sensitive primary sensory neurones synthesize several neuropeptides (tachykinins, calcitonin gene-related peptide, CGRP) which are transported to both central and peripheral nerve endings at which level the peptides are released to produce their sensory and 'efferent' function, respectively (Szolcsányi, 1984; Maggi & Meli, 1988; Holzer, 1988). Pharmacological evidence indicates that these sensory nerve endings are endowed with various receptors for transmitters and autacoids (opioids, γ-aminobutyric acid (GABA), prostaglandins etc.), the activation of which produces changes in excitability, thereby regulating the amount of released transmitter (Yaksh *et al.*, 1980; Go & Yaksh, 1987; Barthó *et al.*, 1987; Saria *et al.*, 1988a,b; Maggi *et al.*, 1988a,b; 1989a; Giuliani *et al.*, 1989). These modulatory influences can be also observed in peripheral tissues indicating that the 'efferent' function of these primary sensory neurones could be regulated by other components of the autonomic nervous system. For instance, nicotinic and $GABA_A$ excitatory receptors and inhibitory α_2-adrenoceptors modulate excitability of capsaicin-sensitive afferents in the periphery (Saria *et al.*, 1988a,b; Maggi *et al.*, 1989a). Neuropeptide Y (NPY) is a 36 amino acid peptide, widely distributed in the mammalian peripheral nervous system, which has been shown to be present in sympathetic nerves where it is co-localized with noradrenaline and released by electrical nerve stimulation (Lundberg *et al.*, 1982; 1983; 1984; Fried *et al.*, 1985; Stjarne *et al.*, 1986). A dense plexus of nerve fibres containing NPY-like immunoreactivity is present in the heart and respiratory tract of various species (Sternini & Brecha, 1985; Lundberg *et al.*, 1983; Uddman *et al.*, 1984; Sheppard *et al.*, 1984; Potter, 1988). Among other effects, NPY has been shown to exert a prejunctional inhibitory influence on transmitter release from both cholinergic and noradrenergic nerves in the guinea-pig heart (Lundberg *et al.*, 1984; Kilborn *et al.*, 1985; Potter, 1987).

Here we present data indicating that NPY exerts a potent inhibitory action on motor responses produced in the guinea-pig atria and bronchi when

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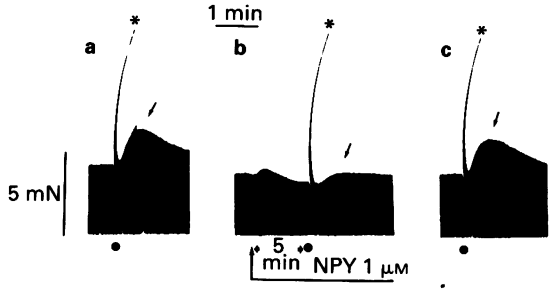


Figure 1 Typical tracings showing the early myogenic potentiating effect (indicated at asterisks) the delayed positive inotropic response (indicated at arrows) to electrical train field stimulation (applied at ●) of the guinea-pig isolated left atria (reserpine pretreated, atropine in the bath) which is due to antidromic activation of capsaicin-sensitive sensory nerves. In (b) neuropeptide Y (NPY, 1 μM) produced transient positive and negative inotropic effects and inhibited the delayed inotropic response to field stimulation. In (c) recovery from the inhibitory effect of NPY after 30 min thorough washing.

these sensory fibres are activated by electrical field stimulation but not by capsaicin. In view of the particular interest in interactions between sympathetic and sensory nerves in the mechanisms of pain generation (Wall & Gutnick, 1974; Levine *et al.*, 1986) the present data provide a further link whereby sympathetic nerves could influence excitability of sensory nerves, at least with regard to their 'efferent' function.

Methods

Guinea-pig atria

Inotropic responses of electrically driven guinea-pig left atria due to antidromic activation of capsaicin-sensitive nerves were obtained in a preparation similar to that described previously (Maggi *et al.* 1988b, 1989a; Giuliani *et al.*, 1989). Male albino guinea-pigs weighing 280–350 g received intraperitoneal reserpine (5 mg kg^{-1}) 48–96 h beforehand and were stunned and bled. The whole heart was rapidly removed and placed in Tyrode solution containing atropine (1 μM). The left atrium was cleaned from adhering tissues and placed in a 5 ml organ bath at 37°C containing oxygenated physiological salt solution of the following composition (mM): NaCl 137, KCl 2.68, $CaCl_2$ 1.8, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 5.5. The atrium was connected, under a resting tension of 5 mN, to an isometric force transducer connected to a Basile 7050 Unirecord. The atrium was stimulated by means of two wire platinum electrodes placed at the top and the bottom of the organ bath connected to a GRASS S11 stimulator. The atria were driven at a frequency of 3 Hz (0.5 ms pulse width, maximal voltage). After a 2 h equilibration period, a train of stimuli was delivered, at 15–20 min intervals, at a frequency of 10 Hz for 2.5 s (1 ms pulse width, 60 V) by means of a second stimulator, which superimposed on the stimuli delivered by the first stimulator. During the train, the atria exhibited a flutter-like response, followed, at the end of the train, by a short (4–5 s) period of increased contractility (poststimulus potentiation or postextrasystolic potentiation) while the neurogenic inotropic response to the train of stimuli appeared 15–30 s and peaked 60–120 s after the end of the train of stimuli. This stimulation produces, in left atria from reserpinized guinea-pigs and in the presence of atropine, a delayed positive inotropic response mediated by antidromic invasion of capsaicin-sensitive sensory nerve terminals.

Guinea-pig bronchi

Zig-zag strips of the main guinea-pig bronchus were prepared as described previously (Maggi *et al.*, 1988b; Giuliani *et al.*, 1989), placed in 5 ml organ baths containing oxygenated (96% O_2 and 4% CO_2) Krebs solution at 37°C plus atropine (1 μM) and indomethacin (5 μM). Tension was recorded by means of an isotonic transducer (load 5 mN) connected to a Basile 7050 pen recorder. Electrical field stimulation was obtained as described above for the guinea-pig atria, using trains of stimuli (2–5 Hz, 60 V, 0.5 ms pulse width for 10 s).

The responses to electrical field stimulation, capsaicin or neurokinin A were expressed as % of the maximal response to KCl (40 mM, added to the bath).

Statistical analysis

Each value is mean ± standard error (s.e.) of the mean. Statistical analysis was performed by means of Student's *t* test for paired or unpaired data, when applicable. Regression analysis was performed by the least squares method. EC_{50} and 95% confidence limits (c.l.) were calculated accordingly.

Drugs

Drugs used were: capsaicin and indomethacin (Sigma), atropine HCl and reserpine (Serva), neuropeptide Y, rat calcitonin gene-related peptide and neurokinin A (Peninsula).

Results

Guinea-pig left atria

NPY produced *per se* a small transient positive inotropic effect (threshold concentration 100 nM) followed by a small negative inotropic effect which peaked at 5 min (Figure 1). At 1 μM, the positive and negative inotropic changes produced by NPY averaged $+9.9 \pm 2$ and $-12 \pm 3\%$ variation of the resting tension ($n = 10$).

In the electrically-driven left atria from reserpine-pretreated guinea-pigs and in the presence of atropine, application of a train of stimuli (10 Hz, 1 ms, 60 V for 2.5 s) produced a delayed positive inotropic response ($57 \pm 7\%$ increase over resting values, $n = 13$) which was fairly reproducible at 15–20 min intervals without any sign of fading. This response has been characterized previously (Goto *et al.*, 1987; Saito *et al.*, 1987; Maggi *et al.*, 1988b; 1989a; Giuliani *et al.*, 1989) as being dependent upon activation of capsaicin-sensitive nerves which release a CGRP-like transmitter responsible for the inotropic effect. NPY produced a concentration- (10 nM–1 μM, 5 min before) dependent inhibition of these non-adrenergic non-cholinergic inotropic responses (Figures 1 and 2). The effect of NPY was fully reversible 30 min later, after thorough washing out (Figure 1). The EC_{50} for this inhibitory effect was 63 nM (35–143 nM, 95% c.l.).

By contrast, NPY had no inhibitory effect on inotropic responses produced by application of capsaicin (30 nM, $n = 5$) or exogenous CGRP (3 nM, $n = 5$) (Figure 3), at doses matched to produce an inotropic response similar to that observed in response to electrical field stimulation. These concentrations of capsaicin and CGRP were submaximal. In the present experimental conditions, maximal inotropic effects were obtained with 1 μM capsaicin and 0.1 μM CGRP. The concentrations used for the NPY experiments (30 nM for capsaicin, 3 nM for CGRP) produced a response which averaged 24 ± 4 and $21 \pm 5\%$ of the maximum for capsaicin and CGRP, respectively ($n = 5$ for each agent).

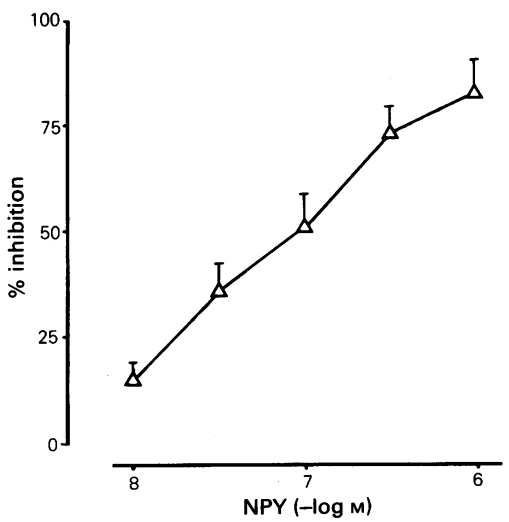


Figure 2 Concentration-dependent inhibition by neuropeptide Y (NPY) of the delayed positive inotropic response produced by electrical train field stimulation in the guinea-pig isolated left atria. Each value is mean of 6 experiments with s.e. shown by vertical bars.

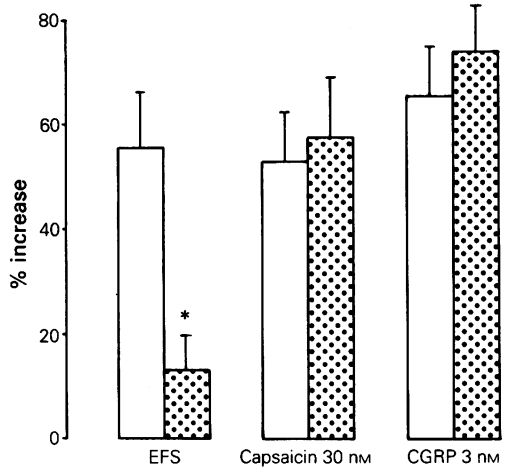


Figure 3 Comparison of the positive inotropic response produced in the guinea-pig isolated left atria by electrical field stimulation (EFS, 10 Hz, 60 V, 1 ms for 2.5 s), capsaicin or exogenously administered calcitonin gene-related peptide (CGRP). Open columns = controls; stippled columns = 5 min after addition of neuropeptide Y (1 μM). * Significantly different from controls $P < 0.01$.

Guinea-pig bronchi

In the presence of atropine (1 μM) and indomethacin (5 μM) electrical field stimulation produced frequency-related, slowly developing non-adrenergic non-cholinergic (NANC) contractions due to antidromic activation of capsaicin-sensitive nerves (Szolcsányi & Barthó, 1982; Barthó *et al.*, 1987; Maggi *et al.*, 1988b; Giuliani *et al.*, 1989). NPY (0.1 μM) did not affect tension. The NANC contractile response to electrical field stimulation (2–5 Hz, 60 V, 0.5 ms for 10 s) was significantly reduced by NPY (0.1 μM, 5 min before) by about the same amount (50–60% inhibition) at both frequencies (Figure 4). NPY (0.1 μM, 5 min) did not affect the response to exogenous neurokinin A (2 nM) or capsa-

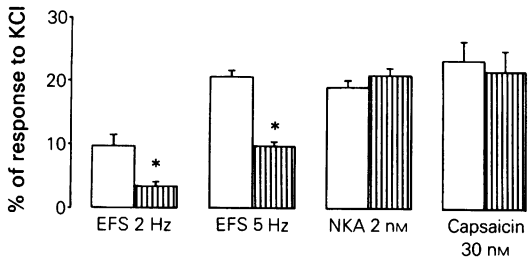


Figure 4 Comparison of the positive inotropic response produced by electrical field stimulation (EFS, 2–5 Hz, 0.5 ms, 60 V for 10 s), neurokinin A (NKA) or capsaicin in the guinea-pig isolated bronchus (atropine and indomethacin in the bath). Open columns = control; lined columns = 5 min after addition of neuropeptide Y (0.1 μM).

icin (30 nM) (Figure 4). These concentrations of neurokinin A and capsaicin were submaximal. In the present experimental conditions, maximal responses were obtained at 3 μM neurokinin A and 1 μM capsaicin which averaged 80 ± 4 and $82 \pm 5\%$ of the maximal response to KCl (40 mM, $n = 4$ for each agent). The concentrations of neurokinin A (2 nM) and capsaicin (30 nM) used for the NPY experiments were matched to produce a contractile response similar to that observed in response to electrical field stimulation at 5 Hz (about 20% of the maximal response to KCl, Figure 4).

Discussion

Using appropriate experimental conditions it is possible to elicit, in the guinea-pig isolated left-atria and bronchi, motor responses produced by antidromic activation of capsaicin-sensitive afferents (Szolcsányi & Barthó, 1982; Goto *et al.*, 1987; Saito *et al.*, 1987; Barthó *et al.*, 1987; Maggi *et al.*, 1988b; 1989a). These responses are highly reproducible and appear particularly suitable for studying the modulatory influence of substances on the excitability of the sensory nerves.

Present findings indicate that NPY exerts a potent inhibitory effect on motor responses produced when the capsaicin-sensitive nerves were activated by electrical field stimulation but not when they were activated by capsaicin. As NPY did not affect responses to CGRP (left atria) or neurokinin A (bronchi), the putative endogenous mediators of the responses produced by activation of capsaicin-sensitive nerves, present data strongly suggest a prejunctional action of NPY. This conclusion is consistent with the well known neuromodulatory action of NPY on autonomic nerves (Lundberg *et al.*, 1984; Wahlestedt *et al.*, 1986; Potter, 1988).

We have previously shown (Maggi *et al.*, 1988b,c; 1989a) that the activation of peripheral endings of capsaicin-sensitive sensory nerves by electrical field stimulation and capsaicin differ in their sensitivity to ω-conotoxin fraction GVIA. In particular, ω-conotoxin suppressed the responses produced by electrical field stimulation while leaving the responses to capsaicin unaffected. From these findings, we proposed that the responses produced by these two stimuli would be dependent on (electrical field stimulation) and independent of (capsaicin) activation of ω-conotoxin-sensitive voltage-dependent calcium channels. A selective inhibition of sensory nerve excitability when they are activated by field stimulation but not when they are activated by capsaicin has been reported previously for opioids (Barthó *et al.* 1987) and for the neuropeptide galanin (Giuliani *et al.*, 1989). This may reflect a peculiar mode of action of capsaicin which is actually capable of concentrating extracellular calcium within sensory neurones (Wood *et al.*, 1988) in such a way that even μmolar calcium concentrations in the medium are sufficient to sustain a full release of sensory neuropeptides from peripheral endings of capsaicin-sensitive nerves (Maggi *et al.*, 1988d; 1989b).

NPY has been shown to inhibit calcium currents in rat dorsal root ganglia and abolish the depolarization-coupled (high K medium) release of substance P (Ewald *et al.*, 1988; Walker *et al.*, 1988). As NPY selectively inhibits the responses produced via the ω-conotoxin-sensitive mode of activation of sensory fibres (Maggi *et al.*, 1988b), the present findings agree with those of Walker *et al.* (1988) suggesting inhibition of voltage-sensitive calcium channels as a likely mechanism for the prejunctional inhibitory action of NPY on excitability of primary sensory neurones.

NPY is released from sympathetic nerves in the peripheral nervous system, particularly at high frequencies of stimulation (Lundberg *et al.*, 1986; 1989). Thus we cannot exclude the possibility that endogenous NPY might have been released during train field stimulation e.g. that the effects of exogeneous NPY observed in this study were superimposed on a background of endogenous peptide. This might well apply to the experiments in the bronchi, while it seems less likely for experiments in the atria because these preparations were obtained from reserpine-pretreated animals. In fact, reserpine pretreatment was shown to produce a marked reduction of the NPY level in the guinea-pig atria (Lundberg *et al.*, 1985; Franco-Cereceda *et al.*, 1987).

The present findings suggest a possible mode of interaction between sympathetic and sensory nerves at the peripheral level (cf. Wall & Gutnick, 1974; Levine *et al.*, 1986), that is, prejunctional inhibition of sensory nerve excitability by NPY. Further studies are needed to assess whether sensory impulse

generation and/or transmission are affected by this neuropeptide concomitantly with inhibition of the 'efferent' function of these sensory nerves.

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Arterial chemoreceptor involvement in salicylate-induced hyperventilation in rats

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1 The extent to which peripheral arterial chemoreceptors are involved in the respiratory stimulant action of salicylates has been investigated in rats.

2 Injection of sodium salicylate (200 mg kg^{-1}, single dose i.v.) caused a rapid transient hyperventilation that was not obtained when the carotid chemoreceptors were denervated by section of the carotid sinus nerves. A delayed (10 min) increase in respiration occurred regardless of whether or not the carotid nerves were sectioned.

3 Intravenous infusions of sodium salicylate (0.5 or 4 mg kg^{-1} min^{-1}) caused hyperventilation in barbiturate-anaesthetized rats. The threshold dose for respiratory stimulation was significantly lower when the carotid sinus nerves were intact than when they were bilaterally sectioned, and the same pattern was observed following intravenous injections of sodium salicylate (cumulative doses) in anaesthetized and conscious rats.

4 Bilateral sectioning of the vagosympathetic nerve trunks did not significantly affect hyperventilation evoked by salicylate, suggesting that this response does not involve actions of salicylate on sensory receptors innervated by these nerves.

5 Administration of salicylate close-arterial to a carotid body, by local perfusion or cross-perfusion of a carotid sinus, led to an increase in respiration when the ipsilateral carotid nerve was intact, but not when it was sectioned.

6 Neuropharmacological studies on anaesthetized rats showed that chemosensory discharge, recorded from a sectioned carotid nerve, increased in response to salicylate injections with a similar dose-response pattern to the hyperventilation. Salicylate had no effect on baroreceptor discharge.

7 We conclude from our experiments that arterial chemoreceptors do contribute to salicylate-induced hyperventilation, and are almost exclusively responsible for the initial phase of the response in rats. Later increases in breathing are independent of reflexes from arterial chemoreceptors and result from actions at other sites, including the CNS. The therapeutic implications of our results are discussed.

Introduction

A common symptom of salicylate toxicity is hyperventilation which causes potentially serious acid-base balance disorders in man (e.g. Smith & Smith, 1966; Proudfoot & Brown, 1969; Vivian & Goldberg, 1982, Proudfoot, 1983). Salicylates have been shown to stimulate respiration via actions within the CNS (Tenney & Miller, 1955; Cameron & Semple, 1968; Millhorn *et al.*, 1983), but there are also data suggesting that salicylate may affect ventilation by stimulating sensory receptors in the periphery (Wright, 1935; Graham & Parker, 1948; Levine, 1976). Evidence favouring a central action appears to be stronger than that for a peripheral action, and it is generally considered that salicylate-induced hyperventilation results from central actions of the drug.

Salicylates would, however, be expected to activate peripheral arterial chemoreceptors and thereby reflexly increase ventilation (Heymans & Neil, 1958). This is because these sensory receptors, being much more sensitive to energy disturbance than most tissues (Dontas, 1955) are readily stimulated by uncouplers of oxidative phosphorylation (e.g. 2,4-dinitrophenol and sodium azide; Jarisch *et al.*, 1952; Anichkov & Belen'kii, 1963), and salicylates can uncouple oxidative phosphorylation (Brody, 1956; Smith & Smith, 1966). Although there is no doubt

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that salicylate can act centrally to increase respiration, it is possible that the drug acts at more than one site, as suggested by Wright (1935). The present study was undertaken to investigate further the pharmacology of salicylate by determining the contribution made by arterial chemoreceptors to salicylate-induced hyperventilation in rats. Preliminary communications of some of these results have been published (Birrell *et al.*, 1987; McQueen & Ritchie, 1988).

Methods

Male Wistar rats (240–360 g) were used for this study. Inactin (a long-acting thiobarbiturate) was injected i.p. in a dose of 100–115 mg kg^{-1} when anaesthesia was required.

Surgical procedures

In acute experiments under anaesthesia the trachea, both femoral arteries and the right femoral vein were cannulated. Input to the CNS from functional arterial chemoreceptors was abolished by bilateral sectioning of the carotid sinus nerve 15 min before the administration of salicylate; this procedure abolished the reflex hyperventilation previously evoked by the chemoreceptor stimulant, sodium cyanide (10–50 μg i.v.). The vago-sympathetic nerve trunks were both sectioned at the mid-cervical level in particular experiments.

For chronic experiments, in rats anaesthetized with halothane (1.5% in oxygen) the right femoral artery and right femoral vein were cannulated under aseptic conditions. The cannulae were led subcutaneously along the animal's back and exteriorised via a small skin incision just below the neck. The protruding catheters were flushed then filled with 80 μl saline containing heparin (500 units ml^{-1}) before being sealed by a knot in the end. In some animals both carotid nerves were sectioned under halothane anaesthesia and 9–12 days recovery allowed for the neck incision to heal before cannulation. Incisions were sutured and the animals allowed to recover for 24–48 h before being used for acute experiments; the drinking water contained 0.8 g l^{-1} chlortetracycline.

Recording of ventilation and arterial blood pressure

In anaesthetized rats respiration was measured by means of an integrating pneumotachograph and electrospirometer (Muercury, CS5), with output displayed on a chart recorder (Devices M4), as previously described (Bond *et al.*, 1982). Arterial blood pressure (BP) was measured by connecting one femoral catheter to a BP transducer, with the output also being displayed on the chart. The other femoral artery catheter was used for taking 0.7 ml samples of blood which commenced 10 min after the start of an infusion of salicylate and continued every 20 min thereafter. Samples were also taken after cumulative doses of 70 and 550 mg kg^{-1} sodium salicylate during the experiments in which salicylate was injected. The arterial blood gas tensions and pH were measured in 0.2 ml of the sample with a Radiometer ABL2, analyser and the remaining 0.5 ml was used for the assay of total salicylate levels in plasma by colorimetry (520 nm, Whatman DC100 Colorimeter) with a Sigma Diagnostics Kit (Procedure # 530).

For experiments on conscious animals the rat was placed in a perspex box (13 × 9 × 9 cm) with a hole at one end, the hole being covered by a double layer of latex sheeting. The head of the rat was passed through a hole in the latex sheets, which formed an air-tight collar around the neck. An arched piece of perspex was wedged over the neck and a small perspex box (4 × 5.5 × 5.5 cm) was fixed over the head and secured to the main box; a rubber gasket provided an air-tight seal. The head-box contained silica gel and soda-lime to absorb moisture and CO_2 respectively, and an outlet was connected to a pneumotachograph. Another pneumotachograph was linked to the airtight lid that closed off the main box. Catheters from the animal passed through holes in the lid, which were sealed with plasticine, and the protruding cannulae connected to fine tubing for withdrawal of blood samples or intravenous administration of drugs.

In five experiments the animals were sedated by an injection of ketamine (100 mg kg^{-1}) and midazolam (5 mg kg^{-1}) i.p. 20 min before they were placed in the box. Although the animals were easier to handle, they were also quite markedly sedated and this technique was abandoned in favour of training rats to sit in the box while respiration was monitored for 30 min. Once the rat had settled, sodium cyanide (10–50 μg i.v.) was injected to check for the presence or absence of chemoreceptor-mediated reflex hyperventilation, then the administration of salicylate was begun. Respiratory data were measured from the chart traces and values derived by computer analysis (IBM PC).

Perfusion experiments

In two heparinised (500 units kg^{-1}) rats under Inactin anaesthesia, a carotid artery was cannulated in both directions, so that blood could be pumped from the cardiac end into the carotid bifurcation at the cephalic end at constant flow by use of a roller pump (Watson Marlow MHRE). Salicylate could be injected into the perfusion tubing at the distal end, just before the carotid bifurcation. The internal

carotid artery was ligated, and in one experiment the ipsilateral carotid nerve was sectioned.

In a further experiment two rats were anesthetized and blood from the carotid artery of one was pumped by the roller pump to the cephalic end of the carotid artery of the recipient. The ipsilateral internal carotid artery of the latter was cannulated and blood from it was pumped back to the donor rat via a cannula in the femoral vein of the donor. There was a 75 s delay in the perfusion system before blood from the donor reached the perfused carotid bifurcation of the recipient. Salicylate was injected into the donor and respiration measured in both donor and recipient. Blood samples were taken for estimation of salicylate levels in both animals.

Arterial chemoreflexes were tested in all these experiments by i.v. or i.a. (via perfused tubing) injections of sodium cyanide (50 μg), which evoked a marked increase in respiration when the carotid nerves were intact, but had no effect when they were cut.

Neural recording

In rats anaesthetized with Inactin the carotid nerve on one side was dissected and sectioned. Chemosensory discharge was recorded from the peripheral end of the nerve by a monopolar platinum-iridium wire electrode. The signal was amplified (Neurolog), displayed on an oscilloscope (Tektronix 5103N) and digitized (Sony PCM 701ES) before it was recorded on video tape (Sony SL-F25UB VCR). The recorded digital signal was subsequently replayed, converted to analogue, and passed through a pulse height (voltage) discriminator (WPI Model 120) whose output was counted and quantified by a microcomputer (IBM-PC) to provide data for individual chemoreceptor action potentials.

In some experiments the contralateral carotid nerve was cut, but in others it was left intact and respiration was recorded concurrent with the neural recording.

Data analysis

Respiratory minute volume (RMV) was plotted against dose of salicylate injected or infused and dose-response curves generated by fitting a logistic expression to the data with a microcomputer (see Barlow, 1983). Variables such as the ED_{75} (dose which increased basal RMV by 75%) could be calculated from the sigmoidal curve, and mean pooled values from different protocols were compared by means of non-parametric statistical tests (Wilcoxon two-sample test; see Colquhoun, 1971) except when sample size was small, in which case the t test was used. Differences between group means were considered statistically significant and the null hypothesis rejected if $P < 0.05$. Results are shown as mean values $\pm$ s.e.mean.

Drug administration

Drugs were administered by either intravenous injection (bolus injections, 3 min interval between doses) or infusion (0.05 ml min^{-1}) via a Braun Unita pump. All drugs were dissolved in isotonic saline, with the following exceptions: aspirin (acetylsalicylic acid)—98.4 mg acetylsalicylic acid dissolved by adding 2–3 ml 10% w/v calcium carbonate suspended in saline. When effervescence ceased excess carbonate was filtered off and the filtrate (calcium aspirin) made up to volume with saline to give a dose for infusion of 0.62 mg kg^{-1} min^{-1}, equivalent to 0.5 mg kg^{-1} of sodium salicylate (sodium and potassium salts of aspirin are unstable, Davison, 1971).

Drugs used

Sodium pentobarbitone (May & Baker), Inactin (Byk Gulden), sodium salicylate and acetylsalicylic acid (Aldrich), midazolam (Roche), ketamine (Parke Davis), chlortetracycline (Lederle).

Results

Effects of salicylate on respiration

Infusions of sodium salicylate The experiments performed at the outset of this project entailed an infusion of sodium salicylate, 4.0 mg kg^{-1} min^{-1} i.v., over 50–80 min with the aim of administering a total dose of 200–300 mg kg^{-1}, which is mildly toxic in man (Brenner & Simon, 1982). Use of the long-acting barbiturate, Inactin, proved very successful in providing stable anaesthesia without a need for supplementation during experiments lasting up to 6 h.

Hyperventilation occurred in anaesthetized rats with intact carotid nerves ($n = 7$) as well as those in which the carotid nerves were sectioned ($n = 8$), and the only significant difference was that the onset was more rapid in the intact group ($P < 0.05$ at 25 min in comparison with denervated). However, a similar hyperventilation was eventually obtained in both groups, and the mean lethal doses were not significantly different (452 ± 58 mg kg^{-1} intact, 475 ± 56 mg kg^{-1} denervated). One notable difference was that whereas the intact rats died from respiratory failure, denervated animals died from cardiac failure.

Salicylate was next infused at a lower dose (0.5 mg kg^{-1} min^{-1}) in order to achieve a better separation between peripheral and central actions of salicylate on respiration. The change in ventilation of

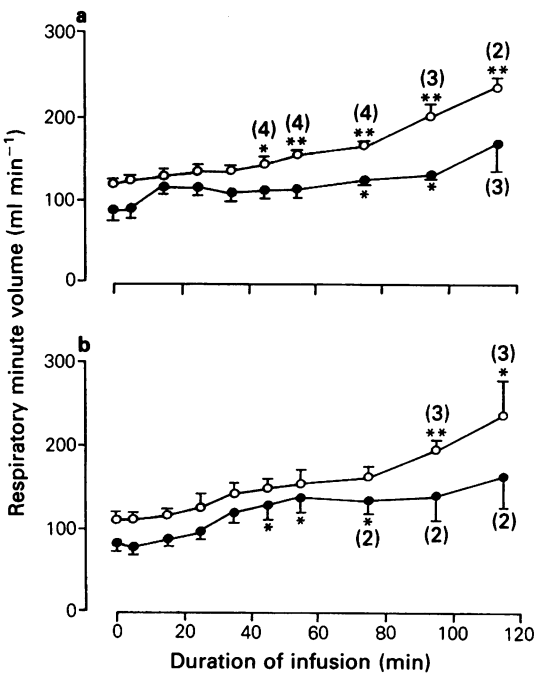


Figure 1 Averaged data for respiratory (RMV) responses to i.v. infusions of (a) $0.5\,mg\,kg^{-1}\,min^{-1}$ sodium salicylate in anaesthetized rats with intact carotid nerves (○, $n = 5$) or bilaterally sectioned carotid nerves (●, $n = 4$) and (b) calcium acetylsalicylate ($0.62\,mg\,kg^{-1}\,min^{-1}$), (○) carotid nerves intact ($n = 4$), (●) carotid nerves sectioned ($n = 3$). Both drugs increased RMV, but responses were bigger when the carotid nerves were intact. $^{*}P < 0.05$, $^{**}P < 0.01$ (t test vs time zero). Numbers in parentheses show survivors from the initial samples. Vertical lines show s.e.mean. Death occurred after an infusion period of 104 ± 15 min (intact) and 178 ± 50 min (denervated) with sodium salicylate, the corresponding values for calcium aspirin were 127 ± 8 and 131 ± 28 min, respectively.

rats with intact or bilaterally sectioned carotid nerves was measured and the results obtained are shown in Figure 1a. The increase in respiratory minute volume (RMV) above basal values began at about 45 min in the intact group and was due to increases in both frequency and tidal volume, which reached a peak ($235 \pm 8\,ml\,min^{-1}$) at 115 min. In contrast, rats with sectioned carotid nerves showed a more delayed onset, with no significant increase above their basal RMV occurring until 75 min, at which time increases in tidal volume occurred and a peak response of $170 \pm 35\,ml\,min^{-1}$ was obtained at 115 min ($P > 0.05$ in comparison with intact group).

Plasma salicylate levels in intact animals were $54.6 \pm 6.1\,mg\,l^{-1}$ at 30 min, $115.1 \pm 10.6\,mg\,l^{-1}$ at 70 min and $146.9 \pm 3.1\,mg\,l^{-1}$ at 110 min after the start of the infusion; these did not differ significantly from corresponding values for denervated rats of $64.3 \pm 5.8\,mg\,l^{-1}$ (30 min), $112.8 \pm 19.7\,mg\,l^{-1}$ (70 min) and $179.9 \pm 35.7\,mg\,l^{-1}$ (110 min). The lethal dose was $52 \pm 7\,mg\,kg^{-1}$ for intact and $89 \pm 25\,mg\,kg^{-1}$ for denervated rats after $0.5\,mg\,kg^{-1}\,min^{-1}$ sodium salicylate, and the cause of death was not notably different – respiratory failure or cardiac collapse were observed in both groups. Arterial pH increased to a mean maximum of 7.45 ± 0.005 units from a pre-infusion value of 7.33 ± 0.02 units in intact rats ($P < 0.01$, $n = 4$) and to 7.44 ± 0.1 from 7.37 ± 0.02 units ($n = 4$, $P < 0.01$) in chemodenervated rats 110 min after the start of the infusion.

Aspirin In order to determine whether the effects of aspirin on respiration differed appreciably from those of salicylate, $0.62\,mg\,kg^{-1}\,min^{-1}$ of calcium aspirin was infused in intact rats and in rats with bilaterally sectioned carotid nerves. Results obtained are shown in Figure 1b, where it can be seen that hyperventilation evoked by calcium aspirin was qualitatively similar to that obtained when sodium salicylate was infused (Figure 1a). However, responses were more variable and because of this the increase in mean RMV that occurred early during the infusion of aspirin in intact rats was not statistically significant, whereas it was later on, in contrast to the denervated group. Salicylate levels intact/cut averaged $49.4 \pm 4.4/58.8 \pm 8.5\,mg\,l^{-1}$ at 30 min post-infusion, $87.6 \pm 8.7/102.9 \pm 14.7\,mg\,l^{-1}$ at 70 min, and $135.1 \pm 2.7/117.7 \pm 14.8\,mg\,l^{-1}$ at 110 min after the start of the infusion.

Injection of sodium salicylate Results from infusion experiments pointed to the possible involvement of arterial chemoreceptors in the early phase of salicylate-induced hyperventilation. In order to separate further the peripheral and central respiratory components, and to determine whether the anaesthetic agent was influencing responses, we undertook a series of short experiments to compare the effect of injected salicylate on respiration both in anaesthetized and in conscious rats, intact or with carotid nerves sectioned.

A single i.v. injection of $500\,mg\,kg^{-1}$ sodium salicylate rapidly killed anaesthetized rats (lethal dose in man $> 500\,mg\,kg^{-1}$; Temple, 1981), whereas they survived a dose of $200\,mg\,kg^{-1}$, in agreement with previous work (Hill, 1971); use of the latter dose revealed significant differences in the respiratory response depending on whether or not the carotid sinus nerves were intact. As shown in Figure 2, a substantial but transient hyperventilation occurred

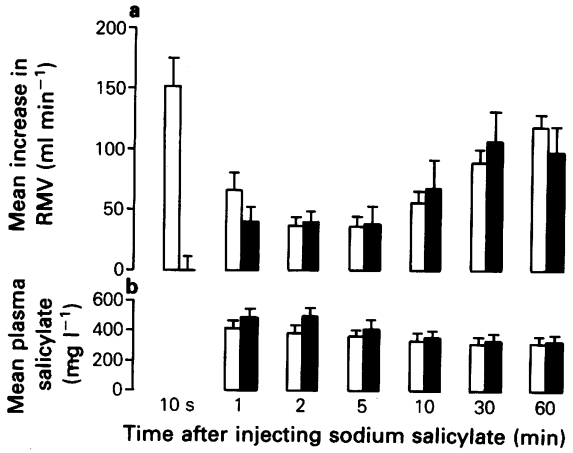


Figure 2 (a) Mean increase in respiratory minute volume (RMV) above pre-injection levels obtained following a single i.v. injection of sodium salicylate 200 mg kg^{-1} in anaesthetized rats with carotid nerves intact (open columns, $n = 6$) or cut (solid columns, $n = 3$). Note the rapid increase when the nerves were intact, and the slower smaller response from the denervated animals peaking at 60 s. A delayed increase started about 10 min after the injection in both groups. Pre-injection RMV averaged 126 ± 14 ml min^{-1} intact, 87 ± 9 ml min^{-1} with the carotid nerves cut (difference NS, $P > 0.05$). (b) Plasma salicylate levels are shown. There was no significant difference between group means for any of the times at which blood samples were taken. In (a) and (b) vertical bars show s.e.mean.

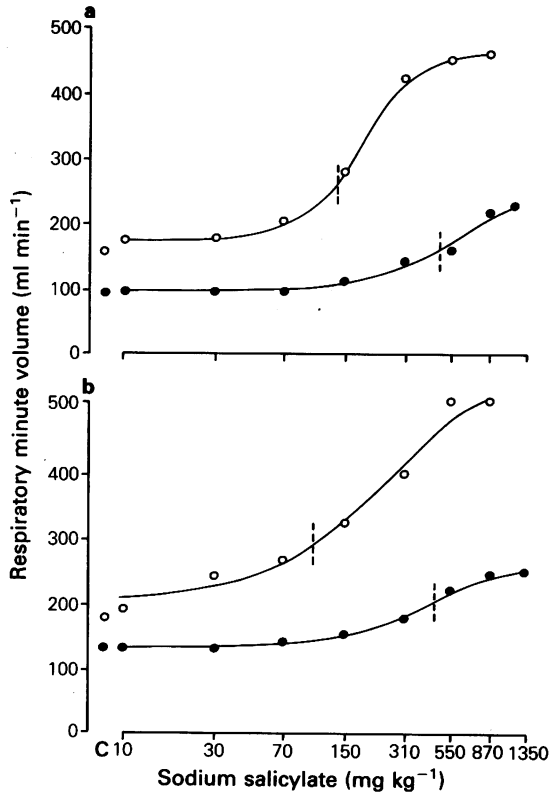


Figure 3 Respiratory responses to i.v. injections of sodium salicylate (applied cumulatively) from four individual experiments in anaesthetized (a) or conscious (b) rats. (○) represent results from rats with intact carotid nerves; (●) those from rats in which both carotid nerves had been sectioned before the salicylate was administered. Log dose-response curves were fitted to the data by computer and the calculated ED_{75} for each line is shown (broken vertical line). Cutting the carotid nerves increased the ED_{75} and reduced the maximum respiratory response (both absolute and incremental) to salicylate; the anaesthetic agent did not appear to influence the results. C = control (pre-salicylate) values.

almost immediately after injection when the nerves were intact, but not when they were sectioned ($P < 0.01$). The RMV in the denervated group began to increase 20 s after the injection and reached a steady-state 60 s post-injection. At this time there was no significant difference between RMV in the two groups, because RMV in the intact group had fallen from the peak levels. About 10 min after salicylate had been given, RMV in both groups increased to the same extent, and the animals died 45–90 min after the injection.

We studied the RMV response to salicylate over a wider dose-range, and to avoid the use of a substantial number of animals we injected increasing doses of salicylate to individual animals and constructed response curves for RMV against cumulative dose of salicylate injected. This was done in a variety of experiments to compare the respiratory response obtained when the carotid sinus nerves were cut, with responses seen under both normal (carotid nerves intact) and other (e.g. vagotomised) conditions.

Typical respiratory responses to sodium salicylate administered by serial bolus i.v. injections to rats with carotid nerves either intact, or sectioned (no respiratory response to i.v. NaCN), anaesthetized or conscious, are shown in Figure 3. Salicylate had similar stimulant effects on ventilation regardless of whether or not the animals were anaesthetized (compare Figure 3a and b). However, in both conscious and anaesthetized rats with sectioned carotid nerves the hyperventilation was less for any given dose of salicylate (in either absolute or relative

terms) than it was in animals with intact nerves. The threshold dose of salicylate for increasing respiration above basal level was also higher in the denervated rats. The rise in RMV resulted from increases in frequency and tidal volume in normal animals (conscious or anaesthetized), whereas in denervated rats it was due to an increased tidal volume but a fall in frequency.

The lethal dose tended to be slightly higher when the carotid nerves were cut, and it was noted that animals with intact nerves died suddenly from respiratory failure following an additional dose of salicylate (i.e. next dose in the sequence shown in Figure 3 would result in zero RMV); in contrast, those with bilaterally sectioned carotid sinus nerves died from cardiac arrest. Basal values for RMV, together with other variables, are shown in Table 1. Measurement of pH, $P_a CO_2$ and $P_a O_2$ made after cumulative doses of 70 and 550 mg kg^{-1} salicylate in anaesthetized intact rats gave mean (±s.e.mean) values ($n = 3$) of pH 7.35 ± 0.02 units, $P_a CO_2$ 42.7 ± 3.3 mmHg, $P_a O_2$ 76.6 ± 2.1 mmHg after 70 mg kg^{-1} and pH 7.44 ± 0.02 units, $P_a CO_2$ 26.9 ± 2.6 mmHg, $P_a O_2$ 80.8 ± 6.3 mmHg after 550 mg kg^{-1}. Corresponding values for anaesthetized rats with both carotid nerves cut were pH 7.22 ± 0.02 units, $P_a CO_2$ 54.9 ± 2.2* mmHg, $P_a O_2$ 71.5 ± 1 mmHg (70 mg kg^{-1}) and pH 7.32 ± 0.04 units, $P_a CO_2$ 46.1 ± 6.3* mmHg, $P_a O_2$ 88.9 ± 10 mmHg (550 mg kg^{-1}) (*$P < 0.05$ in comparison with intact group, *t* test).

Control RMV was significantly lower in anaesthetized animals with intact carotid nerves in comparison with their conscious equivalents ($P < 0.05$), but there was no statistically significant difference in the case of animals with sectioned carotid nerves. Basal RMV tended to be lower in rats with both carotid nerves cut in comparison with intact controls, but whereas this difference was not statistically significant ($P > 0.05$) in anaesthetized animals, it was in conscious rats ($P < 0.05$). There was no significant difference between body weights of the intact or bilaterally denervated groups, either anaesthetized or conscious.

To facilitate comparison of results from different experiments ED_{75} values were calculated from the computer-generated log dose-response curves (see Figure 3). The ED_{75} is defined as the cumulative dose of salicylate required to increase basal (i.e. pre-salicylate) RMV by 75%, a figure selected because it was the lowest common maximum increase. ED_{75} values are summarised in Figure 4 and there was no significant difference between anaesthetized, sedated or conscious rats in the dose of salicylate required to evoke a 75% increase in RMV when the carotid nerves were intact. Values were less variable in sedated rats, but although the mean ED_{75} for sali-

Table 1 Control (pre-salicylate) values for rats with carotid sinus nerves (CSN) and vagi either intact or cut

	Anaesthetized						*Conscious*	
	Intact	*Cut vagi*	*Cut 1 CSN*	*Cut 2 CSN*	*Cut 2 CSN* *Cut vagi*	*Intact*	*Intact* *sedated*	*Cut 2 CSN*
n =	5	3	3	6	3	4	4	3
Weight (g)	357 ± 9	390 ± 39	339 ± 22	319 ± 21	406 ± 7	326 ± 8	306 ± 26	297 ± 14
Basal RMV (ml min^{-1})	131 ± 11*	129 ± 8	102 ± 9	109 ± 8	118 ± 11	164 ± 6	143 ± 6	127 ± 12
pH (units)	7.34 ± 0.01*	7.25 ± 0.02	7.29 ± 0.03	7.30 ± 0.03	7.27 ± 0.02	7.39 ± 0.02	7.35 ± 0.003	†
$P_a CO_2$ (mmHg)	45.1 ± 3.2	39.2 ± 5.2	35.7 ± 7.2	40.4 ± 4.5	32.8 ± 4.3	36.5 ± 1.7	42.2 ± 1.8	†
$P_a O_2$ (mmHg)	73 ± 5	74 ± 3	84 ± 4	78 ± 4	86 ± 5	87 ± 2	69 ± 3	†

Mean ± s.e.mean values from different groups of *n* rats which recieved i.v. injections of sodium salicylate.
RMV, respiratory minute volume; CSN, carotid nerve.
† No data.
* $P < 0.05$ vs equivalent conscious group.

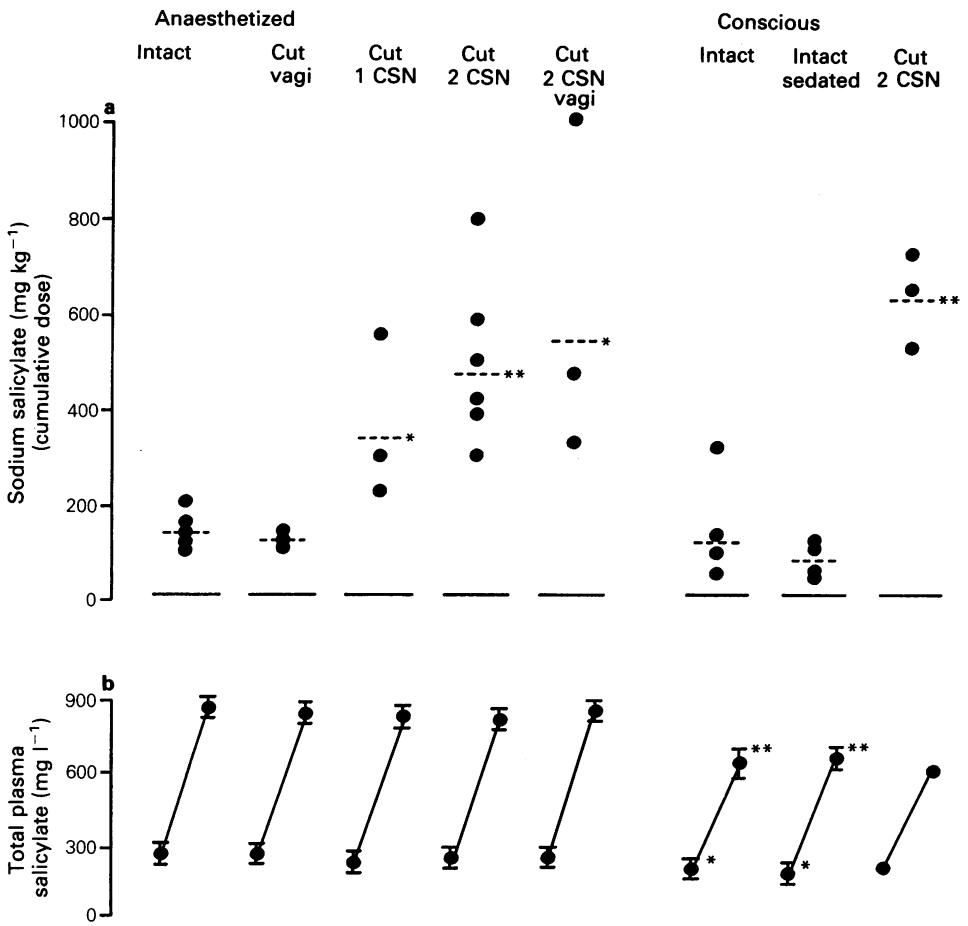


Figure 4 Summary of respiratory responses to cumulative i.v. doses of sodium salicylate obtained from experiments in anaesthetized and in conscious rats under a variety of experimental conditions. (a) Individual ED_{75} values, with dashed lines indicating the geometric mean for each group. Cutting one or both carotid nerves (CSN) significantly increased the dose of salicylate needed to raise basal respiratory minute volume (RMV) by 75% (*$P < 0.05$; **$P < 0.01$ in comparison with intact group). The pattern was similar in conscious and anaesthetized animals. (b) Plasma salicylate levels in blood samples taken following the 70 mg kg^{-1} (left) and 550 mg kg^{-1} (right) cumulative doses of sodium salicylate. Each point represents the mean and vertical lines show s.e.mean. In both conscious and anaesthetized states the levels did not differ significantly between groups; in absolute terms levels were significantly lower at each dose in the conscious animals (*$P < 0.05$; **$P < 0.01$ in comparison with the intact anaesthetized group).

cylate was slightly lower than in conscious animals with no premedication, the difference was not statistically significant ($P > 0.05$). In three anaesthetized animals, cutting the vagi (leaving the carotid nerves intact) had no significant effect on the respiratory response ($P > 0.05$), and cutting the vagi in addition to the carotid nerves gave results which did not differ significantly from those obtained when only the carotid nerves were cut (see also Table 1).

A comparison of ED_{75} values obtained in anaesthetized rats with either one or both carotid nerves cut showed that a significantly higher dose of salicylate was required to cause a 75% increase in RMV in comparison with control animals. Similarly, the salicylate ED_{75} in conscious rats with sectioned carotid nerves was significantly higher than that in the intact conscious or intact sedated animals. Measurement of plasma salicylate levels in the different groups of anaesthetized rats (see Figure 4b) showed there were no significant inter-group differences after either 70 or 550 mg kg^{-1} of salicylate, nor were there differences between group results in conscious

animals. Absolute levels were significantly lower after each salicylate dose in conscious as compared with anaesthetized rats, possibly reflecting a slower metabolism and clearance of salicylate secondary to reduced hepatic blood flow in barbiturate anaesthesia.

Sodium cyanide

The peripheral chemoreceptor stimulant sodium cyanide was injected (10–50 μg i.v.) to test for chemoreflex activity. In both conscious and anaesthetized rats with intact carotid nerves cyanide evoked the expected reflex hyperventilation. However, when both carotid nerves had been cut either during the experiment (anaesthetized) or chronically (conscious group), no respiratory reflex was obtained, which confirmed that the functional arterial chemoreceptors had been denervated. Bilateral vagotomy (at mid-cervical level, which left the carotid nerves intact) did not reduce the cyanide-induced hyperventilation, and if anything increased it, suggested a lack of any significant input from aortic or abdominal arterial chemoreceptors.

Perfusion experiments in anaesthetized rats

Because intravenous administration of sodium salicylate results in the drug acting throughout the body (Hill, 1971; 1973), the possibility existed that the peripheral actions might be dependent on, or influenced by, central effects. We therefore administered sodium salicylate locally into the carotid arterial circulation on one side of an anaesthetized rat and found an immediate substantial increase in RMV when the carotid nerve was intact, but not when it was sectioned (Table 2), showing that actions of salicylate within the CNS, or peripheral sites other than those innervated by the carotid nerves, were not involved in the fast respiratory response. This was confirmed by performing experiments in which blood from an anaesthetized donor rat perfused the carotid bifurcation of an anaesthetized recipient; most of the blood was returned to the donor, but some did reach the systemic circulation of the recipient. Salicylate caused hyperventilation in the donor and also increased ventilation in the recipient after a delay accounted for by the lag in the perfusion circuit (Table 2); plasma salicylate levels were below the level of detection in the systemic blood of the recipient, so actions at sites other than the carotid bifurcation were unlikely to be involved in the respiratory responses obtained.

Neural recordings

The respiratory experiments provided indirect evidence that sodium salicylate stimulated carotid body arterial chemoreceptors. Direct evidence confirming this was obtained from five experiments on anaesthetized rats in which chemosensory discharge was recorded from the peripheral end of a sectioned carotid nerve. Salicylate injections caused a dose-related increase in chemosensory discharge which began within 10 s of making the injection and lasted for 1–2 min in all the experiments. Basal discharge gradually increased in response to cumulative doses, but there was no evidence for any action of the salicylate on baroreceptor afferents. Results from one of the experiments in which RMV and neural discharge were recorded are shown in Figure 5 and it can be seen that the pattern of response was broadly similar for the two variables; threshold doses of salicylate were similar, but the ED_{50} (half-maximum response) was lower for neural discharge than it was for RMV, reflecting the steeper log dose-response curve for the former.

Effect of 100% oxygen

Experiments in which the rats continually breathed 100% oxygen instead of room air made no signifi-

Table 2 Respiratory responses to injections of sodium salicylate (70 mg kg^{-1}, cumulative dose)

	A	B	C	
	Carotid nerve intact	*Carotid nerves sectioned*	*Donor*	*Recipient*
% increase in RMV	129	8	44	14
Plasma [salicylate] (mg l^{-1})	290	240	290	<5

A: intracarotid injection close to an innervated bifurcation. B: intracarotid injection close to a bifurcation, carotid nerve sectioned. C: data from a cross-perfusion experiment in which salicylate was injected into a donor animal whose blood perfused the carotid bifurcation of a recipient (intact carotid nerve). Respiration minute volume (RMV) was measured over 13 s post-injection period and expressed as the percentage increase above the pre-injection level (allowing for the 75 s delay in the perfusion circuit before blood from the donor reached the recipient in C).

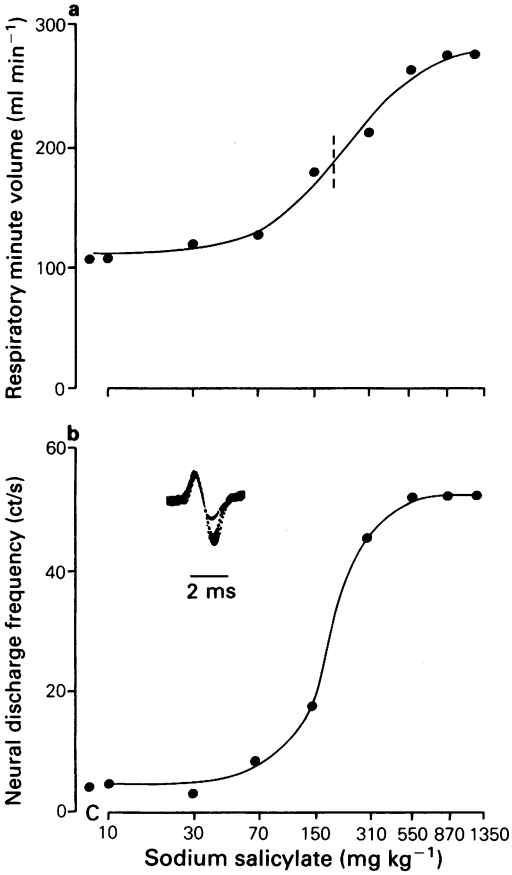


Figure 5 Concurrent recordings of (a) respiration and (b) chemosensory discharge obtained in an anaesthetized rat during injections of sodium salicylate (doses injected cumulatively i.v. at 3 min intervals). (a) The respiratory response to salicylate (one carotid nerve cut) with the ED_{75} (225 mg kg^{-1}) indicated by a dashed line. (b) Illustrates the response of two chemoreceptors recorded from the peripheral end of the cut carotid nerve (inset: 20 superimposed oscilloscope sweeps showing the two action potentials; the sweeps were triggered by the upward deflections, which also activated a counter). Dose-response curves were fitted to the data by use of a logistic function, and the ED_{50} calculated as 198 mg kg^{-1} for neural discharge, 266 mg kg^{-1} for respiratory minute volume (based on the computer-derived maximum responses).

cant difference to the RMV during either the 0.5 mg kg^{-1} min^{-1} infusion ($n = 3$) or serial injection up to 500 mg kg^{-1} of sodium salicylate ($n = 4$). A switch from air to 100% O_2 30 min after the start of an infusion of sodium salicylate (0.5 mg kg^{-1} min^{-1}) caused an immediate 15% reduction in RMV, but breathing recovered over the next 10 min, despite the continued presence of oxygen.

Discussion

Salicylate-induced hyperventilation in rats

Salicylates are known to cause hyperventilation in the cat (Wright, 1935; Phillippot & Dallemagne, 1949; Millhorn *et al.*, 1983), dog (Tenney & Miller, 1955; Levine, 1976), rabbit (Cameron, 1968) and man (Riley *et al.*, 1977). Our results show that peripheral arterial chemoreceptors make a significant contribution to the hyperventilation evoked by intravenous salicylate in rats, being particularly involved in the early phase of the response. Experiments in which various doses of salicylate were injected or infused into animals with either one or both carotid nerves sectioned, demonstrated that the onset of hyperventilation evoked by salicylate was delayed and the ED_{75} significantly increased in comparison with the intact controls (see Figure 2). The anaesthetic agent did not appear to influence the respiratory response to salicylate because results from conscious, sedated and anaesthetized rats were all similar. Neural recording of carotid chemosensory discharge in anaesthetized rats confirmed that sodium salicylate causes a dose-dependent increase in chemosensory discharge, without affecting carotid baroreceptors, and showed that the $\log_{10}$ dose-response curve for chemosensory input to the CNS was qualitatively similar to that for the respiratory output (RMV) in response to salicylate injections (see Figure 5).

Cutting both carotid nerves totally abolished the respiratory response to cyanide (1–100 μg i.v.), whereas bilateral sectioning of the vagosympathetic nerve trunks at mid-cervical level, which eliminates vagally innervated thoracic or abdominal chemoreceptors, did not diminish respiratory responses to either cyanide or salicylate. Overall, our evidence suggests that carotid chemoreceptors are the only functional chemoreceptors in rats under the conditions of our experiments (i.e. experiments which involved either acute denervation, or denervation performed up to 8 days before the experiments on conscious rats). This is in agreement with the consensus view that aortic and/or abdominal chemoreceptors do not appear to be functional in rats (Colinet-Lagneaux *et al.*, 1967; Sapru & Krieger, 1977; Martin-Body *et al.*, 1986). Cardenas & Zapata (1983) also considered the carotid chemosensors to be the main source of chemoreflex activity, but argued for a slight extra-carotid contribution from vagal and non-vagal afferents. However, their results are difficult to interpret because of possible non-specific effects of the stimulants used, namely hypoxia (see Martin-Body *et al.*, 1986) or high doses of cyanide (see Wright, 1935).

The evidence for involvement of carotid chemoreceptors in the hyperventilation evoked by sali-

cylate was further strengthened by showing that local administration of salicylate to an innervated carotid bifurcation, but not to a denervated one, led to hyperventilation. Cross-perfusion experiments showed that reflex hyperventilation could be obtained from a carotid bifurcation perfused with salicylate-containing blood from a donor, at a time when there was no measurable salicylate in the systemic circulation of the recipient. This demonstrated that this hyperventilation evoked by salicylate was not dependent on direct or indirect actions of the drug within the CNS, or at peripheral sites other than the chemoreceptors.

Results from experiments in which salicylate was injected or infused show that the early phase of salicylate-induced hyperventilation is due to a respiratory reflex arising from stimulation of carotid chemoreceptors. A slower phase of respiratory stimulation occurs even in the absence of sensory input from the carotid and vagosympathetic nerves, implying that salicylates affect respiration by at least two mechanisms, and the delayed phase is independent of actions on arterial chemoreceptors.

Mechanism of peripheral chemoreceptor stimulation

It was not surprising to find that arterial chemoreceptors are stimulated by salicylate because it is known that salicylate uncouples oxidative phosphorylation, and carotid chemoreceptors are stimulated by uncouplers of oxidative phosphorylation (see Introduction). Experiments in man (Riley *et al.*, 1977) have shown that hyperventilation persisted throughout the nine days during which aspirin was taken (3.9 g daily for nine days; plasma salicylate level averaged 186 $mg\,l^{-1}$, threshold for detectable RMV increases being 137–224 $mg\,l^{-1}$; serum salicylate levels of 150–300 $mg\,l^{-1}$ are achieved during therapy of rheumatoid arthritis, Furst *et al.*, 1987) and there was a rapid rise in the hypoxic ventilatory response. Hypoxic drive to respiration was enhanced rapidly, which is what would be expected if peripheral arterial chemoreceptors were being stimulated by salicylate, whereas the centrally-mediated increase in hypercapnic ventilatory response took a long time (32 h) to develop, presumably reflecting the slow entry of salicylate into the CNS (see Mayer *et al.*, 1959).

Delayed hyperventilation not caused by a peripheral chemoreflex

We have shown, in agreement with others, that profound hyperventilation can occur even in the absence of functional peripheral chemoreceptors, and it is probable that direct actions of salicylate within the medulla oblongata (Tenney & Miller, 1955; Millhorn *et al.*, 1982) are responsible for this effect. However, although it is undoubtedly the case that salicylate administered directly into the brain can cause hyperventilation, the question is whether the drug enters the CNS quickly enough and in high enough concentrations to evoke this effect in the type of acute experiments we performed. Hill (1971; 1973) showed that salicylate levels in rat brain are fairly high 20 min after an injection of a lethal dose, and 90 min after a 200 $mg\,kg^{-1}$ i.v. infusion of sodium salicylate mean levels in plasma were 400 $mg\,l^{-1}$ (which is not dissimilar to our values 60 min post-injection of this dose) and 100 $mg\,kg^{-1}$ in brain. Hill (1973) also showed that brain levels of $\sim$500 $mg\,kg^{-1}$ were lethal and suggested that animals die when this critical level is reached, irrespective of salicylate levels in blood. Our experiments demonstrated that although infusion at a low dose (0.5 $mg\,kg^{-1}\,min^{-1}$ sodium salicylate) took longer to kill an animal than did a higher dose (4 $mg\,kg^{-1}\,min^{-1}$), the lethal dose of salicylate was much lower, suggesting that it is the amount of salicylate getting into the brain that matters, rather than the absolute amount infused or present in plasma. Distribution of salicylate between brain and serum is dependent on plasma protein binding and at high salicylate levels more salicylate is unbound and able to cross the blood brain barrier (Snodgrass, 1980); it can then enter the CSF and exert a local action on respiratory neurones (Brem *et al.*, 1973). Our results with close-arterial injection of salicylate to the carotid bifurcation demonstrated a lack of any rapid effect on RMV in chemodenervated animals and argue against actions within the CNS contributing to the fast response. The slow responses (see Figures 2, 3) may be centrally mediated, but it has been suggested that much of the respiratory increase caused by salicylate is not due to actions within the CNS or on arterial chemoreceptors, but on thoracic receptors (e.g. Levine, 1976). It was not the aim of our study to establish where salicylate acted to cause the delayed increases in RMV, but it appears not to involve sensors whose afferent fibres run in the carotid nerves or the vago-sympathetic trunk. The simplest explanation for the delayed hyperventilation is to opt for an action of salicylate within the brain, but we cannot exclude the possibility that the drug acted at some peripheral site (e.g. cell bodies in nodose (X) or petrosal (IX) ganglia, which remain intact after the peripheral nerves associated with them have been cut).

Therapeutic considerations

It is always a problem to know whether experiments in animals provide information of relevance to man, but our results from rats showed that salicylate

caused hyperventilation, and hyperventilation does occur in man at plasma salicylate levels similar to those measured in our experiments. It seems reasonable to conclude that the initial hyperventilation after acute administration of salicylate in moderate-high doses will be mainly due to activation of arterial chemoreceptors in man as in rat. Other reflexes (e.g. cardiovascular, adrenal, see Heymans & Neil, 1958; Anichkov & Belen'kii, 1963) would also be expected to arise from stimulation of peripheral chemoreceptors, and could explain the differences we observed between the causes of death in rats with intact or sectioned carotid nerves. To prevent chemoreflexes during treatment of salicylate poisoning, it would be necessary to suppress chemosensory discharge, but we found that ventilating rats with oxygen caused only a transient decrease in the hyperventilation. Oxygen-rich gas mixtures normally almost totally depress chemosensory discharge (McQueen, 1983), so the failure to do this after salicylate suggests that the action of the drug in uncoupling oxidative phosphorylation produces metabolic disturbances that cannot be overcome by breathing 100% oxygen. An alternative, therefore, would be to reduce the elevated chemosensory discharge pharmacologically, without producing non-selective depression of the CNS. Drugs which could do this include dopamine or dopamine D_2-receptor agonists (Mir *et al.*, 1984) and δ-opioid receptor agonists (Kirby & McQueen, 1986).

In conclusion, our results provide evidence for the involvement of peripheral arterial chemoreceptors in salicylate-induced hyperventilation. These sensory receptors are almost exclusively responsible for the initial respiratory response, but are not essential for the delayed increase in ventilation which results from actions of salicylate at other sites (e.g. Tenney & Miller, 1955; Millhorn *et al.*, 1983) and is seen when the drug is administered directly into the brain (Cameron & Semple, 1968; Millhorn *et al.*, 1983). Our evidence shows that it would be wrong to dismiss arterial chemoreceptors from involvement in the hyperventilation of salicylism which results from acute or chronic ingestion of salicylates.

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The X-ray crystal structure of 4-diphenylcarbamyl-*N*-methyl-piperidine methobromide (the carbamate analogue of 4-DAMP methiodide)

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1 Crystals of 4-diphenylcarbamyl-*N*-methylpiperidine methobromide contain 2 conformers, one with the ester group equatorial and the other with the ester group axial to the piperidine ring.

2 In both conformers and diphenylcarbamyl nitrogen is trigonally substituted and flat compared with the tetrahedral substitution of the corresponding carbon atom in 4-diphenylacetoxy-*N*-methyl-piperidine (4-DAMP) methiodide.

3 The low activity and selectivity of the carbamate at muscarinic receptors is comparable with that of compounds containing only one phenyl ring, e.g. phenylacetyl-*N*-methyl piperidine methiodide or phenylacetylcholine. It is suggested that the high affinity of 4-DAMP metho-salts and their selectivity depend upon interactions with groups arranged in three dimensions and that the trigonal arrangement about the nitrogen atom in the carbamate makes it impossible for both benzene rings to interact at once.

Introduction

The methiodide and methobromide of 4-diphenylacetoxy-*N*-methylpiperidine (4-DAMP) have high affinity for muscarinic receptors but show some selectivity, being more active on guinea-pig ileum than on guinea-pig atria (Barlow *et al.*, 1976). The diphenylcarbamyl analogue, however, has less than one-thousandth of the affinity and is not selective (Barlow *et al.*, 1988). The two compounds differ only in the replacement of $Ph_2CHCOO-$ by Ph_2NCOO-. The carbamyl compound is detectably less lipophilic when tested by paper chromatography but the difference is small (Barlow *et al.*, 1988) and it is important to compare the size and shape of the two structures. An X-ray crystal analysis of 4-DAMP methiodide has already been made (Barlow *et al.*, 1987): this paper describes the results of a similar analysis of the carbamate, 4-diphenylcarbamyl-*N*-methylpiperidine methobromide.

Methods

4-Diphenylcarbamyl-*N*-methylpiperidine methobromide, part of the original sample made by Dr M.K. Shepherd (Barlow *et al.*, 1988), was crystallized from a mixture of butanone and ethanol and had m.p. 228–230°C.

Crystal structure determination

Crystal data for 4-diphenylcarbamyl-*N*-methylpiperidine methobromide (I) $C_{20}H_{25}O_2N_2^+ \cdot Br^-$, $M_r = 405.38$, monoclinic, $P2_1/n$ (non-standard setting of No: 14), $a = 9.873(2)$, $b = 20.651(4)$, $c = 38.492(6)$ Å, $\beta = 93.27(1)°$, $U = 7839(2)$ Å^3, $Z = 16$, $D_x = 1.37\,g\,cm^{-3}$, Mo K_α X-radiation ($\lambda = 0.71073$ A), $\mu = 20.89\,cm^{-1}$, $F(000) = 3360$, $T = 298$ K, $R(R_w) = 0.0704$ (0.0677) for 4320 reflections with $I > 3\sigma(I)$.

Diffracted intensity data were collected on a Nicolet four-circle automated diffractometer by $\theta/2\theta$ variable speed scans. Table 1 gives the data collection parameters and details of data reduction, structure solution and refinement. Structure solution was by direct methods and Fourier difference synthesis. An empirical absorption correction based on the intensity variation of 270 azimuthal scan data was applied. Minimum and maximum transmission coefficients for the whole data set were 0.286 and 0.378, respectively. No extinction correction was applied. Refinement was by blocked full-matrix least-squares

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on a Digital MicroVAX II computer with the SHELXTLplus package (Nicolet Instrument Corp., 1988). All non-hydrogen atoms (excluding the phenyl ring carbons) were refined with anisotropic thermal parameters. The phenyl rings were refined isotropically as regular hexagons (C-C 1.395 A). Hydrogen atoms were incorporated at geometrically idealised positions (C-H 0.96 Å, fixed U_{iso} of 1.2 $\times U_{eq}$ of supporting C) and refined by a riding model. Residual peaks and troughs in the final ΔF map were $<1.05\,e\,Å^{-3}$. The function minimized during refinement was $\Sigma w(F_o - |F_c|)^2$, where $w^{-1} = [\sigma^2(F) + gF^2]$ and the value of g is given in Table 1. Atomic scattering factors and corrections for anomalous dispersion were taken from *International Tables for X-ray Crystallography*, Vol. IV.

Table 1. Data collection parameters, structure solution and refinement details for 4-diphenylcarbamyl-*N*-methylpiperidine methobromide

Crystal colour	colourless
habit	rounded tablet
size (mm)	0.5 × 0.4 × 0.2
Diffractometer	R3m/V
Unit cell determination	
No. of reflections	39
2θ range (°)	24–26
Scan range (°2θ)	2.9–50.0
Range of *hkl*	0/12, −25/0, −46/46
Scan type	$\theta/2\theta$
Scan speeds (°2θ min^{-1})	
min.	2.00
max.	29.30
No. of standard reflections	3
Frequency of standards	100
Variation/decay (%)	negligible
Data collected	15,684
Independent data	13,869
R_{int}	0.0067
Solution method	Direct/ΔF
Programme system	SHELXTLplus
Refinement method*	blocked fmls
Refined data	4320
$I > n\sigma(I)$: n	3
R	0.0704
R_w	0.0677
$w^{-1} = [\sigma^2(F) + gF^2]$;	
g-value	0.0008
Goodness-of-fit	1.638
Data: parameter ratio	7
Max. Δ/σ (final)	1.4
Max. $\Delta\rho$ final (eA^{-3})	
positive	1.03
negative	0.47
Hydrogen atoms:	
location	geometric
refinement	riding

*fmls = full-matrix least-squares.

Results

Final fractional atomic coordinates are given in Table 2. The surprisingly large unit cell contains 16 molecules. The asymmetric unit of the structure consists of two essentially identical axial conformers (Figure 1a) and two essentially identical equatorial conformers (Figure 1b). In all forms the two benzene rings are twisted so that they lie in planes approximately at right angles to each other and the arrangement about the carbamyl nitrogen atom (N9) is trigonal with the sum of the three angles being 359.9, 359.2, 359.2 and 360.0° in the four conformers. The ester group does not lie in the same plane as either of the benzene rings, being twisted about 30° relative to one and about 60° relative to the other in all conformers.

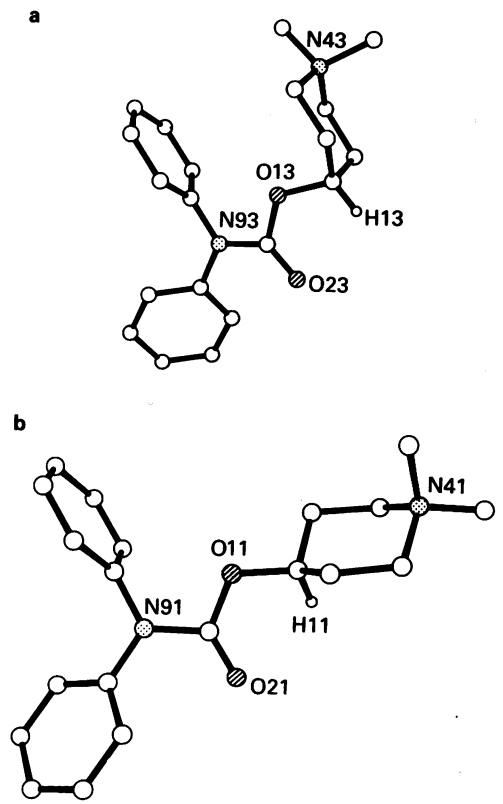


Figure 1 (a) Axial conformer: note the benzene ring (top left) is roughly parallel to the piperidine ring (top right) and the molecule is roughly symmetrical about the plane of the paper. The bond angles about the carbamyl nitrogen are 117, 121, 121° in one conformer and 116, 119, 125 in the other. (b) Equatorial conformer: the molecule is more extended but is roughly symmetrical about a plane dividing the piperidine ring through the nitrogen atom.

Table 2 Atomic coordinates ($\times 10^4$) for 4-diphenylcarbamyl-*N*-methylpiperidine methobromide

	x	y	z
Br(1)	9605(2)	7289(1)	2221(1)
Br(2)	14676(2)	5261(1)	2243(1)
Br(3)	9553(2)	3396(1)	1719(1)
Br(4)	4436(2)	9168(1)	1682(1)
C(11)	3832(13)	7074(7)	1649(3)
C(21)	5282(13)	7217(7)	1647(4)
C(31)	6006(13)	6941(7)	1980(3)
N(41)	5485(12)	7244(5)	2307(3)
C(51)	3934(13)	7159(7)	2295(4)
C(61)	3239(14)	7391(6)	1964(3)
C(71)	5855(16)	7938(7)	2332(4)
C(81)	6078(17)	6888(7)	2607(4)
O(11)	3171(10)	7346(5)	1338(2)
O(21)	1395(11)	6661(5)	1377(3)
C(91)	1978(15)	7063(9)	1236(5)
N(91)	1500(12)	7339(5)	917(3)
C(121)	−873(9)	7060(4)	969(2)
C(131)	−2140	6905	812
C(141)	−2309	6860	450
C(151)	−1211	6971	246
C(161)	56	7126	402
C(111)	225	7171	764
C(221)	3500(10)	7485(3)	563(3)
C(231)	4316	7884	370
C(241)	4016	8541	335
C(251)	2900	8801	493
C(261)	2084	8402	685
C(211)	2383	7744	720
C(12)	8903(16)	5468(7)	1695(4)
C(22)	8363(14)	5162(7)	2017(4)
C(32)	9068(15)	5426(7)	2325(3)
N(42)	10568(12)	5338(6)	2335(3)
C(52)	11101(13)	5605(7)	2004(3)
C(62)	10420(13)	5335(7)	1677(3)
C(72)	10943(17)	4620(7)	2367(5)
C(82)	11143(18)	5674(8)	2651(4)
C(92)	7115(17)	5480(7)	1250(4)
O(12)	8294(10)	5217(4)	1372(3)
O(22)	6555(9)	5894(4)	1409(2)
N(92)	6649(10)	5209(5)	942(3)
C(122)	8622(10)	5065(3)	605(3)
C(132)	9440	4658	418
C(142)	9108	4005	378
C(152)	7958	3758	526
C(162)	7140	4165	713
C(112)	7472	4818	753
C(222)	4218(10)	5465(4)	998(2)
C(232)	2945	5623	846
C(242)	2776	5707	486

Table 2—(continued)

	x	y	z
C(252)	3881	5633	279
C(262)	5154	5476	432
C(212)	5323	5392	791
C(13)	−1510(14)	10224(7)	1759(4)
C(23)	−1866(14)	9619(7)	1937(4)
C(33)	−1293(14)	9008(7)	1770(4)
N(43)	204(11)	9046(6)	1726(3)
C(53)	546(15)	9664(8)	1541(4)
C(63)	24(17)	10261(8)	1715(4)
C(73)	987(14)	9005(7)	2076(3)
C(83)	605(20)	8504(7)	1501(5)
O(13)	−2066(11)	10180(5)	1402(3)
O(23)	−3804(11)	10819(5)	1499(3)
C(93)	−3309(19)	10442(7)	1316(5)
N(93)	−3688(13)	10254(6)	993(3)
C(123)	−6149(10)	10513(5)	1041(2)
C(133)	−7416	10652	879
C(143)	−7562	10701	518
C(153)	−6440	10613	318
C(163)	−5173	10475	480
C(113)	−5028	10425	842
C(223)	−3237(9)	9145(5)	821(3)
C(233)	−2449	8704	647
C(243)	−1341	8920	470
C(253)	−1023	9578	467
C(263)	−1811	10020	641
C(213)	−2918	9803	818
C(14)	3623(17)	2340(8)	1795(4)
C(24)	3271(15)	2950(8)	1989(4)
C(34)	3791(14)	3530(8)	1798(4)
N(44)	5285(12)	3496(5)	1767(3)
C(54)	5646(16)	2883(6)	1578(4)
C(64)	5106(16)	2283(6)	1763(4)
C(74)	6091(15)	3548(8)	2112(4)
C(84)	5683(16)	4052(7)	1537(3)
C(94)	1829(17)	2063(8)	1361(4)
O(14)	3028(11)	2383(5)	1439(3)
O(24)	1264(11)	1716(5)	1556(3)
N(94)	1358(11)	2260(5)	1042(3)
C(124)	3265(10)	2512(4)	671(3)
C(134)	4033	2958	495
C(144)	3700	3615	500
C(154)	2599	3824	682
C(164)	1830	3377	859
C(114)	2163	2721	853
C(224)	−64(8)	2017(5)	522(2)
C(234)	−1333	1874	362
C(244)	−2456	1806	563
C(254)	−2311	1882	924
C(264)	−1041	2025	1083
C(214)	82	2093	882

In the axial forms of the carbamate one benzene ring is stacked more or less parallel to the piperidine ring with the second ring lying in the plane of the ester group, which forms a plane of symmetry. A similar arrangement in the diphenylacetoxy compound (4-DAMP) would have the second ring projecting out of the plane and there would be no symmetry.

Discussion

The results confirm the suggestion (Barlow *et al.*, 1988) that 'the two benzene rings could well be arranged differently about the ester group in diphenylcarbamyl from the arrangement in diphenylacetyl' and the differences are illustrated in Figure 2 in which 4-DAMP methiodide is superimposed on the equatorial forms of its carbamate analogue. The trigonal (sp^2-hybridized) nitrogen atom, is relatively flat compared with the corresponding tetrahedral (sp^3-hybridized) carbon atom in 4-DAMP methiodide but the most striking difference in Figure 2 is in the twist of the second phenyl ring (which appears almost edge-on). Rotation of the rings is not free: both cannot lie in the same plane, for instance, and if one is fixed relative to the rest of the molecule this largely determines the position of the second.

The different position of the phenyl rings in the carbamate and the acetate, seen in Figure 2, may explain the low affinity of the carbamate and the absence of selectivity. Although weak compared with 4-DAMP methobromide (log K 9 for ileum and 8 for atria), the carbamate is not totally inactive (log K 5.8 for ileum or atria) but similar to compounds containing only one phenyl ring. It is stronger than phenylacetylcholine (log K 4.5 for ileum: Abramson *et al.*, 1969) but weaker than 4-phenylacetoxy-*N*-methylpiperidine methiodide (log K 6.2 for ileum: Abramson *et al.*, 1974). If the properties of 4-DAMP methiodide involve the binding of both phenyl rings as well as the charged nitrogen atom, the second phenyl ring in the carbamyl analogue would be wrongly placed for binding, reducing its affinity to that of a compound with only one phenyl ring.

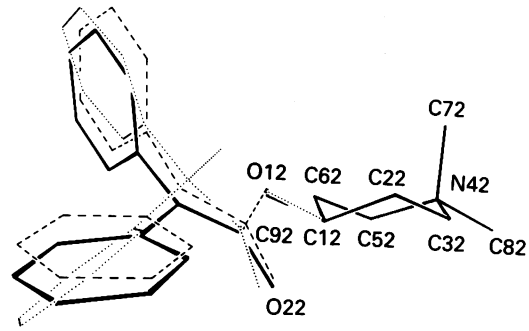


Figure 2. Superimposition of the piperidine rings of the equatorial forms of the carbamate (solid and broken lines) on 4-DAMP methiodide (dotted line). Note that the two conformers of the carbamate are very similar and that one phenyl ring (upper left) is similarly placed in all 3 compounds but the second phenyl ring (lower left) is different in 4-DAMP methiodide, being twisted almost edge-on.

We wish to thank the Medical Research Council for support and Professor F.G.A. Stone and Dr J.A.K. Howard for X-ray crystallography facilities.

Note: additional material available from the Cambridge Crystallographic Data Centre comprises thermal parameters and bond lengths and angles.

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An examination of the putative σ-receptor in the mouse isolated vas deferens

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1 The effects of several ligands which interact with the σ-binding site were studied on the electrically-evoked (0.1 Hz) neurogenic twitch contractions of the mouse isolated vas deferens.

2 (+)-3-(3-Hydroxyphenyl)-N-(1-propyl)piperidine ((+)-3-PPP) (10^{-8}–10^{-5} M), inhibited the neurogenic twitch contractions. This inhibitory action was unaffected by naloxone (10^{-6} M), idazoxan (10^{-6} M), cocaine (10^{-6} M) or tyramine (10^{-4}–3×10^{-4} M), but was abolished by the dopamine D_2-antagonist, sulpiride (10^{-6} M). Therefore, in order to study the potentiating actions of σ ligands, sulpiride (10^{-6} M) was used to prevent any inhibitory actions mediated via dopamine D_2-receptors.

3 In the presence of sulpiride (10^{-6} M), haloperidol (10^{-6}–10^{-5} M), (+)-3-PPP (10^{-6}–3×10^{-4} M) and (+)-N-allyl-N-normetazocine ((+)-SKF 10,047) (10^{-5}–10^{-4} M) each reversibly potentiated the neurogenic twitch contractions in a concentration-dependent manner. The rank order of potency was haloperidol > (+)-3-PPP > (+)-SKF 10,047.

4 The stereoisomers of 3-PPP displayed stereoselectivity with (+)-3-PPP being more potent than (−)-3-PPP.

5 At a concentration that did not potentiate the twitch contractions, (3×10^{-7} M), haloperidol did not antagonize the potentiating action of (+)-3-PPP (3×10^{-5} M).

6 1,3-Di-*O*-tolyguanidine (DTG) (10^{-8}–10^{-5} M) had no effect on the amplitude of twitch contractions and did not affect the potentiating action of (+)-3-PPP (10^{-5}–3×10^{-5} M).

7 It is concluded that σ-ligands potentiate neurogenic twitch contractions of the mouse isolated vas deferens via a site that is different from the central σ-binding site.

Introduction

The existence of the σ-receptor was first postulated by Martin *et al.* (1976) on the basis of *in vivo* studies using the prototypic sigma agonist, N-allyl-N-normetazocine (SKF 10,047). Although it was proposed that the σ-receptor and the phencyclidine (PCP) receptor were the same entity (Zukin & Zukin, 1981), it is now clear that these represent two distinct sites with different pharmacological profiles and distributions (see Quirion *et al.*, 1987; Sonders *et al.*, 1988 for reviews). PCP binds to a site associated with the N-methyl-D-aspartate receptor/channel complex, which has a relatively low affinity for (+)-SKF 10,047 (Anis *et al.*, 1983; Tam, 1983; Largent *et al.*, 1986). In contrast, the σ-binding site has been defined by the high affinity binding of [^{3}H]-(+)-SKF 10,047 which displays a high sensitivity to haloperidol, a much lower sensitivity to PCP and is insensitive to naloxone and sulpiride (Su, 1982; Tam, 1983; Ferris *et al.*, 1986; Largent *et al.*, 1986).

Until recently the possible responses evoked by σ-ligands could not be easily characterized due to the lack of an *in vitro* pharmacological bioassay. However, the isolated vasa deferentia from the mouse and the guinea-pig have been suggested as suitable bioassays since some of the ligands which bind to the σ-binding site in brain membranes also potentiate neurogenic twitch contractions in these tissues (Campbell *et al.*, 1987; Vaupel & Su, 1987) and increase electrically-evoked noradrenaline release from the mouse vas deferens (Campbell *et al.*, 1987).

The aim of the present study was to determine the effects of a number of σ-ligands at the putative σ-receptor in the mouse isolated vas deferens. We have

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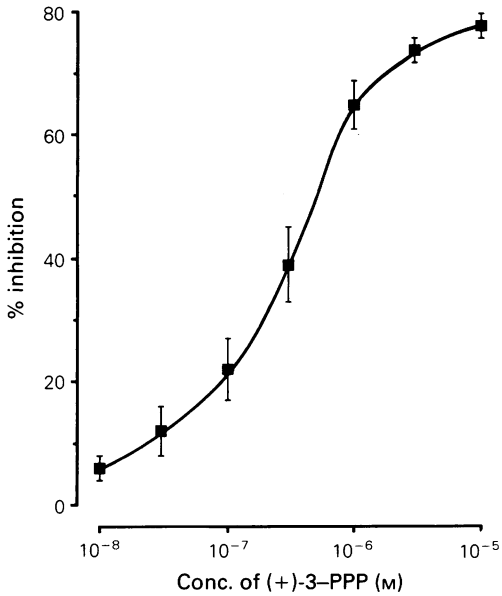


Figure 1 Inhibition of electrically-evoked (0.1 Hz) neurogenic twitch contractions of the mouse vas deferens *in vitro* by (+)-3-PPP (10^{-8}–10^{-5} M). Each point is the mean of seven observations and vertical bars represent s.e.mean. The curve was fitted by eye.

studied the effects of proposed agonists, especially (+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine ((+)-3-PPP) which binds to the central sigma site with higher affinity and selectivity than (+)-SKF 10,047 (Largent *et al.*, 1984; 1986; Church *et al.*, 1986) and of the putative antagonists haloperidol (Vaupel & Su, 1987; Bell *et al.*, 1988) and 1,3-di-*O*-tolylguanidine (DTG) (Bobker, Campbell, Leslie & Weber, communicated to the International Narcotics Research Conference in Adelaide, 1987; Malouf *et al.*, 1988). A preliminary account of the results has been presented to the International Narcotics Research Conference (Kennedy *et al.*, 1989).

Methods

Male mice (DBA/1A and Swiss Webster strains) (10–20 g) were killed by cervical dislocation. The vasa deferentia were excised, mounted vertically in a 2 ml organ bath under isotonic conditions and bathed in a modified Krebs solution of the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.5, KH_2PO_4 0.93, $NaHCO_3$ 24, glucose 11, maintained at 37°C and bubbled with 95% O_2, 5% CO_2. Contractions were evoked by electrical field stimulation (square wave pulses at 0.1 Hz, maximal voltage, 2 ms duration) via two platinum wire electrodes and were recorded by a Washington Isotonic Lever Transducer T2 connected to a pen recorder.

Agonists were added either as single doses or cumulatively and left in contact with the tissue until a steady-state response was obtained. Antagonists were added 10–20 min before addition of agonist. Changes in the amplitude of twitches were calculated as a percentage of the control twitches immediately prior to drug addition. Results are expressed as the mean ± s.e.mean and the geometric mean and its 95% confidence intervals calculated for IC_{50} values. Where appropriate, results were analysed by either Student's paired or unpaired *t* test. A probability of less than 0.05 was considered significant. Over the course of most experiments a slow downwards drift in the baseline tension was seen due to tissue relaxation. In the traces shown in Figures 2, 3, 4 and 6 the drift has been offset between each section of trace.

Drugs used and abbreviations

The following drugs were used: clonidine hydrochloride, cocaine hydrochloride, guanethidine sulphate, naloxone hydrochloride, sulpiride, tetrodotoxin, tyramine hydrochloride (Sigma Chemical Company); [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAGOL) and [D-Sar^2,Leu^5]enkephalin-Thr (DSLET) (Cambridge Research Biochemicals); 1,3-di-*O*-tolylguanidine (DTG) (Aldrich Chemical Company); haloperidol (Searle Pharmaceuticals); idazoxan (Reckitt & Coleman); (+)-N-allyl-N-normetazocine hydrochloride ((+)-SKF 10,047) (R.L. Walsh, NIDA, U.S.A.); phentolamine mesylate (Ciba-Geigy); quinpirole (Eli Lilly) and (+)- and (−)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine ((+)- and (−)-3-PPP) (Research Biochemicals Incorporated). Drugs were dissolved in distilled water, apart from DTG (2×10^{-2} M stock solution in ethanol) and sulpiride (2×10^{-2} M stock solution in methanol). Subsequent dilutions of drugs were made in Krebs solution.

Results

DBA/1A mice

Inhibitory action of (+)-3-PPP (+)-3-PPP (10^{-8}–10^{-5} M) inhibited the neurogenic twitch contractions of the vas deferens evoked by electrical field stimulation (Figures 1, 2, 3). Inhibition was concentration-dependent ($IC_{50} = 2.9 \times 10^{-7}$ M, 95% confidence intervals = $1.5–5.7 \times 10^{-7}$ M, $n = 7$), with rapid

onset, was maximal within 3 min and readily reversed on washout of the drug. Maximum inhibition of the twitch produced was 78 ± 2%.

This result was unexpected as (+)-3-PPP has previously been found to potentiate rather than inhibit neurogenic twitch contractions of the vas deferens (Campbell *et al.*, 1987). Since the inhibition resembled that previously reported to be evoked by agonists at opioid receptors and α-adrenoceptors in this preparation (Henderson *et al.*, 1972; Hughes *et al.*, 1975) we examined the effects of the opioid receptor antagonist, naloxone and the α_2-adrenoceptor antagonist idazoxan. Naloxone (10^{-6} M) had no effect either on the neurogenic twitches or on the inhibitory action of (+)-3-PPP (3×10^{-7} M), but abolished the inhibitory actions of the μ-receptor agonist, DAGOL (10^{-7} M) and of the δ-receptor agonist, DSLET (10^{-9} M) ($n = 3$) (Figure 2a). Likewise, idazoxan (10^{-6} M) had no effect on the neurogenic twitches or on the depression of the twitch by (+)-3-PPP (10^{-6} M), but abolished the inhibition evoked by the α_2-agonist, clonidine (10^{-9} M), ($n = 4$)

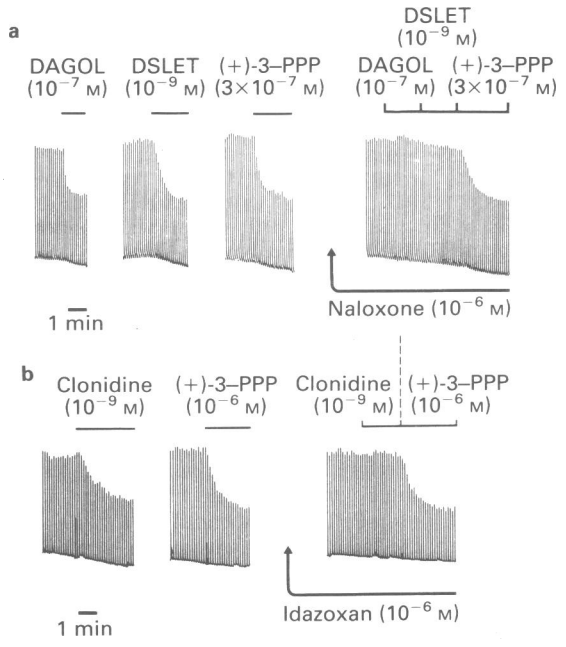


Figure 2 Mouse vas deferens *in vitro*: (a) naloxone (10^{-6} M) abolishes the inhibition of electrically-evoked (0.1 Hz) neurogenic twitch contractions elicited by DAGOL (10^{-7} M) and DSLET (10^{-9} M) but has no effect on that due to (+)-3-PPP (3×10^{-7} M). (b) Idazoxan (10^{-6} M) abolishes the inhibition of electrically-evoked (0.1 Hz) neurogenic twitch contractions elicited by clonidine (10^{-9} M) but not (+)-3-PPP (10^{-6} M).

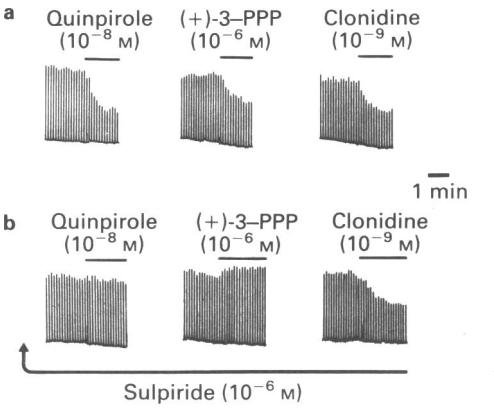


Figure 3 Effect of quinpirole (10^{-8} M), (+)-3-PPP (10^{-6} M) and clonidine (10^{-9} M) on the electrically-evoked (0.1 Hz) neurogenic twitch contractions of the mouse vas deferens *in vitro* (a) in the absence and (b) in the presence of sulpiride (10^{-6} M). These recordings were obtained in the same tissue.

(Figure 2b). Thus, the inhibitory action of (+)-3-PPP does not appear to be mediated via prejunctional μ- and δ-opioid receptors or α_2-adrenoceptors. In addition, the inhibition induced by (+)-3-PPP (3×10^{-7}–10^{-6} M) was unaffected by prior addition of cocaine (3×10^{-6} M) ($n = 2$) or tyramine (10^{-4}–3×10^{-4} M) ($n = 2$).

Next we tested the effects of sulpiride, an antagonist at dopamine D_2-receptors. Sulpiride (10^{-6} M) produced a small (<10%) potentiation of the neurogenic twitches. Subsequently, it abolished the depression of the twitch evoked by either the D_2-agonist, quinpirole (10^{-8} M) or (+)-3-PPP (10^{-6} M) ($n = 4$), but had no effect on that evoked by the α_2-agonist, clonidine (10^{-9} M) ($n = 2$) (Figure 3). Thus, (+)-3-PPP inhibits neurogenic twitch contractions by an action at dopamine D_2-receptors. Therefore, subsequent experiments on the potentiating actions of (+)-3-PPP and other putative sigma receptor ligands were performed in the continuous presence of sulpiride (10^{-6} M) to eliminate this action.

Excitatory actions of (+)-3-PPP In the presence of sulpiride (10^{-6} M), (+)-3-PPP (10^{-6}–3×10^{-4} M) potentiated the neurogenic twitch contractions ($n = 6$) (Figures 4, 5, 6). This was rapid in onset, reached a maintained level within 2–5 min and was reversible on washout of the drug. The potentiation was concentration-dependent over this range (Figures 4a, 5) but a full concentration-response curve could not be obtained since, addition of 3×10^{-4} M (+)-3-PPP elicited a large increase in the amplitude of the twitches and these varied to such

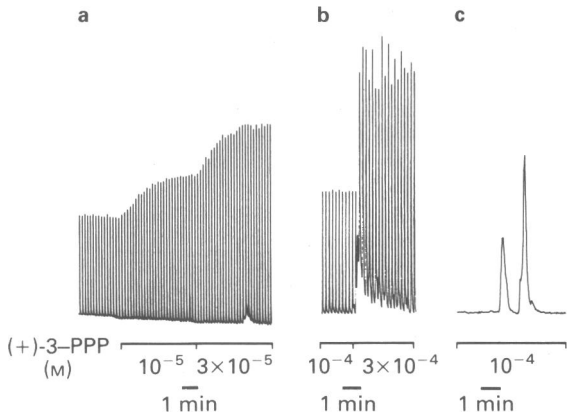


Figure 4 Mouse vas deferens *in vitro*: potentiation of the electrically-evoked (0.1 Hz) neurogenic twitch contractions (in the presence of sulpiride (10^{-6} M)) by (+)-3-PPP at (a) 10^{-5} and 3×10^{-5} M and (b) 10^{-4} and 3×10^{-4} M. (c) Contractions of the smooth muscle elicited by (+)-3-PPP (10^{-4} M) in the absence of electrically-evoked neurogenic twitch contractions and sulpiride. Recordings in (a), (b) and (c) were obtained from different preparations.

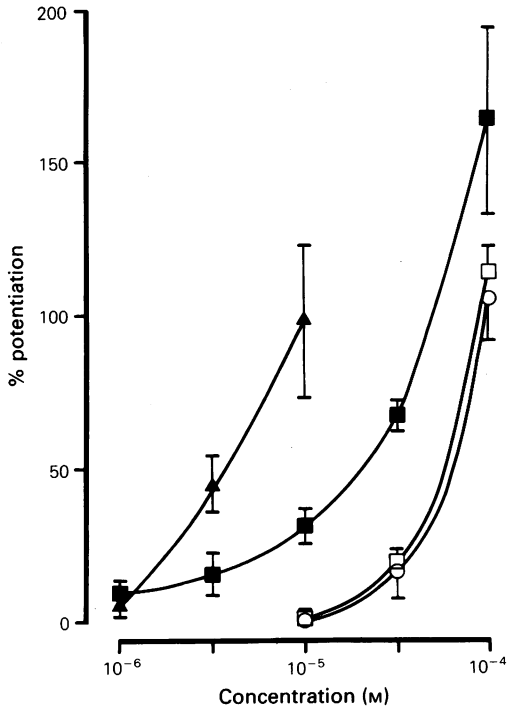


Figure 5 Potentiation of electrically-evoked (0.1 Hz) neurogenic twitch contractions of the mouse vas deferens *in vitro*, in the presence of sulpiride (10^{-6} M), by haloperidol (10^{-6}–10^{-5} M) (▲), (+)-3-PPP (10^{-6}–10^{-4} M) (■), (−)-3-PPP (10^{-5}–10^{-4} M) (□) and (+)-SKF 10,047 (10^{-5}–10^{-4} M) (○). Each point is the mean of six observations and vertical bars represent s.e.mean. The curves were fitted by eye.

an extent that a consistent response was not obtained (Figure 4b). Consequently, EC_{50} values could not be calculated.

In many preparations, high concentrations of (+)-3-PPP (10^{-4}–3×10^{-4} M) also evoked spontaneous contractions of the smooth muscle, with variable latency, amplitude and duration (Figure 4b,c). When studied in the absence of nerve stimulation and sulpiride (Figure 4c) the spontaneous contractions were unaffected by tetrodotoxin (10^{-6} M) ($n = 2$) and guanethidine (3×10^{-6} M) ($n = 1$) but were abolished by phentolamine (10^{-5} M) ($n = 2$). The mechanism underlying these contractions was not studied further.

Effects of other putative σ-agonists The stereoisomer of (+)-3-PPP, (−)-3-PPP (10^{-5}–10^{-4} M) also potentiated the neurogenic twitch contractions in a reversible and concentration-dependent manner ($n = 6$) (Figure 5). However, the concentration-response curve for (−)-3-PPP was to the right of that for (+)-3-PPP (Figure 5) and when tested in the same preparation (−)-3-PPP was always less potent than (+)-3-PPP. Thus, 3-PPP potentiates the neurogenic twitch contractions in a stereoselective manner.

The prototypic σ-agonist, (+)-SKF 10,047 (10^{-5}–10^{-4} M) likewise potentiated the neurogenic twitches ($n = 6$) (Figure 5). The concentration-response curve for (+)-SKF 10,047 was to the right of that for (+)-3-PPP (Figure 5) and when tested in the same preparation (+)-SKF 10,047 was always less potent than (+)-3-PPP.

Effects of putative σ-antagonists Both haloperidol and DTG have been suggested as antagonists at the σ-receptor; therefore, these compounds were tested against the twitch contractions and the potentiating action of (+)-3-PPP. Haloperidol (10^{-6}–10^{-5} M) potentiated the neurogenic twitches in a manner similar to (+)-3-PPP but its concentration-response curve was to the left of that for (+)-3-PPP ($n = 6$) (Figure 5). Higher concentrations of haloperidol (3×10^{-5} M and 10^{-4} M) produced a rapid and profound depression of the twitches (83 ± 6% and 100% decreases respectively, $n = 6$), which quickly reversed on washout of the drug. The mechanism underlying this inhibitory action was not studied further.

At a concentration of 3×10^{-7} M, haloperidol did not increase the twitch amplitude and did not inhibit the potentiation evoked by (+)-3-PPP (3×10^{-5} M)

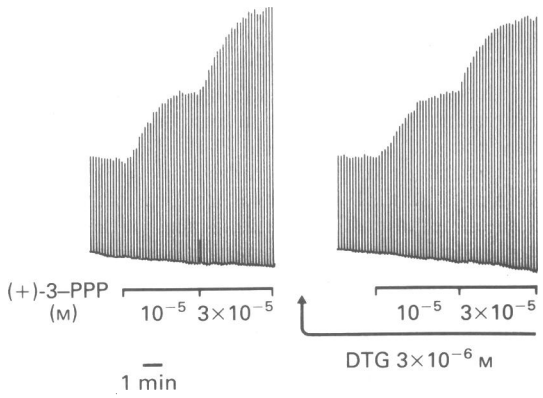


Figure 6 DTG (3×10^{-6} M) does not antagonise the potentiation of electrically-evoked (0.1 Hz) neurogenic twitch contractions of the mouse vas deferens *in vitro* by (+)-3-PPP (10^{-5} and 3×10^{-5} M). Sulpiride (10^{-6} M) was present throughout.

($n = 4$). Thus, a concentration of haloperidol that is subthreshold for potentiation does not produce antagonism of the potentiating actions of (+)-3-PPP in this preparation.

DTG (10^{-8}–10^{-5} M) had no significant effect on twitch amplitude ($n = 4$). Also, preincubation with 3×10^{-6} M DTG ($n = 6$) or with 10^{-5} DTG M ($n = 4$) did not inhibit the potentiation elicited by (+)-3-PPP (10^{-5} M and 3×10^{-5} M) (Figure 6). Thus, over the concentration-range used, DTG exhibits neither agonism nor antagonism in this tissue. Higher concentrations of DTG (3×10^{-5} and 10^{-4} M) rapidly and reversibly inhibited twitch amplitude ($13 \pm 5\%$, $72 \pm 10\%$ decrease respectively, $n = 4$). This action may be due to the local anaesthetic action of DTG previously reported by Malouf *et al.* (1988) and has not been studied further.

Effect of (+)-3-PPP on vasa deferentia from Swiss Webster mice

The results described above differ from those of Campbell *et al.* (1987) in that these authors did not find an inhibitory action of (+)-3-PPP. Therefore, to exclude the possibility that this difference is due to the different strains of mice used, we repeated our experiments using vasa deferentia from Swiss Webster mice.

(+)-3-PPP (10^{-8}–3×10^{-6} M) inhibited neurogenic twitch contractions with a potency similar to that found for DBA/1A mice ($IC_{50} = 3.0 \times 10^{-7}$ M, 95% confidence intervals = 1.8–5.6×10^{-7} M, maximum inhibition = $73 \pm 5\%$ decrease, $n = 4$). Sulpiride (10^{-6} M), but not naloxone (10^{-6} M), abolished this action ($n = 4$) and subsequently (+)-3-PPP (3×10^{-7}–3×10^{-5} M) potentiated the twitch contractions ($n = 4$) in a manner similar to that described above for DBA/1A mice and by Campbell *et al.* (1987).

Discussion

The results of this study have further characterized the actions of σ-receptor ligands in the mouse isolated vas deferens. Comparison of our results with those from ligand binding studies on brain membranes indicates that whilst the site mediating the potentiating action in the vas deferens displays some characteristics of the central σ-binding site, it is not identical in all respects. Thus, at present we are unable to conclude whether or not this action of the σ-ligands represents an interaction with the σ-receptor.

Haloperidol, (+)- and (−)-3-PPP and (+)-SKF 10,047 each reversibly potentiated neurogenic twitch contractions in a time- and concentration-dependent manner and although full concentration-response curves could not be obtained, the linear portion of the curves each had a similar slope, consistent with the drugs acting at the same site. The observed rank order of potency of haloperidol > (+)-3-PPP > (+)-SKF 10,047 and the stereoselectivity displayed by (+)- and (−)-3-PPP are the same as those seen in σ-site binding studies (Largent *et al.*, 1984; Weber *et al.*, 1986; Quirion *et al.*, 1987). However, the concentrations of these drugs which were required to elicit potentiation were 2–3 orders of magnitude greater than their K_D values in binding studies (Su, 1982; Tam, 1983; Largent *et al.*, 1984). This could argue against the co-identity of the sigma site in the brain and the site of action of sigma ligands in the vas deferens. However, note that the incubation media used in binding studies are designed to optimise binding and are dissimilar to the bathing solutions used in *in vitro* organ bath studies. This could perhaps explain the apparent difference in the potency of the ligands in the two experimental systems.

Receptors are best classified by use of specific, competitive antagonists rather than through agonist action. But neither of the two proposed antagonists used here acted as such. Haloperidol was in fact the most potent agonist tested and is unlikely to be a partial agonist since the slope of the concentration-response curve to haloperidol was similar to that of the other sigma ligands and at a concentration which was subthreshold for potentiation haloperidol did not antagonize the potentiating action of (+)-3-PPP. In a recent study, haloperidol was also reported to have a full agonist action at a putative

σ-site since it decreased carbachol-induced phosphoinositide metabolism in rat brain with a potency and concentration-response curve slope similar to those of several other σ-ligands (Bowen *et al.*, 1988a). Furthermore, haloperidol, like (+)-SKF 10,047, produced marked dystonia when micro-injected into the red nucleus of rats (Walker *et al.*, 1988). In contrast, haloperidol has been reported to antagonize both the potentiating action of (+)-SKF 10,047 on neurogenic twitch contractions of the guinea-pig isolated vas deferens, in a non-competitive manner (Vaupel & Su, 1987) and also the electrophysiological actions of (+)-SKF 10,047 and (+)-3-PPP on the NCB-20 cell line (Bell *et al.*, 1988).

The other putative antagonist tested here, DTG, at nanomolar and low micromolar concentrations, had no effect either on twitch contraction amplitude or on the potentiating action of (+)-3-PPP. (Initial findings of potentiation of the twitch contractions by DTG were found to be due to the presence of hydrochloric acid in the stock solution, Kennedy & Henderson, unpublished observations). A similar lack of effect on its own or against (+)-3-PPP is also seen in the rat isolated tail artery (T. Massamiri & S.P. Duckles, personal communication). These results are surprising as DTG has an affinity for the σ-binding site similar to that of (+)-3-PPP (Weber *et al.*, 1986; Bowen *et al.*, 1988b). Like haloperidol, DTG has been observed to display agonist action at a putative σ-site in that it depresses carbachol-induced phosphoinositide metabolism in rat brain (Bowen *et al.*, 1988a). DTG also produces a marked dystonia in rats when micro-injected into the red nucleus (Walker *et al.*, 1988), and this is associated with a decrease in the action potential firing rate of neurones within the red nucleus (Matsumoto & Walker, 1988). DTG, like (+)-3-PPP, also decreases action potential firing in A9 dopamine neurones (Steinfels & Tam, 1988). Finally, DTG elicits contra-lateral turning when micro-injected into the rat substantia nigra (Walker *et al.*, 1988). In contrast, DTG has been reported to antagonize the increase in evoked release of noradrenaline elicited by (+)-3-PPP in the mouse vas deferens (Bobker *et al.*, communicated to the International Narcotics Research Conference in Adelaide, 1987). DTG also antagonizes the excitatory actions of (+)-3-PPP on rat hippocampal neurones (Malouf *et al.*, 1988).

Thus, it is clear that haloperidol and DTG can, in some preparations, mimic the actions of (+)-SKF 10,047 and (+)-3-PPP, whilst in other preparations they are antagonists of these compounds or have neither effect. This mixture of activity hinders the elucidation and classification of σ-receptors and the study of their function. Recently, several other novel compounds (e.g. rimcazole, BMY 14802, HR 375) have been reported to displace potently the binding of σ-ligands (Ferris *et al.*, 1986; Taylor & Dekleva, 1988; Largent *et al.*, 1988) and so may be more useful in characterizing σ-receptors.

It has been suggested that σ-ligands potentiate the neurogenic twitches of the mouse vas deferens by a prejunctional action as (+)-3-PPP increases the evoked release of [^{3}H]-noradrenaline in this tissue (Campbell *et al.*, 1987). This is not due to the inhibition of noradrenaline uptake since in the present study cocaine neither potentiated contractions *per se* nor affected the potentiation elicited by σ-ligands. Note, however, that in the mouse vas deferens noradrenaline is a cotransmitter with adenosine triphosphate and mediates the second, tonic phase rather than the initial, twitch phase of neurogenic contractions (Allcorn *et al.*, 1986). Thus, an increase in evoked noradrenaline release may not underlie the potentiation of the twitch seen in this study. However, a prejunctional site of action cannot be ruled out as intracellular electrophysiological studies show that (+)-3-PPP depolarizes the cell bodies of the postganglionic sympathetic neurones which innervate the vas deferens (the hypogastric ganglion) *in vitro* and this is associated with an increase in membrane input resistance. Further voltage-clamp studies show that (+)-3-PPP inhibits two potassium currents in these neurones, the M-current and a fast, calcium-activated potassium current (Kennedy & Henderson, 1989 and unpublished observations). These actions of (+)-3-PPP may underlie the increase in evoked release of motor transmitter since the inhibition of the potassium currents is likely to lead to an increase in neuronal action potential duration. This in turn would lead to an increase in calcium influx into the synaptic bouton during the action potential and consequently an increase in neurotransmitter release.

An unexpected finding in the present study was the inhibitory action on neurogenic twitches of low concentrations of (+)-3-PPP via dopamine D_2-receptors, even though (+)-3-PPP was originally studied as a D_2-agonist (Hjorth *et al.*, 1981). Firstly, such an action was not reported by Campbell *et al.* (1987). This was not due to the difference in the strains of mice used as we found a similar effect of (+)-3-PPP in Swiss Webster mice, the strain used by Campbell *et al.* Thus, the reason for this difference in results is not known, but one possibility may be the age of the mice used, as we noted that in several older mice (+)-3-PPP had a much smaller inhibitory action (unpublished observations). Secondly, it has been reported that D_2-receptors are absent from the mouse vas deferens (Gibson & Samini, 1979; Hurst *et al.*, 1979), though a recent report contradicts this and agrees with our findings (Smith & Rowland, 1989). Thus, it is clearly important when using (+)-3-PPP as a σ-ligand to eliminate the D_2-receptor as

a possible site of action. This can be ensured by the co-administration of a D_2-antagonist, such as sulpiride, which does not interact with the sigma site (Ferris *et al.*, 1986).

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Regulation of the substance P-induced contraction via the release of acetylcholine and γ-aminobutyric acid in the guinea-pig urinary bladder

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1 The action of substance P (SP) on the release of γ-aminobutyric acid (GABA) and acetylcholine (ACh) and on contraction were studied in strips of the guinea-pig urinary bladder. Substance P induced a dose-dependent contraction of strips of guinea-pig urinary bladder ($EC_{50} = 1.2 \times 10^{-9}$ M). This contraction was not altered by tetrodotoxin, but with a dose of 10^{-9} M and less, there was a complete inhibition by 10^{-6} M atropine. Contractions initiated by 3×10^{-9} M SP or more were partly inhibited by atropine. The EC_{50} value of substance P in the presence of atropine was 7.0×10^{-9} M.

2 Substance P induced a Ca^{2+}-dependent and tetrodotoxin-resistant release of [^{3}H]-acetylcholine (ACh) from strips of urinary bladder preloaded with [^{3}H]-choline ($EC_{50} = 4.9 \times 10^{-10}$ M), and this release was antagonized by [D-Pro^2,D-Trp^{7,9}]substance P.

3 Bicuculline increased the substance P-induced contraction and the release of [^{3}H]-ACh from the strips.

4 Substance P induced a Ca^{2+}-dependent and tetrodotoxin-sensitive release of [^{3}H]-γ-aminobutyric acid (GABA) from strips preloaded with [^{3}H]-GABA ($EC_{50} = 2.6 \times 10^{-9}$ M), and this release was antagonized by [D-Pro^2,D-Trp^{7,9}]substance P.

5 Therefore, substance P appears to exert excitatory effects on the contractility of urinary bladder predominantly by stimulating its own receptor located on the cholinergic nerve terminals. GABA released by substance P inhibits stimulation of the cholinergic neurone. However, the direct action of substance P on the cholinergic neurone is more potent that the indirect action via GABA release.

Introduction

Vesical ganglia of the urinary bladder receive input from both sympathetic and parasympathetic nervous systems and play a key role in the regulation of bladder motility (El-Badawi & Schenk, 1971; Dixon & Gosling, 1974; de Groat & Booth, 1980). Adenosine triphosphate (Burnstock *et al.*, 1978; Fujii, 1988) and peptides, including substance P (Alm *et al.*, 1978; Hökfelt *et al.*, 1978; Husted *et al.*, 1981; McConnell *et al.*, 1982), vasoactive intestinal polypeptide (Alm *et al.*, 1977) and somatostatin (Hökfelt *et al.*, 1978) are considered to be putative non-cholinergic, non-adrenergic excitatory neurotransmitters. Immunohistochemical studies have shown that substance P-containing neurones exist in the genito-urinary tract (Alm *et al.*, 1978; Hökfelt *et al.*, 1978; Husted *et al.*, 1981; McConnell *et al.*, 1982), and capsaicin-sensitive sensory cells (P1 cells) have been found to contain substance P as the neurotransmitter in the rat urinary bladder (Maggi & Meli, 1988). Substance P applied exogenously causes a contraction of the urinary bladder (Sjögren *et al.*, 1982). However, the site of the action of substance P in these tissues is not well understood. We have demonstrated that γ-aminobutyric acid (GABA) is an inhibitory neurotransmitter in the urinary bladder (Taniyama *et al.*, 1983; Kusunoki *et al.*,

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1984). GABA is released by stimulation of neurones and inhibits the motility of the urinary bladder of guinea-pig by reducing the release of acetylcholine (ACh) via $GABA_A$-receptors located on postganglionic cholinergic neurones (Kusunoki *et al.*, 1984). The inhibition of electrical stimulation-induced contraction via $GABA_B$- receptors has been shown in the rabbit urinary bladder muscle (Santicioli *et al.*, 1984). Intravenous application of GABA has been found to suppress the spontaneous contractions of rat urinary bladder (Maggi *et al.*, 1983). Electrophysiological studies showed that GABA caused a reduction in the resting membrane potential and a depression of the membrane excitability of the vesical ganglion cells (de Groat, 1970; Simonds *et al.*, 1983). The present study was carried out to elucidate the involvement of cholinergic neurones in the substance P-induced contraction of the urinary bladder as in the case of intestinal tissue (Yau & Youther, 1982; Tanaka & Taniyama, 1985). Interactions of the excitatory neurotransmitter, substance P and an inhibitory neurotransmitter, GABA were also examined.

Methods

Adult guinea-pigs of either sex, weighing between 300 and 350 g, were killed by cervical dislocation. The urinary bladder was immediately excised and 1.5 cm long strips of the upper body were prepared by cutting in a circular fashion. The number of ganglion cells, GABA content and GAD activity were highest in the upper body of this organ (Kusunoki *et al.*, 1984).

Measurement of mechanical activity of strips from urinary bladder

The bladder strips were placed in a 20 ml organ bath in the presence of Krebs solution of the following composition (in mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.19, $NaHCO_3$ 25.0, KH_2PO_4 1.18 and glucose 11, which was maintained at 35°C and continually bubbled with 95% O_2–5% CO_2. Approximately 1 g of resting tension was applied and was kept constant by readjustment during the equilibration period. Following an equilibration period, responses of the bladder strips were elicited by adding substance P to the bath. Mechanical activity was recorded by means of an isometric transducer (Nihon Kohden, SD-1T).

Measurement of [^{3}H]-acetylcholine release

Strips were incubated with [^{3}H]-choline at a final concentration of 10^{-8} M in Krebs solution for 60 min. After washing in fresh Krebs solution for 30 min, these strips were mounted and superfused at 1.2 ml min^{-1} with Krebs solution containing 10^{-5} M hemicholinium-3 to prevent the uptake of choline formed from acetylcholine (ACh), and gassed with 95% O_2–5% CO_2. The superfusate was collected at 1 min intervals, and the radioactivity was measured in a liquid scintillation spectrometer. Ca^{2+}-free medium was Krebs solution from which $CaCl_2$ was omitted and which contained 10^{-3} M ethylene glycol *bis* (β-aminoethylether)-N,N,N′,N′,-tetraacetic acid (EGTA). At the end of the experiments, the tissue was dissolved in Soluene and the radioactivity measured. Extraction and separation of [^{3}H]-ACh and [^{3}H]-choline were carried out by the method of Potter & Murphy (1967). The superfusates were collected in 1 ml of 3-heptanone-tetraphenylboron (10 mg ml^{-1}) on ice. [^{3}H]-ACh and [^{3}H]-choline were extracted with 1 N formic acid-acetone 15:85 (v/v). The samples were then subjected to electrophoresis to separate ACh and choline. The spots were identified with iodine and the radioactivity was measured in a liquid scintillation spectrometer. Since over 81, 85 and 83% of the total radioactivity in the superfusates from substance P-, high K^+-stimulated and non-stimulated tissues proved to be [^{3}H]-ACh, respectively, the total radioactivity in the superfusates from substance P-, high K^+-stimulated and non-stimulated preparations were considered to approximate the amount of [^{3}H]-ACh and was therefore denoted as [^{3}H]-ACh release. When substance P 10^{-9} M was added to the perfusion medium for 30 s at 30 min intervals, the decline in [^{3}H]-ACh release evoked by substance P was marked from the first (S_1) to the second application (S_2) period, whereas the decay was only slight from the second (S_2) to the fifth applications (S_5). Therefore, the release evoked by the second application of substance P was used as the control.

In the experiments of high K^+-evoked release of [^{3}H]-ACh, tissues were pretreated with tetrodotoxin for 15 min before the application of KCl. In the experiments in which electrical transmural stimulation (ETS)-evoked release of [^{3}H]-ACh was measured, strips were mounted between two parallel platinum electrodes to stimulate the intramural nerves (Nihon Kohden, MSE-3R) with pulses of 0.2 ms duration, 15 V intensity and 10 Hz frequency. The superfusate was collected at 1 min intervals, and the radioactivity was measured in a liquid scintillation spectrometer. When KCl 40 mM was added to the perfusion medium for 30 s and ETS was applied for 30 s at 30 min intervals, the decline in [^{3}H]-ACh release evoked by KCl and ETS was marked from the first (S_1) to the second application (S_2) period, whereas the decay was only slight from the second to the fourth applications (S_4). Therefore, the release

evoked by the second application of KCl and ETS was used as the control.

Measurement of [^{3}H]-GABA release

Strips of urinary bladder were incubated at 37°C for 60 min with [^{3}H]-GABA at a final concentration of 5×10^{-8} M in Krebs solution. After washing in fresh Krebs solution for 20 min, the strips were mounted in an apparatus and superfused at a rate of 1.2 ml min^{-1} with Krebs solution. The Krebs solution used throughout contained 10^{-5} M aminooxyacetic acid (AOAA), to prevent the metabolism of [^{3}H]-GABA, and 10^{-3} M β-alanine to prevent the glial uptake of [^{3}H]-GABA, and was gassed with 95% O_2–5% CO_2. The superfusate was collected every 30 s and the radioactivity measured in a liquid scintillation spectrometer. To determine the proportion of unchanged GABA in the tritium efflux, 100 μl of the superfusates with added unlabelled GABA were subjected to high-voltage electrophoresis on Whatman 3 mm chromatography paper. The chromatograms were developed with ninhydrin, and the radioactivity in the spot area corresponding to GABA, determined in a scintillation counter, represented the percentage of the total radioactivity found in the superfusate. Even at 120 min after the start of superfusion, over 93% of the total radioactivity in the superfusates from both substance P-stimulated and non-stimulated samples was associated with unchanged [^{3}H]-GABA. Accordingly the total radioactivity in the superfusate seems to represent the approximate amount of [^{3}H]-GABA. When substance P 3×10^{-9} M was added to the perfusion medium for 30 s at 30 min intervals, the decline in [^{3}H]-GABA release evoked by substance P was marked from the first (S_1) to the second application (S_2) period, whereas the decay was only slight from the second (S_2) to the fourth applications (S_4). Therefore, the release evoked by the second application (S_2) of substance P was used as the control.

Calculations

The release of tritium was expressed as the fractional rate, in which the amount of radioactivity in the superfusate was divided by the total radioactivity present in the tissue, in the same collection period. The tritium content of the tissue at each period was calculated by adding cumulatively the amount of tritium in each fraction to the tritium content of the tissue at the end of the experiment. The evoked release was expressed as % increase over spontaneous release, that is the percentage of release during the application of substance P compared to the release just before its application. Data were analysed by Student's t test and a $P \leqslant 0.05$ was considered statistically significant.

Drugs and chemicals

The drugs and chemicals used were as follows: [^{3}H]-choline (80 Ci mmol^{-1}) and [^{3}H]-GABA (57 Ci mmol^{-1}) (New England Nuclear, Boston, Mass.), aminooxyacetic acid (AOAA), bicuculline (Sigma, St. Louis, MO, U.S.A.), tetrodotoxin (Sankyo, Tokyo, Japan), ethylene glycol *bis* (β-aminoethylether)-N,N,N',N',-tetraacetic acid (EGTA), atropine sulphate and β-alanine (Nacalai tesque, Inc., Kyoto, Japan), [D-Pro^2,D-Trp^{7,9}]substance P and substance P (Peptide Institute, Osaka, Japan), Soluene (Packard, Downers Groves, IL, U.S.A.). Other chemicals used were of reagent grade. The drugs were dissolved in Krebs solution immediately before use and were then added to the perfusate.

Results

Mechanical activity of strips of urinary bladder

Substance P, 3×10^{-10} M to 10^{-6} M, contracted the strip in a concentration-dependent manner. The maximum response was obtained with 3×10^{-7} M SP and the EC_{50} value was 1.2×10^{-9} M (Figure 1). Pretreatment with tetrodotoxin at 3×10^{-7} M for 15 min did not alter the substance P-induced contraction (Figure 1a). Atropine 10^{-6} M abolished the contractions induced by substance P at concentrations of 10^{-9} M and less, partially inhibited those induced by the peptide at 3×10^{-9} M to 3×10^{-7} M, and did not alter those induced by the peptide at 10^{-6} M (Figure 1a), thereby suggesting that the cholinergic neurone of the urinary bladder participates to some extent in the contractions induced by substance P (3×10^{-10} M to 3×10^{-7} M). The cholinergic component of the substance P-induced contraction was gradually decreased by increasing the concentration of substance P. Bicuculline (10^{-6} M), a $GABA_A$- receptor antagonist, increased the contraction induced by substance P at 10^{-7} M and less (Figure 1b), with no effect on the basal tone. Yohimbine (10^{-7} M) did not significantly alter the concentration-response curve for the substance P-induced contraction and there was no effect on the basal tone (Figure 1b).

Release of [^{3}H]-ACh induced by substance P

The spontaneous release of [^{3}H]-ACh declined in a multiphasic exponential course and approached a fairly constant level at a fractional rate of

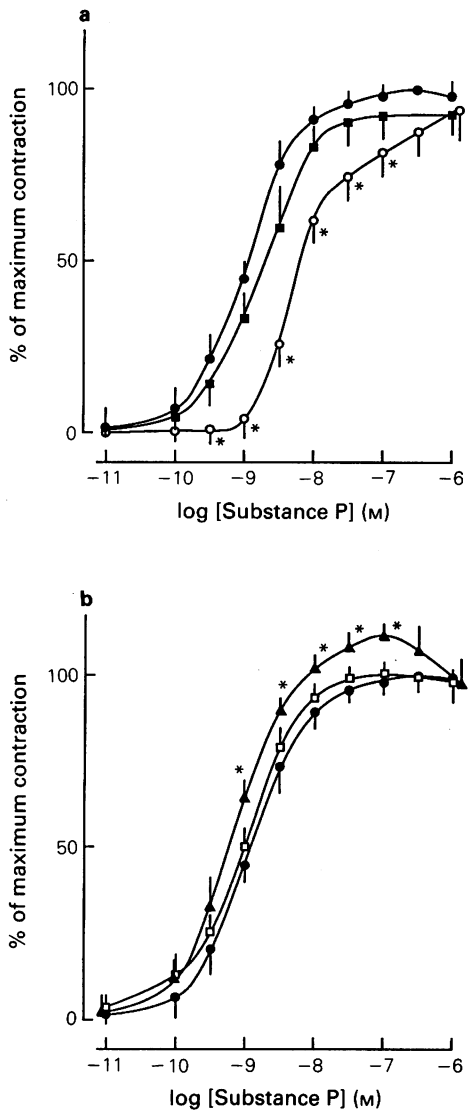


Figure 1 Concentration-response curves for substance P-induced mechanical contraction in the presence of (a) atropine and tetrodotoxin and (b) bicuculline and yohimbine. Atropine (10^{-6} M), tetrodotoxin (3×10^{-7} M), bicuculline (10^{-6} M) and yohimbine (10^{-7} M) were added to the medium 10 min before and during the application of substance P. The concentration-response curve is expressed as a % of the maximum contraction induced by substance P. Each point represents the mean from tissues from 5 or 7 animals; vertical lines show s.e.mean. (●) In the absence of agents; (○) in the presence of atropine; (■) in the presence of tetrodotoxin; (▲) in the presence of bicuculline; (□) in the presence of yohimbine. * Significantly different from the value in the absence of agents.

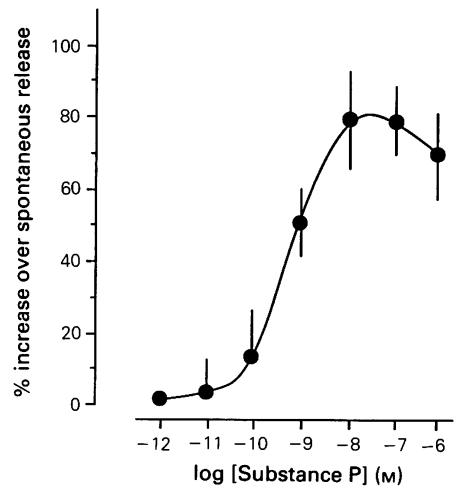


Figure 2 Effect of substance P on the release of [^{3}H]-acetylcholine ([^{3}H]-ACh). Strips of the urinary bladder were exposed to substance P for 30 s and the evoked release of [^{3}H]-ACh was expressed as a % increase over the spontaneous release. Effects of substance P at concentrations of 10^{-12} M, 10^{-10} M, 10^{-8} M and 10^{-6} M were examined in one preparation, that at concentrations of 10^{-11} M, 10^{-9} M and 10^{-7} M in another preparation. Each point represents the mean from tissues from 6 or 7 animals; vertical lines show s.e.mean.

$0.0034 \pm 0.0004\ \text{min}^{-1}$ ($n = 30$ animals) 60 min after superfusion. Superfusion with medium containing substance P, 10^{-10} M to 10^{-6} M for 30 s increased the release of [^{3}H]-ACh in a concentration-dependent manner, the EC_{50} value being 4.9×10^{-10} M (Figure 2). The substance P-induced release of [^{3}H]-ACh was antagonized by [D-Pro^2,D-Trp^{7,9}]substance P (Table 1), thereby indicating that substance P acts on its own receptor. Ca^{2+}-free medium containing 10^{-3} M EGTA completely inhibited the release of [^{3}H]-ACh induced by substance P. However, 3×10^{-7} M tetrodotoxin did not alter the induced release (Table 1). Bicuculline 10^{-6} M increased the substance P (10^{-9} M)-induced release of ACh by $33.9 \pm 4.6\%$.

Effect of GABA on the release of [^{3}H]-ACh induced by electrical transmural stimulation and high K^+

Electrical transmural stimulation (ETS, 15 V, 10 Hz, 0.2 ms, for 30 s) and high K^+ (4×10^{-2} M, for 30 s) induced a release of [^{3}H]-ACh. These stimulation-induced releases of [^{3}H]-ACh were abolished by pretreatment with Ca^{2+}-free medium. The ETS-induced release of [^{3}H]-ACh was not obtained in the presence of 3×10^{-7} M tetrodotoxin, and the high K^+-induced release was reduced by tetrodotoxin to

Table 1. Effect of various agents on substance P-evoked release of [^{3}H]-acetylcholine

Agents	*Fractional rate of* [^{3}H]*-ACh release* ($\times 10^{-3}$ per min) *Spontaneous* (A)	*SP-evoked* (B)	*B/A × 100*
Control (4)	3.48 ± 0.41	5.29 ± 0.53	152.0
Ca^{2+}-free (4)	3.44 ± 0.21	3.62 ± 0.15	105.2*
Control (4)	3.71 ± 0.32	5.79 ± 0.36	156.1
Tetrodotoxin (3×10^{-7} M) (4)	3.65 ± 0.28	5.45 ± 0.35	149.3
Control (7)	3.41 ± 0.29	5.46 ± 0.25	160.1
[D-Pro^2, D-Trp^{7,9}]SP (3×10^{-6} M) (7)	3.42 ± 0.12	3.71 ± 0.18	108.2*

Strips of guinea-pig urinary bladder were stimulated by substance P (SP, 10^{-9} M) for 30 s. Tetrodotoxin (3×10^{-7} M), Ca^{2+}-free medium containing 10^{-3} M EGTA and [D-Pro^2,D-Trp^{7,9}]substance P (3×10^{-6} M) were added 15 min before and during the application of substance P. Values are mean ± s.e. for the number of experiments shown in parentheses. In each experiment, control and experimental responses were obtained for every strip used.

* Significantly different from the control value, calculated by Student's t test ($P \leq 0.05$).

73.7%. GABA 10^{-5} M inhibited the ETS-induced release of [^{3}H]-ACh, but not the high K^+-induced release in the presence of tetrodotoxin (Table 2).

Release of [^{3}H]*-GABA induced by substance P*

The fractional rate of spontaneous release of [^{3}H]-GABA from strips preloaded with [^{3}H]-GABA after 60 min superfusion was $0.0014 \pm 0.0002\ min^{-1}$ ($n = 30$ animals). Substance P, 10^{-10} M to 10^{-7} M, induced release of [^{3}H]-GABA in a concentration-dependent manner, the EC_{50} value being 2.6×10^{-9} M (Figure 3). The substance P-induced release of [^{3}H]-GABA was antagonized by [D-Pro^2, D-Trp^{7,9}]substance P, and was inhibited by superfusion with Ca^{2+}-free medium containing 10^{-3} M EGTA or 3×10^{-7} M tetrodotoxin (Table 3).

Discussion

Substance P contracted strips of the urinary bladder. As part of this contraction was sensitive to atropine, the release of ACh must have occurred. This notion was supported by the finding that substance P induced a Ca^{2+}-dependent release of ACh from

Table 2 Effect of γ-aminobutyric acid (GABA) on electrical transmural stimulation (ETS) and high K^+-evoked release of [^{3}H]-acetylcholine

Agents	*Fractional rate of* [^{3}H]*-ACh release* ($\times 10^{-3}$ per min) *Spontaneous* (A)	*Evoked* (B)	*B/A × 100*
ETS-evoked release			
Control (4)	3.44 ± 0.38	6.20 ± 0.36	180.2
Ca^{2+}-free (4)	3.52 ± 0.33	3.96 ± 0.35	112.5*
Control (4)	3.62 ± 0.16	6.15 ± 0.25	169.9
GABA (10^{-5} M) (4)	3.64 ± 0.28	4.51 ± 0.15	123.9*
High K^+-evoked release in the presence of tetrodotoxin			
Control (4)	3.71 ± 0.37	5.94 ± 0.53	160.1
Ca^{2+}-free (4)	3.82 ± 0.33	4.01 ± 0.35	150.0*
Control (4)	3.53 ± 0.31	5.72 ± 0.53	162.0
GABA (10^{-5} M) (4)	3.64 ± 0.28	6.03 ± 0.15	165.7

Strips of guinea-pig urinary bladder were stimulated by ETS (15 V, 10 Hz, 0.2 ms for 30 s) or by high K^+ (40 mM for 30 s) in the presence of tetrodotoxin (3×10^{-7} M). GABA (10^{-5} M) was added 5 min before and during the application of ETS or high K^+. Values are mean ± s.e. for the number of experiments shown in parentheses. In each experiment, control and experimental responses were obtained for every strip used.

* Significantly different from the control value, calculated by Student's t test ($P \leq 0.05$).

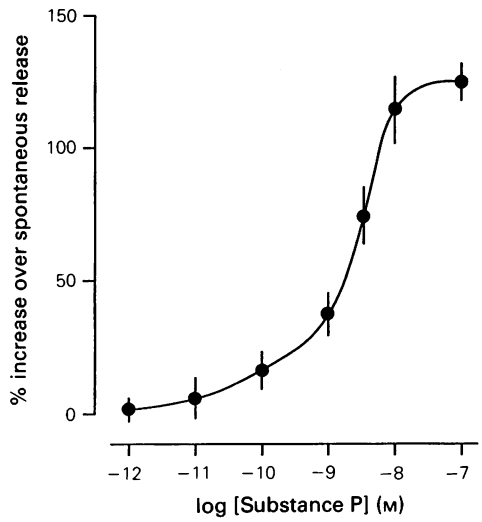


Figure 3 Effect of substance P on the release of [^{3}H]-γ-aminobutyric acid ([^{3}H]-GABA). Strips of urinary bladder were exposed to substance P for 30 s. The evoked release was expressed as a % increase over spontaneous release. Effect of substance P at concentrations of 10^{-12} M, 10^{-10} M and 10^{-8} M were examined in one preparation, that at concentrations of 10^{-11} M, 10^{-9} M and 10^{-7} M in another preparation. Each point represents the mean from tissues from 6 or 7 animals; vertical lines show s.e.mean.

strips of the urinary bladder. Both the contraction and the release of ACh induced by substance P were tetrodotoxin-resistant. Since it is assumed that the tetrodotoxin-resistant release of neurotransmitter is due to stimulation of nerve terminals (Gonella *et al.*, 1980; Starke, 1981; Alberts *et al.*, 1982), the atropine-sensitive and tetrodotoxin-resistant contraction is thought to occur via the release of ACh due to the stimulation of cholinergic nerve terminals by substance P. As regards excitatory presynaptic receptors in the peripheral nervous systems, β-adrenoceptors have been located on adrenergic nerve terminals (Langer, 1981) and nicotinic receptors are present on the cholinergic nerve terminals of the neuromuscular junction (Wessler, 1988). Substance P receptors are located on cholinergic nerve terminals in the vesical ganglia of the urinary bladder, and excitatory substance P receptors have been observed in cell bodies of the myenteric cholinergic neurones (Tanaka & Taniyama, 1985). The atropine-resistant contraction may be mediated by the direct stimulation of smooth muscle, although the possibility that the contraction is mediated by the release of non-cholinergic excitatory substances cannot be excluded.

The $GABA_A$-receptor antagonist, bicuculline increased the substance P-induced release of ACh and the atropine-sensitive contraction. Substance P induced a neuronal release of GABA, and GABA inhibited the ETS-induced release of ACh from the urinary bladder, as already noted (Kusunoki *et al.*, 1984). Therefore, our observations suggest that bicuculline prevents the inhibitory action of GABA released by substance P on the release of ACh. As the substance P-induced release of GABA was tetrodotoxin-sensitive, substance P may act on the soma-dendritic region of the GABAergic neurone rather than at the nerve terminals. The site of action of GABA appears to be the soma-dendritic region of the postganglionic cholinergic neurone, because exogenously applied GABA inhibited the tetrodotoxin-sensitive release of ACh, but did not alter the tetrodotoxin-resistant release of ACh

Table 3 Effect of various agents on the substance P (SP)-evoked release of [^{3}H]-γ-aminobutyric acid ([^{3}H]-GABA)

Agents	*Fractional rate of [^{3}H]-GABA release* ($\times 10^{-3}$ per 30 s) *Spontaneous* (A)	*SP-evoked* (B)	*B/A × 100*
Control (4)	1.32 ± 0.12	2.19 ± 0.21	175.8
CA^{2+}-free (4)	1.29 ± 0.15	1.44 ± 0.13	111.6*
Control (4)	1.45 ± 0.11	2.47 ± 0.18	170.3
Tetrodotoxin (3×10^{-7} M) (4)	1.38 ± 0.17	1.45 ± 0.20	105.1*
Control (7)	1.37 ± 0.09	2.26 ± 0.13	165.0
[D-Pro^2,D-Trp^{7,9}]SP (3×10^{-6} M) (7)	1.41 ± 0.14	1.70 ± 0.15	120.6*

Strips of guinea-pig urinary bladder were stimulated by substance P (3×10^{-9} M) for 30 s. Tetrodotoxin (3×10^{-7} M), Ca^{2+}-free medium containing 1 mM EGTA and [D-Pro^2,D-Trp^{7,9}]substance P (3×10^{-6} M) were added 15 min before and during the application of substance P. Values are mean ± s.e. for the number of experiments shown in parentheses. In each experiment, control and experimental responses were obtained for each strip used.

* Significantly different from the control value, calculated by Student's *t* test ($P \leq 0.05$).

induced by direct depolarization of cholinergic nerve terminals by high K^+. The α_2-adrenoceptor antagonist, yohimbine did not alter the concentration-response curve for the substance P-induced contraction. In the urinary bladder the adrenergic neruone is inhibitory and does not seem to participate in the response to substance P.

The present study indicates that there are three sites of action of substance P: the cholinergic nerve terminals, the soma-dendritic region of GABAergic neurones and the smooth muscle of the guinea-pig urinary bladder. The affinity of substance P for the cholinergic neurone was 5.3 times higher than that for the GABAergic neurone, as calculated from the EC_{50} value, therefore a relatively low concentration of substance P may act predominantly on the cholinergic rather than on the GABAergic neurone. If such is indeed the case, then the excitatory effect of substance P on the cholinergic neurone would be more potent than the inhibitory effect via the release of GABA. As substance P acted on the smooth muscle at concentrations approximately 14.3 times higher than those required to act on the cholinergic neurone, this peptide may exert its effects predominantly on the cholinergic neurones, and thereby induce contraction of the urinary bladder. It seems feasible that the cholinergic neurone in the guinea-pig urinary bladder is regulated by substance P by a direct action and an indirect action via the GABAergic neurone.

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A comparison of haemoglobin and erythrocytes as inhibitors of smooth muscle relaxation by the NANC transmitter in the BRP and rat anococcygeus and by EDRF in the rabbit aortic strip

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1 The inhibitory effect of erythrocyte suspensions and haemoglobin solutions on the response of the bovine retractor penis muscle (BRP) and the rat anococcygeus to field stimulation of their non-adrenergic non-cholinergic (NANC) nerves has been compared. Haemoglobin 3 μM greatly reduced the relaxant response in both tissues whereas a haemoglobin-equivalent suspension of erythrocytes was without effect.

2 A similar comparison of erythrocytes and haemoglobin on the response of the rabbit aortic strip to EDRF liberated by acetylcholine (ACh) showed that both reduced EDRF-mediated relaxation, though haemoglobin was significantly more effective.

3 These results suggest that the NANC transmitter may not be as freely diffusible through the erythrocyte membrane as EDRF and may therefore not be nitric oxide.

Introduction

Haemoglobin is known to block smooth muscle relaxation by the endothelium-derived relaxant factor (EDRF) in the rabbit aorta and the non-adrenergic non-cholinergic (NANC) inhibitory neurotransmitter in both the bovine retractor penis muscle (BRP) and the rat anococcygeus (Bowman & Gillespie, 1982; Bowman *et al.*, 1982; Martin *et al.*, 1985). Recently we have shown that erythrocytes are as effective as haemoglobin in blocking the relaxant effect of EDRF released into the superfusing fluid in a cascade system (Gillespie & Sheng, 1988a,b). Erythrocytes also blocked the effect of nitric oxide. These results are consistent with the suggestion that EDRF is nitric oxide (Palmer *et al.*, 1987) and that the mechanism of action of either haemoglobin or erythrocytes is physically to bind the nitric oxide, either directly or after the nitric oxide has diffused through the erythrocyte membrane.

The identity of the neurotransmitter of the NANC inhibitor nerves in the BRP and anococcygeus is unknown. It has, however, several properties in common with EDRF. For example, both relax the BRP (Gillespie & Sheng, 1988a,b), both increase cyclic GMP (Rapoport & Murad, 1983; Diamond & Chu, 1983; Bowman & Drummond, 1984), both are abolished by anoxia (Furchgott & Zawadzki, 1980; Bowman & McGrath, 1985) and by borohydride (Griffith *et al.*, 1984; Gillespie & Sheng, unpublished observation), as well as by haemoglobin. If EDRF is nitric oxide, it is possible that the NANC transmitter is also nitric oxide. If this is so, then not only haemoglobin but also erythrocytes might block the response to nerve stimulation. A possible error in this argument lies in the short diffusion distance between varicosities and the smooth muscle cells and the likely inability of erythrocytes to penetrate to any significant extent into the tissue. In such a system the erythrocytes would be at a considerable disadvantage in binding the neurotransmitter, in comparison with a cascade system in which EDRF is released into the superfusion fluid and has several seconds for binding before this fluid reaches the test tissue. Aortic strips retaining their endothelium and directly relaxed by EDRF, released from that endothelium by acetylcholine, represent a situation closer to the conditions in the BRP and anococcygeus. We have, therefore, compared the relative abilities of haemoglobin and erythrocytes to abolish the NANC

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nerve response in the BRP and rat anococcygeus with their effect on the relaxant action of acetylcholine in the rabbit aortic strip.

Methods

New Zealand rabbits (2–3 kg) were killed by CO_2 and bled. The abdominal aorta was removed, cleaned and spiral strips cut at an angle of 30° to the line of the artery to produce strips 2–3 mm wide and 2 cm long. These strips were prepared under microscopic observation and care was taken to avoid damaging the endothelium. Strips were suspended in 10 ml organ baths under a resting tension of 1 g measured by Grass FTD3 tension transducers. Responses were displayed on a Grass polygraph. The organ bath contained Krebs solution at 36°C and was bubbled with 95% O_2 + 5% CO_2. Tone was induced by adding 5-hydroxytryptamine (5-HT, 10^{-5} M). Acetylcholine 10^{-6} M was added to each preparation at the beginning of the experiment, to check that relaxation was produced and the endothelium was present. All preparations produced 90% or more relaxation of the 5-HT tone in response to acetylcholine.

BRP muscles were obtained from the local abattoir and normally used within 3 h of the animals' death. Muscles were cleaned of connective tissue and thin strips, 2–3 mm in diameter and 2 cm in length, were cut and removed. The rat anococcygeus muscles were isolated as previously described (Gillespie, 1972). Both the BRP preparation and the rat anococcygeus were suspended in pairs of Ag-AgCl ring electrodes under 1 g tension. Tone was raised by adding guanethidine 3×10^{-5} M. Field stimulation through the ring electrodes was applied from a Grass S44 stimulator at supramaximal voltage, 1 ms pulses and trains of stimuli lasting 10 s. The composition of the Krebs solution was (in mM) Na^+ 145, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 127, HCO_3^- 25, HPO_4^{2-} 1.2, SO_4^{2-} 1.2 and dextrose 11.

Preparation of erythrocytes and haemoglobin solutions

The technique for preparing fresh haemoglobin solution from rat blood has already been described (Bowman *et al.*, 1982). Briefly, blood was collected through a polythene cannula in one carotid artery of an anaesthetised rat into heparin containing tubes. The erythrocytes were separated by centrifugation, washed in isotonic phosphate buffer and resuspended after further centrifugation in a volume of buffer equal to the original volume of blood. One ml of this suspension was added to 19 ml of 20 mosmol hypotonic phosphate buffer, pH 7.4, to lyse the cells. Cell membranes were removed by centrifugation and the concentration of haemoglobin in the supernatant measured spectrophotometrically as methaemoglobin. To prepare a suspension of erythrocytes, the same first stage of centrifuging the heparinised blood, decanting the plasma and resuspending the erythrocytes in isotonic phosphate buffer was used. The resuspended erythrocytes were again centrifuged, the supernatant decanted and the erythrocytes again suspended in isotonic buffer solution. Such washed erythrocytes proved more fragile than unwashed cells, so that when they were added to an oxygenated organ bath the turbulence caused a variable degree of lysis. This was tested at the end of each experiment by removing the bath fluid, centrifuging to remove unlysed cells, then assaying spectrophotometrically the haemoglobin content of the supernatant. Nevertheless, the blocking effect of the released haemoglobin interfered with several experiments and limited the concentrations of erythrocytes which could be used. Whole blood added to the organ bath was much more stable and, since previous experiments (Bowman & Gillespie, 1982) showed plasma to be quite ineffective against the NANC relaxation, in the present experiments suspensions of erythrocytes were produced by adding an appropriate volume of heparinised whole blood to the bath. The concentration of free haemoglobin in the bath fluid was still measured at the end of each experiment and on all occasions was below the level of detection (10^{-7} M).

Drugs

The following drugs were used: acetylcholine (Sigma), 5-HT (Sigma), guanethidine (CIBA).

Results

The effect of haemoglobin or erythrocytes on NANC relaxation

The effect of haemoglobin 3 μM and a suspension of erythrocytes with an equivalent concentration of haemoglobin on NANC relaxation is illustrated in Figure 1. Haemoglobin measurement at the end of this experiment demonstrated the absence of lysis of the erythrocytes. In these circumstances, while haemoglobin produced a 50–60% reduction in relaxation in response to NANC nerve stimulation in both the BRP and the rat anococcygeus, erythrocytes were without effect. In some experiments, particularly with washed erythrocytes or higher concentrations of erythrocytes, a variable degree of inhibition of the NANC response was observed, apparently as a result of haemolysis. The degree of

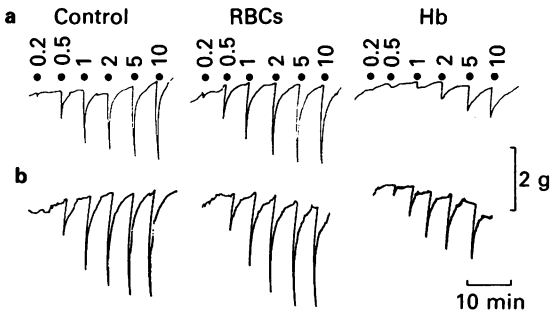


Figure 1 The response of the bovine retractor penis (a) and the rat anococcygeus (b) to field stimulation of their non-adrenergic non-cholinergic (NANC) nerves. The nerves were stimulated for 10 s at the marker (●) at the frequencies shown. Guanethedine 30 μM was present throughout to block the motor adrenergic nerves and raise tone. Haemoglobin (Hb) 3 μM in the last panel reduces responses at all frequencies and in both preparations by more than 50% whereas a comparable concentration of haemoglobin but held within erythrocytes (RBCs) is without effect.

haemolysis depended, among other things, on the time the erythrocytes spent in the bath, the vigour of oxygenation and the gravitational forces used for centrifugation of the bath fluid. Figure 2 compares the free haemoglobin concentration at the end of the experiment with the inhibition of the nerve response and shows a resonable correlation. In some experiments there was no inhibition in spite of free haemoglobin concentrations which would be expected to produce inhibition. In these experiments further haemolysis presumably occurred during centrifugation of the bath fluid. Where whole blood was used to produce the erythrocyte suspension, it was possible to compare the inhibitory effect of haemoglobin (Hb) and erythrocytes both at a concentration equivalent to 3×10^{-6} M Hb without significant haemolysis. The results are shown in Figure 3. Whereas haemoglobin produced about 50% inhibition of nerve-induced relaxation in both the anococcygeus and the BRP, suspensions of erythrocytes were completely ineffective. As the next section of results will show these results are quite different from the effects on EDRF where both erythrocyte suspensions and haemoglobin inhibited the response. The sensitivity of EDRF to inhibition by haemoglobin was higher than that of the NANC response and it was possible that the apparent resistance of the nerve responses was simply a concentration effect. If this were true, then higher concentrations of erythrocytes, with a haemoglobin content which if free would produce an inhibition equivalent to that of EDRF, would be effective against NANC nerve stimulation. Experimentally this was difficult to test because of the

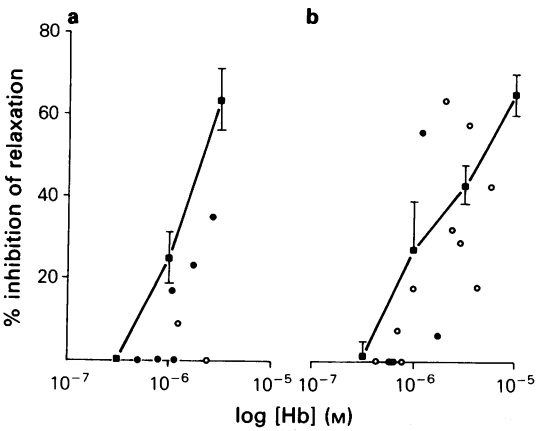


Figure 2 Comparison of the inhibitory effect of haemoglobin solutions on the responses of the (a) bovine retractor penis muscle (BRP) and (b) rat anococcygeus muscle to field stimulation of their NANC nerves, with the inhibition produced by suspensions of erythrocytes which have undergone a variable degree of haemolysis. The solid line represents the inhibitory effect of free haemoglobin at the concentrations shown on the BRP (a) and rat anococcygeus (b). The individual values show the degree of haemolysis of erythrocyte suspensions in terms of the free haemoglobin they gave rise to in comparison, with the inhibitory effect such suspensions had on the response. The erythrocyte suspensions had a haemoglobin-equivalent of either 3 μM (●) or 10 μM (○). The inhibition by the erythrocyte suspensions correlates reasonably well with the free haemoglobin they give rise to, though most values lie below the haemoglobin dose-response curve presumably because some additional cell rupture takes place during centrifugation. The NANC nerves in these experiments were stimulated at 5 Hz for 10 s.

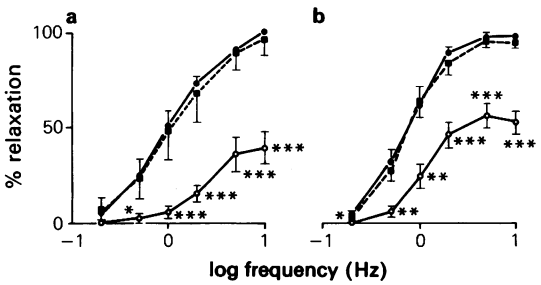


Figure 3 Frequency-response curves to field stimulation of NANC nerves at frequencies between 0.2 and 10 Hz in the (a) bovine retractor penis muscle (BRP) and (b) the rat anococcygeus. Control responses (●——●) and responses in the presence of erythrocytes with a haemoglobin concentration equal to 3 μM (■- - -■ were indistinguishable whereas haemoglobin solutions of 3 μM (○——○) produced a significant reduction at all frequencies. Vertical lines represent s.e.mean, $n = 5$; *P < 0.05, **$P < 0.01$, ***$P < 0.001$.

problem of haemolysis of higher erythrocyte concentrations. By reducing the range of frequencies tested, so as to shorten the time the erythrocytes were in the bath, and reducing the vigour of oxygenation it was possible to compare the effect of 10^{-5} M free haemoglobin with an equivalent suspension of erythrocytes without haemolysis. The results are shown in Figure 5 for the rat anococcygeus. The free haemoglobin solution produced a degree of inhibition similar to that shown for EDRF. However, the suspension of erythrocytes remained ineffective.

The effects of haemoglobin and erythrocytes on EDRF-mediated relaxation

The effect of haemoglobin solutions or erythrocyte suspensions, both at a concentration equivalent to 3×10^{-6} M haemoglobin, on the relaxation of the rabbit aortic strip to acetycholine 10^{-7} and 10^{-6} M is illustrated in Figure 4. The relaxation to the lower dose of acetylcholine was completely abolished by

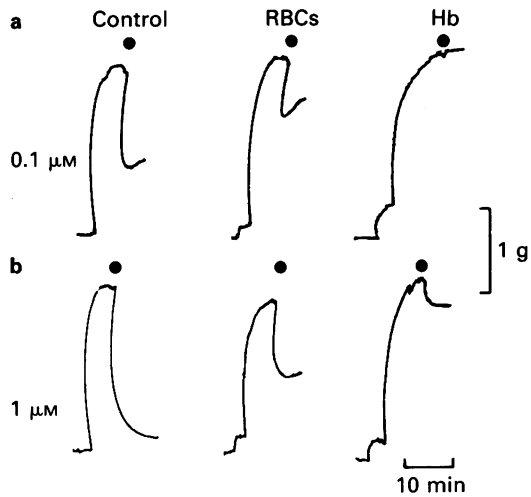


Figure 4 The response of a rabbit aortic strip to endothelium-derived relaxant factor (EDRF) liberated by two dose levels of acetylcholine, (a) 0.1 and (b) 1.0 μM, and the effect on this of haemoglobin (Hb) 3 μM or a suspension of erythrocytes (RBCs) with an equivalent haemoglobin content. Haemoglobin abolished the response to the low concentration of EDRF and greatly reduced that to the high concentration (end panels). Erythrocytes also reduced the response at both concentrations (middle panels) though the reduction was less than with haemoglobin. Tone was raised with 5-hydroxytryptamine (5-HT). In the presence of erythrocytes and haemoglobin the sensitivity to 5-HT was increased. To produce contractions of comparable magnitude the 5-HT concentration in controls, 10 μM, was reduced to 1 μM in the presence of erythrocytes and 0.1 μM in the presence of haemoglobin solution.

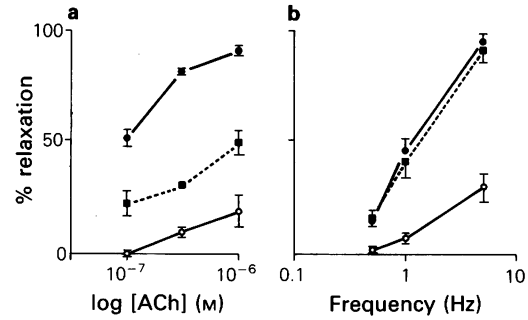


Figure 5 A comparison of the effects of free haemoglobin and a suspension of erythrocytes with an equivalent haemoglobin content on the response of the rabbit aortic strip to endothelium-derived relaxant factor (EDRF) (a) and the rat anococcygeus to NANC nerve stimulation (b). EDRF was liberated by acetylcholine (ACh) at the concentrations shown; the NANC nerves were supramaximally stimulated at 0.5, 1 and 10 Hz for 10 s. For the rabbit aortic strip the concentration of haemoglobin was 3 μM and for the rat anococcygeus 10 μM. These concentrations were chosen to produce similar inhibition of the responses to EDRF and nerve stimulation. However, the corresponding suspension of erythrocytes reduced only the response to EDRF and had no effect on the nerve response. Tone was raised with 5-hydroxytryptamine (5-HT) in the aortic strip and with guanethidine in the anococcygeus. In the presence of erythrocytes and haemoglobin the sensitivity to 5-HT was increased. To produce contractions of comparable magnitude the 5-HT concentration in controls, 10 μM, was reduced to 1 μM in the presence of erythrocytes and 0.1 μM in the presence of haemoglobin solution. Vertical lines show s.e.mean. $n = 6$ for the anococcygeus and 9–15 for the aorta. (●——●) Control responses; (■- - -■) responses in the presence of erythrocyte suspension and (○——○) those in the presence of haemoglobin.

haemoglobin but only reduced by a haemoglobin-equivalent suspension of erythrocytes. The higher concentrations of acetylcholine produced a greater relaxation in the control which, though greatly reduced, could not be completely blocked even by the solution of haemoglobin and again was much less affected by the suspension of erythrocytes. The results of all experiments with EDRF are shown in Figure 5. Both erythrocytes and haemoglobin significantly reduced the EDRF-mediated relaxation at all concentrations, but the haemoglobin solution was significantly more effective than its equivalent in erythrocytes.

Discussion

We have previously shown that haemoglobin or erythrocytes are equally effective in abolishing the

response to EDRF when the two are exposed to one another in the transfer fluid in a cascade-type experiment. The present experiments show that erythrocytes are significantly less effective than haemoglobin in abolishing the response of an aortic strip to the release of EDRF from its own intact endothelium. The most obvious explanation is the inability of the erythrocytes to bring their haemoglobin content into as close contact to the sites of release of the EDRF, particularly on the deep surface of the endothelium facing the smooth muscle. Nevertheless, erythrocytes did reduce by more than 50% the response to even the higher concentrations of acetylcholine. In contrast, erythrocytes were completely ineffective in reducing the response to NANC nerve stimulation in either the BRP or the rat anococcygeus. Does this mean the transmitter, while able to react with haemoglobin, is unable to penetrate the erythrocyte membrane, or is it simply that the sites of release are too deeply buried within the muscle preparation for erythrocytes to be effective? Two factors should be relevant. First, the diffusional distance between the endothelium or nerve varicosity and the muscle layer and, secondly, the distance between the endothelial cell or varicosity and the bathing fluid. The mean gap between nerve varicosities and muscle cells in the rat anococcygeus is about 260 nm (Gillespie & Lullmann-Rauch, 1974) and between the deep surface of the endothelium cell and the underlying muscle is between 50–100 nm (Simionescu & Simionescu, 1977). In terms of the immediate diffusional distance, therefore, the endothelium would be the more difficult site to block. On the other hand, the total distance between erythrocytes and the luminal surface of the endothelium is negligible, whereas there is a substantial distance between the muscle surface and the nerve varicosities in the BRP and anococcygeus muscles. Nevertheless, the rat anococcygeus is a flat relatively thin tissue averaging 250–300 μm in thickness and the nerves are uniformly distributed through the tissue. Some reduction, at least in superficial muscle bundles, might have been expected. The results may indicate either that the NANC transmitter is not nitric oxide or that the nitric oxide is bound in a form not easily diffusible through the erythrocyte membrane.

The ability of erythrocytes with a haemoglobin equivalent of 3 μM, i.e. about 600 times less than whole blood, to reduce the response to EDRF raises a question mark against the physiological role of EDRF. To test this, in some experiments much higher concentrations of haemoglobin equivalent (500 μM) were used. These completely abolished the EDRF response. Unfortunately, some haemolysis always occurred as measured by the haemoglobin in solution at the end of the experiment. Nevertheless, in some preparations that free concentration was less than the 3 μM haemoglobin used in the other experiments, yet the inhibition of the EDRF response was much greater. Further experiments are needed to clarify this observation.

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Lorazepam discontinuation promotes 'inverse agonist' effects of benzodiazepines

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1 The effects of lorazepam discontinuation on responses to benzodiazepine agonists and antagonists were studied in mice.

2 The convulsant dose of pentylenetetrazol was decreased after an acute dose of lorazepam (0.5 $mg\,kg^{-1}$) at 4 days after drug discontinuation, compared to 1 or 7 days after discontinuation or to vehicle treatment.

3 The percentage of mice undergoing convulsions after an acute dose of FG 7142 (40 $mg\,kg^{-1}$) was increased at 4 days after lorazapam discontinuation, compared to 1 or 7 days after discontinuation or to vehicle treatment.

4 After an acute dose (0.5 $mg\,kg^{-1}$), lorazepam concentrations in cortex tended to be greater in lorazepam-treated compared to vehicle-treated mice at 4 days after discontinuation compared to 1 and 7 days.

5 These data indicate a shift toward reduced agonist sensitivity and increased inverse agonist sensitivity in mice 4 days after lorazepam discontinuation.

Introduction

Discontinuation syndromes associated with benzodiazepine administration have been reported widely in man and may limit clinical use of benzodiazepines (Petursson & Lader, 1981; Owen & Tyrer, 1983; Woods *et al.*, 1987; 1988; Roy-Byrne & Hommer, 1988). However, the mechanism of benzodiazepine discontinuation syndromes remains uncertain. Studies in animal models have reproduced a behavioural discontinuation syndrome (File, 1982; Miller *et al.*, 1988c), in some cases indicating an increase in sensitivity to benzodiazepine 'inverse agonists' after discontinuation of flurazepam (Little *et al.*, 1987a,b; Nutt *et al.*, 1988). Indeed, one group suggested the presence of a 'withdrawal shift' in the direction of inverse agonist effects associated with benzodiazepine discontinuation (Little *et al.*, 1986).

Benzodiazepines exert their effects by binding at a specific site on the $GABA_A$ receptor complex located on postsynaptic neurones (Haefely *et al.*, 1985). Alterations in this site associated with chronic benzodiazepine use have been described by several groups. Although prior studies addressing receptor alterations after benzodiazepine discontinuation in animals produced conflicting results (Crawley *et al.*, 1982; Scharf & Feil, 1983), we have recently described a model of lorazepam discontinuation which produces behavioural changes consistent with prior studies and changes in binding and function at the $GABA_A$ receptor (Miller *et al.*, 1988c). The functional effects of benzodiazepines in this model, and the occurrence of a 'withdrawal shift', have not been evaluated.

To assess the efficacy of benzodiazepine agonists and inverse agonists in a model of benzodiazepine discontinuation, we evaluated the anticonvulsant response of an acute dose of lorazepam and the convulsant response of an acute dose of the inverse agonist FG-7142 in mice after discontinuation of chronic lorazepam.

Methods

Male CD-1 mice, 6–8 weeks of age, were obtained from Charles River laboratories (Wilmington, MA), housed under a 12 h light-dark cycle, and given laboratory chow and water *ad libitum*.

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Lorazepam, 2 mg kg^{-1} daily, was administered chronically as previously described (Miller *et al.*, 1988b). Briefly, lorazepam was dissolved in PEG 400 and placed in Alzet 2001 or 2002 osmotic pumps. Pumps were implanted subsutaneously under brief ether anaesthesia. Control mice received pumps containing vehicle alone. Pumps were removed after 7 days. To ensure that behavioural changes occurred as previously described, open-field activity was performed in groups of lorazepam- and vehicle-treated mice 4 days after drug discontinuation. Open-field activity was greater in lorazepam-treated compared to vehicle-treated mice at this time (LRZ, 1353 ± 61 cm, $n = 9$; Vehicle, 1160 ± 83 cm, $n = 7$; mean ± s.e. mean, $P < 0.05$), consistent with prior results demonstrating increased motor activity 4 days after discontinuation of chronic lorazepam infusion (Miller *et al.*, 1988c).

For determination of benzodiazepine agonist efficacy, mice were injected i.p. with a single dose of lorazepam, 0.5 mg kg^{-1}. After 20 min, animals were injected i.v. via the tail vein with a continuous infusion of pentylenetetrazol, 10 mg kg^{-1} at a rate of 0.6 ml min^{-1} (Nutt *et al.*, 1986). The infusion was terminated at the onset of a full tonic-clonic seizure, and the amount required for seizure induction was recorded. Experimental groups were analyzed at 1, 4 and 7 days after the discontinuation of chronic treatment.

To control for possible differences in brain uptake of lorazepam, groups of chronically lorazapam- and vehicle-treated mice were injected with an acute dose of lorazepam as above. After 20 min, animals were killed and brains rapidly removed and dissected on ice. Cortices were homogenized in 1 ml water with a Polytron (setting 7, 10 s) and frozen at −20°C for subsequent lorazepam determinations. Lorazepam concentrations in tissue were determined by gas-liquid chromatography as previously described, with oxazepam used as the internal standard (Greenblatt *et al.*, 1978).

To determine benzodiazepine inverse agonist efficacy, chronically treated mice were injected with a single dose of FG-7142, 40 mg kg^{-1} i.p. (Little *et al.*, 1984). The occurrence of a full tonic-clonic seizure was recorded. Groups were similar to those evaluated for benzodiazepine agonist efficacy.

Data were analyzed by analysis of variance with Dunnett's correction for continuous data, or Chi-Square for discrete data, or the Mann-Whitney test for non-parametric data.

Materials

Osmotic pumps (models 2001, 2002) were obtained from Alza (Palo Alto, CA). FG-7142 (n-methyl-β-carboline-3-carboxamide) was obtained from Research Biochemicals (Natick, MA). Pentylenetetrazol was obtained from Sigma (St. Louis, MO). Lorezepam was a generous gift from Wyeth Laboratories (Radnor, PA). PEG-400 was obtained from J.T. Baker (St. Louis, MO). All other reagents were obtained from standard commercial sources.

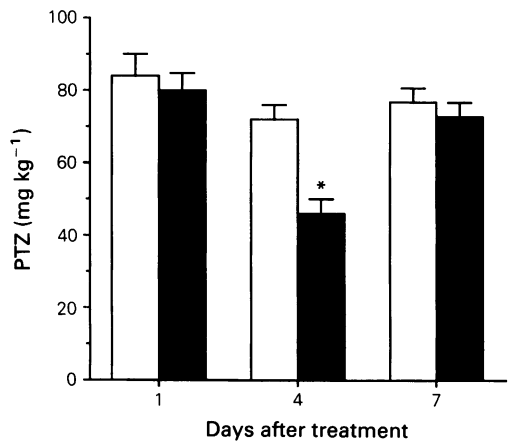


Figure 1 Effects of lorazepam discontinuation on anticonvulsant effects of acute lorazepam. Mice were treated with vehicle or lorazepam as described in the text. Mice then received lorazepam, 0.5 mg kg^{-1}, and 20 min later an infusion of pentylenetetrazol(PTZ), 10 mg ml^{-1} at 0.6 ml min^{-1}. Infusion was terminated at the onset of a full tonic-clonic seizure. Open columns represent vehicle, solid columns represent lorazepam treatment. Results are mean, $n = 15$ for each group; s.e. mean shown by vertical bars. Asterisk denotes $P < 0.05$ vs. vehicle and lorazepam at days 1 and 7.

Results

At 1, 4, and 7 days after drug discontinuation, mice treated with vehicle had similar seizure thresholds for pentylenetetrazol after a single dose of lorazepam (Figure 1). Results in lorazepam-treated mice 1 and 7 days after drug discontinuation produced similar results; similar doses of pentylenetetrazol were required to induce seizures at these time points, and results were similar to those in vehicle-treated mice. However, 4 days after lorazepam discontinuation, significantly less pentylenetetrazol was required for seizure induction, indicating a decreased anticonvulsant effect of lorazepam in these mice. Results at 4 days were significantly different from lorazepam-treated mice 1 and 7 days after drug discontinuation, and from all vehicle-treated groups.

Four days after drug discontinuation, lorazepam concentrations after an acute dose in lorazepam-treated mice tended to be greater than concentra-

tions in vehicle-treated mice (vehicle, $206 \pm 34\,ng\,g^{-1}$; chronic lorazepam $314 \pm 19\,ng\,g^{-1}$; mean $\pm$ s.e.mean, $n = 5$ in each group, $P = 0.06$ Mann-Whitney). Thus, brain uptake of benzodiazepines may be altered in mice after benzodiazepine discontinuation. However, the finding of possibly increased lorazepam concentrations in brain concurrent with decreased anticonvulsant effect further emphasizes the decreased efficiacy of lorazepam in these experiments.

In groups of mice at 1, 4, and 7 days after vehicle discontinuation, administration of the inverse benzodiazepine agonist FG 7142 caused tonic-clonic seizures in similar numbers of mice (Day 1: 20%; Day 4: 21%; Day 7: 25%; Figure 2). Similar results were observed in mice 1 (18.5%) and 7 (27%) days after discontinuation of chronic lorazepam. However, at 4 days after lorazepam discontinuation, a significantly greater percentage of animals (58%) sustained tonic-clonic seizures compared to days 1 and 7 post-lorazepam, and compared to all vehicle-treated groups. Although the group size at 7 days was less than 1 and 4 days (7 days $n = 12$, 1 and 4 days $n = 15$–18), the results are significant ($P < 0.05$) in the lorazepam group.

Discussion

A discontinuation syndrome associated with benzodiazepine use has been described in man and in several animal models (Petursson & Lader, 1981; File, 1982; Owen & Tyrer, 1983; Roy-Byrne & Hommer, 1988; Woods *et al.*, 1987; 1988; Miller *et al.*, 1988c). In animals, this syndrome is characterized by increased motor activity (Miller *et al.*, 1988c), and increased sensitivity to the effects of inverse agonist

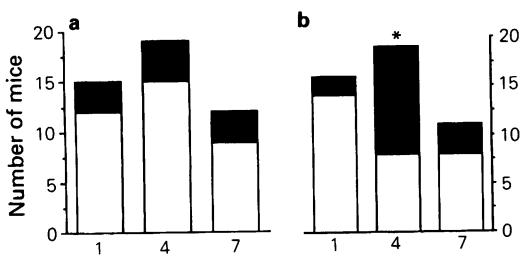


Figure 2 Effects or lorazepam discontinuation on convulsant responses to FG 7142. Mice were treated with vehicle (a) or lorazepam (b) as described in the text. Mice then received FG 7142, $40\,mg\,kg^{-1}$, and were observed for the development of a full tonic-clonic seizure. Solid columns represent number of mice sustaining seizures; open columns represent mice without seizures. Asterisk denotes $P < 0.05$ vs. vehicle and lorazepam at days 1 and 7.

compounds (Little *et al.*, 1987a,b). Indeed, Little and colleagues coined the term 'withdrawal shift' to describe the increased hypothermic and convulsant effects of inverse agonists after flurazepam discontinuation in mice (Little *et al.*, 1986). We have used the term 'discontinuation syndrome' rather than 'withdrawal syndrome' to avoid connotations associated with opiate withdrawal (Miller, 1988). Discontinuation syndromes have not been observed in all systems or for some behavioural effects (Nutt *et al.*, 1988). This may be due in part to use of various benzodiazepines and to choice of time points for testing. In general, benzodiazepine discontinuation syndromes appear to occur at increasing intervals after the last drug dose as drug half-life increases (Greenblatt & Shader, 1978). Symptoms may not be present until days after discontinuation in animals treated with agents with prolonged half-lives or persistent metabolites, such as diazepam or flurazepam (Greenblatt *et al.*, 1983). Thus, assessment of symptoms soon after discontinuation of these agents may fail to detect behavioural or neurochemical alterations. For example, few alterations in seizure susceptibility were observed in mice 24 and 48 h after discontinuation of chronic flurazepam (Nutt *et al.*, 1988). However, the metabolite *n*-desalkyl flurazepam has high receptor occupancy (low K_i) and a prolonged half-life, so that symptons may not occur until days after drug discontinuation (Miller *et al.*, 1988a). It is possible that substantial concentrations of the metabolites were present in brain at 24 h and perhaps 48 h after drug termination.

Our results indicate that 4 days after a course of lorazepam previously demonstrated to induce behavioural tolerance, mice exhibited a decrease in the anticonvulsant effect of acute lorazepam to a pentylenetetrazol stimulus. To exclude a pharmacokinetic explanation for this effect, concentrations of lorazepam were determined in brain after the acute dose. Cortical lorazepam concentrations tended to be increased in lorazepam-treated mice, making it likely that brain uptake of lorazepam was increased at this time point. These data emphasize the decrease in efficacy of lorazepam after drug discontinuation, since increased concentrations would be expected to enhance anticonvulsant effects. Our results are consistent with a shift toward reduced agonist sensitivity as suggested by Little *et al.* (1986).

We also observed an increase in the number of mice exhibiting seizures after a fixed dose of the inverse agonist FG 7142 in mice 4 days after lorazepam discontinuation. These results are consistent with those obtained using pentylenetetrazol, also indicating enhanced efficacy of inverse agonist compounds at this time point. Alterations in brain uptake may contribute to the differential effects of FG 7142. Since acute lorazepam concentrations

tended to be increased in mice after chronic lorazepam discontinuation, it is possible that brain uptake of FG-7142 is also increased. Such an alteration could contribute to the increase in convulsant effect observed in lorazepam compared to vehicle-treated mice.

These data indicate that several indices of $GABA_A$ receptor function are altered after lorazepam discontinuation. Behavioural effects at 4 days post-lorazepam include altered open-field activity (Miller *et al.*, 1988c) and benzodiazepine efficacy. As previously reported, this time point is associated with increased benzodiazepine- and GABA-related chloride uptake (Miller *et al.*, 1988c). The mechanism for increased inverse agonist effects remains uncertain. It is possible that the observed increases in receptor number or availability might also be associated with altered coupling between benzodiazepine and GABA binding, so that an inverse agonist effect predominates. Alternatively, alterations in putative endogeneous ligands might result in an imbalance in favour of inverse agonist compounds (Medina *et al.*, 1989). The simultaneous occurrence of behavioural and neurochemical alterations after lorazepam discontinuation indicates that this system may be valuable as a model for benzodiazepine discontinuation.

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The key role of sodium in the ouabain-mediated potentiation of potassium-evoked catecholamine release in cat adrenal glands

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1 The effect of $[Na]_o$ on the catecholamine release evoked by K in ouabain pretreated, isolated adrenal glands of the cat, was investigated.

2 Reduction of $[Na]_o$ to 70, 50 and 25 mM, with sucrose as a substitute, did not modify the spontaneous catecholamine release but progressively increased the K (17.7 mM)-evoked secretory response.

3 Ouabain pretreatment (100 μM; 10 min) greatly increased the K (17.7 mM)-evoked catecholamine secretory response in glands perfused with normal Krebs. Such an increase was still seen in glands perfused with 70 mM Na-containing solution but disappeared when $[Na]_o$ was reduced to 25 and 10 mM.

4 Preperfusion of non-ouabain treated glands with Li-enriched Krebs, for a 40 min period, caused an increase in the K (17.7 mM)-evoked secretory response which was dependent on $[Li]_o$ and essentially similar to that induced by ouabain pretreatment.

5 Ouabain treatment (100 μM; 10 min) of glands perfused with normal Krebs evoked a long lasting catecholamine release, which reached a plateau at about 36 min and amounted to 0.68 ± 0.25 μg 2 min^{-1} ($n = 9$). Such a secretory response was dramatically increased, and its shape modified, when glands were preperfused with K (17.7 mM)-Krebs: a peak of 3.77 ± 0.42 μg 2 min^{-1} ($n = 7$) was reached at 18 min. This response was drastically reduced in the presence of nitrendipine (1 μM).

6 In summary, our results indicate that both $[Na]_o$ reduction or Na accumulation into the chromaffin cell by inhibition of the Na pump with ouabain, greatly enhance the secretory efficacy of small increments of $[K]_o$, and suggest that sodium may play a role in the regulation of catecholamine release mediated by voltage-dependent Ca channels in adrenal glands.

Introduction

It is well known that ouabain induces catecholamine release from perfused adrenal glands of various species (Banks, 1967; García *et al.*, 1980; 1981a; Wakade, 1981) and from isolated chromaffin cells of bovine adrenal medulla (Aunis & García, 1981; Pocock, 1983). This effect seems to be mediated by ionic redistribution secondary to inhibition of the Na pump, as the induction of secretion is strongly dependent on extracellular Na (Esquerro *et al.*, 1980).

Previous experiments from our laboratory demonstrated that ouabain pretreatment of perfused adrenal glands of the cat not only increased spontaneous catecholamine release, but also dramatically potentiated the secretion evoked by small increments of $[K]_o$ (García *et al.*, 1981b). These results have been corroborated by other groups (Sorimachi & Nishimura, 1983; Nakazato *et al.*, 1986). Since the ouabain-mediated potentiation of the K-evoked secretory response observed in normal Krebs was maintained in low-Na Krebs perfused glands, it was concluded that this effect was essentially independent of extracellular Na (García *et al.*, 1981b). However, new findings from our laboratory showing that K-evoked catecholamine release is also potentiated in non-ouabain pretreated glands perfused with low-Na Krebs, cast doubts about this conclusion.

The purpose of the present study was to re-

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examine the role of Na in this phenomenon. Our results show that ouabain potentiates the K-evoked catecholamine secretory response if a sufficient electrochemical gradient of Na is maintained, and are consistent with the view that Na accumulation into the chromaffin cell, after inhibition of Na pump, is the critical factor involved in the ouabain-mediated potentiation of K-evoked release.

Methods

Perfusion of isolated adrenal glands of the cat

Cats of either sex weighing 2.5–4 kg were anaesthetized with ether followed by chloralose (40 to 60 mg kg^{-1} i.v.). Both cat adrenal glands were isolated and prepared for retrograde perfusion with Krebs-bicarbonate solution at room temperature as previously described (García *et al.*, 1980). The perfusion rate was adjusted to 1 ml min^{-1}.

Perfusion media

The normal Krebs-bicarbonate solution had the following composition (mM): NaCl 119, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11. This solution was equilibrated with 95% O_2 and 5% CO_2 the final pH being 7.4–7.5. Low-Na solutions were prepared by substituting NaCl by equivalent amounts of sucrose. Other Na-substitutes employed are specified in the results. When $[Na]_o$ was decreased to less than 25 mM, tris-hydroymethyl-amino methane (10 mM), instead of bicarbonate, was used as a buffer. Under these circumstances the modified Krebs solution was bubbled with pure O_2; the final pH was adjusted to 7.4 to 7.5 by adding HCl. Lithium-enriched solutions were prepared by substituting LiCl for equivalent amounts of NaCl. Calcium-free solutions were made up simply by omitting $CaCl_2$ from the perfusion medium without osmotic adjustments. High-Mg (20 mM) solutions were prepared by adding $MgCl_2$ to normal Krebs without osmotic adjustments. Potassium-rich solutions (17.7 mM; 59 mM) were prepared by the addition of KCl and reduction of similar amounts of NaCl to maintain isotonicity. These solutions will be referred to in the text as 17.7 K-Krebs or 59 K-Krebs. All experiments concluded with a 2 min perfusion period with a solution containing an excess of K (59 mM), in order to ascertain the functional viability of the glands.

Collection of perfusate samples

After 1 h of initial perfusion with normal Krebs solution, collection of perfusate samples at 2 min intervals was initiated. The first two samples were collected to determine the spontaneous catecholamine output; then ouabain-containing or high-K solutions were introduced in different $[Na]_o$-media. Ouabain was always given for periods of 10 min whereas high-K solutions were usually perfused for 2 min; in some experiments longer perfusion periods of high-K containing Krebs solution were used (see Results). Samples were collected in ice-cold assay tubes containing enough perchloric acid to give a final concentration of 0.05 N and assayed the same day for their total catecholamine content.

Catecholamine assay

The total catecholamine content of the samples (noradrenaline plus adrenaline) was determined according to the method of Anton & Sayre (1962), without further purification on alumina. The catecholamines present in each collection tube were expressed as $\mu g\, 2\, min^{-1}$ perfusion period. Net catecholamine release was calculated by subtracting the basal spontaneous output from the evoked release, and was expressed as $\mu g\, 2\, min^{-1}$ or $\mu g\, 8\, min^{-1}$.

Drugs

Ouabain was purchased from Sigma Chem. Co., St Louis, MO. U.S.A.; nitrendipine was a gift from Bayer AG, Wuppertal, F.R.G. Ouabain was dissolved directly in Krebs solutions. Nitrendipine was dissolved in 96% ethanol at a 10^{-2} M concentration; appropriate dilutions were made in Krebs solutions. The ethanol final concentration was smaller than 0.1% and had no effects on basal or evoked catecholamine release rates. All these solutions were carefully protected from light during preparation and throughout the experiments.

Statistical analysis

The data are expressed as means ± s.e.mean. Tests for significance were carried out by use of Student's *t* test. *P* values of less than 0.05 were considered significant.

Results

The effect of $[Na]_o$ on catecholamine release evoked by K

Perfusion of Krebs solution containing 17.7 mM K for 2 min caused a slight net increase in the release of catecholamines which amounted to 0.79 ± 0.14 μg $8\, min^{-1}$ ($n = 4$). Reduction of $[Na]_o$ to 70 mM, 50 mM and 25 mM did not change the spontaneous release

but progressively increased the K-evoked secretion ($1.77 \pm 0.23\,\mu g\ 8\,min^{-1}$; $3.45 \pm 0.11\,\mu g\ 8\,min^{-1}$; and $5.42 \pm 0.47\,\mu g\ 8\,min^{-1}$, respectively; $n = 4$) (Figure 1). This secretory response was strictly $[Ca]_o$-dependent and completely abolished by Mg (20 mM) (Figure 2).

In another group of paired experiments, the effect of nitrendipine (1 μM) on K (17.7 mM)-evoked catecholamine release in glands perfused with 25 mM Na-Krebs was explored. Two pulses (S_1 and S_2) of 17.7 K-Krebs (2 min) were applied, 30 min apart. Nitrendipine (1 μM) was perfused 10 min before and during S_2 in the experimental gland. Under these conditions, the net catecholamine release evoked by S_2 in control glands reached $63 \pm 4\%$ of S_1 ($n = 3$), while in the experimental gland only $16 \pm 2\%$ of S_1 ($n = 3$) ($P < 0.01$) was obtained.

Because NaCl was always substituted by sucrose, experiments were conducted in order to investigate whether the increase of K-evoked catecholamine release in low-Na media (25 mM) was also observed with other Na substitutes. Figure 3 shows that Na substitution by choline chloride increased, as sucrose, the rates of catecholamine release induced by K (17.7 mM). However, the substitution of NaCl by LiCl failed to enhance the K-evoked release.

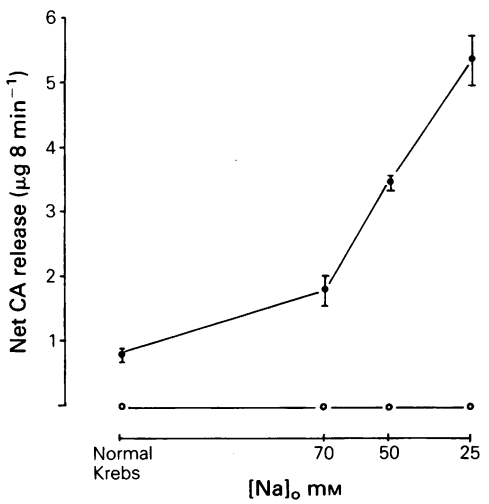


Figure 1 Effect of $[Na]_o$ on the catecholamine (CA) release evoked by K (17.7 mM). Four 2 min pulses of 17.7 mM K were given at 30 min intervals, each one in a different $[Na]_o$. Extracellular Na concentration was reduced in a step-wise manner 10 min before each K stimulus was applied. (●) Represent the net release of catecholamines during the 2 min period of stimulation plus that collected in the following 6 min. (○) The effect of $[Na]_o$ reduction on spontaneous basal release. Results are mean values of 4 experiments and vertical lines show s.e.mean.

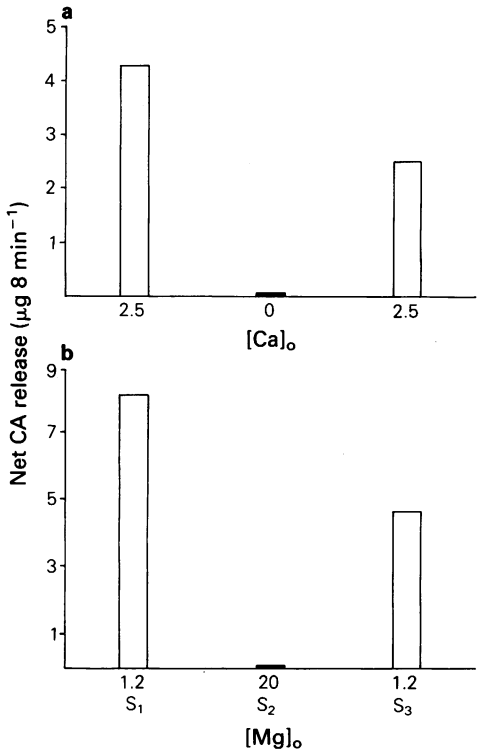


Figure 2 Effect of Ca deprivation and excess of Mg on the K-evoked catecholamine (CA) secretion in low Na-Krebs. Three 2 min pulses of 17.7 mM K (S_1, S_2 and S_3), 30 min apart, were given to glands perfused with 25 mM Na-Krebs. S_1 and S_3 pulses served as controls. Ten min before and during S_2 Ca was removed in (a) while $[Mg]_o$ was increased to 20 mM in (b). Results are from a typical experiment out of 3.

Effect of $[Na]_o$ on the K-evoked secretory response in ouabain pretreated glands

The K (17.7 mM)-evoked secretory response is known to be dramatically potentiated, in a time-dependent manner, by ouabain pretreatment (100 μM; 10 min), reaching a plateau 30 min later (García *et al.*, 1981b). Based on these data, a 40 min period after initiation of ouabain treatment was systematically used in the following experiments, in which the effect of $[Na]_o$ was examined (Figure 4). In normal Krebs, the K (17.7 mM)-evoked catecholamine release ($1 \pm 0.46\,\mu g\ 8\,min^{-1}$; $n = 5$) was markedly increased after ouabain exposure ($7.69 \pm 1.08\,\mu g\ 8\,min^{-1}$; $n = 5$). In low-Na Krebs (70, 25 and 10 mM Na), the catecholamine release evoked by K (17.7 mM) was increased, as previously shown (Figure 1), before ouabain perfusion. Treatment with the glycoside (100 μM; 10 min) further increased the K-evoked secretory response in

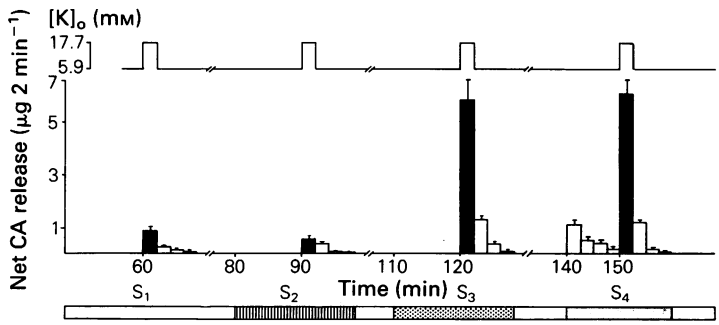


Figure 3 Effect of several Na-substitutes on K (17.7 mM)-evoked catecholamine (CA) release in low Na-Krebs. Four K (17.7 mM) pulses were given at 30 min intervals under different conditions. The first pulse (S_1) was applied to glands perfused with normal Krebs (open bar) and served as control; the second (S_2) in 25 mM Na-119 mM Li-Krebs (hatched bar); the the third (S_3) in 25 mM Na-238 mM sucrose-Krebs (spaced-stippled bar); and the fourth (S_4) in 25 mM Na-119 mM choline-Krebs (close stippled bar). All modified solutions were perfused 10 min before and during the K (17.7 mM) stimulus, as specified. Note that perfusion with choline-Krebs increased the spontaneous rate of catecholamine release, which is thought to be mediated by stimulation of nicotinic receptors. Each column represents the mean of 3 experiments and vertical lines show s.e.mean.

70 mM Na Krebs ($4.04 \pm 1.2\,\mu g\ 8\,min^{-1}$ before vs $12.2 \pm 1.5\,\mu g\ 8\,min^{-1}$ after ouabain treatment; $n = 3$), but did not when glands were perfused with 25 mM Na Krebs ($8.38 \pm 1.89\,\mu g\ 8\,min^{-1}$ before vs $8.87 \pm 1.33\,\mu g\ 8\,min^{-1}$ after ouabain treatment, $n = 6$) or with 10 mM Na Krebs ($7.45 \pm 0.97\,\mu g\ 8\,min^{-1}$ before vs $7.22 \pm 0.84\,\mu g\ 8\,min^{-1}$ after ouabain treatment; $n = 4$).

Effect of lithium on the K-evoked secretory response in non ouabain-treated glands

Lithium, a monovalent cation that shares many features with Na (Schou, 1976), is a poor substrate for the Na pump (Keynes & Swan, 1959), and easily accumulates inside the cells. Lithium accumulation would lead to an ionic distribution similar, in a sense, to that obtained by inhibition of the Na pump in adrenal glands perfused with normal Krebs, the only difference being that the prevalent intracellular monovalent cation, in this particular case, is Li instead of Na. In fact, perfusion of cat adrenal glands with 119 mM Li-containing Krebs induces a catecholamine secretory response with essentially the same profile as that caused by ouabain, provided that a perfusion period of at least 15 min is used (Abajo *et al.*, 1987). Therefore, it was considered of interest to see if preperfusion of non-ouabain pretreated glands with Li-enriched Krebs would increase the K (17.7 mM)-evoked secretory response. This was indeed the case. As is shown in Figure 5, preperfusion with Li-enriched Krebs for 40 min increased the K-evoked catecholamine release in a concentration-dependent manner. Although Li-enriched Krebs was prepared by substitution of LiCl for NaCl, the potentiation of K-evoked secretion found in these experiments cannot be ascribed to the concomitant $[Na]_o$ reduction because preperfusion with 119 mM Li-25 mM Na Krebs for shorter periods (10 min), which prevents an effective Li accumulation, did not enhance the K-evoked secretion (Figure 3).

The effect of predepolarization on the catecholamine secretory response evoked by ouabain

Ouabain treatment (100 μM; 10 min) of glands perfused with normal Krebs, causes a long-lasting release of catecholamines (García *et al.*, 1980), which reaches a plateau 30–40 min after initiation of ouabain treatment. The net release at 36 min amounted to $0.68 \pm 0.25\,\mu g\ 2\,min^{-1}$ ($n = 9$). This response was dramatically increased when glands were preperfused with a solution containing 17.7 mM K for a 60 min period (see Figure 6). Under these conditions the secretory response reached the maximum earlier and amounted to $3.77 \pm 0.42\,\mu g\ 2\,min^{-1}$ at 18 min ($n = 7$).

Effect of nitrendipine on the catecholamine release induced by ouabain in predepolarized glands

It has recently been suggested that the catecholamine secretory response evoked by ouabain in cat adrenal glands results from a dual mechanism: (a) chromaffin cell depolarization with secondary opening of voltage-dependent calcium channels, and (b) activation of a membrane Na/Ca exchange system in reverse mode (Artalejo & García, 1986). In order to explore which one of these mechanisms was involved in the increased secretory efficacy of ouabain in predepolarized adrenal glands, the effect of nitrendipine

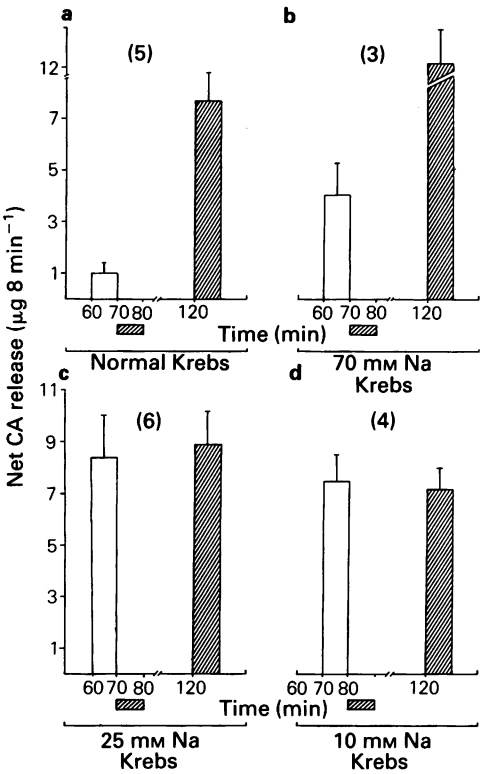


Figure 4 Effect of $[Na]_o$ on the ouabain-mediated potentiation of K (17.7 mM)-evoked catecholamine (CA) release. Glands received two pulses of 17.7 mM K (2 min); the second one was preceded 40 min before by a short pulse of ouabain (100 μM; 10 min) (horizontal hatched bar). The experiments were carried out in four different $[Na]_o$; (a) Normal Krebs, (b) 70 mM Na-Krebs, (c) 25 mM Na-Krebs and (d) 10 mM Na-Krebs. The basic perfusion medium used in this group of experiments was Krebs-Tris. Each column represents the mean from the number of experiments shown in parentheses and vertical lines show s.e.mean.

on such a response was investigated. The results are shown in Figure 7. The presence of nitrendipine (1 μM) dramatically reduced the secretion evoked by ouabain (100 μM; 10 min) in predepolarized glands; the secretion reached a maximum of 0.30 ± 0.02 μg $2\,min^{-1}$ ($n = 4$) at 42 min after initiation of ouabain treatment, whereas in the control gland a maximum of 4.03 ± 0.31 μg $2\,min^{-1}$ ($n = 4$) was reached at 16 min.

Discussion

In the present study the role of Na in ouabain-mediated potentiation of K-evoked secretory response has been examined. The results of our experiments show four main findings: (1) the reduction of $[Na]_o$ potentiates by itself the K-evoked catecholamine release; (2) the potentiation effect on the K-evoked secretory response mediated by ouabain pretreatment, is strictly dependent on the electrochemical gradient of Na during the perfusion period before stimulation; (3) perfusion of glands with Li-enriched Krebs for a long period (40 min) leads to a potentiation of K-evoked secretion in a concentration-dependent manner; and (4) in glands predepolarized with 17.7 mM K-Krebs the secretory efficacy of ouabain is remarkably increased, being greatly inhibited by nitrendipine (1 μM).

Potentiation of K-evoked secretion in low-Na media

The catecholamine secretory response evoked by 17.7 mM K increased as $[Na]_o$ decreased. This effect seems to be specifically due to $[Na]_o$ reduction since it could be observed when NaCl was substituted either by sucrose or choline chloride. The fact that the replacement of NaCl by LiCl, 10 min before stimulation, did not modify the K secretory response (note that longer perfusion periods markedly increased the response by accumulation into the chromaffin cell) indicate that adrenal glands cannot discriminate between Na and Li ions at the extracellular level, as has been suggested previously (Lastowecka & Trifaro, 1974; Abajo *et al.*, 1987).

The results of our experiments show that the K-evoked catecholamine release in low-Na media was strictly $[Ca]_o$-dependent and inhibited by Mg (20 mM) and nitrendipine (1 μM), suggesting that voltage-dependent calcium channels are involved in this phenomenon and that Na plays a role in its regulation. Sorimachi & Nishimura (1982) have suggested that monovalent together with divalent cations regulate the opening of calcium channels by screening the membrane surface negative charges. Our results are consistent with this hypothesis.

Ouabain-mediated potentiation of K-evoked secretory response

When our group described for the first time this effect of ouabain (García *et al.*, 1981b), we concluded that it was essentially independent of $[Na]_o$ because a similar potentiation was observed in glands perfused with 25 mM Na-Krebs (NaCl was substituted by choline chloride). However, such an interpretation has to be reconsidered in the light of the data obtained in this study, which demonstrate that the potentiation of the K secretory response observed in low-Na media (25 mM or 10 mM Na-Krebs) can be explained almost solely by the $[Na]_o$ reduction (see Figure 4). On the other hand, these results indicate that a sufficient electrochemical gradient of Na

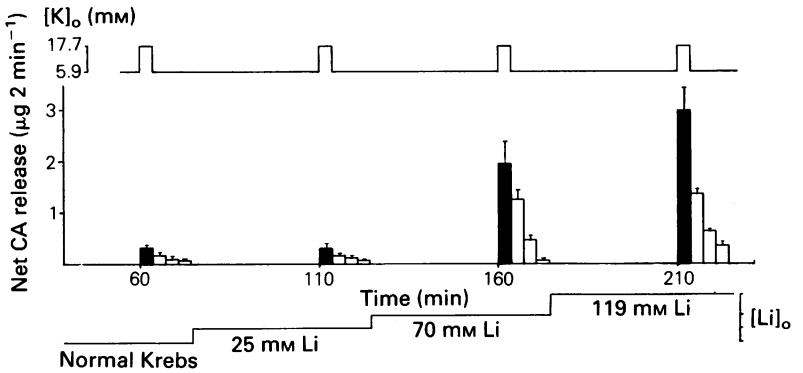


Figure 5 Effect of preperfusion with Li-enriched solutions on the K (17.7 mM)-evoked catecholamine (CA) release. Each gland received four pulses of 17.7 mM K (2 min). The first was applied in normal Krebs, which served as control; the second in 25 mM Li-119 mM Na Krebs; the third in 70 mM Li-74 mM Na-Krebs; and the fourth in 119 mM Li-25 mM Na Krebs. Lithium-enriched solutions were perfused for 40 min before each K (17.7 mM) pulse. Each column represents the mean from 4 experiments and vertical ines show s.e.mean.

during the perfusion period, after ouabain treatment, is required to potentiate the K-evoked catecholamine secretory response. Similar conclusions were reached by Nakazato *et al.* (1986) in the guinea-pig perfused adrenal gland. They showed that the effect of ouabain on the catecholamine release evoked by excess K, acetylcholine or splachnic nerve stimulation was time-dependent and increased as the $[Na]_o$ was increased from 25 to 154 mM. These and our data, taken together, suggest that Na accumulation inside the chromaffin cell is the critical factor involved in this phenomenon. A sodium-independent action of the glycoside seems not to play a role. The finding that preperfusion with Li-enriched Krebs solutions for long periods (40 min) led to a similar potentiation in non-ouabain-treated glands, strongly supports this assumption.

Potentiation of ouabain-evoked secretion in predepolarized glands

An interesting finding of this study was that the predepolarization of chromaffin cells with 17.7 mM K-containing Krebs, greatly enhanced the ouabain-evoked secretory response. The fact that the interaction of ouabain and K results in potentiation of

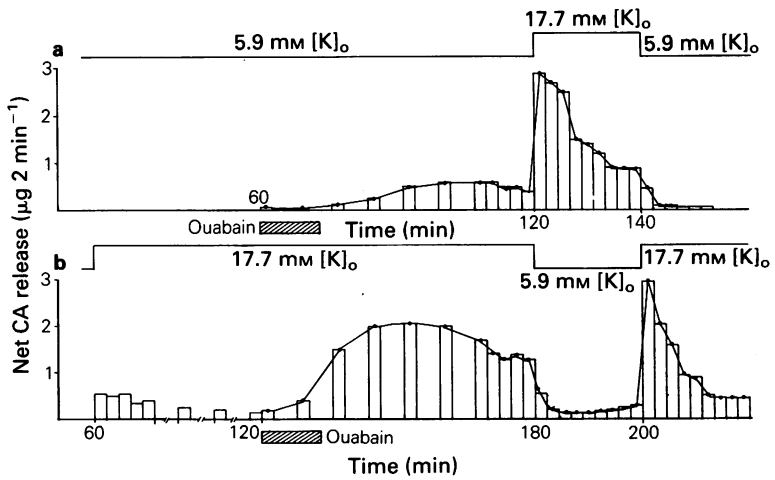


Figure 6 Effect of predepolarization with 17.7 mM K-containing Krebs on the ouabain-induced secretory response. In (a) ouabain (100 μM; 10 min) (horizontal hatched bar) was perfused in normal Krebs; this solution was later changed to 17.7 K-Krebs. In (b) the gland was perfused from the beginning of the experiment with 17.7 K-Krebs; 60 min later a ouabain pulse (100 μM; 10 min) was given. Results are from a typical paired experiment out of 3.

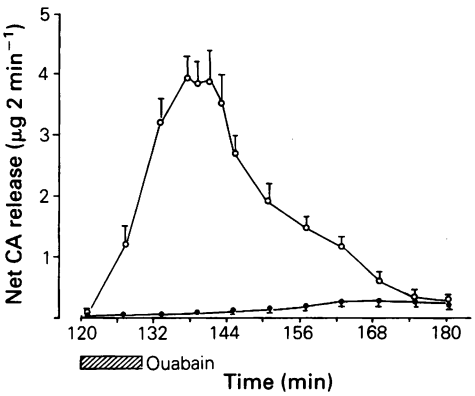


Figure 7 Effect of nitrendipine (1 μM) on the ouabain-evoked secretory response in glands predepolarized with 17.7 mM K-containing Krebs. Paired glands were perfused with 17.7 mM K-Krebs from the beginning of the experiment; 60 min later a ouabain pulse (100 μM; 10 min) (horizontal hatched bar) was given in both glands, as in Figure 6b. In the experimental gland, nitrendipine (1 μM) was present 10 min before ouabain pulse and throughout the experiment. (○) Control glands; (●) experimental glands. Each point represents the mean catecholamine (CA) release from 4 experiments and vertical lines show s.e.mean.

the secretory response regardless of the order they are applied to the gland, suggests that membrane potential may modulate the secretory efficacy of cardiac glycosides.

Artalejo & García (1986) have proposed that ouabain enhances the spontaneous rates of catecholamine release by a dual mechanism: (1) chromaffin cell depolarization which subsequently would open voltage-dependent calcium channels and (2) activation of a Na-Ca exchange system in reverse mode. Both mechanisms showed a strong dependence on $[Na]_o$, which indicates that they must be related to the ionic redistribution secondary to the inhibition of Na pump caused by the cardiac glycoside. Further membrane depolarization, induced by an increment of $[K]_o$, would obviously increase the efficacy of the first mechanism, but it might also potentiate the second since it has been demonstrated that the Na-Ca exchange system can be modulated by changes of membrane potential (Mullins & Brinley, 1975); hyperpolarization activating the direction which extrudes calcium from cell, and depolarization favouring the Na intracellular-dependent Ca influx (Baker, 1986). However, the dramatic inhibition of such a secretory response by nitrendipine (1 μM), found in our experiments (Figure 7), does not support a contribution of Na-Ca exchange system to the predepolarization-mediated enhancement of secretory response evoked by ouabain. These results are consistent with previous findings which demonstrated that Mg (García *et al.*, 1981b; Nakazato *et al.*, 1986), methoxyverapamil (García *et al.*, 1981b) and Co (Nakazato *et al.*, 1986) inhibited the K-evoked secretion in ouabain-pretreated glands.

Our results show that predepolarization with 17.7 mM K-containing Krebs not only increased the ouabain-evoked secretory response, but also changed its shape: the maximum was reached earlier and the inactivation developed faster. It is interesting to note that Bay K 8644, a calcium channel agonist, which also enhances the ouabain-secretory response, has been demonstrated to produce a very similar change in the shape of secretion induced by the cardiac glycoside (Artalejo & García, 1986). This adds further evidence to the idea that the potentiation resulting from the interaction between ouabain and K mainly involves voltage-dependent calcium channels.

In summary, our experiments demonstrate that the reduction of the $[Na]_o$ or the Na accumulation inside chromaffin cells in which the Na-K pump has been inhibited by ouabain, are two different experimental procedures that greatly enhance the secretory efficacy of small increments of $[K]_o$, and in both cases voltage-dependent Ca channels are involved. These findings suggest that sodium plays a role in regulating the catecholamine secretory response mediated by voltage-dependent Ca channels in adrenal glands.

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Cyclosporin therapy *in vivo* attenuates the response to vasodilators in the isolated perfused kidney of the rabbit

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1 The endothelium releases a number of vasoactive compounds, including the vasodilator prostaglandins, prostacyclin (PGI_2) and prostaglandin E_2 (PGE_2) and endothelium-derived relaxing factor (EDRF), which play an important role in the regulation of vascular tone in the microcirculation. Nephrotoxicity is the major complication of cyclosporin (CS) therapy and is related to an increase in intrarenal vascular tone. Endothelial cell generation of PGI_2 is inhibited by CS although this cannot fully explain the changes in vascular tone observed. We have investigated the possibility that EDRF-dependent vasodilatation is also affected by CS therapy *in vivo*.

2 CS nephrotoxicity was induced in rabbits with CS ($15\,mg\,kg^{-1}$ per day s.c. for 20 days ($n = 6$)); 6 rabbits were given CS vehicle (Veh) and 9 animals were studied without any treatment. Creatinine clearance fell significantly during treatment in the CS-treated rabbits ($11.78 \pm 1.5\,ml\,min^{-1}$, mean ± s.e.mean, to 7.79 ± 1.2 after 20 days treatment) but did not change in the vehicle-treated animals.

3 The responses to the endothelium-dependent (acetylcholine (ACh)) and endothelium-independent (nitroprusside (NP) and PGI_2) vasodilators were assessed in indomethacin-treated isolated perfused kidneys (IPKs) from untreated, CS- and Veh-treated animals. Vascular tone was induced with a constant infusion of noradrenaline 150 nM and the perfusion rate adjusted to produce a perfusion pressure of 90 mmHg. Perfusate flow rate (22.3 ± 4.6 vs $20.4 \pm 3.1\,ml\,min^{-1}$) and glomerular filtration rate (2.04 ± 0.37 vs $1.88 \pm 0.16\,nl\,min^{-1}$) did not differ between IPKs from CS- and Veh-treated animals.

4 The vasodilator response to ACh was reduced in the kidneys from CS-treated animals compared with those from untreated and Veh-treated animals (mean reduction $35.3 \pm 2.3\%$ compared with Veh) as were the responses to both NP ($42.8 \pm 3.6\%$) and PGI_2 ($27.7 \pm 7.4\%$).

5 This suggests that CS nephrotoxicity is not mediated via an effect on endothelium-dependent responses and that it is more likely that CS has a direct effect on vascular smooth muscle.

Introduction

The nephrotoxicity associated with the use of cyclosporin (CS) limits the usefulness of this potent immunosuppressive agent. Initially this toxicity was assumed to result from a direct toxic effect on renal tubules (Whiting *et al.*, 1982), but there is now evidence that intrarenal haemodynamic disturbances play a primary role, at least in the acute reversible toxicity that occurs within a few weeks of treatment. Total renal vascular resistance is increased by CS therapy both in animals (Murray *et al.*, 1985) and man (Curtis *et al.*, 1986), due to an increase in tone of the afferent and, to a lesser extent, efferent glomerular arterioles (Thiel, 1986; English *et al.*, 1987). This leads to a reduction in both glomerular blood flow and intraglomerular capillary hydrostatic pressure and hence to a fall in glomerular filtration rate (GFR). The mechanism underlying this change in vascular tone is unclear. Plasma renin activity (PRA) is increased early in CS nephrotoxicity but this is probably a secondary phenomenon as PRA later falls without an improvement in the nephrotoxicity, and blockade of the renin-angiotensin axis with captopril does not influence the changes in intrarenal vascular tone (Murray *et al.*, 1985). An alteration in

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the neural control of vessel tone is also unlikely as the denervated transplanted kidney readily develops nephrotoxicity.

The endothelium releases a variety of factors which influence vascular tone, including the vasodilator prostaglandins such as prostacyclin (PGI_2) and prostaglandin E_2 (PGE_2) and also the more recently described endothelium-derived relaxing factor (EDRF). Although the synthesis of the vasodilator prostaglandin PGI_2 by endothelial cells is impaired by CS (Neild *et al.*, 1983; Brown & Neild, 1987), a major role for prostacyclin in CS nephrotoxicity is unlikely as cyclo-oxygenase inhibitors such as indomethacin do not affect glomerular filtration rate (GFR) in normal man (Schnermann & Briggs, 1981). EDRF is an unstable, short-lived substance which is released by endothelial cells in response to a variety of pharmacological (Furchgott & Zawadski, 1980; Griffith *et al.*, 1984) and mechanical (Griffith *et al.*, 1987) stimuli. It produces relaxation of vascular smooth muscle by activating soluble guanylate cyclase (Radomski *et al.*, 1987) which leads to increased production of guanosine 3':5'-cyclic monophosphate (cyclic GMP). EDRF also inhibits platelet aggregation *in vitro* by stimulating intraplatelet guanylate cyclase (Radomski *et al.*, 1987). There is now good evidence that EDRF is nitric oxide (NO) (Palmer *et al.*, 1987; Ignarro *et al.*, 1987), probably derived from L-arginine (Schmidt *et al.*, 1988; Palmer *et al.*, 1988).

EDRF is a potent endogenous vasodilator which plays a major role in the regulation of blood flow within capillary beds (Griffith *et al.*, 1987). EDRF is released by the renal microcirculation both *in vitro* (Bhardwaj & Moore, 1988) and *in vivo* (Conger *et al.*, 1988), although its role in the regulation of renal vascular resistance in normal and pathological conditions is unknown. We have investigated the possibility that inhibition of endothelium-dependent vasodilatation may be involved in the changes of vascular tone that occur in CS nephrotoxicity.

Methods

Assays

CS blood concentrations were measured on 24 h trough whole blood samples by means of a commercially available polyclonal radioimmunoassay (Sandoz). Plasma and urinary creatinines were measured by the continuous flow Jaffe technique and creatinine clearance calculated.

Induction of nephrotoxicity

New Zealand white rabbits, initial weight 2.5–3.0 kg, were kept in individual cages and had free access to standard rabbit chow (Special Diet Services, Witham, Essex, UK) and water. Animals were treated with CS $15\,mg\,kg^{-1}\,day^{-1}$ s.c. for 20 days (CS group) or an equivalent volume of CS vehicle (Veh group) ($n = 6$ for each group). Twenty-four hour urine collections for urinary creatinine were performed before treatment was started and then every 5 days. Blood was sampled via an ear vein at the end of each urine collection and creatinine clearance calculated.

Isolated perfused kidney model

Each rabbit was anaesthetized with pentobarbitone (Rhone Poulenc), $40–60\,mg\,kg^{-1}$ i.v., and ventilated with air following surgical tracheal intubation. Frusemide (4 mg) which partially protects the kidney against ischaemic damage (Myers & Moran, 1986), was administered intravenously at induction of anaesthesia. Laparotomy via a midline incision was performed and the left kidney exposed. Following ligation the left renal artery was cannulated directly with polythene tubing (Portex 800\100\260\100): average warm ischaemic time 30–60 s. The kidney was initially perfused *in situ* at 4°C with 100 ml of Krebs-Henseleit solution supplemented with amino acids (KHB/AA) containing indomethacin 5.6 μM and PGI_2 10^{-9} M. Cold perfusate was used as we found that chilling the kidney prior to handling and removal improved the function of the isolated perfused kidney (IPK) of normal animals. The left ureter was cannulated with polythene tubing (Portex 800\100\340\100) and the kidney was then removed and placed in an organ chamber where it was perfused with KHB/AA containing indomethacin 5.6 μM, warmed to 37°C and gassed with 95% O_2/5% CO_2. Venous effluent was discarded, i.e. a non-return system was used.

Measurement of renal function in IPK

GFR of each isolated kidney was determined by clearance of [^{51}Cr]-EDTA; 0.5 MBq of [^{51}Cr]-EDTA were added to the KHB/AA and urine was collected over 5 min intervals, volume being measured gravimetrically. Urine and perfusate counts were determined and GFR calculated (Ross *et al.*, 1973).

Vascular tone in the IPK

Vascular tone in the IPK is absent or very low when the kidney is perfused with a cell-free perfusate (Swanson *et al.*, 1981). Therefore noradrenaline 150 nM was infused into each kidney throughout each experiment to induce vascular tone. Noradrenaline was used because it produces approximately

equal increases in vascular tone in intralobular, afferent and efferent glomerular arterioles in the rabbit kidney (Edwards, 1985). Perfusion pressure was monitored continuously throughout each experiment via a Statham pressure transducer. After a 20–30 min period of stabilisation, the perfusion rate was adjusted for each kidney to produce a perfusion pressure of 90 mmHg, adjusted for the pressure drop across the cannula.

Measurement of vascular response to vasodilators

Bolus injections of each agent were administered in a volume of 100 μl and the maximum drop in perfusion pressure recorded. Dose-response curves were established to the endothelium-dependent vasodilator acetylcholine (ACh, dose range 0.27–13.75 nmol) and the endothelium-independent dilators nitroprusside (NP, range 9.5–380 nmol) and PGI_2 (range 0.028–2.8 nmol) in kidneys from untreated rabbits (n = 9). ACh, NP and PGI_2 were then used in kidneys from CS- and vehicle (Veh)-treated rabbits.

Materials

CS for intravenous use (50 mg ml^{-1}) was provided by Sandoz (Basle, Switzerland). Its vehicle, cremophor, a polyoxylated castor oil (Sandoz), was used in equivalent volumes in the control (Veh) group.

The Krebs-Henseleit perfusate for the IPK had the following final electrolyte concentrations (mmol l^{-1}); NaCl 118, KCl 4.75, $MgSO_4$ 1.19, $NaHCO_3$ 25, K_2HPO_3 1.19, $CaCl_2$ 1.9, glucose 11.1. Twenty essential and non-essential amino acids were added to the perfusate (Epstein *et al.*, 1982) by using 100 ml of Vamin 14 Electrolyte Free solution (Kabi-Vitrium) per 10 l of final solution (KHB/AA), supplemented with additional L-cysteine, L-tyrosine, L-glutamine and glycine (Sigma) to produce the required final concentrations (Epstein *et al.*, 1982). Indomethacin (Sigma) was dissolved in absolute alcohol to a concentration of 2 mg ml^{-1} and then added to KHB/AA to a final concentration of 5.6 μM (2 μg ml^{-1}) which is sufficient to inhibit completely cyclo-oxygenase activity (Ferreira *et al.*, 1971). The perfusate was then filtered through a 0.45 μm filter (Millipore, UK).

Noradrenaline, the endothelium-dependent vasodilator acetylcholine (ACh) and the endothelium-independent vasodilator sodium nitroprusside (NP) were all obtained from Sigma. Prostacyclin (PGI_2), an endothelium-independent vasodilator which acts via adenylate cyclase and cyclic AMP, was a gift from Wellcome. All were dissolved in 0.9% saline at 4°C to appropriate concentrations and stored on ice before use. Exposure to light of the NP-containing solutions was kept to a minimum. Noradrenaline was infused in each IPK experiment at a rate of 90 μl min^{-1} for each 7.5 ml min^{-1} of KHB/AA to produce a final concentration in the kidney of 150 nM.

Statistical analysis

Results are expressed as mean ± s.e.mean. The Wilcoxon signed-pairs rank-sum test and the Mann-Whitney U test were used to analyse results from paired and unpaired samples respectively. $P < 0.05$ was accepted as indicating statistical significance.

Results

Induction of cyclosporin nephrotoxicity

Whole blood CS concentrations reached a peak after 5 days of treatment with a mean of 689 ± 12 ng ml^{-1} (mean ± s.e.mean); falling slightly thereafter; mean concentrations on days 10, 15 and 20 were 462 ± 94, 278 ± 57 and 356 ± 89 respectively (therapeutic range in our laboratory in human renal transplant recipients is 200–400 ng ml^{-1}). Creatinine clearance decreased significantly in the CS-treated rabbits during treatment compared with the Veh animals in which creatinine clearance did not change during treatment (Figure 1).

Renal function in the isolated perfused kidney

Perfusate flow rate to produce a perfusion pressure of 90 mmHg with a continuous infusion of noradrenaline 150 nM did not differ between the CS- and Veh-treated groups; CS 22.3 ± 4.6 vs Veh 20.4 ± 3.1 ml min^{-1}. The GFRs of perfused kidneys averaged approximately 2.0 ml min^{-1} after an initial

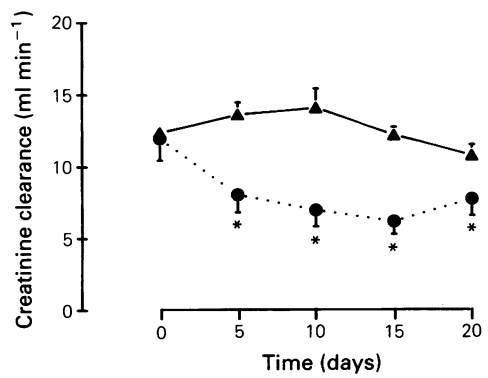


Figure 1 Creatinine clearance of rabbits at different times after treatment with cyclosporin or its vehicle. (●- - -●) Cyclosporin group ($n = 6$), (▲——▲) control vehicle group ($n = 6$). Each point represents the mean and vertical lines shown s.e.mean. * $P < 0.05$ vs value at time 0.

period of stabilization and then fell by approximately 20% during the period of study. GFRs did not differ significantly between the CS- and Veh-treated groups (after 1 h—CS group, 2.04 ± 0.37 ml min^{-1}; Veh group, 1.88 ± 0.16 ml min^{-1}; after 2.5 h—CS, 1.61 ± 0.26 ml min^{-1}; Veh, 1.53 ± 0.16 ml min^{-1}).

Vascular responses in the isolated perfused kidney

Vascular responses to vasodilators did not change during the perfusion period in kidneys from untreated rabbits (maximum response to ACh 1.375 nmol after 30 min was 15.4 ± 1.3 mmHg and after 150 min 15.9 ± 1.6 mmHg, NS (n = 5)).

Vasodilatation following bolus injections of the endothelium-dependent vasodilator ACh (doses 0.27 nmol, 1.375 nmol and 13.75 nmol) was significantly reduced in kidneys from CS-treated animals compared with those from both untreated and Veh-treated animals (Figure 2); mean reduction in drop in perfusion pressure 35.3 ± 2.3% compared with Veh group. A similar pattern occurred following injection of the endothelium-independent dilators NP (Figure 3) and PGI_2 (Figure 4); mean reductions 42.8 ± 3.6% and 27.7 ± 7.4%, respectively.

Discussion

Nephrotoxicity is the major problem associated with the use of CS. This toxicity is related to an increase in renal vascular resistance and a consequent fall in renal blood flow, the cause of which is unclear. In the present study we have demonstrated that CS 15 mg kg^{-1} per day s.c. in rabbits produces whole blood CS concentrations similar to those found in man and induces nephrotoxicity, as shown by a significant fall in creatinine clearance. The vasodilator responses to both endothelium-dependent and -independent vasodilators were similarly reduced in isolated perfused kidneys from CS-treated rabbits compared with those from both untreated and vehicle-treated animals. This suggests that CS does not influence vascular tone solely by an effect on EDRF-dependent vasodilatation.

CS undoubtedly has important effects on the endothelium. Elevated factor VIII-related antigen (Brown *et al.*, 1986), an increased thrombotic tendency (Vanrenterghem *et al.*, 1985) and cases of a haemolytic uraemic-like syndrome (Van Buren *et al.*, 1985) in patients treated with CS all indicate that the

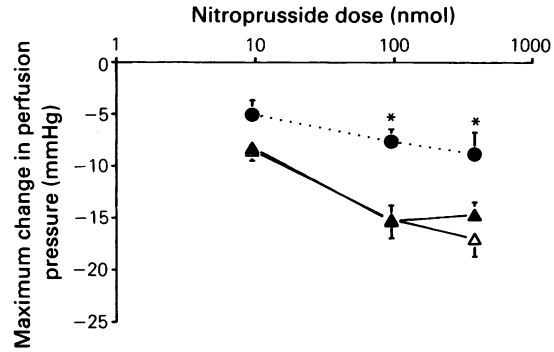


Figure 3 Effect of nitroprusside on the maximum change in perfusion pressure of the isolated perfused kidney from (△——△) untreated (n = 9), (●- - -●) cyclosporin-treated (n = 6) and (▲——▲) vehicle-treated (n = 6) rabbits. Each point represents the mean and vertical lines show s.e.mean. * $P < 0.05$ vs vehicle-treated group.

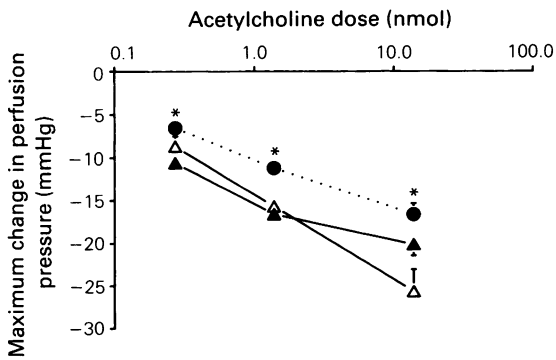


Figure 2 Effect of acetylcholine on the maximum change in perfusion pressure of the isolated perfused kidney from (△——△) untreated (n = 9), (●- - -●) cyclosporin-treated (n = 6) and (▲——▲) vehicle-treated (n = 6) rabbits. Each point represents the mean and vertical lines show s.e.mean. * $P < 0.05$ vs vehicle-treated group.

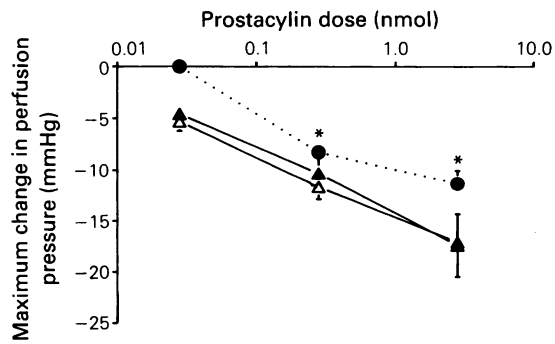


Figure 4 Effect of prostacyclin on the maximum change in perfusion pressure of the isolated perfused kidney from (△——△) untreated (n = 9), (●- - -●) cyclosporin-treated (n = 6), (▲——▲) vehicle-treated (n = 6) rabbits. Each point represents the mean and vertical lines show s.e.mean. * $P < 0.05$ vs vehicle-treated group.

endothelium is altered by CS. As the endothelium releases several important regulators of vascular tone including prostacyclin and EDRF, this suggests that the increased vascular tone of CS nephrotoxicity may be the result of reduced activity of endothelial-derived vasodilators. Although our data do not exclude an effect on EDRF-dependent vasodilatation, the broadly similar reductions in vasodilatation seen with ACh, NP and PGI_2 indicate that this is unlikely.

Although decreased endothelial prostacyclin is unlikely to explain fully the nephrotoxicity of CS, several recent studies indicate that there is an increase in the renal release of vasoconstrictor eicosanoids during treatment with CS (Coffman *et al.*, 1987; Rogers *et al.*, 1988) and that reducing production of these factors may improve the nephrotoxicity (Rogers *et al.*, 1988; Smeesters *et al.*, 1988). The origins of these constrictors is unclear as the endothelium does not produce significant quantities of vasoconstrictor eicosanoids (Ali *et al.*, 1980), although mesangial cells may (Kriesberg *et al.*, 1985). As indomethacin was used throughout our experiments, it is unlikely that increased intrarenal generation of vasoconstrictor eicosanoids can explain the attenuation of the vasodilator response seen in the kidneys from CS-treated animals.

The vasodilator response which occurs with acetylcholine is largely due to the release of EDRF by the endothelium; this is true both for isolated vessels (Furchgott & Zawadski, 1980; Griffith *et al.*, 1984) and the microcirculation, including the kidney (Bhardwaj & Moore, 1988). A component of this response may, however, be the result of vasodilator prostaglandin release (e.g. PGI_2). Nevertheless, the use of indomethacin in our system excludes reduced PGI_2 release as an explanation for the attenuation of the vasodilator responses seen in the CS group.

Recent work from other authors indicates that CS has a direct effect on vascular smooth muscle. Large vessels from CS-treated rats have a reduced responsiveness to both NP and ACh (Huang *et al.*, 1987; Luscher *et al.*, 1988; Rego *et al.*, 1988). More recently CS has been shown to increase the elevation of cytosolic calcium in response to angiotensin II in cultured aortic smooth muscle cells (Pfeilschifter & Ruegg, 1987) and, similarly, to increase cytosolic free calcium elevation and contraction induced by vasopressin in cultured mesangial cells (Meyer-Lehnert & Schrier, 1988), a specialised smooth muscle cell. Such an effect on vascular smooth muscle would be consistent with our findings. However, in contrast to the *in vitro* effects of CS on isolated vessels or cells, Monaco *et al.* (1988) have demonstrated a reduced contractile response to noradrenaline in the IPK from CS-treated rabbits. This could be an explanation for our finding of reduced vasodilatation to all agents tested; if the vascular tone induced by noradrenaline is less in the CS kidneys, the potential for vasodilatation may therefore be reduced. Nevertheless, the broadly similar reductions in vascular resistance to both EDRF-dependent and -independent vasodilators indicate that CS does not specifically have an effect on EDRF-dependent vasodilatation.

In summary, our results indicate that it is unlikely that CS nephrotoxicity is mediated solely via an inhibition of endothelium-dependent vasodilatation and more likely that CS has a direct effect on vascular smooth muscle.

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Nitroglycerin relaxes canine coronary arterial smooth muscle without reducing intracellular Ca^{2+} concentrations measured with fura-2

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1 Changes in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured simultaneously with force by a microfluorometric method using a calcium indicator, fura-2, in canine coronary arterial smooth muscle cells.

2 Depolarization by high (30–90 mM) KCl-physiological salt solution (PSS) produced concentration-dependent increases in force and $[Ca^{2+}]_i$.

3 The KCl-induced increase in $[Ca^{2+}]_i$ was abolished by Ca^{2+}-free conditions and almost abolished by verapamil 10^{-5} M, suggesting that it was entirely due to the increased Ca^{2+} influx through voltage-dependent Ca^{2+} channels.

4 The $[Ca^{2+}]_i$-force relationship in the presence of verapamil was not distinguishable from that of control.

5 Nitroglycerin produced a concentration-dependent, reversible contraction of the coronary artery that had been contracted by high KCl-PSS, without reduction of the increased $[Ca^{2+}]_i$.

6 The KCl-induced increase in $[Ca^{2+}]_i$ was not affected by nitroglycerin and in the presence of nitroglycerin it was abolished by 10^{-5} M verapamil suggesting that it was caused by the influx of extracellular Ca^{2+}.

7 The $[Ca^{2+}]_i$-force curve was shifted to the right by nitroglycerin.

8 It is likely that nitroglycerin relaxes the coronary arterial smooth muscle by reducing the amount of myosin light chain phosphorylation even in the presence of raised $[Ca^{2+}]_i$ produced by increased Ca^{2+} influx following depolarization.

Introduction

Although nitroglycerin, the prototypical nitrate, has been used for more than a century, the subcellular mechanism of action of this class of vasodilators has become understood only in the last decade. Nitrates including nitroglycerin increase the amount of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) in smooth muscle tissues (Schultz *et al.*, 1977; Kukovetz *et al.*, 1979) by activating guanylate cyclase (Kimura *et al.*, 1975; Ignarro and Kadowitz, 1985). The activation of cyclic GMP-dependent protein kinase by increased cyclic GMP is thought to be primarily responsible for the vasodilator effect of nitrates (Waldman & Murad, 1987). Although the first intracellular mediator of nitrates has been identified as above, the final step directly responsible for vasodilatation remains to be elucidated. The candidates for this step are membrane hyperpolarization (Ito *et al.*, 1978), inhibition of Ca^{2+} influx (Thorens & Haeusler, 1979; Karaki *et al.*, 1984), increase in Ca^{2+} extrusion (Itoh *et al.*, 1985), Ca^{2+} sequestration (Imai & Kitagawa, 1981; Dubé *et al.*, 1988) and inhibition of the contractile elements (Pfitzer *et al.*, 1984) in vascular smooth muscle. It seems essential to know the effect of nitroglycerin on $[Ca^{2+}]_i$ in order to test these possibilities. We have recently developed a microfluorometric method, using a Ca^{2+} indicator, fura-2 (Grynkiewicz *et al.*, 1985), to measure $[Ca^{2+}]_i$ simultaneously with force in isolated vascular rings. Fluorometric methods using fura-2 have been developed so as to record mechanical activity simultaneously (Ozaki *et al.*, 1987; Bruschi *et al.*, 1988). Using the new method we investigated whether nitroglycerin changes $[Ca^{2+}]_i$ simultaneously with its relaxant effect on isolated

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canine coronary arterial rings. We compared the effects of nitroglycerin with those of verapamil which is known to inhibit Ca^{2+} influx by blocking Ca^{2+} channels opened by depolarization (Godfraind *et al.*, 1986).

Methods

Hearts were excised from mongrel dogs of either sex, weighing 5 to 13 kg, anaesthetized with pentobarbitone sodium (30 mg kg^{-1}, i.v.). Coronary arterial rings (0.5–1.5 mm in diameter, about 1 mm in width) were dissected and connective tissues were carefully removed in a dissecting chamber under a binocular microscope. Endothelium was removed by gentle rubbing and the luminal side was turned outwards. The composition (mmol $litre^{-1}$) of the physiological salt solution (PSS) was as follows: NaCl 140, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2.5, glucose 11.1 and HEPES 3 (pH 7.4). The solution was equilibrated with 100% O_2 at 37°C. High K^+ solution was made by substituting NaCl with equimolar KCl. Ca^{2+}-free solution was made by removing $CaCl_2$ from the PSS. Coronary arterial rings were exposed to 10 μM fura-2 acetoxymethyl ester (fura-2 AM) for about 2 h at 37°C. The noncytotoxic detergent, Pluronic F-127, was premixed in the loading PSS (0.1%) to help solubilize fura-2 AM in PSS (Poenie *et al.*, 1986). After the fura-2 loading, muscles were rinsed with normal PSS for more than 1 h and used for experiments.

Experiments were performed with a fluorometer using the dual wavelength excitation method (CAM-200, Japan Spectroscopic, Tokyo, Japan) specially designed to measure the fluorescence of muscle cells conjugated to the inverted microscope (TMD, Nikon, Tokyo, Japan) (Figure 1a). The excitation light was obtained from a xenon high-pressure lamp (150 W) equipped with a monochrometer. Ultraviolet light of alternating 340 and 380 nm (band width: 10 nm) was obtained by a chopping wheel (100 to 1000 Hz) placed in front of a monochrometer. To minimize the photobleaching of fura-2, the excitation light was reduced by an ND filter to 10% and the preparation was exposed to the excitation light intermittently (usually 2 s excitation and 8 s darkness) (Becker & Fay, 1987). The ratio of the fluoroscence due to excitation at 340 nm (F_{340}) to that at 380 nm (F_{380}) was calculated from successive illumination periods and referred to as the ratio (F_{340}/F_{380}). The fluorescence image, using a Nikon CF UV (Fluor) 10X, was obtained from the smooth muscle cells in the media layer by focusing on them. The muscle ring was placed horizontally in a temperature-controlled 0.4 ml tissue bath which was mounted on the inverted microscope and perfused with PSS at a rate of 4 ml min^{-1} (Figure 1b). The muscle ring was stretched to a resting tension of about 5 mN between two tungsten needles, one of which was glued to a transducer element (AE801, AME, Horten, Norway). The fluorescence signals, F_{340}, F_{380} and their ratio, were sampled and held by using S/H amplifiers (SHM-IC-1, Datel, Tokyo, Japan). The photosignals and the mechanical activity were measured simultaneously and recorded on a chart recorder (Rectihoriz-8k, NEC-San-ei, Tokyo, Japan). The fluorescence signals and tension signal were also digitized by A/D converters and input into a personal computer (PC-9801, NEC, Tokyo, Japan). Lotus 1-2-3 (Lotus Development Co., Tokyo, Japan) was used for the calculation and graphical analysis (Himpens & Somlyo, 1988). Every experiment was started with perfusion with 90 mM KCl-PSS for 10 min. At the end of every experiment, external Ca^{2+} was removed and $[Ca^{2+}]_i$ was minimized by replacing PSS with 10 mM EGTA dissolved in Ca^{2+}-free PSS neutralized with HCl. The minimum ratio was obtained by this procedure. After removing the EGTA solution, 0.3 ml normal PSS containing 1% Triton X-100 was applied, by which procedure the maximum ratio was obtained. After adding 0.3 ml 10 mM EGTA solution, the fluorescence due to fura-2 was abolished by addition of 0.1 ml of 200 mM $MnCl_2$. The remaining fluorescence was regarded as autofluorescence whose intensity and ratio were very similar to that of unloaded muscle. We subtracted the corresponding value due to autofluorescence from F_{340} and F_{380} to derive a recalculated $ratio_{rc}$. Changes in $ratio_{rc}$ and force were expressed by taking the differences between basal values and those of the responses to 90 mM KCl-PSS at 10 min as 100%.

The following drugs and chemicals were used: nitroglycerin (Nippon Kayaku, Tokyo, Japan), verapamil hydrochloride (Eisai, Tokyo, Japan), EGTA (glycoletherdiaminetetraacetic acid), fura-2 AM, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) and Pluronic F-127 (Dojin, Kumamoto, Japan). DMSO (dimethyl sulphoxide) and Triton X-100 (Wako, Tokyo, Japan). Fura-2 AM was dissolved in DMSO at a concentration of 1 mM. Pluronic F-127 was dissolved in 25% w/w in DMSO. Other drugs were dissolved in distilled water in the desired concentrations.

The concentration-effect curves for verapamil and nitroglycerin were expressed as % reduction in the pre-drug values of force of contracture or increased $[Ca^{2+}]_i$ and computer fitted to a logistic equation:

$$E = 100 - 100 \times A^p/(A^p + K^p)$$

where E is normalized response. A is drug concentration, K is EC_{50} value of each drug and p is slope parameter (Parker & Waud, 1971).

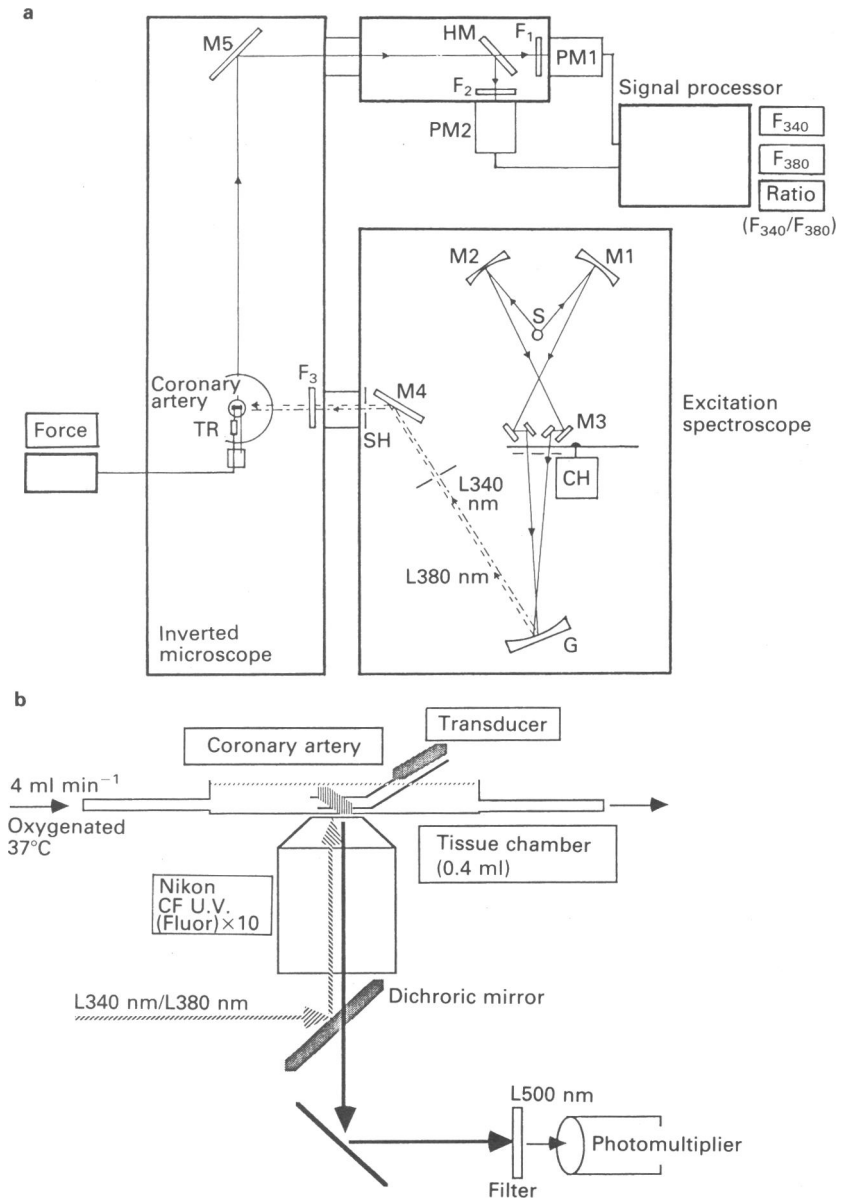


Figure 1 (a) A diagram of a microscopic fluorometer using the dual wavelength excitation method. The excitation light was obtained from a xenon high-pressure lamp (S) equipped with a monochrometer (G). Alternating u.v. lights of 340 and 380 nm (band width: 10 nm) were obtained by the chopping wheel (CH) placed in front of a monochrometer. To minimize photobleaching of fura-2, the excitation light was reduced by an ND filter (F3) to 10% and the preparation was exposed to the excitation light intermittently (electromechanical shutter: SH); 500 nm fluorescent light through a half mirror (HM) and a filter (F1) was obtained by a photomultiplier (PM1). Another photomultiplier (PM2) and a filter (F2) were used when the preparations were loaded with another Ca^{2+} indicator, Indo-1. The fluorescence due to excitation at 340 nm (F_{340}), that at 380 nm (F_{380}) and the ratio (F_{340}/F_{380}) were sampled and held by using S/H amplifiers and continuously recorded on a chart recorder and digitized and input into a personal computer. (b) The fluorescence image, using a Nikon CF U.V. (Fluor) 10X, was obtained from the smooth muscle cells in the media layer by focusing on them. The muscle ring was placed horizontally in a temperature-controlled 0.4 ml tissue bath which was mounted on the inverted microscope and perfused with PSS at a rate of 4 ml min^{-1}.

Table 1 Changes in force of contracture, $ratio_{rc}$ and $[Ca^{2+}]_i$ induced by KCl-PSS in canine coronary artery

KCl (mM)	n	*Force* (mN)	(%)	*Ratio*	$[Ca^{2+}]_i$ (%)
5	23	0	0	0.90 ± 0.05	0
30	11	1.71 ± 0.25	46.8 ± 4.1	1.08 ± 0.07	73.3 ± 6.1
45	9	3.40 ± 0.46	67.8 ± 11.5	1.14 ± 0.12	80.1 ± 15.1
60	12	2.57 ± 0.31	92.8 ± 9.5	1.18 ± 0.10	88.4 ± 8.2
90	23	3.81 ± 0.34	100	1.20 ± 0.06	100

The concentration-effect curves for extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) were also computer fitted to a logistic equation:

$$E = M \times A^p/(A^p + K^p)$$

where E is response normalized by the differences between basal values and those of 90 mM KCl at 10 min, A is $[Ca^{2+}]_o$ concentration, K is EC_{50} value of $[Ca^{2+}]_o$ and p is the slope parameter. EC_{50} values were presented as pD_2 ($pD_2 = -\log EC_{50}$).

Experimental values are given as means or means ± s.e.mean. Statistical significance of differences between mean values was estimated by Student's *t* test. A *t* test for the paired comparison was used when it was applicable. A *P* value smaller than 0.05 was considered to be significant.

Results

Changes in mechanical activity and $[Ca^{2+}]_i$ induced by depolarization with high KCl-PSS in canine coronary artery

In fura-2 unloaded muscles, 90 mM KCl-PSS caused an increase in tension with concomitant slight increases in F_{340} and F_{380}, but the ratio was not changed, because the increases in F_{340} and F_{380} were proportional (Figure 2a). F_{340} and F_{380} increased more slowly than did tension but the maxima of F_{340} and F_{380} occurred at the time when tension reached maximum. Thus, even if movements of muscles influence F_{340} and F_{380}, this movement artifact could be circumvented by taking their ratio (Sato *et al.*, 1988). Basal F_{340} and F_{380} increased 2–4 times higher in fura-2 loaded muscles than in unloaded muscles, and decayed at a rate of 0.1–0.4% per min; 90 mM KCl-PSS elevated tension with a rapid increase in F_{340} but a decrease in F_{380}. Thus, the ratio was increased and the peak of the increment was attained at about 1 min after perfusion with 90 mM KCl-PSS. The ratio remained high during the high KCl-induced contracture which tended to increase with time (Figure 2b). In canine coronary artery, depolarization produced by 20 mM KCl-PSS slightly increased $[Ca^{2+}]_i$ but it produced no contracture. The threshold increase in $[Ca^{2+}]_i$

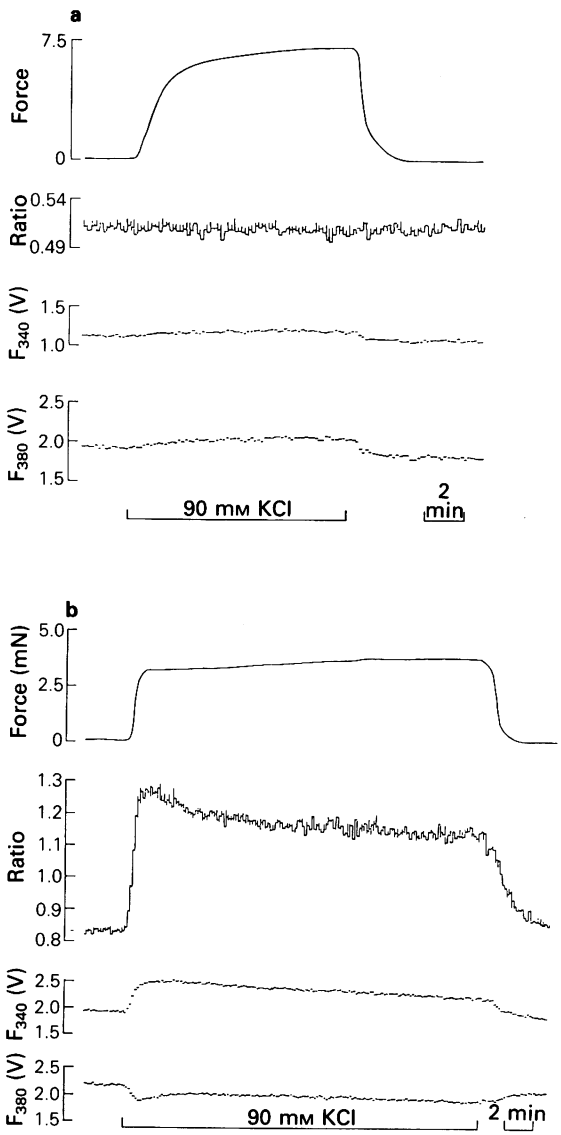


Figure 2 Typical recordings of changes in fluorescence and force development produced by 90 mM KCl measured simultaneously in a coronary arterial muscle without (a) or with (b) fura-2 loading.

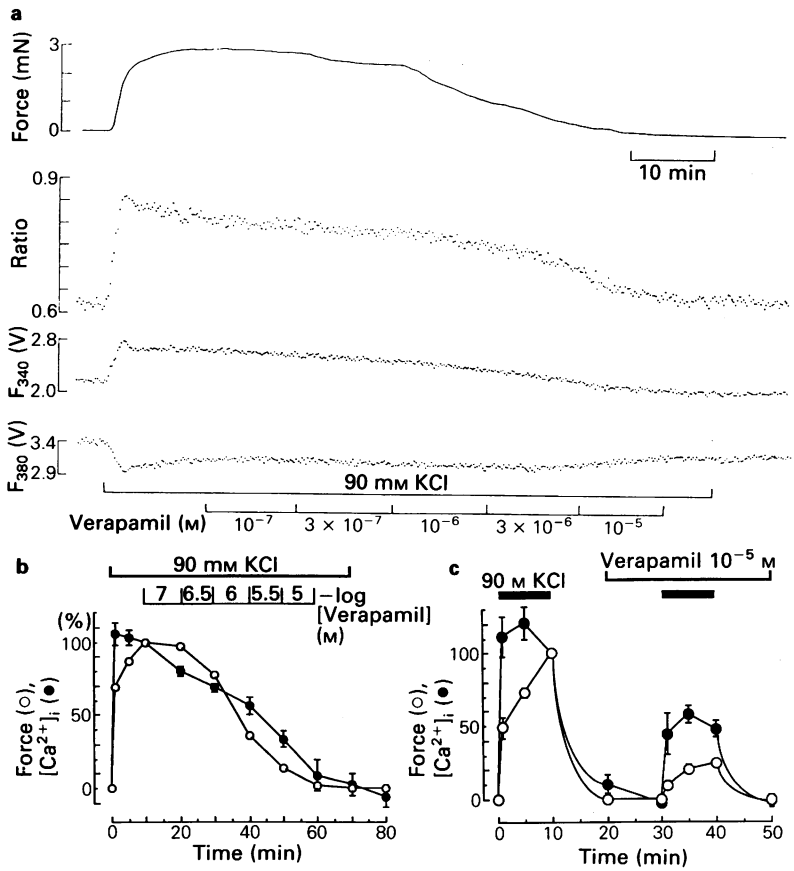


Figure 3 (a) Effects of verapamil (10^{-7}–10^{-5} M) on force and changes in fluorescence produced by 90 mM KCl-PSS in a canine coronary artery. (b) Summarized data which were normalized in relation to the values of 90 mM KCl at 10 min ($n = 6$). (c) Effects of 10^{-5} M verapamil on the basal force and $[Ca^{2+}]_i$ (5 mM KCl-PSS) and 90 mM KCl-induced changes in $[Ca^{2+}]_i$ and force ($n = 5$).

required to generate force was 10–20%. The increase in $[Ca^{2+}]_i$ was less than 10% and the reduction of $[Ca^{2+}]_i$ by reducing $[Ca^{2+}]_o$ in canine coronary artery did not change the force. Table 1 shows changes in $[Ca^{2+}]_i$ and force of contracture produced by 30–90 mM KCl-PSS at 10 min normalized to the effects by 90 mM KCl-PSS.

Effect of verapamil on KCl-induced changes in mechanical activity and $[Ca^{2+}]_i$ in canine coronary artery

Figure 3a shows the effect of verapamil on 90 mM KCl-PSS-induced changes in mechanical activity and $[Ca^{2+}]_i$ signal in canine coronary artery. Verapamil (10^{-7}–10^{-5} M) reduced in a concentration-dependent manner both force and $[Ca^{2+}]_i$ which had been increased by 90 mM KCl-PSS. The results of analysis of the concentration-effect curves for verapamil to reduce the contracture and the increased $[Ca^{2+}]_i$ due to 45 mM KCl-PSS are summarized in Table 2. The pD_2 ($-\log EC_{50}$) values of verapamil producing both effects were not different at one concentration of KCl, although the slope parameters of both curves were different. Interestingly, these values of verapamil were also not different between 45 mM and 90 mM KCl in spite of the difference of the degree of membrane depolarization. When the muscle was exposed to 10^{-6} M verapamil, the basal $[Ca^{2+}]_i$ tended to decrease, although the basal force was not changed. However the slight reduction of $[Ca^{2+}]_i$ by verapamil could not be distinguished from the gradual decline of $[Ca^{2+}]_i$ following a previous challenge. The increased $[Ca^{2+}]_i$ and force of contracture due to 90 mM KCl-PSS were decreased by 10^{-6} M verapamil to 47.8 ± 4.3% and

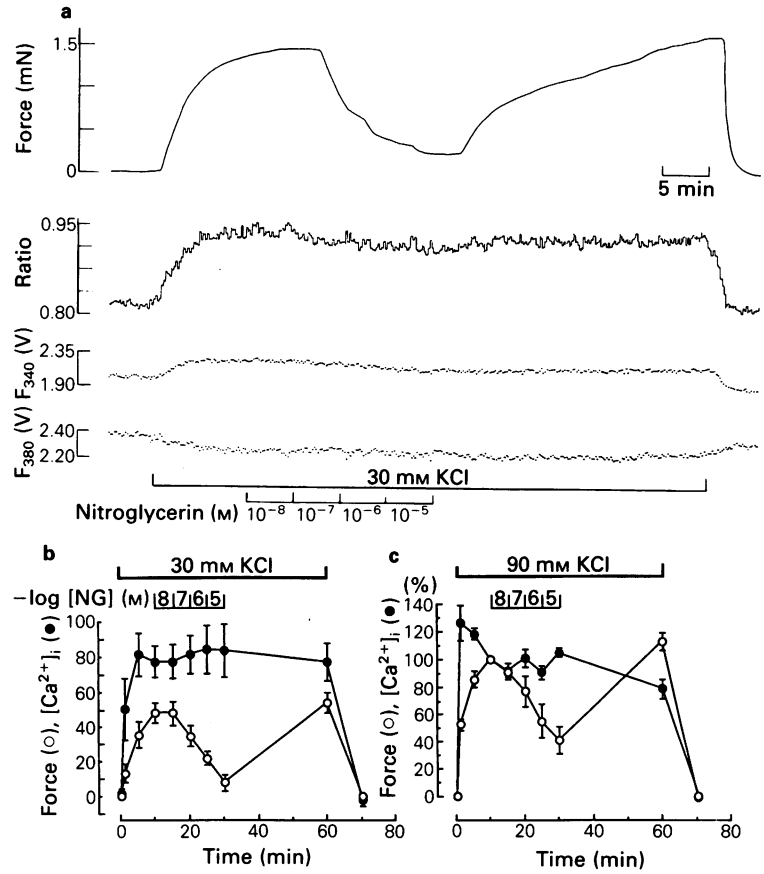


Figure 4 (a) Effects of nitroglycerin (NG) (10^{-8}–10^{-5} M) on the 30 mM KCl-PSS induced changes in $[Ca^{2+}]_i$ and force. The effects of nitroglycerin could be reversibly washed out by 30 min perfusion with 30 mM KCl-PSS. (b) Summarized data obtained from 6 experiments with 30 mM KCl-PSS. (c) Summarized data obtained from 5 experiments with 90 mM KCl-PSS.

Table 2 Effects of verapamil and nitroglycerin in reducing the force of contracture and the increase in $[Ca^{2+}]_i$ induced by KCl

		Reduction of force		*Reduction of* $[Ca^{2+}]_i$	
KCl (mM)	n	pD_2	*Slope parameter* (*p*)	pD_2	*Slope parameter* (*p*)
Verapamil					
45	5	6.26 ± 0.06	1.45 ± 0.15	6.34 ± 0.24	0.87 ± 0.10
90	5	6.15 ± 0.08	1.47 ± 0.18	5.99 ± 0.16	0.72 ± 0.28
Nitroglycerin					
30	6	6.25 ± 0.19	0.65 ± 0.06	No effect ($<10^{-5}$ M)	
60	6	5.54 ± 0.10*	0.62 ± 0.09	No effect ($<10^{-5}$ M)	
90	5	5.35 ± 0.17*	0.54 ± 0.06	No effect ($<10^{-5}$ M)	

The concentration-effect curves for verapamil and nitroglycerin were analysed by computer fitting to a logistic equation: $E = 100 - 100 \times A^p/(A^p + K^p)$ where E is normalized response, A is drug concentration, K is EC_{50} value of each drug and p is the slope parameter. EC_{50} values were presented as pD_2 ($pD_2 = -\log EC_{50}$). * $P < 0.05$ compared with 30 mM KCl.

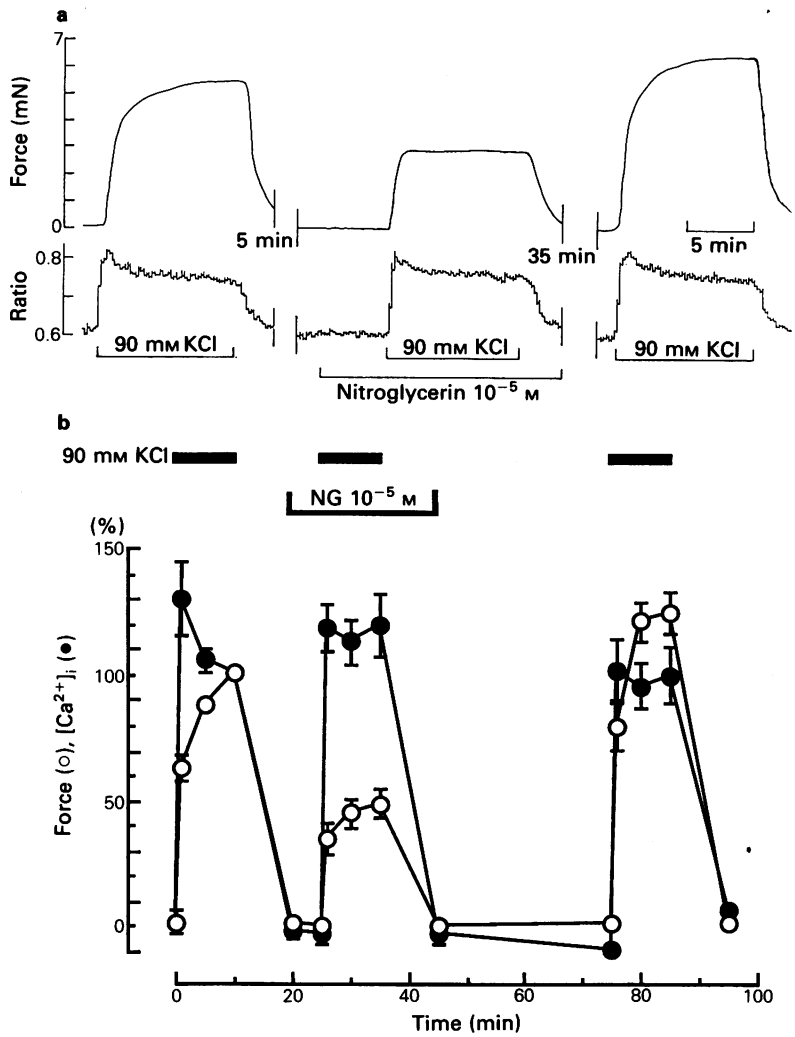


Figure 5 (a) Effects of 10^{-5} M nitroglycerin (NG) on the basal force and $[Ca^{2+}]_i$ (5 mM KCl-PSS) and 90 mM KCl-PSS induced changes in $[Ca^{2+}]_i$ and force. The effect of nitroglycerin on force of contracture was washed out by perfusion with normal PSS. (b) Summarized data obtained from 7 experiments.

23.8 ± 7.9%, respectively ($n = 5$) (Figure 3b). Similar reductions were produced by 10^{-6} M verapamil of the contracture previously induced by 90 mM KCl-PSS (Figure 3c).

Effect of nitroglycerin on KCl-induced changes in mechanical activity and $[Ca^{2+}]_i$ in canine coronary artery

Figure 4a shows the effect of nitroglycerin on force of contracture and fluorescence signals from fura-2 induced by 30 mM KCl-PSS in a canine coronary artery. Nitroglycerin (10^{-8}–10^{-5} M) reduced the force of contracture in a concentration-dependent manner, but did not affect $[Ca^{2+}]_i$ at all. The effect of nitroglycerin on the force of contracture could be reversibly washed out by perfusion with 30 mM KCl-PSS. Even during the washout of nitroglycerin $[Ca^{2+}]_i$ did not change at all. These results indicate that, in canine coronary artery, nitroglycerin relaxes the KCl-PSS-induced contracture without reducing $[Ca^{2+}]_i$. Summarized data of force and $[Ca^{2+}]_i$ are

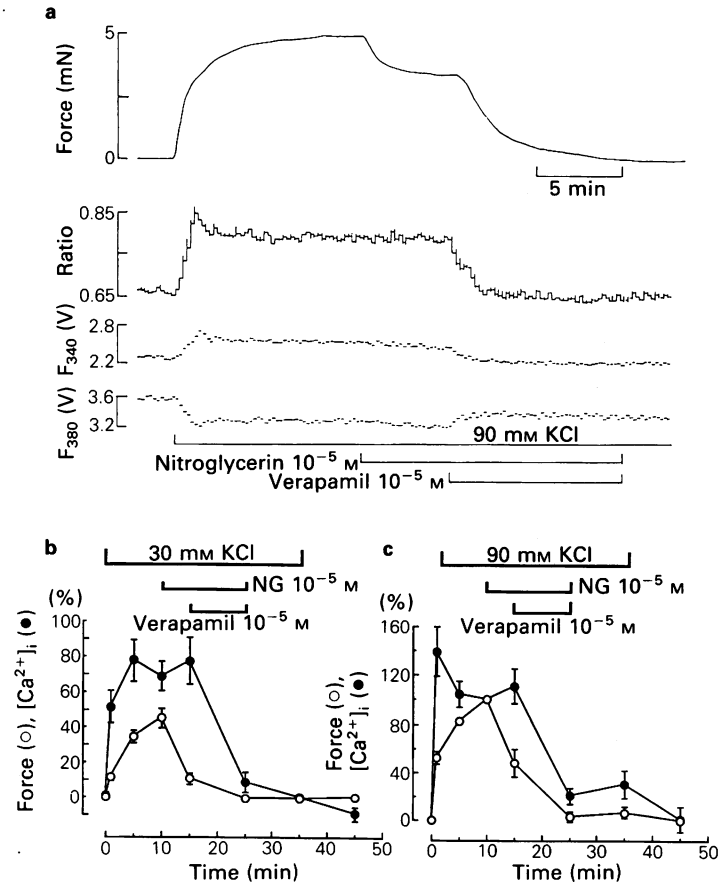


Figure 6 (a) Effects of 10^{-5} M nitroglycerin (NG) and 10^{-5} M verapamil on changes in $[Ca^{2+}]_i$ and force of contracture produced by 90 mM KCl-PSS. The $[Ca^{2+}]_i$ which remained increased in the presence of nitroglycerin was reduced to the basal level by the application of 10^{-5} M verapamil. (b) Summarized data obtained from 5 experiments with 30 mM KCl-PSS. (c) Summarized data obtained from 6 experiments with 90 mM KCl-PSS.

shown in Figure 4b (30 mM KCl-PSS) and 4c (90 mM KCl-PSS) and analysed data of the concentration-response curves for nitroglycerin to reduce the force of contracture are also shown in Table 2. The potency of nitroglycerin in causing relaxation was dependent on KCl concentrations, e.g., the pD_2 values of nitroglycerin became greater when the muscle was depolarized to a smaller extent or when increases in $[Ca^{2+}]_i$ in muscles were smaller. Figure 5 shows the effect of nitroglycerin on the basal force and $[Ca^{2+}]_i$ and the force of contracture and the increased $[Ca^{2+}]_i$ due to 90 mM KCl-PSS. Nitroglycerin (10^{-5} M) did not change basal $[Ca^{2+}]_i$. Although it reduced the force of contracture induced by 90 mM KCl-PSS, the increased $[Ca^{2+}]_i$ was not affected at all. The reduction in force produced by nitroglycerin could also be reversibly washed out by perfusion with normal PSS.

Effect of verapamil on the increase in $[Ca^{2+}]_i$ induced by high KCl-PSS in the presence of nitroglycerin in canine coronary artery

The results showing that nitroglycerin relaxed the coronary artery contracted by KCl without changing $[Ca^{2+}]_i$ were so interesting that we examined whether the increased $[Ca^{2+}]_i$ due to high KCl-PSS which remained unaffected by nitroglycerin is susceptible to verapamil, as was the case with the absence of nitroglycerin. Figure 6 shows such an experiment and summarized data for 30 mM or

90 mM KCl-PSS. Without changing $[Ca^{2+}]_i$, nitroglycerin (10^{-5} M) reduced the force of contracture induced by 30 mM and 90 mM KCl-PSS to 22.4 ± 6.3% and 47.9 ± 11.6% (force produced by 30 mM or 90 mM KCl-PSS at 10 min = 100%), respectively. Perfusion with KCl-PSS containing both 10^{-5} M nitroglycerin and 10^{-5} M verapamil abolished the remaining force and the increased $[Ca^{2+}]_i$. Thus, the result that $[Ca^{2+}]_i$ remained increased in the presence of nitroglycerin but was reduced by verapamil almost to the basal level indicates that the increased $[Ca^{2+}]_i$ was derived from the influx of Ca^{2+} through Ca^{2+} channels opened by KCl-PSS-induced depolarization and that nitroglycerin can relax the coronary artery without reducing $[Ca^{2+}]_i$.

Effect of nitroglycerin on extracellular $[Ca^{2+}]_o$-induced changes in mechanical activity and $[Ca^{2+}]_i$ in depolarized coronary artery

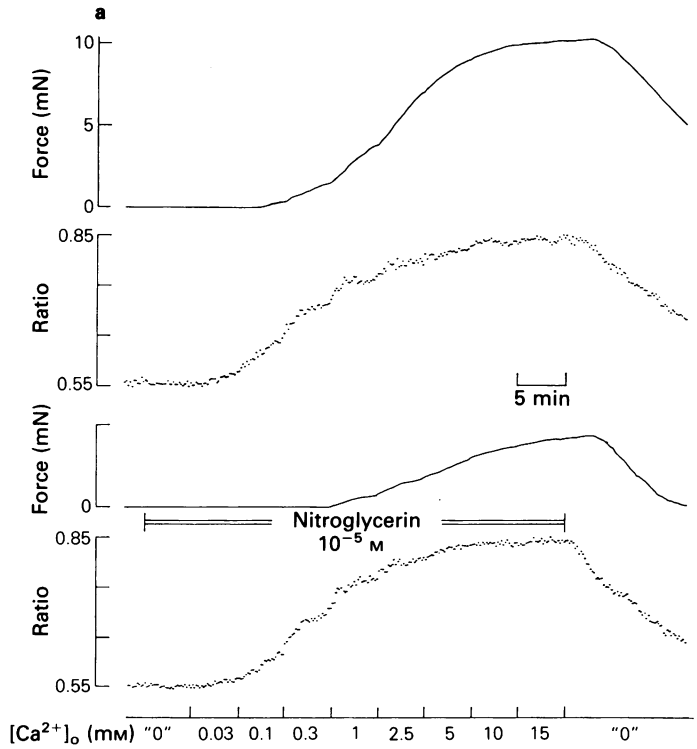
As described in the previous section, the relaxant potency of nitroglycerin was dependent on KCl concentrations, e.g., the pD_2 values of nitroglycerin were greater when the muscle was depolarized to a smaller extent or when the increases in $[Ca^{2+}]_i$ in muscles were smaller (Table 2). To investigate whether the degree of depolarization or the increase in $[Ca^{2+}]_i$ influences the relaxant potency of nitroglycerin, coronary muscle was at first perfused with Ca^{2+}-free PSS for 10 min and then with Ca-free 90 mM KCl-PSS. Removing extracellular Ca^{2+} produced a reduction of $[Ca^{2+}]_i$ (−27 ± 2%), whereas the force was not changed. After removal of extracellular Ca^{2+} by perfusion with Ca^{2+}-free PSS, depolarization of the muscle by 90 mM KCl failed to produce any increase in $[Ca^{2+}]_i$ (−31 ± 2%) or any change in force. In the presence of 90 mM KCl-PSS, the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) was increased from 0.03 to 15 mM. As $[Ca^{2+}]_o$ was increased, $[Ca^{2+}]_i$ was also increased from 0.03 mM $[Ca^{2+}]_o$ and force was generated at 0.1 mM $[Ca^{2+}]_o$; both $[Ca^{2+}]_i$ and force became saturated at 15 mM $[Ca^{2+}]_o$ (Figure 7). The relation between $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ shows that of $[Ca^{2+}]_o$ and Ca^{2+} influx through voltage-sensitive Ca^{2+} channels in vascular smooth muscle. The results of computer fitting of the curve to the logistic equation are summarized in Table 3. The maximum is the difference between values at $[Ca^{2+}]_o$-free and 15 mM $[Ca^{2+}]_o$. The pD_2 values for $[Ca^{2+}]_o$ of 3.19 ± 0.13 indicate the apparent K_D values of $[Ca^{2+}]_o$ for the binding constant of Ca^{2+} channel opened by depolarization. The relationship between $[Ca^{2+}]_o$ and force of contracture was also analysed and is summarized in Table 3. The maximum force produced by KCl depolarization was 225 ± 44% and pD_2 values for $[Ca^{2+}]_o$ were 2.73 ± 0.05 and the slope parameter was 1.17 ± 0.04. The difference in pD_2 values for $[Ca^{2+}]_o$ between increase in $[Ca^{2+}]_i$ and increase in force seemed to be due to the steepness of the curve of $[Ca^{2+}]_i$-force relationship (Figure 8).

When nitroglycerin 10^{-5} M was applied to coronary arterial muscle perfused with Ca^{2+}-free 90 mM KCl-PSS, $[Ca^{2+}]_i$ was not changed. Although increases in $[Ca^{2+}]_o$ produced the same increases in $[Ca^{2+}]_i$, the force produced by the increased $[Ca^{2+}]_i$ was much smaller than that of control and the threshold values of $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ to generate force were increased (Figure 7). The pD_2 values in the presence of nitroglycerin 10^{-5} M, the maximum force and the pD_2 values for $[Ca^{2+}]_o$ were significantly reduced. Since the relationship between $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ was not changed by nitroglycerin, in the 90 mM KCl-PSS the membrane potential may have not been changed by nitroglycerin. Figure 7 also shows the relaxant effect of nitroglycerin was larger in low concentrations of $[Ca^{2+}]_o$ than in high concentrations. The effect of nitroglycerin was reversibly washed out and the muscle responded to an increase in $[Ca^{2+}]_o$ in 90 mM KCl-PSS as in the control (Figure 7 and Table 3). Thus, these results clearly show that nitroglycerin does not affect Ca^{2+} influx through Ca^{2+} channels, that its relaxant effect is operative without

Table 3 Effects of nitroglycerin (NG) on the $[Ca^{2+}]_o$-force and $[Ca^{2+}]_o$-$[Ca^{2+}]_i$ relationship in 90 mM KCl-depolarized canine coronary artery

	Increase in force			*Increase in $[Ca^{2+}]_i$*		
	Max (%)	*pD_2*	*p (Slope parameter)*	*Max* (%)	*pD_2*	*p (Slope parameter)*
Control	225 ± 44	2.73 ± 0.05	1.17 ± 0.04	190 ± 37	3.19 ± 0.13	0.83 ± 0.19
NG 10^{-5} M	155 ± 32*	2.32 ± 0.04*	1.19 ± 0.04	189 ± 29	3.23 ± 0.09	1.00 ± 0.16
Washout	251 ± 61	2.83 ± 0.01	1.12 ± 0.01	174 ± 30	3.17 ± 0.10	0.81 ± 0.22

Increase in $[Ca^{2+}]_i$ was calculated from the difference between that obtained by Ca^{2+}-free 90 mM KCl-PSS and the maximum value of $[Ca^{2+}]_i$ in each experiment (usually at 15 mM $[Ca^{2+}]_o$). * $P < 0.05$ compared with control values.



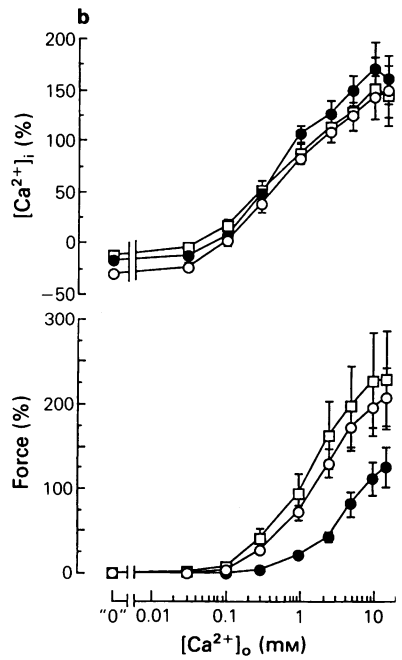


Figure 7 (a) Changes in $[Ca^{2+}]_i$ and force produced by increasing $[Ca^{2+}]_o$ in canine coronary arterial muscle depolarized 90 mM KCl-PSS and the effects of nitroglycerin on these changes. (b) Summarized data obtained from 6 experiments. Control (○), in the presence of 10^{-5} M nitroglycerin (NG) (●), washout (□).

reducing $[Ca^{2+}]_i$ and that the relaxant potency of nitroglycerin is dependent on $[Ca^{2+}]_i$ but not on the degree of depolarization.

Effects of nitroglycerin and verapamil on the relationship between $[Ca^{2+}]_i$ and force in depolarized canine coronary artery

Figure 8 shows the relationship between $[Ca^{2+}]_i$ and force in the absence and the presence of nitroglycerin (10^{-5} M) or verapamil in depolarized canine coronary artery. The relationship between $[Ca^{2+}]_i$ and force obtained by changing the degree of depolarization by 30, 45, 60 and 90 mM KCl (Table 1) was similar to that produced by changing $[Ca^{2+}]_o$ in 90 mM KCl-depolarized muscles (control). The curve in the presence of verapamil (10^{-7}–10^{-5} M), which was constructed from the data obtained from 45 and 90 mM KCl-depolarized coronary muscles (Figure 3b), was not different from that in the absence of the drugs (control). The curve in the presence of nitroglycerin (10^{-5} M) was different from that of control and it was shifted to the right.

Discussion

In the present study, we were able to measure changes in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) simultaneously with force in isolated canine coronary arterial rings. Measurement of $[Ca^{2+}]_i$ in smooth muscle cells has been successful with quin-2 (Kobayashi *et al.*, 1985; Williams & Fay, 1986). Simultaneous measurement of $[Ca^{2+}]_i$ with force has been performed in vascular smooth muscle by use of fura-2 (Ozaki *et al.*, 1987; Bruschi *et al.*, 1988). In the present study we measured $[Ca^{2+}]_i$ microfluorometrically using the dual wavelength excitation method. There are several advantages to this method (Yagi *et al.*, 1988). First, we were able to observe smooth muscle cells through a microscope and obtain the fluorescence signals from the cells by focusing on them. Second, the simultaneous measurement of $[Ca^{2+}]_i$ with force allowed us to determine the precise relationship between $[Ca^{2+}]_i$ and force in the same coronary artery. Third, in the present system, the reduction of fluorescence signal due to photobleaching of fura-2 (Becker & Fay, 1987) was minimized by the attenuation of excitation intensity and by giving the excitation only while collecting data. This enabled us to run the same muscle for a long period, e.g., we were able to examine the washout effect of nitroglycerin. Fourth, the fluorescence signals were not disturbed by the extracellular leakage of fura-2, which was removed, if any occurred, by continuous perfusion of the tissue bath. Although we were unable to determine absolute values of $[Ca^{2+}]_i$ because of a relatively large amount of autofluorescence (about 25–50% in fura-2 loaded preparations) and uncertainty of absolute concentrations of fura-2 in the smooth muscle, the semiquantification of $[Ca^{2+}]_i$ could be performed by % changes in ratio, normalized by the difference between the basal value and the 90 mM KCl-induced value.

In canine coronary artery, depolarization induced by 30–90 mM KCl-PSS produced KCl concentration dependent increases in $[Ca^{2+}]_i$ and force. The threshold increase in $[Ca^{2+}]_i$ to generate force was 10–20%. An increase in $[Ca^{2+}]_i$ of less than 10% and reduction of $[Ca^{2+}]_i$ by reducing $[Ca^{2+}]_o$ in canine coronary artery did not change the force. These results indicate that the basal force is due to the passive force generated by the elastic component of tissue but not due to the active force generated by smooth muscle, and that there is an influx of Ca^{2+} into smooth muscle cells by its electrochemical gradient even in the resting condition. The relationship between $[Ca^{2+}]_i$ and force obtained by changing the degree of depolarization by 30, 45, 60 and 90 mM KCl (Table 1) was similar to that obtained by changing $[Ca^{2+}]_o$ in 90 mM KCl-depolarized muscles. The increase in $[Ca^{2+}]_i$ produced by depolarization seems to be entirely due to the increased influx of Ca^{2+} through voltage-dependent Ca^{2+} channels, since depolarization of smooth muscle which had been perfused with Ca^{2+}-free PSS failed to induce any increase in $[Ca^{2+}]_i$ and the increased $[Ca^{2+}]_i$ was reduced to the basal level by 10^{-5} M verapamil which is known to block the voltage-dependent Ca^{2+} channels (Godfraind *et al.*, 1986). This result is different from that obtained in cultured smooth muscle cells of rat aorta in which depolarization induced release of Ca^{2+} from an intracellular storage site (Kobayashi *et al.*, 1985), and from that in smooth muscle of guinea-pig aorta in which ryanodine reduced $[Ca^{2+}]_o$-induced contraction related to KCl-depolarization (Ito *et al.*, 1986). Although in aortic smooth muscle cells, KCl-depolarization may have released Ca^{2+} from its storage sites, the KCl-depolarization-induced contraction has been commonly thought to be the result of influx of extracellular Ca^{2+} and not the release of sequestered Ca^{2+} (Kuriyama *et al.*, 1982).

Verapamil (10^{-7}–10^{-5} M) reduced the increased force and $[Ca^{2+}]_i$ due to 45 or 90 mM KCl-PSS in a concentration-dependent manner. Although the pD_2 values for verapamil to reduce $[Ca^{2+}]_i$ and force were not different from each other at each KCl concentration, the slope parameter (p) for reduction of $[Ca^{2+}]_i$ was smaller than that at force. This result seems to be due to the steepness of $[Ca^{2+}]_i$-force relationship observed in various skinned smooth

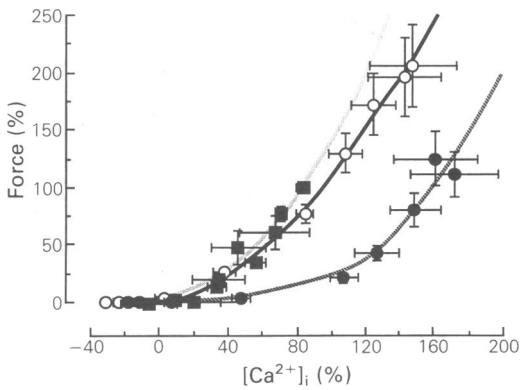


Figure 8 Relationship between $[Ca^{2+}]_i$ (%) and force (%) in the absence (control, ○), and in the presence of 10^{-5} M nitroglycerin (●) or 10^{-8}–10^{-5} M verapamil (■). Each point in the absence and presence of nitroglycerin was adopted from Figure 7(b) and that in the presence of verapamil from the experiments with 45 and 90 mM KCl. Vertical and horizontal bars are s.e.mean. Curves are 3 order polynomial least squares regression fits ($Y = a_1X + a_2X^2 + a_3X^3 + b$). The curve in the presence of verapamil was not different from the control, whereas that in the presence of nitroglycerin was distinctly different from the control.

muscle preparations (Iino, 1981 for guinea-pig taenia coli; Itoh *et al.*, 1983; 1985 for rabbit mesenteric artery). When the pD_2 values were compared among different KCl concentrations, those obtained from 45 mM KCl-depolarized muscles were slightly but not significantly larger than those from 90 mM KCl. In the present study, the $[Ca^{2+}]_i$-force relationship in the presence of verapamil was not distinguishable from that of control. Thus, the relaxant effect of verapamil was entirely due to its well-known Ca^{2+} channel blocking effect (Godfraind *et al.*, 1986), without any influence on Ca^{2+} sensitivity of myofibrils.

Unlike verapamil, nitroglycerin relaxed coronary artery contracted by high KCl-PSS without reducing the increased $[Ca^{2+}]_i$. The relaxant effect of nitroglycerin was concentration-dependent and reversible (Figure 4). In the presence of nitroglycerin, $[Ca^{2+}]_i$ was not changed at all, whereas the increased $[Ca^{2+}]_i$ was reduced by 10^{-5} M verapamil to the basal level (Figure 6). Thus, the increased $[Ca^{2+}]_i$ which was unaffected in the presence of nitroglycerin was due to the influx of extracellular Ca^{2+}. Nitroglycerin failed to affect the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels (Figure 7). Thus, it is highly likely that nitroglycerin interfered with the genesis of force by $[Ca^{2+}]_i$.

Nitrates including nitroglycerin increase the amount of cyclic GMP in various vascular smooth muscle tissues (Schultz *et al.*, 1977; Kukovetz *et al.*, 1979) by activating guanylate cyclase (Kimura *et al.*, 1975; Ignarro & Kadowitz, 1985). The activation of cyclic GMP-dependent protein kinase is thought to be responsible for the vasodilator effect of nitrates (Waldman & Murad, 1987). It has been suggested that the final mechanism of action of organic nitrates for vasodilatation is hyperpolarization of the membrane (Ito *et al.*, 1978), inhibition of Ca^{2+} influx (Thorens & Haeusler, 1979; Karaki *et al.*, 1984), increase in Ca^{2+} extrusion (Itoh *et al.*, 1985), Ca^{2+} sequestration (Imai & Kitagawa, 1981; Dubé *et al.*, 1988) or inhibition of contractile elements (Pfitzer *et al.*, 1984). When the canine coronary arterial muscle was exposed to KCl-PSS, the depolarized membrane potential was not affected by nitroglycerin (Ito *et al.*, 1980). Thus, it is unlikely that hyperpolarization is responsible for the vasodilator effect of nitroglycerin in canine coronary artery. Although in aortic smooth muscle cells nitrates (Kobayashi *et al.*, 1985; Karaki *et al.*, 1988) and 8-bromo cyclic GMP (Kai *et al.*, 1987) reduce $[Ca^{2+}]_i$ by stimulation of Ca^{2+} extrusion via the Ca pump through activation of cyclic GMP-dependent protein kinase (Popescu *et al.*, 1985; Furukawa *et al.*, 1988), in KCl-induced depolarized canine coronary artery, $[Ca^{2+}]_i$ measured by fura-2 was not changed at all by nitroglycerin in all experiments while the relaxation was observed. Although the Ca^{2+} releasable storage sites, probably the sarcoplasmic reticulum (SR), may be developed in rat aorta and its cultured cells, in canine coronary artery they may be sparse. Thus, the vasodilator mechanism must be considered to be due to $[Ca^{2+}]_i$-independent mechanisms. There are several lines of evidence to support this supposition. In rat aorta, sodium nitroprusside relaxes KCl-induced contraction accompanied by a small decrease in $[Ca^{2+}]_i$ and a small decrease in ^{45}Ca uptake (Karaki *et al.*, 1988). In skinned porcine coronary artery, cyclic GMP inhibits contraction at intermediate $[Ca^{2+}]_i$ concentrations (Pfitzer *et al.*, 1984; Itoh *et al.*, 1985). The amount of myosin light chain phosphorylation was reduced by sodium nitroprusside and 8-bromo cyclic GMP (Rapoport *et al.*, 1982; Draznin *et al.*, 1986). The phosporylation of myosin light chain which is activated by its kinase via Ca^{2+} and calmodulin complex is correlated with the amount of tension (Kamm & Stull, 1985). Thus, it is likely that nitroglycerin relaxes smooth muscle cells by reducing the amount of myosin light chain phosphorylation even in the presence of increased $[Ca^{2+}]_i$ following Ca^{2+} influx by depolarization.

The relaxant potency of nitroglycerin is known to be reduced by higher concentrations of KCl (see Ito *et al.*, 1980; Itoh *et al.*, 1983) (Table 2). This phenomenon seems to be due to the relationship between $[Ca^{2+}]_i$ and force in the presence of nitroglycerin

being shifted to the right (Figure 8). The rightward shift of $[Ca^{2+}]_i$-tension relationship by cyclic GMP has also been reported (Pfitzer *et al.*, 1984; Itoh *et al.*, 1985). The inhibitory effect of nitroglycerin on myosin light chain phosphorylation may be counteracted by the increase in $[Ca^{2+}]_i$.

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Effects of endothelin on the mechanical activity and cytosolic calcium levels of various types of smooth muscle

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1 Effects of porcine/human endothelin (endothelin-1), a novel vasoconstrictor peptide, on various smooth muscles were examined.

2 In rat aorta, endothelin (1 pM–30 nM) induced contraction in a concentration-dependent manner. Removal of endothelium shifted the concentration-response curve to the left. When added during the sustained contraction induced by 0.1 μM noradrenaline, endothelin (1 nM) induced a relaxation that was inhibited by removing endothelium or by methylene blue.

3 In rat aorta without endothelium, endothelin (1–30 nM) increased cytosolic Ca^{2+} level ($[Ca^{2+}]_{cyt}$) followed by contraction. Endothelin induced less contraction than high K^+ at a given $[Ca^{2+}]_{cyt}$ when the concentration of endothelin was lower (1–3 nM) and/or during the early phase of the contraction (< 10 min). In contrast, endothelin induced a greater contraction than KCl after prolonged exposure to high concentrations (> 10 nM).

4 The increase in $[Ca^{2+}]_{cyt}$ due to endothelin was strongly inhibited by 10 μM verapamil or 0.3 μM nicardipine although muscle contraction was only partially inhibited.

5 In Ca^{2+}-free solution, endothelin (30 nM) induced a transient increase in $[Ca^{2+}]_{cyt}$ and a slow increase in muscle tension. After a prolonged incubation in Ca^{2+}-free solution, endothelin (30 nM) still induced a slow increase in tension without changing $[Ca^{2+}]_{cyt}$. This contraction was inhibited by 1 μM sodium nitroprusside or 10 μM forskolin.

6 In canine trachea and guinea-pig uterus, endothelin (30 nM) induced sustained contraction with an increase in $[Ca^{2+}]_{cyt}$. In the absence of external Ca^{2+}, endothelin (30 nM) induced a sustained contraction in canine trachea without changing $[Ca^{2+}]_{cyt}$. In guinea-pig vas deferens, taenia caeci and ileal longitudinal muscle, endothelin induced small increases in $[Ca^{2+}]_{cyt}$ and tension.

7 In permeabilized smooth muscles, endothelin (30 nM) did not change the muscle tone.

8 These results suggest that endothelin acts on the endothelium and increases the synthesis or release of endothelium-derived relaxing factor (EDRF). These results also suggest that endothelin acts directly on smooth muscle and increases $[Ca^{2+}]_{cyt}$ by releasing Ca^{2+} from storage sites and increasing Ca^{2+} influx through the verapamil- and 1,4-dihydropyridine-sensitive pathway. Endothelin seems to decrease Ca^{2+}-sensitivity of contractile elements at lower concentrations and/or during the early phase of the contraction, whereas it increases Ca^{2+}-sensitivity at higher concentrations during the sustained phase of the contraction. Furthermore, endothelin induces a contraction that is not dependent on $[Ca^{2+}]_{cyt}$.

Introduction

The supernatant of cultured endothelial cells has been shown to cause contraction in vascular smooth muscles (Hickey *et al.*, 1985; Gillespie *et al.*, 1986; O'Brien *et al.*, 1987). Recently, a novel and potent vasoconstrictor peptide, endothelin, has been isolated from the culture supernatant of porcine aortic endothelial cells and its amino acid sequence has been determined (Yanagisawa *et al.*, 1988). Endothelin induces contraction in a variety of isolated vascular smooth muscles at picomolar concentrations

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(Auquet *et al.*, 1988; Yanagisawa *et al.*, 1988). Since the endothelin-induced contraction is reduced by a Ca^{2+} channel blocker (Yanagisawa *et al.*, 1988) and endothelin increases the cytosolic Ca^{2+} level ($[Ca^{2+}]_{cyt}$) in cultured vascular smooth muscle cells (Hirata *et al.*, 1988), it is suggested that its vasoconstrictor effect is due to an increased Ca^{2+} influx through the activation of voltage-dependent Ca^{2+} channels. On the other hand, endothelin induces contraction even in the absence of extracellular Ca^{2+} in rat aorta (Auquet *et al.*, 1988).

In order to improve our understanding of the contractile effect of endothelin, we have measured $[Ca^{2+}]_{cyt}$ using the fura-2 method (Ozaki *et al.*, 1987; Sato *et al.*, 1988a) and correlated it with the mechanical activity in isolated vascular smooth muscle tissue of rat aorta and also in non-vascular smooth muscle tissues, such as canine trachea, guinea-pig uterus, vas deferens, taenia caecum and ileum.

Methods

Tissue preparation and solutions

Male Wistar rats (250–300 g) were killed by a blow on the neck. The thoracic aorta was isolated, cut into a rectangular strip 2 mm wide and 7 mm long and placed in normal physiological salt solution (see below). In some strips, endothelium was removed by gently rubbing the intimal surface with a finger moistened with normal solution. Taenia caecum, ileal longitudinal smooth muscle and vas deferens were isolated from male guinea-pigs (250–300 g) and cut into strips 1 mm wide and 5 mm long. Female guinea-pigs were given a s.c. injection of oestradiol ($0.1\ mg\ kg^{-1}$) in order to get rid of possible effects of changes in hormonal state. Twenty hours later, the animal was killed and uterine longitudinal muscle was cut into a strip 1 mm wide and 5 mm long. Male mongrel dogs were killed by a rapid infusion of sodium pentobarbitone ($50\ mg\ kg^{-1}$) and the trachea was excised. The smooth muscle layer was dissected free of epithelium, cartilage and connective tissue, and cut into a strip 1 mm wide and 5 mm long. The mesenteric artery was also isolated from dog and a spiral strip 0.5 mm wide and 3 mm long was prepared.

Normal physiological salt solution contained (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 23.8, ethylenediamine tetraacetic acid (EDTA) 0.01 and glucose 5.5. High K^+ (65.4 mM) solution was made by substituting NaCl with equimolar KCl. Ca^{2+}-free solution was made by removing $CaCl_2$ from normal solution and adding ethyleneglycol *bis*(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (0.5 mM). These solutions were saturated with 95% O_2 and 5% CO_2 mixture at 37°C and pH 7.4. Muscle strips were suspended in a 20 ml volume organ bath and contractions were recorded isometrically with a strain gauge transducer (Nihon-Koden, Tokyo, Japan).

Measurement of $[Ca^{2+}]_{cyt}$

$[Ca^{2+}]_{cyt}$ was measured with a fluorescent Ca^{2+}-indicator fura-2 (Grynkiewicz *et al.*, 1985). In these experiments, $NaHCO_3$ in the normal solution was replaced with 20 mM N-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES) and this solution was saturated with 100% O_2 at 37°C and pH 7.4. Muscle strips were treated with acetoxymethyl ester of fura-2 (fura-2/AM) (5 μM) for 3–5 h at room temperature. The non-cytotoxic detergent, cremophor EL (0.02%), was also added to increase the solubility of fura-2/AM. Experiments were performed with a fluorimeter designed to measure both the fura-2-Ca^{2+} fluorescence and contractile tension of muscle tissues (CAF-100, Japan Spectroscopic, Tokyo, Japan) as described previously (Ozaki *et al.*, 1987; Sato *et al.*, 1988a). The muscle strip was held horizontally in a 7 ml volume organ bath at 37°C. One end of the muscle strip was connected to a strain gauge transducer (Toyo Boldwin, Tokyo, Japan). A part of the muscle was excited by light obtained from xenon high pressure lamp (75 W) equipped with rotating filter wheel (48 Hz) which contained 340 nm (±10 nm) and 380 nm (±10 nm) interference filters. The ratio of the fluorescence due to excitation at 340 nm (F_{340}) to that at 380 nm (F_{380}) was calculated from successive illumination periods. The emitted light from the muscle strip was collected by a photomultiplier through a 500 nm (±20.0 nm) filter. The time constant of the optical channels was 0.25 s.

Absolute Ca^{2+} concentration was not calculated in these experiments because a large part of the intracellular fura-2 binds to soluble proteins and the dissociation constant of fura-2 for Ca^{2+} (Konishi *et al.*, 1988) and fluorescent characteristics of fura-2 (Abe & Karaki, 1989) may be changed from that obtained *in vitro*. Instead, the ratio of F_{340} to F_{380} was used as an indicator of relative $[Ca^{2+}]_{cyt}$, as has been reported previously (Ozaki *et al.*, 1987; 1988; Himpens & Somlyo, 1988; Sato *et al.*, 1988a).

Preparation of permeabilized muscle

Permeabilization of smooth muscle membrane was performed according to the method described by Sparrow *et al.* (1981). A thin bundle of guinea-pig taenia or canine mesenteric artery was soaked for 30 min in a solution containing 20 mM imidazole (pH 7.4), 5 mM EGTA, 50 mM KCl and 150 mM

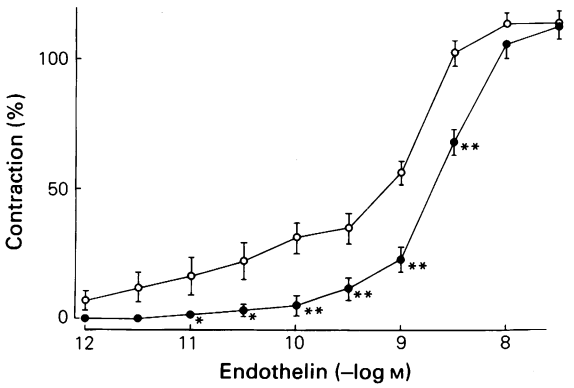


Figure 1 Effect of cumulative addition of endothelin on muscle tension in rat aorta with (●) or without endothelium (○). Each point represents the mean of 5 experiments and s.e.mean is shown by vertical bar; 100% represents the magnitude of contraction induced by 65.4 mM KCl. The magnitude of the high K^+-induced contraction was not affected by removal of endothelium. * and ** indicate values significantly different from the values in the strip without endothelium with $P < 0.05$ and $P < 0.01$, respectively. Concentration of endothelin is shown as −log M.

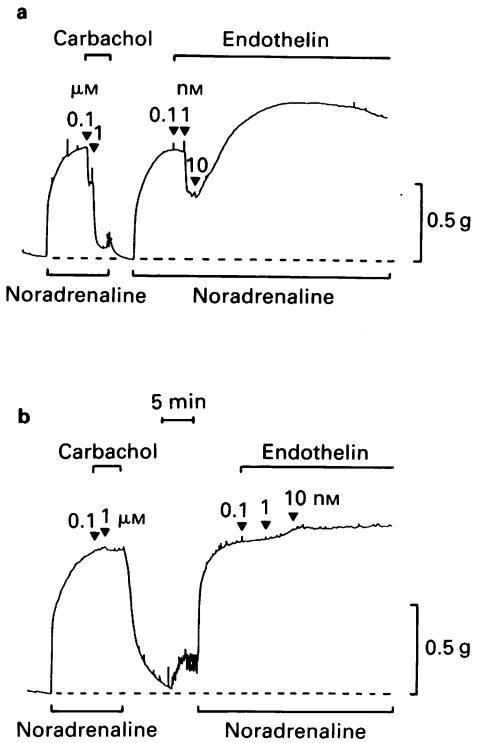


Figure 2 Effect of carbachol and endothelin on noradrenaline-induced contraction in rat aorta with (a) or without endothelium (b). Carbachol (0.1 and 1 μM) or endothelin (0.1, 1 and 10 nM) was added during the sustained contraction induced by 0.1 μM noradrenaline.

sucrose. Then, 1% (v/v) Triton X-100 and 0.5 mM dithioerythritol were added for 30 min at 4°C. After rinsing for 15 min in the solution without Triton X-100, muscle bundles were stored in 20 mM imidazole (pH 7.4), 4 mM EGTA, 10 mM $MgCl_2$, 7.5 mM adenosine triphosphate (ATP), 1 mM NaN_3 and 0.5 mM dithioerythritol with 50% glycerol at −20°C for up to 10 days. Relaxing solution contained 20 mM imidazole, 50 mM KCl, 8 mM $MgCl_2$, 5 mM ATP, 1 mM NaN_3, 0.2 μM calmodulin, 1 mM creatine phosphate, 10 u ml^{-1} phosphocreatine kinase and 2 mM EGTA at pH 6.8 and 20–24°C. Ca^{2+} concentrations were changed by adding an appropriate amount of $CaCl_2$. The apparent binding constant of EGTA for Ca^{2+} was considered to be 1×10^{-6} M at pH 6.8 and 20°C (Harafuji & Ogawa, 1980).

Chemicals

Porcine/human endothelin (endothelin-1) was purchased from Peptide Institute Inc., Osaka, Japan. Other drugs used were sodium nitroprusside, (−)-noradrenaline bitartrate, methylene blue trihydrate (Wako Pure Chemicals, Tokyo, Japan), verapamil hydrochloride, nicardipine (Sigma Chemicals, St. Louis, MO, U.S.A.), forskolin (Hoechst Japan, Tokyo, Japan), cremophor EL (Nakarai Chemicals, Kyoto, Japan), HEPES, EGTA, EDTA, fura-2/AM (Dojindo Laboratories, Kumamoto, Japan), creatine phosphate and phosphocreatine kinase (Boehringer Mannheim, F.R.G.). Calmodulin was isolated from swine testes as described by Walsh *et al.* (1983).

Statistics

Results of the experiments are expressed as mean ± s.e.mean. Values were considered to be significantly different when P value was less than 0.05 by use of Student's t test.

Results

Effects of endothelin on rat aorta without or with endothelium

In rat isolated aorta with endothelium, cumulative addition of endothelin induced contractions in a concentration-dependent manner and the concentration required to induce 50% contraction (EC_{50}) was calculated to be 2.16 nM (95% confidence limits: 1.91–2.29 nM, $n = 5$) (Figure 1). The concentration-

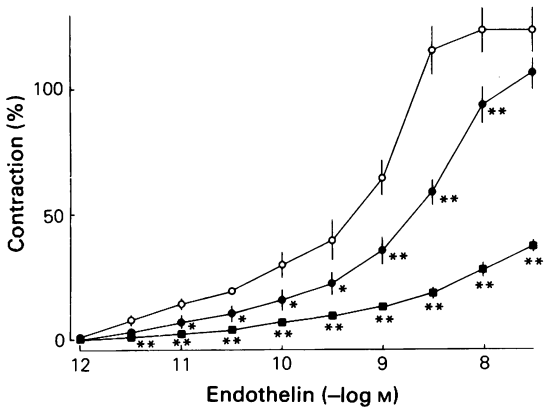


Figure 3 Effect of verapamil and Ca^{2+}-depletion on endothelin-induced contractions in rat aorta without endothelium. Tissues were pretreated with 10 μM verapamil or Ca^{2+}-free solution (with 0.5 mM EGTA) for 5 min and endothelin was cumulatively added. Each point represents the mean of 4 experiments and s.e.mean is shown by vertical bar; 100% represents the magnitude of contraction induced by 65.4 mM KCl. (○) Control; (●) with verapamil; (■) in Ca^{2+}-free solution. * and ** indicate values significantly different from the values in the strip without endothelium with $P < 0.05$ and $P < 0.01$, respectively. Concentration of endothelin is shown as −log M.

response curve for endothelin was shifted to the left and the EC_{50} was decreased to 0.99 nM (0.89–1.07 nM, $n = 5$) by removing the endothelium from the aorta. In the aorta with endothelium, cumulative application of carbachol (0.1 and 1 μM) during the sustained contraction induced by 0.1 μM noradrenaline inhibited the contraction by 14.2 ± 7.0% and 95.8 ± 2.5% ($n = 4$), respectively (Figure 2a). In this preparation, endothelin (1 nM) induced relaxation (by 44.7 ± 14.1%, $n = 4$) whereas a higher concentration (10 nM) induced contraction. In the aorta without endothelium, endothelin (1 nM) did not relax but rather potentiated the noradrenaline-induced contraction ($n = 4$) (Figure 2b). Endothelin-induced relaxation was completely inhibited by a 15 min pretreatment of the muscle with 10 μM methylene blue ($n = 4$).

Effects of verapamil and Ca^{2+}-depletion on endothelin-induced contraction in rat aorta without endothelium

Pretreatment of the muscle with 10 μM verapamil only partially inhibited the cumulative concentration-response curve for endothelin (Figure 3). In Ca^{2+}-free solution (with 0.5 mM EGTA), cumulative addition of endothelin (1 pM–30 nM) induced graded contractions (Figure 3).

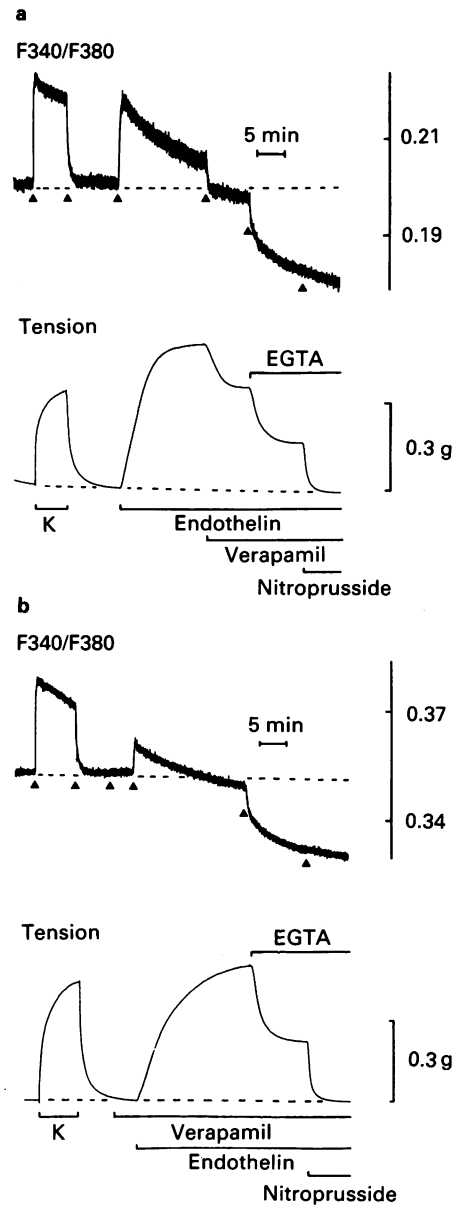


Figure 4 Effect of endothelin on $[Ca^{2+}]_{cyt}$ (indicated by F_{340}/F_{380}; upper trace) and muscle tension (lower trace) in rat aorta without endothelium. (a) After the effects of 72.7 mM KCl were examined, 30 nM endothelin was added. When the contractile tension reached a steady level, 10 μM verapamil, 4 mM EGTA and 1 μM sodium nitroprusside were sequentially added. (b) Endothelin (30 nM) was added 5 min after the addition of 10 μM verapamil. When the contractile tension reached a steady level, 4 mM EGTA and 1 μM sodium nitroprusside were sequentially added.

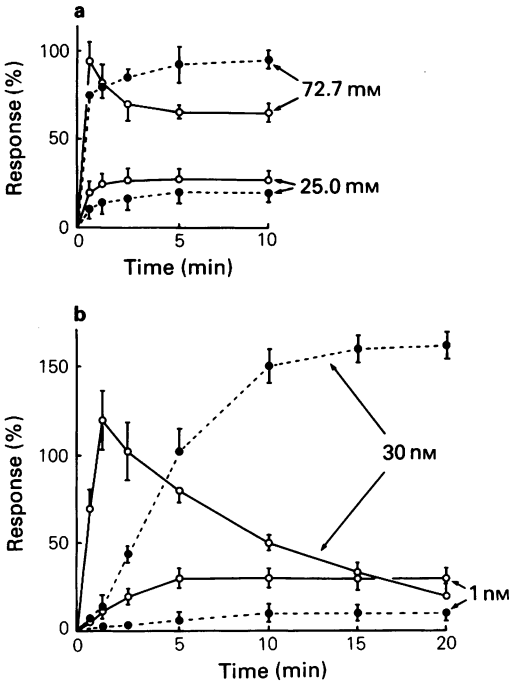


Figure 5 Time course of the changes in $[Ca^{2+}]_{cyt}$ (indicated by F_{340}/F_{380}; upper trace) and muscle tension (lower trace) in the presence of 25.0 and 72.7 mM KCl (a) or 1 and 30 nM endothelin (b) in rat aorta without endothelium. Each point represents the mean of 4 experiments and s.e.mean is shown by a vertical bar. 100% represents the levels of muscle tension and $[Ca^{2+}]_{cyt}$ stimulated by 72.7 mM KCl which was applied before starting the experiments, and 0% represents the resting levels. (○) F_{340}/F_{380}; (●) muscle tension.

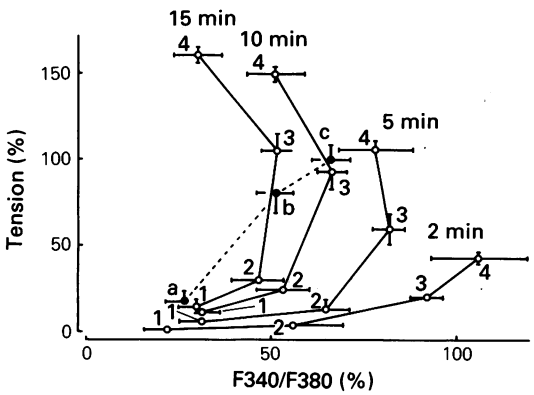


Figure 6 The time-dependent changes in the $[Ca^{2+}]_{cyt}$ (indicated by F_{340}/F_{380})-tension relationship in the presence of various concentrations of endothelin in rat aorta without endothelium. Experiments were done as shown in Figure 5. Muscle tension and $[Ca^{2+}]_{cyt}$ at 2, 5, 10 and 15 min after the addition of endothelin was plotted. Concentration of endothelin (○) is shown by numbers (1) 1 nM; (2) 3 nM; (3) 10 nM and (4) 30 nM. $[Ca^{2+}]_{cyt}$-tension relationship for high K^+ obtained after a 10 min incubation is also shown (●). Concentration of KCl is shown by letters: (a) 25 mM; (b) 40 mM; (c) 72.7 mM.

Effects of endothelin on $[Ca^{2+}]_{cyt}$ and tension in rat aorta without endothelium

In fura-2-loaded rat aorta, high K^+ (25.0–72.7 mM) increased $[Ca^{2+}]_{cyt}$ which was followed by an increase in muscle tension (Figure 4 and 5a). Addition of 1–30 nM endothelin also increased $[Ca^{2+}]_{cyt}$ and contractile tension of the vascular strip (Figure 4a and 5b). Although the rate of rise of $[Ca^{2+}]_{cyt}$ due to 30 nM endothelin was almost identical to that induced by 72.7 mM KCl, the rate of rise of the endothelin-induced contraction was slower (Figure 5).

Figure 6 shows the $[Ca^{2+}]_{cyt}$-tension relationship obtained by single applications of endothelin (1–30 nM) or high K^+ (25.0–72.7 mM). There was a positive correlation between $[Ca^{2+}]_{cyt}$ and tension measured 10 min after the application of KCl, as has been reported (Sato *et al.*, 1988a). In contrast to this, $[Ca^{2+}]_{cyt}$ increased with little effect on muscle tension 2 min after the application of endothelin. Although muscle tension increased gradually with time thereafter, $[Ca^{2+}]_{cyt}$ did not increase but rather decreased. Fifteen min after the application, the contractions induced by 1 to 3 nM endothelin were still smaller than that induced by KCl at a given $[Ca^{2+}]_{cyt}$ whereas 10 to 30 nM endothelin induced a greater contraction than that due to KCl at a given $[Ca^{2+}]_{cyt}$.

The endothelin-stimulated $[Ca^{2+}]_{cyt}$ was completely inhibited by 10 μM verapamil, although this inhibitor only partially suppressed the endothelin-induced contraction (Figure 4a). Nicardipine (0.3 μM) showed an effect similar to verapamil ($n = 4$). Subsequent addition of 4 mM EGTA decreased $[Ca^{2+}]_{cyt}$ to the level below the resting level although the muscle tension was only partially inhibited.

Figure 4b shows the effect of endothelin on the aorta pretreated with 10 μM verapamil. Endothelin (30 nM) induced only a transient increase in $[Ca^{2+}]_{cyt}$ but induced a sustained contraction with a slow rate of rise. Addition of EGTA decreased $[Ca^{2+}]_{cyt}$ and partially inhibited contraction.

Figure 7 shows the effect of endothelin in the absence of external Ca^{2+}. After the muscle strip was treated with 72.7 mM K^+ for 5 min, the external solution was replaced with Ca^{2+}-free solution containing 0.5 mM EGTA. The Ca^{2+} removal rapidly decreased

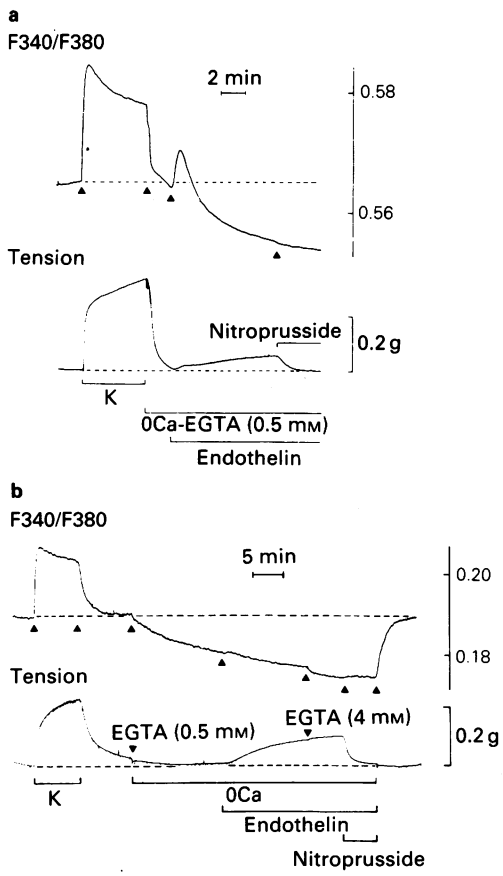


Figure 7 Effect of endothelin on $[Ca^{2+}]_{cyt}$ (indicated by F_{340}/F_{380}; upper trace) and muscle tension (lower trace) in rat aorta without endothelium in the absence of external Ca^{2+}. (a) After the addition of 72.7 mM KCl, external solution was changed to Ca^{2+}-free solution (with 0.5 mM EGTA). After $[Ca^{2+}]_{cyt}$ decreased to the resting level, 30 nM endothelin was added. When the contractile tension reached a steady level, 1 μM nitroprusside was added. (b) After a 15 min incubation with Ca^{2+}-free solution (with 0.5 mM EGTA), 30 nM endothelin was added. When the contractile tension reached a steady level, 4 mM EGTA and 1 μM nitroprusside were sequentially added.

$[Ca^{2+}]_{cyt}$ which was accompanied by a decrease in muscle tension. After $[Ca^{2+}]_{cyt}$ reached a resting level, endothelin (30 nM) was added and a transient increase in the $[Ca^{2+}]_{cyt}$ was induced. In contrast, endothelin induced a slow and sustained contraction in Ca^{2+}-free solution (Figure 7a). After incubation for approx. 15 min with Ca^{2+}-free solution, endothelin induced a sustained contraction without changing $[Ca^{2+}]_{cyt}$, as shown in Figure 7b. Addition of 4 mM EGTA further decreased $[Ca^{2+}]_{cyt}$ but had no effect on the contraction. The contraction remaining

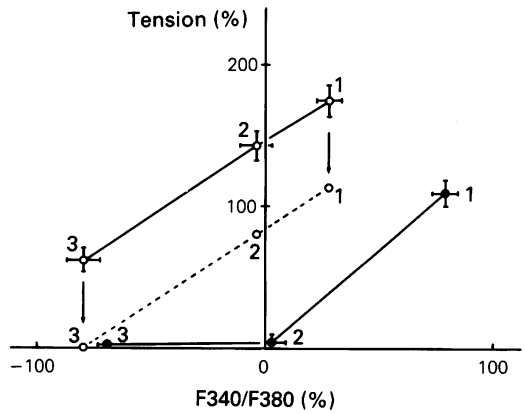


Figure 8 $[Ca^{2+}]_{cyt}$ (indicated by F_{340}/F_{380})-tension relationship in the presence of endothelin (15 min or longer incubation) and KCl in rat aorta without endothelium. Experiments were done as shown in Figure 4a; 100% represents the levels of muscle tension and $[Ca^{2+}]_{cyt}$ stimulated by 72.7 mM KCl which were measured before starting the experiments, and 0% represents the resting levels. Each point represents the mean of 6 experiments and s.e.mean is shown by vertical bar. (1) Control; (2) with 10 μM verapamil; (3) with 10 μM verapamil and 4 mM EGTA. (○) Endothelin 30 nM; (●) KCl 72.7 mM. Dotted line indicates the endothelin-response after subtracting the Ca^{2+}-independent contraction from the control contraction.

in Ca^{2+}-free solution was inhibited by 1 μM sodium nitroprusside (Figure 4 and 7) or by 10 μM forskolin ($n = 2$). Washing the muscle with normal PSS increased $[Ca^{2+}]_{cyt}$ to the resting level without changing the muscle tension (Figure 7b).

The effects of verapamil and Ca^{2+}-depletion on the $[Ca^{2+}]_{cyt}$-tension relationship in the KCl- or endothelin-stimulated aorta are summarized in Figure 8. This figure indicates that high K^+-stimulated $[Ca^{2+}]_{cyt}$ and muscle tension were completely inhibited by verapamil. Further, the steady level of the endothelin-stimulated $[Ca^{2+}]_{cyt}$, but not muscle tension (obtained with 15 min or longer incubation), was completely inhibited by verapamil. Addition of EGTA decreased the KCl- or endothelin-stimulated $[Ca^{2+}]_{cyt}$ to similar levels although a part of the endothelin-induced contraction was resistant to EGTA. Thus, the $[Ca^{2+}]_{cyt}$-tension curve for steady state effects of 30 nM endothelin was located at lower $[Ca^{2+}]_{cyt}$ levels than that due to KCl.

Effects of endothelin on non-vascular smooth muscles

In fura-2-loaded canine trachea, high K^+ (72.7 mM) rapidly increased $[Ca^{2+}]_{cyt}$ which was followed by an increase in muscle tension. Addition of 30 nM endothelin increased both tension and $[Ca^{2+}]_{cyt}$

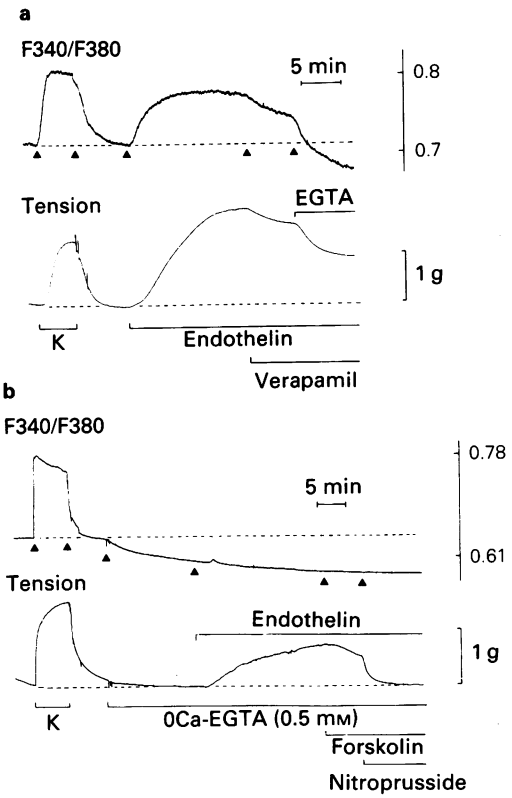


Figure 9 Effect of endothelin on $[Ca^{2+}]_{cyt}$ (indicated by F_{340}/F_{380}; upper trace) and muscle tension (lower trace) in dog trachea in the presence or absence of external Ca^{2+}. (a) After the effects of 72.7 mM KCl were examined, 30 nM endothelin was added. When the contractile tension reached a steady level, 10 μM verapamil and 4 mM EGTA were sequentially added. (b) After a 15 min incubation with Ca^{2+}-free solution (with 0.5 mM EGTA), 30 nM endothelin was added. When the contractile tension reached a steady level, 10 μM forskolin and 1 μM nitroprusside were sequentially added.

(Figure 9a). In two out of five tracheal strips, increase in muscle tension preceded the increase in $[Ca^{2+}]_{cyt}$ by approximately 30 s. The increase in $[Ca^{2+}]_{cyt}$ and contraction induced by endothelin were inhibited by 10 μM verapamil although the rates of decrease of $[Ca^{2+}]_{cyt}$ and muscle tension in trachea were markedly slower than those in rat aorta. Subsequent addition of 4 mM EGTA decreased $[Ca^{2+}]_{cyt}$ below the resting level, but only slightly inhibited the contraction. In Ca^{2+}-free solution, high K^+ changed neither contraction nor $[Ca^{2+}]_{cyt}$ ($n = 6$). In contrast, endothelin (30 nM) induced a slow increase in muscle tension without changing $[Ca^{2+}]_{cyt}$ (Figure 9b).

Figure 10 shows the effects of endothelin on $[Ca^{2+}]_{cyt}$ and muscle tension in fura-2-loaded guinea-pig uterus (a), vas deferens (b) and ileal longitudinal muscle (c). In the uterus, high K^+ (43.9 mM) rapidly increased $[Ca^{2+}]_{cyt}$ followed by an increase in muscle tension. Addition of 30 nM endothelin increased $[Ca^{2+}]_{cyt}$ and spike-like changes were superimposed on the tension curve (Figure 10a). Verapamil (10 μM) suppressed the spike-like activity but not the sustained levels of $[Ca^{2+}]_{cyt}$ and tension. Addition of 4 mM EGTA decreased $[Ca^{2+}]_{cyt}$ below the resting level and decreased muscle tension to the resting level (Figure 10b). In vas deferens, high K^+ (140 mM) induced a transient increase followed by a sustained increase in $[Ca^{2+}]_{cyt}$. Addition of 30 nM endothelin induced only a slight increase in $[Ca^{2+}]_{cyt}$ and tension. Verapamil (10 μM) inhibited the increase in $[Ca^{2+}]_{cyt}$ and partially inhibited the muscle tension (Figure 10b). In guinea-pig ileal muscle, high K^+ (43.9 mM) increased $[Ca^{2+}]_{cyt}$ and tension. Endothelin (30 nM) slightly increased $[Ca^{2+}]_{cyt}$ and tension. Verapamil (10 μM) and EGTA (4 mM) decreased the endothelin-stimulated $[Ca^{2+}]_{cyt}$ and muscle tension, as shown in Figure 10c. In guinea-pig taenia caecum, endothelin (30 nM) induced small increase in $[Ca^{2+}]_{cyt}$ and muscle tension (less than 10% of the high K^+-induced responses; $n = 4$).

Permeabilized muscles

Since endothelin induced a contraction without increase in $[Ca^{2+}]_{cyt}$ in Ca^{2+}-free solution, we examined the effect of this compound on permeabilized muscles. Both in guinea-pig taenia and canine mesenteric artery pretreated with Triton X-100, cumulative addition of Ca^{2+} (0.3–100 μM) induced graded contraction in the presence of ATP ($n = 2$ each). Endothelin, at a concentration of 30 nM, did not induce contraction in the absence of Ca^{2+} (with 2 mM EGTA) and in the presence of ATP ($n = 2$ each).

Discussion

The present experiments demonstrated that porcine/human endothelin shows a contractile effect in vascular and non-vascular smooth muscles. Endothelin-induced contraction in rat aorta was potentiated by removal of vascular endothelium. Endothelin transiently relaxed the noradrenaline-induced contraction and this effect was inhibited by removal of the endothelium or by methylene blue, procedures known to inhibit EDRF-mediated relaxation (Holzmann, 1982; Furchgott, 1984). From these results, it is suggested that endothelin acts on the endothelium, increases the synthesis or release of EDRF which attenuates the contractile response to endothelin itself.

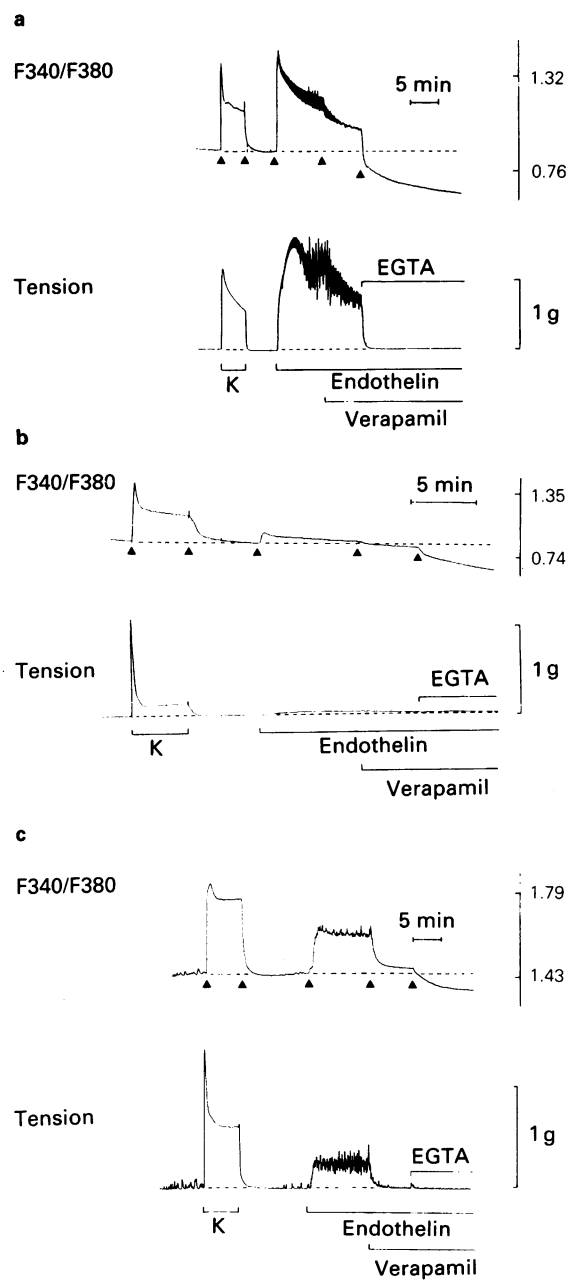


Figure 10 Effect of endothelin on $[Ca^{2+}]_{cyt}$ (indicated by F_{340}/F_{380}; upper trace) and muscle tension (lower trace) in guinea-pig uterus (a), vas deferens (b) and ileal longitudinal muscle (c). After the effects of KCl (43.9 mM for uterus and ileum and 140 mM for vas deferens) were examined, 30 nM endothelin was added. When the contractile tension reached a steady level, 10 μM verapamil and 4 mM EGTA were sequentially added.

In rat aorta, endothelin increased $[Ca^{2+}]_{cyt}$ and muscle tension. In the presence of low concentrations of endothelin and/or shortly after the addition of endothelin, however, endothelin induced a smaller contraction than KCl at a given $[Ca^{2+}]_{cyt}$. These results suggest that although the contractile effect of endothelin is basically attributable to the increase in $[Ca^{2+}]_{cyt}$, endothelin seems to decrease the Ca^{2+}-sensitivity of contractile elements at low concentrations and/or during the early phase of the contraction.

In the absence of external Ca^{2+}, endothelin induced a transient increase in $[Ca^{2+}]_{cyt}$ indicating that endothelin releases Ca^{2+} from intracellular storage site(s) as does noradrenaline (Sato *et al.*, 1988a,b). In cultured vascular smooth muscle cells, endothelin also releases Ca^{2+} from intracellular storage sites (Miasiro *et al.*, 1988). However, endothelin did not induce or induced only a small initial contraction in Ca^{2+}-free solution. Under similar experimental conditions, noradrenaline and caffeine induced a transient increase in $[Ca^{2+}]_{cyt}$ which was followed by a transient contraction (Sato *et al.*, 1988b). These results support the suggestion that endothelin decreases Ca^{2+}-sensitivity of the contractile elements during the early phase of the contraction.

In contrast, after a prolonged exposure to high concentrations of endothelin, endothelin-stimulated $[Ca^{2+}]_{cyt}$ gradually decreased whereas muscle tension increased. This endothelin-induced sustained increase in $[Ca^{2+}]_{cyt}$ was strongly inhibited by the Ca^{2+} channel blockers, verapamil and nicardipine, whereas, endothelin-induced sustained contraction was only partially inhibited by these agents. From these results it is suggested that endothelin increases Ca^{2+} influx through the opening of the Ca^{2+} channel, although this mechanism is only partly responsible for the endothelin-induced contraction in rat aorta. The Ca^{2+} channel blocker-insensitive portion of this contraction seems to be supported by the resting $[Ca^{2+}]_{cyt}$. In contrast to the results in aorta, verapamil only partially inhibited the endothelin-stimulated $[Ca^{2+}]_{cyt}$ in guinea-pig uterus and dog trachea suggesting that endothelin may stimulate different pathways for Ca^{2+} entry in different smooth muscles.

Dissociation between $[Ca^{2+}]_{cyt}$ and muscle tension was also observed in the presence of EGTA. EGTA decreased $[Ca^{2+}]_{cyt}$ to the level below the resting $[Ca^{2+}]_{cyt}$ but the contraction was not totally inhibited. In dog trachea, furthermore, we sometimes observed that endothelin induced a contraction before the increase in $[Ca^{2+}]_{cyt}$. Thus, a part of the endothelin-induced contraction may be attributable to a Ca^{2+}-independent mechanism. In order to evaluate the contribution of the endothelin-induced,

Ca^{2+}-independent contraction to the total contraction in rat aorta, $[Ca^{2+}]_{cyt}$-tension curve was corrected for the Ca^{2+}-independent portion, as shown in Figure 6 (dotted line). This figure indicates that endothelin still induces a greater contraction than KCl at a given $[Ca^{2+}]_{cyt}$ after subtracting the Ca^{2+}-independent portion from the total contraction. These results suggest that, at higher concentrations and after a prolonged exposure period, endothelin induces a contraction by three mechanisms of action: (1) an increase in $[Ca^{2+}]_{cyt}$; (2) an increase in Ca^{2+}-sensitivity of contractile elements; and (3) stimulation of a Ca^{2+}-independent mechanism.

The Ca^{2+}-independent portion of the endothelin-induced contraction does not seem to be due to direct activation by endothelin of contractile elements since endothelin failed to induce contraction in permeabilized smooth muscles. This contraction was inhibited by sodium nitroprusside and forskolin. We have suggested that sodium nitroprusside (Karaki *et al.*, 1988b) and forskolin (Abe & Karaki, 1989) inhibit contractile elements in smooth muscle. Both cyclic AMP- and GMP-dependent protein kinases phosphorylate myosin light chain kinase to inhibit the activity of this kinase (Adelstein *et al.*, 1978; Nishikawa *et al.*, 1984) and thus decreases the activity of contractile elements (Pfitzer *et al.*, 1985; 1986). These results suggest that the endothelin-induced Ca^{2+}-independent contraction is due to the mechanism which is inhibited by the cyclic AMP- and cyclic GMP-dependent protein phosphorylation.

Receptor agonists such as noradrenaline induce contraction by the release of Ca^{2+} from storage sites and also by increasing Ca^{2+} influx (Karaki & Weiss, 1984). It has been reported that noradrenaline in rat aorta (Karaki *et al.*, 1988a; Sato *et al.*, 1988b), carbachol in bovine trachea (Takuwa *et al.*, 1987) and phenylephrine in ferret vascular smooth muscles (Morgan & Morgan, 1984; DeFeo & Morgan, 1985) induces greater contraction than high K^+ at a given $[Ca^{2+}]_{cyt}$, possibly by the increase in Ca^{2+}-sensitivity of contractile elements. In addition, noradrenaline and prostaglandin $F_{2\alpha}$ induce sustained contraction without changing $[Ca^{2+}]_{cyt}$ in Ca^{2+}-free solution (Karaki *et al.*, 1989). The similarities between the actions of endothelin and these receptor agonists may indicate that endothelin has a similar mechanism of action to these receptor agonists.

After submission of this manuscript, de Nucci *et al.* (1988) reported that endothelin increased perfusion pressure in mesenteric artery which was potentiated by the removal of endothelium or in the presence of methylene blue or oxyhemoglobin. This result is consistent with the present findings. Furthermore, endothelin was shown to stimulate the breakdown of phosphatidylinositol in cultured vascular smooth muscle cells (Van Renterghem *et al.*, 1988; Resink *et al.*, 1988; 1989; Marsden *et al.*, 1989; Sugiura *et al.*, 1989). In vascular smooth muscle, receptor agonists, such as noradrenaline, angiotensin II, vasopressin and 5-hydroxytriptamine, induce breakdown of phosphatidylinositol (Abdel-Latif, 1986). These results support the suggestion that endothelin induces smooth muscle contractions by mechanisms similar to those activated by these receptor agonists.

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Caffeine-induced inhibition of calcium channel current in cultured smooth muscle cells from pregnant rat myometrium

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1 The inhibitory effect of caffeine on the calcium channel current was investigated in cultured myometrial cells isolated from pregnant rats.

2 Caffeine inhibited the calcium channel current elicited from a holding potential of −70 mV in a concentration-dependent manner. The IC_{50} was estimated to be 35 mM.

3 The caffeine inhibition was not enhanced when calcium channels were opened by a conditioning depolarizing pulse sequence or when the number of inactivated calcium channels was increased at depolarized holding potentials.

4 Caffeine antagonized the specific binding of (+)-[^{3}H]-isradipine to myometrial membranes. The IC_{50} value found in binding experiments was similar to the IC_{50} value for half-maximal inhibition of calcium channel current. Caffeine decreased the maximal binding capacity of (+)-[^{3}H]-isradipine to myometrial membranes without any significant change in the dissociation constant.

5 The results indicate that caffeine interacts with a site closely associated with the voltage-dependent calcium channels in myometrial cells and, in turn, inhibits calcium influx.

Introduction

In skeletal and cardiac muscle, caffeine is known to release calcium ions from the sarcoplasmic reticulum, resulting in a muscle contraction (Endo, 1977; Fabiato & Fabiato, 1977). In vascular smooth muscle, caffeine also releases cellular calcium to induce a transient contraction (Leijten & Van Breemen, 1984; Karaki *et al.*, 1987). In addition to these contractile effects, caffeine has an inhibitory action in various smooth muscles, including myometrium (Ito & Kuriyama, 1971; Ashoori *et al.*, 1985; Ahn *et al.*, 1988; Small *et al.*, 1988). Caffeine also inhibits cyclic adenosine 3',5'-monophosphate (cyclic AMP) phosphodiesterase leading to accumulation of intracellular cyclic AMP (Ahn *et al.*, 1988; Bray *et al.*, 1989) and decrease of cytoplasmic calcium concentration (Sato *et al.*, 1988). Further, caffeine seems to inhibit stimulated calcium influx (Ahn *et al.*, 1988). However, it is not currently known whether caffeine interacts with the voltage-dependent calcium channels in the plasma membrane of smooth muscles. In the present study we have examined the inhibitory effect of caffeine on calcium channel current in the plasmalemma of isolated smooth muscle cells of rat myometrium. We have compared the ability of caffeine to inhibit calcium channel current with its ability to displace (+)-[^{3}H]-isradipine from its binding sites in the plasmalemma.

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Methods

Electrophysiological experiments

Primary cultures of single rat myometrial cells (19–20 days of pregnancy) were used for electrophysiological experiments as described previously (Amédée *et al.*, 1987). Calcium channel currents of myometrial cells were recorded by means of the whole-cell patch-clamp technique (Honoré *et al.*, 1989). The cells were bathed in an external solution containing (mM): NaCl 130, CsCl 5.6, $CaCl_2$ 2.5, $MgCl_2$ 0.24, glucose 11, Tris 8.3 (pH 7.4). In most experiments, $CaCl_2$ was substituted by $BaCl_2$ (5 mM). The bath solution was maintained at 30 ± 1°C. The patch pipettes (2–3 MΩ) contained (mM): CsCl 130, Cs-pyruvate 5, Cs-succinate 5, Cs-oxaloacetate 5,

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EGTA 10, HEPES 10 (pH 7.3 with CsOH). Calcium channel currents were digitally corrected for leakage currents by subtraction of scaled current traces obtained when hyperpolarizing or small depolarizing pulses from the holding potential were applied to the cells.

Preparation of membranes

Uteri were removed from pregnant rats (19–20 days of pregnancy) and the endometrium was scraped off. Membranes were prepared in the presence of 1 mM iodoacetamide and 0.1 mM phenylmethylsulphonylfluoride to minimize proteolytic degradation (Dacquet *et al.*, 1988). Protein content was determined using gamma-globulin as standard (Bradford, 1976).

Measurement of (+)-[^{3}H]-isradipine binding to membranes

Membrane proteins (0.08 to 0.15 mg ml^{-1}) were incubated for 45 min at 25°C with various concentrations of (+)-[^{3}H]-isradipine in 1 ml of 20 mM HEPES buffer (pH 7.4) containing 0.1% bovine serum albumin (Dacquet *et al.*, 1989). Non-specific binding was defined as the amount of radioligand bound in the presence of 2 μM unlabelled nitrendipine and subtracted from the total binding. All binding experiments were performed under a dim light to minimize photolysis of the dihydropyridines.

Drugs

Drugs used were: (+)-isradipine (Sandoz, Switzerland), (D)-myoinositol-1,4,5-triphosphate ($InsP_3$, Sigma, U.S.A.), (±)-propranolol (Sigma, U.S.A.), caffeine (Merck, F.R.G.), ryanodine (Calbiochem, U.S.A.) and phentolamine (Ciba-Geigy, France). (+)-[^{3}H]-isradipine (specific activity 84 Ci mmol^{-1}) was obtained from Amersham (France).

Data analysis

Scatchard plots for binding of (+)-[^{3}H]-isradipine were obtained by a non linear least square ligand programme (Munson & Rodbard, 1980). The experimental results were expressed as mean ± s.e.mean and significance was tested by Student's *t* test.

Results

Effects of caffeine on the calcium channel current

Figure 1 shows the effects of caffeine (20 mM) on the current-voltage relationships of the inward calcium

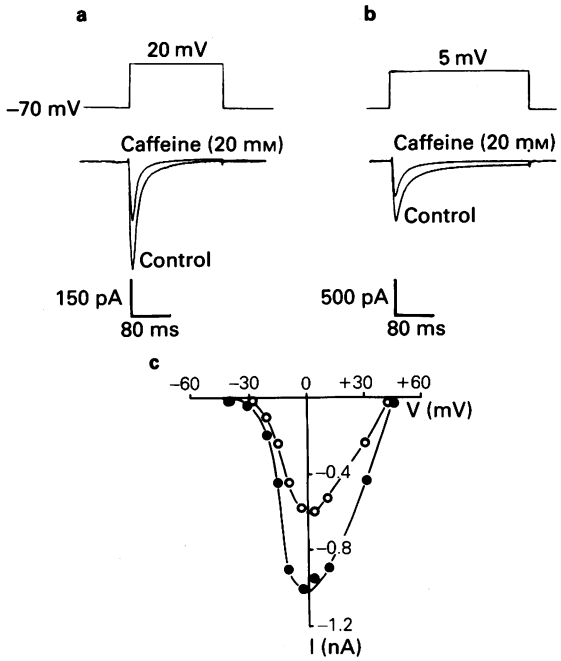


Figure 1 Pregnant rat myometrium: effects of 20 mM caffeine on the calcium channel current recorded from single cells. Two records of the inward currents before and after application of caffeine are shown superimposed. External solutions contained 2.5 mM Ca^{2+} (a) or 5 mM Ba^{2+} (b). The membrane was held at −70 mV and stepped to +20 mV (a) or +5 mV (b). (c) Current-voltage relationships of the inward Ba^{2+} current in the absence (●) or presence (○) of 20 mM caffeine. The amplitude of the inward current in the presence of caffeine was measured after the inhibition had reached a steady-state by repetitive applications of command pulses (20 s intervals).

channel current. The maximal inward current was generated by stepping to +20 mV (in 2.5 mM calcium-containing solution or to +5 mV (in 5 mM Ba-containing solution) from a holding potential of −70 mV. Application of caffeine (20 mM) reduced the amplitude of each current to a similar extent. Two records of the maximal inward currents, before and 5 min after caffeine application, are shown superimposed (Figure 1a,b). At 20 mM caffeine consistently reduced the amplitude of the peak inward Ba current observed at any given potential (−30 to +50 mV) without affecting the apparent reversal potential (Figure 1c). Figure 2a shows the relationship between amplitude of the inward current and concentrations of caffeine. In these experiments, the transmembrane potential stepped to zero mV from a holding potential of −70 mV. Command pulses were delivered at 20 s intervals and were of 300 ms dura-

tion. The peak amplitude of the inward current in the absence of caffeine was normalized as 1. The concentration-dependency of caffeine action in inhibiting the inward barium current was studied by the construction of a sequential concentration-effect curve. This curve had a sigmoidal shape (Figure 2a). The half-maximal blocking concentration for caffeine was estimated to be 35 mM. The slope of the log concentration-effect curve was not shallow, nor did the central portion of the curve exhibit points of inflection. Accordingly, it may be suggested that caffeine bound to a single binding site.

When dihydropyridine derivatives were tested for their ability to block calcium channels (Sanguinetti & Kass, 1984; Terada *et al.*, 1987; Dacquet *et al.*, 1988; Hering *et al.*, 1988), their effects were observed to depend on depolarized holding potentials rather than on the frequency or number of stimuli used to promote calcium channel opening. Accordingly, in the present study, we examined whether the action of caffeine depended on the number of command pulses applied. Command pulses (150 ms duration; 20 s intervals) were used to step membrane potential from a holding potential of −70 mV to zero mV. When such pulses were applied, amplitudes of the inward current remained constant before application of caffeine. In Figure 2b, the amplitude of inward current recorded by the last pulse before application of caffeine was normalized as 1. Caffeine (20 mM) was then added to the solution and the pattern of command pulses was either maintained (solid circles) or recommenced after a 2.8 min rest period (solid triangles). In the presence of caffeine the first post-rest peak inward current did not differ significantly from the peak inward current at the end of the maintained pulse train. A similar observation was made in 4 other experiments. After removal of caffeine, the inward current progressively recovered and 3 min after drug wash-out, its amplitude regained the control value. Addition of phentolamine (1 μM) or propranolol (100 μM) had no effect on the action of caffeine on calcium channel currents.

The effects of caffeine on the voltage-dependency of the calcium channel current were examined by studying both activation and inactivation curves. Inactivation was characterized by use of the classical two-pulse protocol. The amplitude of the current elicited by a test pulse of fixed amplitude (zero mV) and duration (150 ms) was measured when a conditioning pulse was varied. Conditioning pulses of 30 s duration were routinely used to investigate the steady-state inactivation process. The diminution of the test current was taken as an index of inactivation of the calcium channel current. Relative inactivation was expressed by plotting the test current against the level of the conditioning pulse. The amplitude of the test current was normalized by its value in the

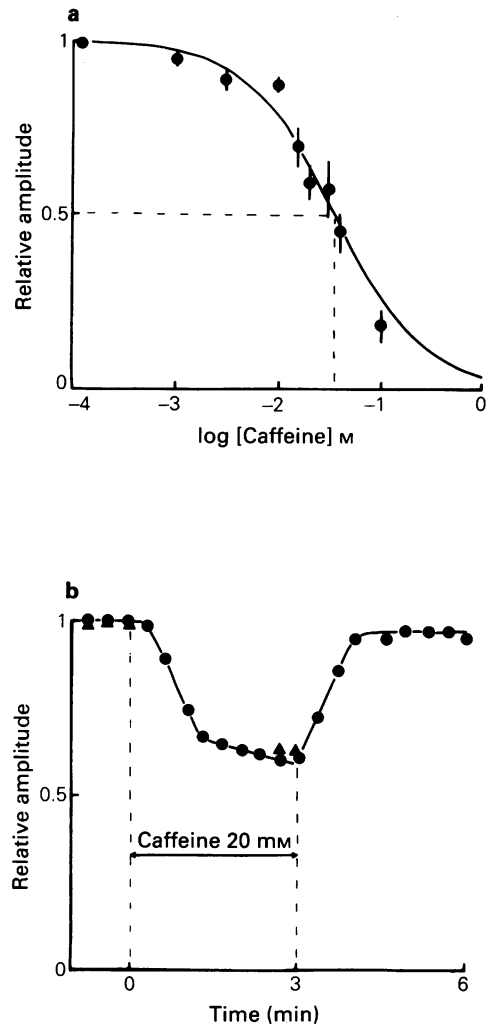


Figure 2 Pregnant rat myometrium: effect of caffeine on the maximal amplitude of the inward current in 5 mM Ba^{2+} solution. The amplitude of the inward current in the absence of caffeine was normalized as 1. The membrane was stepped to zero mV (300 ms duration) from a holding potential of −70 mV. (a) Concentration-response curve showing the inhibition of the inward current evaluated at 5 min after caffeine application with repetitive applications of command pulses (20 s intervals). Each data point is the mean of 3–12 experiments; vertical lines show s.e.mean. Glucose was added in order to have [caffeine] + [glucose] = 100 mM. Glucose by itself was without effect on the calcium channel current amplitude. (b) Effects of 20 mM caffeine with (●) and without (▲) repetitive applications of command pulses (20 s intervals). Steady-state effect of caffeine was obtained within 3 min. After 3 min of washing out, the amplitude of the current was restored to 97% of control.

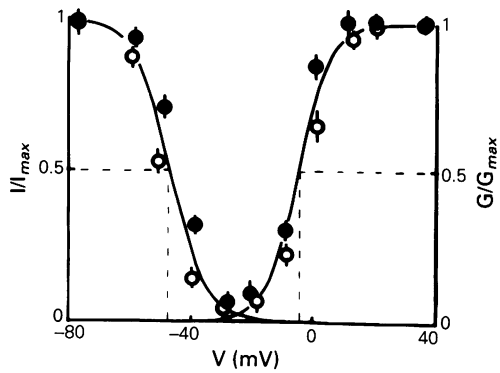


Figure 3 Pregnant rat myometrium: steady-state inactivation and activation parameters of the inward current in 5 mM Ba^{2+} solution against membrane potential in the absence (●) and presence (○) of 20 mM caffeine. The activation (G/G_{max}) and inactivation (I/I_{max}) curves are fitted by an expression of the form $1/[1 + \exp(V_m - V_h)/k]$ where V_m is the membrane potential, V_h is the mid potential and k is the slope factor of the curves. The parameters are: $V_h = -5.5$ mV, $k = -5.2$ mV for the activation curve; $V_h = -47$ mV and $k = 5.8$ mV for the inactivation curve. Each data point is the mean of 4 to 8 experiments; vertical lines show s.e.mean.

absence of a conditioning pulse (I/I_{max}). Figure 3 shows that the relative inactivation of calcium channel current was similarly enhanced in the voltage range from −80 to −20 mV in the absence and presence of 20 mM caffeine. The data were fitted by a Boltzmann distribution. Half-maximal inactivation was obtained at a membrane potential of -45 ± 4 mV ($n = 8$). The activation curves were expressed by plotting the peak membrane conductance (calculated as peak current divided by the difference between membrane potential and apparent reversal potential) against voltage and fitted by a Boltzman distribution. Figure 3 shows that the activation curves are similar in the absence and presence of 20 mM caffeine. Half-maximal and complete activation were obtained at membrane potentials of -5 ± 3 mV and $+35 \pm 5$ mV ($n = 7$).

Inhibitory effect of caffeine is independent of intracellular calcium release

In myometrium, calcium channel inactivation is largely dependent on an increase in cytoplasmic calcium concentration (Jmari *et al.*, 1986; Amédée *et al.*, 1987). It was therefore of interest to examine the inhibitory effect of caffeine on calcium channel current after depletion of intracellular calcium stores. We tested this hypothesis by applying intracellularly a mixture of 20 μM $InsP_3$ and 10 μM ryanodine. These substances are known to induce a release of calcium from the sarcoplasmic reticulum (Ohya *et al.*, 1988; Hisayama & Takayanagi, 1988; Ehrlich & Watras, 1988). In the presence of ryanodine and $InsP_3$ for 6 min, the Ba^{2+} current was slightly reduced. Addition of 20 mM caffeine reduced the amplitude of the inward current by $38.6 \pm 4.5\%$ ($n = 5$), a value similar to that obtained in the absence of intracellular calcium release activators.

Effects of caffeine on (+)-[^{3}H]-isradipine equilibrium binding

The binding of (+)-[^{3}H]-isradipine to myometrial membranes at 25°C was saturable and specific (Honoré *et al.*, 1989). Scatchard analysis of the saturation isotherms was consistent with an interaction at a single class of high affinity sites with an equilibrium dissociation constant K_D of 80 ± 3 pM ($n = 8$) and a binding site density of 106 ± 4 fmol mg^{-1} of protein. Caffeine inhibited (+)-[^{3}H]-isradipine binding with an IC_{50} value estimated to be 30 mM. The Hill coefficient was close to 1, suggesting that caffeine bound to a single binding site (Figure 4a). (+)-[^{3}H]-isradipine saturation isotherms were determined in the absence and in the presence of caffeine. As shown in Figure 4b, caffeine (20 and 50 mM) decreased the maximal binding capacity of the membranes to 58 ± 3 and 34 ± 3 fmol mg^{-1}, respectively ($n = 5$, $P < 0.001$) but did not change the apparent affinity of the radioligand ($P > 0.05$). These results indicate that caffeine behaves as a non competitive inhibitor of (+)-[^{3}H]-isradipine binding to myometrial membranes.

Discussion

It has recently been proposed that in single myometrial cells the calcium channel inward current may be mainly an L-type current (Amédée *et al.*, 1987; Honoré *et al.*, 1989). The present results indicate that caffeine, at concentrations between 1 and 100 mM inhibits in a concentration-dependent manner the calcium channel current recorded on stepping to less negative values from a holding potential of −70 mV. The calcium channel current is reduced by caffeine without any modification of the apparent reversal potential suggesting that caffeine decreases the calcium conductance of the myometrial membrane. As the inward current through calcium channels is involved, in part, in spike firing and activation of the contraction (Mironneau, 1973), our data indicate that inhibition of calcium entry can contribute to the relaxant actions of caffeine observed at concentration of 1 mM and above.

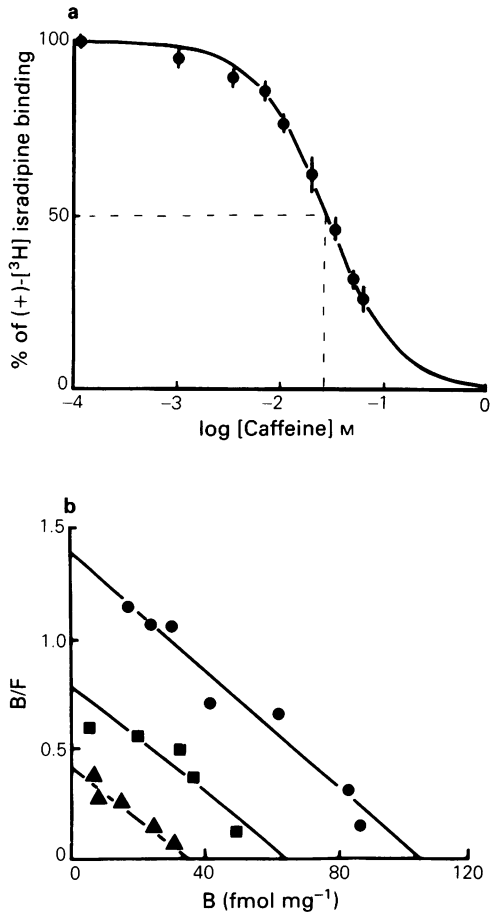


Figure 4 Pregnant rat myometrium: effect of caffeine on the high affinity binding site for (+)-[^{3}H]-isradipine. (a) Binding of (+)-[^{3}H]-isradipine (0.1 nM; 0.08 to 0.15 mg ml^{-1}) in the presence of various concentrations of caffeine. Non-specific binding was defined in the presence of 2 μM nitrendipine and represented 15% of total binding. Results are expressed as a percentage of the specific binding in the absence of caffeine, and are means of 6 experiments with s.e.mean shown by vertical lines. The Hill coefficient was 1.1. Glucose was added in order to have [caffeine] + [glucose] = 100 mM. Glucose by itself was without effect on the (+)-[^{3}H]-isradipine binding assay. (b) Scatchard analysis of the specific binding in the absence (●) and presence of 20 mM (■) and 50 mM (▲) caffeine. Similar results were obtained from 4 other experiments; triplicate estimates were used for each point.

When calcium channels were opened by a conditioning depolarizing pulse sequence, the inhibitory effect of caffeine was similar to that obtained in the absence of stimulation. Increasing the number of inactivated calcium channels at depolarized holding potentials had no further effect on the caffeine-induced blockade. These results suggest that caffeine does not show preferential binding to open or inactivated calcium channels. Thus, the blocking effects of caffeine differ from these of dihydropyridines and phenylalkylamines in smooth muscles (Terada *et al.*, 1987; Dacquet *et al.*, 1988; Hering *et al.*, 1988). It can be suggested that caffeine affects all states of the calcium channels. This assumption is supported by the caffeine inhibition of specific (+)-[^{3}H]-isradipine binding for isolated membranes which are, of course, depolarized.

Several lines of evidence support the idea that caffeine has a direct action on calcium channels: (1) After depletion of internal calcium stores with $InsP_3$ and ryanodine, the inhibitory effect of caffeine was identical to that observed in the absence of intracellular calcium activators. This observation indicates that the action of caffeine is not related to the inactivation of calcium channels by calcium release from intracellular stores. In myometrium, both calcium-dependent and membrane potential-dependent mechanisms contribute to calcium channel inactivation (Jmari *et al.*, 1986). (2) The slope coefficients of the inhibition curves obtained from both electrophysiological and binding experiments were close to 1, suggesting that caffeine bound to a single binding site. (3) The apparent affinity of caffeine for the calcium channel current was nearly identical to that obtained on the high affinity site for (+)-[^{3}H]-isradipine. (4) Although caffeine completely displaced (+)-[^{3}H]-isradipine binding from its high affinity receptors, the inhibitory effect of caffeine was seen as a decrease in B_{max} of isradipine binding without any significant change in affinity. These observations indicate that caffeine behaves as a non competitive inhibitor of (+)-[^{3}H]-isradipine binding to myometrial membranes.

In conclusion, our results show that caffeine, at concentrations between 1 and 100 mM, inhibits the L-type calcium current of myometrial cells by binding to a site associated with the calcium channels. This effect may contribute, in part, to the relaxant action of caffeine in myometrium.

This work was supported by grants from Etablissement Public Régional d'Aquitaine, Institut National de la Santé et de la Recherche Médicale, Association Française des Myopathes and Fondation pour la Recherche Médicale, France. We thank Drs Small and Hollingsworth (University of Manchester) for improving the manuscript and Ms N. Biendon for her excellent secretarial assistance.

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The interaction of hexamethonium with muscarinic receptor subtypes *in vitro*

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1 The action of hexamethonium has been studied at a range of muscarinic receptors *in vitro* by use of both functional and radioligand binding studies.

2 In functional studies, hexamethonium exhibited little or no significant ($P < 0.05$) antagonism of contractile responses to carbachol at muscarinic receptors in the guinea-pig ileum, oesophageal muscularis mucosae, urinary bladder and trachea. However, antagonism was observed at muscarinic receptors in the guinea-pig left atria mediating negative inotropic responses and the calculated pK_B value was 3.80. Hexamethonium also antagonized contractile responses to carbachol in the canine saphenous vein. The pK_B value at these receptors was 3.75.

3 In the presence of 3.2 mM hexamethonium, the pA_2 value for methoctramine at atrial muscarinic receptors was reduced by approximately 10 fold (control pA_2 value was 7.81 ± 0.05; pA_2 value in hexamethonium was 6.73 ± 0.04). In contrast at tracheal muscarinic receptors, the pA_2 values for methoctramine were unaffected in the presence of 3.2 mM hexamethonium (control $pA_2 = 5.58 \pm 0.07$; pA_2 value in hexamethonium was 5.63 ± 0.12). All values quoted are mean ± s.e.mean, $n = 8$.

4 In competition radioligand binding studies, hexamethonium exhibited a higher affinity for cardiac M_2 receptors ($pK_i = 3.68$) than for cerebrocortical M_1 receptors ($pK_i = 3.28$) or for submaxillary gland M_3 receptors ($pK_i = 2.61$). At M_2 receptors hexamethonium at concentrations of 0.1–10 mM, increased the half life of the dissociation rate of [^{3}H]-N-methylscopolamine 1.6–4.3 fold. This was observed at M_3 receptors only at 10 mM, when the half life was increased 1.7 fold.

5 We conclude that hexamethonium, in addition to its well characterized nicotinic antagonist properties, can act as a weak muscarinic antagonist and differentiates between cardiac M_2 receptors and glandular/smooth muscle M_3 receptors. However, hexamethonium differentiates less clearly between M_1 and M_2 receptors. The selectivity between M_2 and M_3 receptors observed in the present study with hexamethonium is comparable to other M_2 selective antagonists such as AF-DX 116 and himbacine.

6 Caution should be exercised with regard to the inclusion of hexamethonium in functional studies of M_2 muscarinic receptor subtypes at concentrations of 0.1 mM and above.

Introduction

Hexamethonium has been shown to act as a nicotinic receptor antagonist and is frequently used to exclude potential nicotinic receptor interactions of muscarinic agonists such as carbachol (e.g. Barlow *et al.*, 1972). A number of workers have also suggested that hexamethonium may act directly at the muscarinic receptor in the ileum (Geddes *et al.*, 1974), ganglia (Brown *et al.*, 1980) and cardiac tissue (Rand & Stafford, 1967; Lüllman *et al.*, 1969; Leung & Mitchelson, 1982; Zonta *et al.*, 1987). It has been shown (Zonta *et al.*, 1987) in functional studies that hexamethonium exhibits a pA_2 value of 4 at atrial muscarinic receptors. Dunlap & Brown (1983) reported an IC_{50} value of 990 μM in competition radioligand binding experiments using atrial membranes. In contrast, hexamethonium, at concentrations of 0.28 mM and above exerted little antagonism at muscarinic receptors in the bronchi or ileum (Barlow *et al.*, 1972; Clague *et al.*, 1985). These data suggest that hexamethonium acts as a selective muscarinic antagonist at receptors mediating nega-

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tive inotropic responses, in comparison to those mediating smooth muscle contraction. In agreement with this hypothesis, it has been shown (Leung & Mitchelson, 1982) that in the presence of hexamethonium, pA_2 values for homatropine and pancuronium at atrial receptors were reduced, whilst no effect was observed on pA_2 values for these antagonists at ileal muscarinic receptors. Similar findings were also observed recently in studies with methoctramine (Eglen *et al.*, 1988a).

The ability of hexamethonium to discriminate between muscarinic receptors in the atria and smooth muscle is consistent with the hypothesis that muscarinic receptors in the atria differ from those in smooth muscle (Barlow *et al.*, 1980). This hypothesis is based upon the differential affinities of a number of antagonists. Those selective for muscarinic receptors in the atria include AF-DX 116, himbacine, methoctramine, gallamine and pancuronium. In addition, those selective for muscarinic receptors in smooth muscle include 4-DAMP (4-diphenylacetoxy-N-methyl piperidine methiodide), hexahydrosiladiphenidol, silahexocylium and silabenzhexol (see Eglen & Whiting, 1986; Mihm & Wetzel, 1987 for reviews).

In the present study we have characterized the action of hexamethonium at muscarinic receptors using both functional and radioligand binding assays. It should be noted that the nomenclature used in this manuscript to describe muscarinic receptor subtypes is based upon proposals by Doods *et al.* (1987) and Hammer *et al.* (1987). Consequently M_1 receptors are characterized by a high affinity for pirenzepine; M_2 receptors exhibit a high affinity for methoctramine, himbacine and AF DX 116 while M_3 receptors exhibit a high affinity for 4-DAMP and hexahydrosiladiphenidol. However, although this classification has yet to encompass muscarinic receptors present on smooth muscle, a number of workers (e.g. Michel & Whiting 1988; Giraldo *et al.*, 1988) have shown similarities between the muscarinic receptor subtype present in glandular and smooth muscle tissue. In the present study, the muscarinic receptors present in smooth muscle are, therefore, referred to as M_3 receptors for the purposes of clarity.

In order to correlate the radioligand binding studies at M_1 receptors, a secondary aim of the study was to examine a functional M_1 receptor assay. O'Rourke & Vanhoutte (1987) have shown that muscarinic receptors mediating contractions of the canine saphenous vein were antagonized by pirenzepine with a high affinity ($pA_2 = 8.1$) and by gallamine with a low affinity ($pA_2 = 4.7$). These data were considered to indicate that M_1 muscarinic receptors mediate contractile responses in this tissue. We have therefore characterized this tissue using pirenzepine, methoctramine and hexamethonium. A preliminary account of this work has been presented at the Dublin meeting of the British Pharmacology Society (Eglen *et al.*, 1988b).

Methods

Functional studies

The following preparations were studied *in vitro*: guinea-pig (Dunkin-Hartley, 300–350 g) ileum, oesophageal muscularis mucosae, trachea, urinary bladder, left atria, and canine saphenous vein. The ileum, oesophageal muscularis mucosae and urinary bladder were placed in Tyrode physiological salt solution (pH 7.4, 37°C); the trachea, left atria, and saphenous were placed in Krebs physiological salt solution (ph 7.4, 37°C). The composition of these salt solutions is given below.

The proximal ileum was prepared according to the method described by Clague *et al.* (1985). Oeso-

Table 1 Muscarinic receptor profiles of preparations used in this study

Functional studies	*Muscarinic receptor subtype*	*Reference*
Canine saphenous vein	M_1	O'Rourke & Vanhoutte (1987)
Guinea-pig left atria	M_2	Clague *et al.* (1985)
Guinea-pig ileum	M_3	Clague *et al.* (1985)
Guinea-pig OMM[a]	M_3	Eglen & Whiting (1988)
Guinea-pig trachea	M_3	Eglen & Whiting (1988)
Guinea-pig urinary bladder	M_3	Eglen & Whiting (1987)
Radioligand binding studies		
Rat cerebral cortex	M_1	Delmendo *et al.* (1989)
Rat cardiac tissue	M_2	Delmendo *et al.* (1989)
Rat submaxillary gland	M_3	Delmendo *et al.* (1989)

[a] OMM = oesophageal muscularis mucosae

phageal muscularis mucosal tissue was prepared according to the method of Kamikawa *et al.* (1985). Zig-zag strips of trachea were prepared according to the method of Eglen *et al.* (1988a). Sheets of urinary bladder tissue were prepared according to methods described by Eglen & Whiting (1987). Two mm rings of canine saphenous vein were prepared according to the method of O'Rourke & Vanhoutte (1987). Left atria were electrically paced (2 Hz, threshold voltage + 20%, 5 ms duration) according to methods described by Blinks (1966). All preparations were suspended under 1.0 g tension with the exception of the left atria which was suspended under 0.5 g tension. In all studies, the preparations were allowed 60 min to equilibrate, during which time the bathing solution was replaced every 15 min.

The experimental protocol was as follows: at the end of the 60 min equilibration period, all smooth muscle preparations were exposed to 50 mM KCl for a period of 30 s or until an equilibrium response was obtained. Preliminary experiments had shown that without prior exposure to KCl, the slope of a second concentration-response curve to carbachol was significantly steeper than the initial curve. The contractions to KCl also provided a measure of the maximal response of the preparation. Thirty min after exposure to KCl, concentration-response curves to carbachol were established. Cumulative curves were constructed in all preparations with the exception of studies using the ileum, in which curves were constructed in a non-cumulative fashion (30 s exposure on a 5 min dose-cycle; Clague *et al.*, 1985). When a 3 fold increase in concentration produced no further increase in response, the tissue was washed at 10 min intervals until the tissue relaxed and baseline tension was re-established.

In experiments in which the effects of antagonists were studied, an equilibration of 60 min was allowed prior to construction of a second concentration-response curve in the presence of antagonist. In experiments studying the effects of hexamethonium alone, 30 min were allowed for equilibration. In all experiments, control tissues were run in which no antagonist was added, in order to check for any change in the sensitivity of the tissue for the agonist.

All responses were determined as changes in isometric tension (mg). The smooth muscle responses were measured with a Hugo Sachs K30 force transducer and displayed on a Graphtec Watanabe Linearecorder. Atrial responses were measured with a Grass FT03 force transducer and displayed on a Grass polygraph.

Radioligand binding studies

Membrane preparations Membranes were prepared as described previously (Michel & Whiting, 1987). EDTA washed cerebrocortical, cardiac and submaxillary gland membranes were prepared from 200–300 g male Sprague-Dawley rats. Tissues were homogenized in 50 mM Tris, 5 mM Na_2EDTA buffer (pH 7.4 at 4°C) in a Polytron P10 tissue disrupter (setting 10; 2 × 10 s bursts). The homogenate was centrifuged at 48,000 *g* for 15 min. The pellet obtained was washed by resuspension and centrifugation, once in homogenizing buffer and twice in 50 mM EDTA buffer (pH 7.4 at 4°C). Membranes were stored under liquid nitrogen until required.

Binding assays Muscarinic receptors in cardiac and submaxillary gland membranes were labelled with [^{3}H]-N-methylscopolamine ([^{3}-H]-NMS) while muscarinic receptors present in rat cerebral cortex were labelled with [^{3}H]-pirenzepine. All binding assays were conducted at 37°C in a final volume of 3 ml of Tris-Krebs assay buffer the composition of which is given below at pH 7.4 at 37°C. In all studies atropine (1 μM) was used to define non-specific radioligand binding (NSB). [^{3}H]-NMS was present at a fixed concentration of 0.1 nM while [^{3}H]-prienzepine was present at a concentration of 0.5 nM. Incubations were for 2 h at 37°C and were terminated by vacuum filtration over Whatman GF/B glass fibre filters using a Brandel 48 well cell harvester. After filtration the filters were washed with 15 ml of ice cold water. The filters were pretreated with 0.1% polyethyleneimine 18 h before use in order to reduce filter binding of the radioligands. Radioactivity retained on the filters was determined by liquid scintillation counting.

In kinetic studies to examine the effect of hexamethonium on the dissociation of [^{3}H]-NMS from muscarinic receptors, 0.2 ml aliquots of a pre-equilibrated solution of muscarinic receptor and [^{3}H]-NMS were added to 20 μl of atropine in the presence and absence of hexamethonium and the dissociation reaction was allowed to proceed for various times before separating bound ligand from free ligand as described above. In all studies the final assay concentration of atropine during dissociation was 1 μM.

Analysis of results

In all functional studies, the data were fitted to a non-linear iterative curve fitting procedure (Parker & Waud, 1971) using RS1 software (BBN corporation). This enabled calculation of the potency (EC_{50}), maximum response and slope of the concentration-response curve. The antagonist affinities were calculated, using at least 4 antagonist concentrations, by the method of Arunlakshana & Schild (1959).

Saturation binding data were analysed using

LIGAND (Munson & Rodbard, 1980) while competition binding data were analysed by a curve fitting programme (Michel & Whiting, 1984) based on the method of Parker & Waud (1971). In the latter case IC_{50} values were converted to K_i values by the Cheng-Prusoff approximation (1973).

Drugs used

The following were used: carbachol (Sigma Chemical Co. Ltd), hexamethonium (Sigma), methoctramine (Research Biochemicals Inc.), [^{3}H]-pirenzepine (NEN), [^{3}H]-NMS (NEN), pirenzepine (Boots plc), AF-DX 116 (Research Biochemicals Inc.) and gallamine (Sigma).

Physiological salt solutions

The compositions of the physiological salt solutions used in the study are given below (mM).

Tyrode solution: NaCl 136.9, KCl 2.7, $MgCl_2 \cdot 6H_2O$ 1.1, $NaH_2PO_4 \cdot 2H_2O$ 0.4, glucose 5.6, $NaHCO_3$ 11.8 and $CaCl_2 \cdot 6H_2O$ 1.8.

Krebs solution: NaCl 118.4, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, glucose 10.0, $NaHCO_3$ 25.0 and $CaCl_2 \cdot 6H_2O$ 2.5.

Tris-Krebs solution: NaCl 118.4, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, glucose 10.0, $NaHCO_3$ 25.0 $CaCl_2 \cdot 6H_2O$ 2.5 and Tris-HCl 10.0.

All solutions were gassed with 5% CO_2 in oxygen.

Results

Functional studies

Hexamethonium (10 μM–3.2 mM) did not significantly antagonize responses to carbachol in the guinea-pig ileum and oesophageal muscularis mucosae. The $-\log EC_{50}$ values in the presence and absence of hexamethonium are shown in Table 2. The pK_B values for hexamethonium at muscarinic receptors in these tissues are therefore less than 2.5. It was, however, observed that a small but significant ($P < 0.05$) dextral shift in the concentration-response curve to carbachol in the presence of 3.2 mM hexamethonium occurred at urinary bladder and tracheal receptors. This was not observed in the presence of lower concentrations of hexamethonium (10 μM–1 mM) in these preparations.

The responses to carbachol in the left atria were significantly ($P < 0.05$) shifted to the right in the presence of hexamethonium. The pA_2 value was 3.75 ± 0.13 and the slope of the Schild plot was 0.57 ± 0.04 (mean $\pm$ s.e.mean, $n = 8$). These data are shown in Table 3 and Figure 1 together with reference pA_2 values and Schild slopes for a range of muscarinic receptor antagonists previously reported by our group (Eglen & Whiting, 1986; Eglen *et al.*, 1988b). Hexamethonium displaced the concentration-response curve to carbachol to the right in a parallel fashion, with no diminution in the maximum responses.

Table 2 Effect of hexamethonium on the potency of carbachol in smooth muscles

Preparation	*Control*	$-\log EC_{50}$ + *Hexamethonium* (3.2 mM)
Ileum	6.83 ± 0.03	6.78 ± 0.08
OMM	6.72 ± 0.05	6.83 ± 0.02
Urinary bladder	6.92 ± 0.03	6.53 ± 0.02*
Trachea	6.13 ± 0.08	5.48 ± 0.12*

Values are mean $\pm$ s.e.mean, $n = 8$.
OMM = oesophageal muscularis mucosae.
* Significantly different from control value ($P < 0.05$; Student's t test).

The pA_2 values for methoctramine were also estimated in the presence and absence of hexamethonium (3.2 mM) at muscarinic receptors present in the left atria and trachea. It was observed that the pA_2 values for methoctramine were significantly ($P < 0.05$) reduced at muscarinic receptors in the left atria but not in the trachea. Control pA_2 values with Schild slopes in parentheses, were 7.81 ± 0.05 (0.83 ± 0.08) and 5.58 ± 0.07 (0.98 ± 0.04); pA_2 values in the presence of 3.2 mM hexamethonium

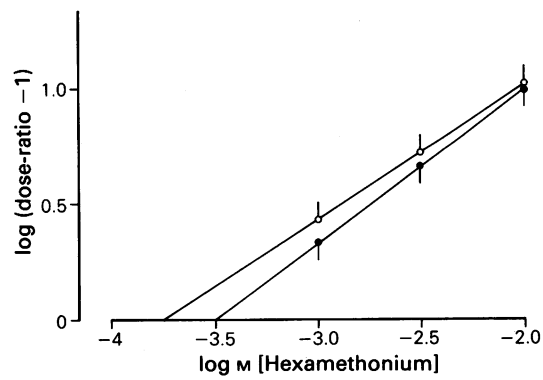


Figure 1 Schild analysis of antagonism of muscarinic responses by hexamethonium in guinea-pig electrically paced atria (○) and canine saphenous vein (●). Values are mean ($n = 4$–8) and vertical lines indicate s.e.mean.

Table 3 Affinity of muscarinic antagonists for muscarinic receptor subtypes in functional studies

	(M_1) *Canine saphenous vein*		(M_2) *Guinea-pig atria*		(M_3) *Guinea-pig ileum*	
Antagonist	pA_2	*Slope*	pA_2	*Slope*	pA_2	*Slope*
AF-DX 116	ND		6.49 ± 0.02	0.96 ± 0.05	5.72 ± 0.03	1.00 ± 0.03
Hexamethonium	3.52 ± 0.13	0.57 ± 0.04*	3.75 ± 0.13	0.57 ± 0.04*	<2.5	—
Himbacine	ND		8.52 ± 0.06	1.00 ± 0.01	7.52 ± 0.08	0.98 ± 0.05
Methoctramine	6.43 ± 0.12	0.81 ± 0.07*	7.81 ± 0.05	0.83 ± 0.08*	5.82 ± 0.07	1.03 ± 0.08
Pirenzepine	8.21 ± 0.04	0.80 ± 0.03*	6.81 ± 0.03	0.93 ± 0.03	6.77 ± 0.05	0.90 ± 0.03

Values are mean (± s.e.mean) from 4–8. The slope of the Schild plot is given in parentheses. Slopes marked with an asterisk indicate significantly different ($P < 0.05$) from unity.
ND indicates data not determined.
Values shown for AF-DX 116 and pirenzepine at atrial and ileal receptors are previously reported by Eglen & Whiting (1986); values for himbacine and methoctramine at these receptors are previously reported by Eglen *et al.* (1988b).

were 6.73 ± 0.04 (1.06 ± 0.13) and 5.63 ± 0.12 (0.91 ± 0.05), respectively; mean ± s.e.mean, $n = 4$–8.

The pA_2 value for pirenzepine, methoctramine and hexamethonium at muscarinic receptors mediating contractions of the canine saphenous vein are shown in Table 3 and Figure 1. The concentration-response curves to carbachol were shifted to the right in a parallel fashion in the presence of hexamethonium. The slope of the Schild plot for hexamethonium, and to a lesser extent, pirenzepine and methoctramine were significantly ($P < 0.05$) less than unity, which indicates deviation from competitive antagonism.

Radioligand binding studies

In ligand binding studies (Figure 2), hexamethonium ($pK_I = 3.68 \pm 0.05$; nH = 0.87 ± 0.04; $n = 3$) displayed higher affinity for the cardiac M_2 muscarinic receptor and the cerebrocortical M_1 ($pK_I = 3.38 \pm 0.12$; nH = 0.83 ± 0.05; $n = 4$) than for the submaxillary gland M_3 receptors ($pK_I = 2.61 \pm 0.05$; nH = 0.63 ± 0.10; $n = 3$). The 11.7 fold selectivity of hexamethonium for M_2 over M_3 receptors was comparable to the 10 fold selectivity of AF-DX 116 for these two receptor subtypes (data not shown).

In these experiments the Hill coefficients for the hexamethonium displacement curves were significantly less than unity. The effect of hexamethonium on radioligand dissociation was also examined for the M_2 and the M_3 receptor. At the cardiac M_2 receptor, hexamethonium at concentrations of 0.1 mM and greater significantly decreased the dissociation rate of [^{3}H]-NMS (Figure 3). The half life of the M_2 receptor-[^{3}H]-NMS complex was increased 1.6, 2.2 and 4.3 fold, respectively, at hexamethonium concentrations of 0.1 mM, 1 mM and 10 mM. Hexamethonium decreased the dissociation rate of [^{3}H]-NMS from the M_3 muscarinic receptor of the rat submaxillary gland 1.7 fold at a concentration of 10 mM. Concentrations of 1 and 0.1 mM did not modify the dissociation rate of [^{3}H]-NMS at the M_3 receptor.

Discussion

In the present study, the interaction of hexamethonium with three subtypes of the muscarinic receptor has been characterized. Hexamethonium has been frequently employed as a tool to exclude

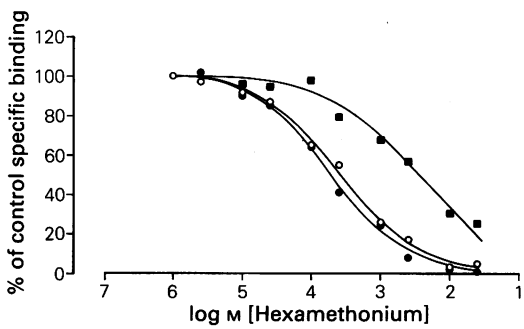


Figure 2 Effect of hexamethonium on the binding of [^{3}H]-pirenzepine to rat cortical muscarinic receptors (○) and [^{3}H]-N-methylscopolamine to rat cardiac (●) and rat submaxillary gland (■) muscarinic receptors. The radioligand and membrane preparation were incubated in the presence or absence of hexamethonium for 2 h prior to separating bound ligand from free ligand. The graphs are from a representative experiment and show the inhibition of control specific binding produced by various concentrations of hexamethonium in the three preparations examined.

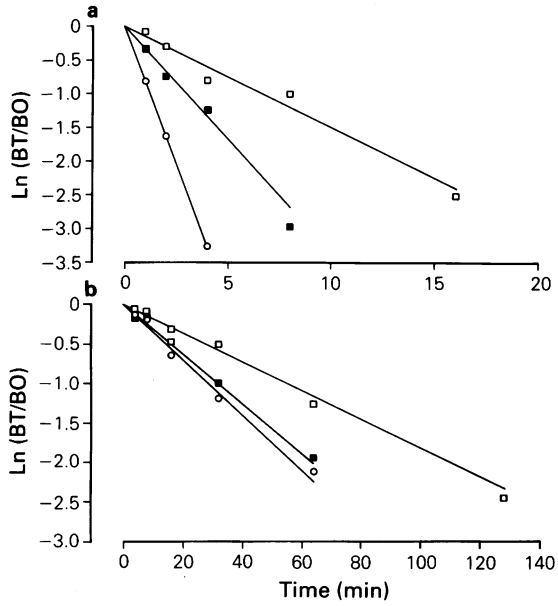


Figure 3 Effect of hexamethonium on the dissociation of [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) from muscarinic receptors present in rat cardiac membranes (a) or in rat submaxillary gland membranes (b). Specific binding of [^{3}H]-NMS was measured at various times (BT) after initiating dissociation of equilibrated receptor-[^{3}H]-NMS complexes by adding atropine to a final concentration of 1 μM and is expressed relative to the level of binding immediately before initiating dissociation (BO). Parallel experiments were conducted in the absence (○) and the presence of 1 mM (■) or 10 mM (□) hexamethonium.

nicotinic effects of muscarinic agonists such as carbachol. However, as shown by Leung & Mitchelson (1982), its inclusion can modify the interaction of antagonists with muscarinic receptors. This was demonstrated with pancuronium (Leung & Mitchelson, 1982) and, in the present study, with methoctramine. The effect of hexamethonium appeared to be specific for cardiac M_2 receptors, since the pA_2 values for methoctramine were not significantly affected by inclusion of hexamethonium at tracheal M_3 receptors. These data indicate that hexamethonium may discriminate between cardiac M_2 receptors and M_3 receptors mediating smooth muscle contraction.

This hypothesis is in accordance with studies in which the effects of hexamethonium were studied alone. Antagonism was observed at cardiac (M_2) receptors, but not at M_3 receptors present in the ileum, oesophageal muscularis mucosae and urinary bladder. The reason for the small but significant shift observed at urinary bladder and tracheal M_3 receptors is at present unknown, and is in contrast to data reported by Barlow *et al.* (1972) using bronchial preparations. The lack of effect of hexamethonium at ileal muscarinic receptors is in agreement with previous studies (Barlow *et al.*, 1972; Leung & Mitchelson, 1982).

The interaction of hexamethonium at cardiac M_2 receptors deviated from competitive antagonism, since the slope of the Schild plot was significantly different from unity. This is in contrast to work by Zonta *et al.* (1982) in which a Schild slope of 1.02 was observed. Leung & Mitchelson (1982), using combination dose-ratio studies with hexamethonium and mecamylamine, observed competitive antagonism at all hexamethonium concentrations except 0.2 mM. Geddes *et al.* (1974), suggested that hexamethonium may act allosterically at ileal receptors, a finding further studied in the radioligand binding studies which are discussed below. The estimates of the affinity of hexamethonium for the M_2 receptor, whilst complicated by the lack of competitive antagonism, were slightly less than the pA_2 values obtained by Zonta *et al.* (1987), and by Lüllman *et al.* (1969), i.e. 4.0 and 3.9 respectively.

Hexamethonium, therefore, in agreement with previous studies (see Introduction), appears to act selectively to antagonize M_2 muscarinic receptors, although the precise nature of the antagonism could not be ascertained from the functional studies.

Hexamethonium also appeared to act as an antagonist of M_1 receptors mediating contractions of the canine saphenous vein. The preparation appears to possess functional M_1 receptors since it exhibited a high pirenzepine pA_2 value in agreement with previous reports by O'Rourke & Vanhoutte (1987). The data obtained with methoctramine and hexamethonium further substantiate the M_1 nature of this receptor since the pA_2 value for methoctramine, in this preparation, was intermediate between its affinity at the M_2 and M_3 receptors. This finding parallels the results obtained in the radioligand binding studies. The pK_i value for hexamethonium at the M_1 receptor in the cerebral cortex, was also similar to that observed in the dog saphenous vein. On the basis of the functional data it would appear that while hexamethonium can differentiate between M_2 and M_3 receptors, it cannot distinguish between M_1 and M_2 receptors. In both the radioligand binding and functional studies only a small difference was observed between the affinity of hexamethonium for the M_1 and M_2 receptors. However, again in good agreement with the functional studies, a lower pK_i value was observed at M_3 glandular receptors. While a pA_2 value could not be determined in the functional studies a pK_i value of 2.7 was obtained in the binding studies.

These data suggest that the selectivity of hexamethonium for M_2 receptors, in comparison to M_3 receptors is approximately 12 fold. As can be seen by inspection of either the pA_2 values (Table 3) or pK_i values this order of selectivity is similar to that reported for AF-DX 116 or himbacine (approx. 10 fold). Whilst the absolute affinity of hexamethonium was very low, only methoctramine has been reported to date to exhibit a greater selectivity (approx. 50-fold; Eglen *et al.*, 1988a).

The nature of the antagonism by hexamethonium at M_1 receptors in the canine saphenous vein resembled that observed at cardiac M_2 receptors, in that it also deviated from competitive antagonism. The functional data suggested that hexamethonium exhibits allosteric properties, since the Schild slopes were less than unity. In the binding studies, the low Hill coefficients also indicated possible allosteric interactions. Although, hexamethonium decreased the dissociation rate of [^{3}H]-NMS at both the M_2 and to a lesser extent the M_3 receptor, previous studies by Freedman *et al.* (1986) have demonstrated that a wide range of compounds also interact with the allosteric site on the muscarinic receptor at concentrations of 0.1 to 1 mM.

In conclusion, hexamethonium acts as muscarinic antagonist which discriminates both the M_1 and M_2 receptor from the M_3 receptor. Caution should therefore be used in the inclusion of hexamethonium at concentrations of 0.1 mM and above in studies designed to study the interaction of antagonists with muscarinic receptor subtypes.

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Metabolism of amines in the isolated perfused mesenteric arterial bed of the rat

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1 Semicarbazide-sensitive amine oxidase (SSAO) activity has been demonstrated in the isolated mesenteric arterial bed of the rat *in vitro* by studying the metabolism of benzylamine (Bz) and tyramine (Tyr) added to the perfusing fluid.

2 Pretreatment of rats with (E)-2-(3',4'-dimethoxyphenyl)-3-fluoroallylamine (MDL72145), a potent inhibitor of SSAO in rat mesenteric blood vessels, reduced the amount of metabolites, following the addition of Bz (25 μM) or Tyr (100 μM) to the perfusing fluid, by 83% and 52% respectively. Inactivation of monoamine oxidase type A (MAO-A) by the addition of clorgyline (10 μM) to the perfusing fluid, had little effect on the appearance of metabolites from Tyr.

3 The presence of 3 μM cocaine in the perfusing fluid increased the amount of metabolites produced from Tyr.

4 The metabolites of Tyr appearing in the perfusion fluid from control preparations were 85% *p*-hydroxyphenylacetic acid and the remainder consisted of a mixture of *p*-hydroxyphenylacetaldehyde and, possibly, *p*-hydroxyphenylethanol.

5 The metabolism of Tyr by homogenates of the rat mesenteric vascular bed was carried out by SSAO (60%) and MAO-A (40%) with very little contribution from MAO-B. Homogenates from rats pretreated with MDL 72145 showed metabolism of Tyr by MAO-A only.

6 These data indicate that SSAO is capable of metabolizing amines present in the fluid perfusing blood vessels to metabolites that are readily released. Histochemical evidence has shown that whereas MAO-A is present in the mitochondria of smooth muscle cells and nerve endings, SSAO is located in the plasma membrane of the smooth muscle cells. This subcellular distribution may explain the differences found between metabolites released from intact vessels and the metabolism seen in homogenates. The identity of the Tyr metabolizing activity in intact vessels that is resistant to both MDL 72145 and clorgyline remains to be determined.

Introduction

Rat blood vessels possess an amine oxidase activity capable of metabolising tyramine (Tyr), which has different properties from monoamine oxidase (MAO; EC 1.4.3.4) enzymes (Coquil *et al.*, 1973). This enzyme activity is insensitive to inhibition by clorgyline but is inactivated by carbonyl reagents such as semicarbazide and, as a result, is called semicarbazide-sensitive amine oxidase (SSAO). It is considered to be a member of the group of enzymes classified as EC 1.4.3.6 (see Callingham & Barrand 1987, for review). SSAO has been shown to reside in the plasma membrane of vascular smooth muscle cells (Wibo *et al.*, 1980; Lyles & Singh, 1985).

Homogenates of blood vessels have been widely used to examine the biochemical properties of this enzyme activity (Lewinsohn *et al.*, 1978; Clarke *et al.*, 1982; Precious *et al.*, 1988; Elliott *et al.*, 1989b). The preferred substrate appears to be benzylamine (Bz) but a number of aromatic and aliphatic amines also have been shown to be substrates. In spite of all the work done with homogenates, this enzyme has not been studied in intact blood vessels of the rat and its physiological significance is poorly understood.

Studies with intact rat caudal artery confirm the long held view that neuronal uptake mechanisms are important for the inactivation of noradrenaline. Catechol-*O*-methyltransferase and extraneuronal uptake make a more significant contribution when the amine enters from the luminal side of the blood vessel rather than the extraluminal side (Venning & de la Lande, 1984; 1985). Amines, that are substrates

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for SSAO, and which enter the blood vessel from the luminal side may be inactivated in a different manner.

The presence of SSAO in the plasma membrane of vascular smooth muscle cells might suggest that this enzyme has ready access to amines present in the circulation without the need for specialised uptake systems (Callingham *et al.*, 1983). There is however, no direct evidence to support this. In order to examine such a possibility, the isolated perfused mesenteric arterial bed of the rat was chosen to determine whether, or not, SSAO is capable of metabolizing substrates present in the fluid perfusing these vessels. Tyr was chosen as a suitable amine for study because it is metabolized by both SSAO and MAO-A in homogenates of these vessels (Coquil *et al.*, 1973). The relative contribution of these two enzymes to the production of metabolites appearing in the perfusate could then be assessed. Preliminary results from these studies have been given elsewhere (Elliott & Callingham, 1988).

Methods

Male Wistar rats (200–350 g), supplied by A. J. Tuck & Son, Rayleigh, Essex were used throughout these studies. Heparin (500 i.u.) was administered by i.p. injection 10 min before the rats were anaesthetized with urethane ($2\,g\,kg^{-1}$; i.p.). The mesenteric vascular bed was isolated and perfused as described by McGregor (1965). Briefly, the cranial mesenteric artery was cannulated with a polyethylene catheter (1.02 mm o.d., Portex Ltd., Hythe, Kent) and the ileocolonic arterial branches were ligated. Perfusion with modified Krebs-Henseleit solution, maintained at 37°C and gassed with 95% O_2 and 5% CO_2, was started as soon as possible after the blood flow had ceased. A constant flow rate of $2\,ml\,min^{-1}$ was delivered by a Harvard 1203A peristaltic pump. The preparation, once isolated from the rat, was placed in a methacrylate dish resting on a block warmed by circulating water and covered with paraffin film for protection against evaporation. The effluent from the preparation was collected. Perfusion pressure was recorded by a Washington PT400 pressure transducer connected via an amplifier to a Kipp & Zonen BD41 two channel X/T pen recorder. Two preparations were tested at the same time. The resting perfusion pressure was consistent between the various preparations. For example, with 36 preparations, in which Tyr was used, it was 8.6 ± 0.28 mmHg, (mean ± s.e.mean). Each preparation was allowed a 45 min equilibration period before the metabolism studies were begun.

Some rats were injected (i.p.) with either 1 or $2\,mg\,kg^{-1}$ (E)-2-(3',4'-dimethoxyphenyl)-3-fluoroallylamine (MDL 72145) 1 h before the dissection. This drug is a potent irreversible inhibitor of SSAO (Lyles & Fitzpatrick, 1985). When used in this way, it has been shown to inhibit SSAO selectively without affecting MAO-A in rat blood vessels (Elliott *et al.*, 1989a). Although this inhibitor also inhibits MAO-B (Zreika *et al.*, 1984), evidence from homogenates of rat mesenteric blood vessels suggests there is little or no MAO-B present in this tissue (Coquil *et al.*, 1973; Fuentes & Neff, 1977; see results below). In those preparations where the inhibition of MAO-A was to be studied, clorgyline (10 μM) was present in the perfusing Krebs solution for 30 min during the equilibration period, after which time, perfusion with drug-free solution was resumed. Clorgyline was used in this way instead of by *in vivo* administration to the rats as we have noted some inhibition of SSAO following clorgyline given *in vivo* (unpublished observations) similar to that reported with pargyline (Elliott *et al.*, 1989a). In those preparations where the effect of cocaine on amine metabolism was examined, cocaine (3 μM) was added to the perfusing fluid after the equilibration period and remained present throughout the experiment. The effects of these drugs on the amine oxidase activities in the mesenteric blood vessels were assessed by assay of MAO-A and SSAO activities in homogenates of the preparations following the perfusion experiments as described below.

Metabolism studies

At the start of each experiment the preparations were stimulated with 10 nmol of noradrenaline administered in Krebs solution (200 μl) through an injection port into the perfusing fluid. This was repeated 5 times at 5 min intervals and served to ensure that the preparations were responding consistently. Subsequently, 10 ml of 25 μM [^{14}C]-Bz ($5\,\mu Ci\,\mu mol^{-1}$) or 20 ml of 100 μM [^{3}H]-Tyr ($6.6\,\mu Ci\,\mu mol^{-1}$) in Krebs solution were perfused. Totals of 30 and 100 ml of perfusate were collected in 0.5 and 1 ml fractions in the Bz and Tyr experiments, respectively. Each fraction was sampled and its total radioactivity measured in a Packard A2020 liquid scintillation spectrometer. Corrections for the efficiency of counting were made by the use of automatic external standardization allowing the amounts of metabolites to be expressed in absolute terms. Deaminated products were extracted into 5 volumes of a water-saturated mixture of ethyl acetate and toluene (1:1) following acidification with 3 M HCl (25 μl or 50 μl). The amount of unchanged Tyr extracted by this method was estimated and found to be less than 0.05% of the total Tyr present. Each fraction was corrected for this when calculating the amount of metabolites extracted. The Tyr concentra-

tion used in these studies (100 μM) was chosen as it is in the same range as the K_m value for the deamination of this amine (86 μM, unpublished observations) in crude homogenates of mesenteric blood vessels.

Chromatographic studies

In some experiments with Tyr, fractions 12 to 36 (6 to 18 min; the period when maximum output of metabolites occurred) were pooled before extraction. The organic solvent was evaporated down to dryness with nitrogen. The residue was dissolved in 0.5 ml ethyl acetate and the metabolites were chromatographed and compared with reference compounds on Whatman no. 1 paper. The phenolic compounds were detected by spraying the paper with a solution of Fast Scarlet GG salt (1 mg ml^{-1} in 0.1 M HCl), followed by 1 M sodium carbonate solution. Radioactivity was located by cutting the chromatogram into 1 cm bands, then eluting with ethoxyethanol and counting. The solvent systems and R_F values of the reference compounds are shown in Table 2.

Amine oxidase activities in homogenates

Following each perfusion experiment, the mesenteric blood vessels were cleaned of adhering fat and frozen until needed. Each mesentery was homogenized 1 in 20 w/v in 1 mM potassium phosphate buffer (pH 7.8) with a mechanical homogenizer (Polytron mark 5, Kinematica) on the day of the assay. The resulting homogenate was centrifuged at 600 g for 10 min to separate unbroken cells and nuclei. A modification of the radiochemical method of Callingham & Laverty (1973) was used to measure the MAO-A and SSAO activity in the supernatant as described elsewhere (Elliott *et al.*, 1989b). The enzyme activity of each sample is the mean of triplicate estimations. The substrates used were 5-hydroxytryptamine (5-HT, 500 μM), to measure MAO-A activity, and Bz (10 μM) to measure SSAO activity. The protein content of each homogenate was estimated by the method of Lowry *et al.* (1951). The results are expressed in nmol of product formed h^{-1} mg^{-1} protein. Some homogenates were pre-incubated at 37°C for 30 min with various concentrations of clorgyline (0.1 nM to 0.5 mM) and the metabolism of Tyr (100 μM) assayed in the resulting homogenates. In these experiments, the metabolism was expressed as a percentage of a control, which had been preincubated in the absence of clorgyline.

Drugs and solutions

The perfusion solution (modified Krebs-Henseleit solution) had the following composition (mM): NaCl 118, KCl 4.57, $CaCl_2$ 1.27, KH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 25 and glucose 5.55. Heparin injection BP was obtained from Paines & Byrne Ltd., Greenford, Middlesex, and urethane from BDH, Ltd. The radiochemicals used were [7-^{14}C]-benzylamine hydrochloride and [G-^{3}H]-tyramine hydrochloride from Dupont U.K. Ltd., Stevenage, Herts., and 5-hydroxy-[G-^{3}H]-tryptamine creatinine sulphate from Amersham International plc., Amersham. Unlabelled amines, *p*-tyramine hydrochloride, 5-hydroxytryptamine creatinine sulphate and (−)-noradrenaline bitartrate and *p*-hydroxyphenylacetic acid were obtained from Sigma Chemical Company Ltd.,

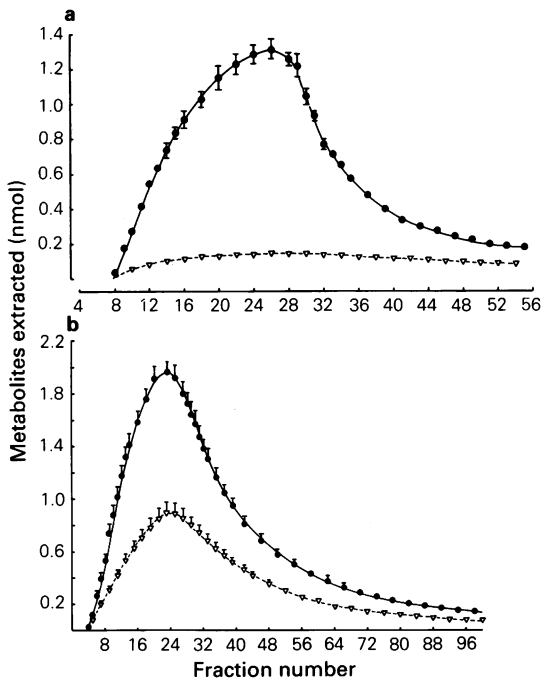


Figure 1 Shows the profile of metabolites extracted from the perfusate of the isolated perfused mesenteric arterial bed of the rat following the addition of radiolabelled benzylamine (Bz) or tyramine (Tyr) to the Krebs solution perfusing the vessels. The deaminated metabolites were extracted following acidification of each fraction of perfusate. Each point represents the mean amount (nmol) of metabolite extracted (s.e.mean shown by vertical bar when larger than the symbol) and has been plotted against the fraction number. Metabolites from control preparations (●) and from preparations from rats treated with MDL 72145 (▽). In (a) 25 μM [^{14}C]-Bz was infused into the preparations for 5 min and fractions of 0.5 ml of perfusate collected for 15 min (30 ml); in (b) 100 μM [^{3}H]-Tyr was perfused through each preparation for 10 min and 1 ml fractions of perfusate collected for 50 min (100 ml).

Poole, Dorset. Benzylamine was purchased from Sigma as the free base and the hydrochloride prepared and crystallised. The alcohol metabolite of tyramine, *p*-hydroxyphenylethanol was obtained from Aldrich Chemical Company, Gillingham, Dorset. The aldehyde, *p*-hydroxyphenylacetaldehyde was prepared, as the Wanzlick derivative, by the method described by Sharman (1987) (m.p. 194° to 195°C, uncorrected, open capillary). This was dissolved in 1 M HCl, the free aldehyde was then extracted into ethyl acetate and the organic solution was used for the chromatography experiments. Clorgyline hydrochloride was a gift from May & Baker Ltd., (now Rhône-Poulenc) Eccles, Manchester, and MDL 72145 was a gift from Merrell Dow Research Institute, Strasbourg, France. All other reagents were of analytical grade where possible.

Statistical analysis of results

Statistical significance was evaluated by Student's *t* test, in which each group was compared with control.

Results

When Bz was added to the fluid perfusing rat mesenteric vascular beds, metabolites appeared in the perfusate within 2 min. The time course for the appearance of the metabolites of Bz in the perfusate is shown in Figure 1a and the quantities of radioactivity recovered and metabolites extracted are given in Table 1. Of the administered radioactivity, 70% was recovered in the perfusate and this did not differ significantly between control preparations and preparations from rats treated with MDL 72145. There was, however, an 83% reduction in the amount of metabolites extracted following MDL 72145 treatment. The profiles of tyramine metabolites appearing from control preparations and preparations from rats treated with MDL 72145 are shown in Figure 1b. The amount of radioactivity recovered and metabolites extracted, from all 5 groups of preparations where the metabolism of Tyr was examined in this way, are given in Table 1. The recovery of radioactivity from these preparations was 80% and again did not differ significantly between the groups. Treatment of the preparations with clorgyline (10 μM) did not reduce the amount of metabolites extracted, whereas treatment of the rats with MDL 72145 caused a reduction of more than 50%. A combination of these two inhibitors gave a small further reduction in the amount of metabolites down to 42% of control. In the presence of cocaine (3 μM), a significant increase in the amount of metabolites was seen.

Table 2 gives the chromatographic data for the metabolites of tyramine appearing in fractions 12 to 36 from control preparations. The recoveries of the radioactivity chromatographed were 78% for solvent system 1 and 68% for solvent system 2. All the radioactivity recovered from chromatograms made with solvent system 1 was found in one peak with an R_F value of 0.91. When solvent system 2 was used, two peaks of radioactivity were recovered, with 83.3% of the recovered activity having the same R_F value as the acid metabolite.

Table 1 Amine metabolism in the isolated perfused mesenteric arterial bed of the rat

Amine	*Group*	(n)	*Total radioactivity recovered* (nmol)	*Amount of metabolites extracted* (nmol)
Benzylamine (25 μM)	Control	(4)	174.7 ± 3.8	30.8 ± 1.0
	MDL	(4)	176.4 ± 6.5	5.4 ± 0.4**
Tyramine (100 μM)	Control	(8)	1562 ± 53	69.2 ± 3.5
	Clorgyline	(6)	1616 ± 55	70.6 ± 4.1
	MDL	(6)	1574 ± 40	33.8 ± 2.4**
	MDL + Clorg	(4)	1549 ± 21	29.2 ± 2.9**
	Cocaine	(6)	1675 ± 40	92.6 ± 9.3*

The isolated perfused mesenteric vascular bed of the rat was perfused with radioactive amines, benzylamine (Bz) and tyramine (Tyr) in modified Krebs-Henseleit solution. Fractions of perfusate collected (0.5 ml or 1 ml) were assessed for their total radioactive content and, following acidification, the amount of radioactivity extracted into an organic solvent. Totals of 30 and 100 ml of perfusate were collected following Bz and Tyr infusion respectively. The values given in the table are the sums of the amounts found in each fraction. The preparations studied were from control rats (control), rats treated with 1 or 2 mg kg^{-1} MDL 72145 1 h before use (MDL), control rats where clorgyline (10 μM) had been added to the perfusing fluid for 30 min during the equilibration period (Clorg), rats treated with MDL 72145 where the preparation was also perfused with clorgyline (MDL + Clorg) and from control rats where cocaine (3 μM) was present in the perfusing fluid throughout the experiment.
(**$P < 0.001$; *$P < 0.05$ vs control by Student's *t* test).

Table 2 Chromatography of tyramine and its metabolites

Compound	R_F value Solvent 1	Solvent 2	Colour reaction
p-Hydroxyphenylacetic acid	0.91	0.54	pink
p-Hydroxyphenylethanol	0.92	0.91	orange
p-Hydroxyphenylacetadehyde	0.94	streaked	red
p-Tyramine	0.58	0.85	yellow
Radioactive metabolites	0.91	(a) 0.54 (83.3%) (b) 0.94 (16.7%)	

Tyramine and its metabolites were chromatographed on Whatman no. 1 paper by ascending chromatography. The solvent systems used were: (1) *n*-butanol-acetic acid-water (25 : 4 : 10) and (2) propan-2-ol-ammonia-water (28 : 1 : 6). The standard compounds were dissolved in ethylacetate (except tyramine which was dissolved in water). The aldehyde was prepared from its Wanzlich derivative on the day of the experiment. The metabolites collected from control mesenteric preparations perfused with radiolabelled tyramine were pooled and extracted as described in the methods section. Each chromatogram was allowed to run to at least 25 cm and the standard compounds detected by spraying the paper with a solution of Fast GG scarlet salt (1 mg ml^{-1} in 0.1 M HCl) followed by 1 M Na_2CO_3. The radioactivity was located by cutting the chromatogram into 1 cm bands and counting by liquid scintillation. The recovery of the radioactivity applied to the chromatogram was 78% from solvent 1 and 68% from solvent 2. The values given in the table are the mean for the metabolites extracted from four preparations and the percentage found at both sites on the chromatogram using solvent system 2 are shown.

The amine oxidase activities detected in homogenates of mesenteric blood vessels from the tyramine experiments are shown in Figure 2 and demonstrate the selectivity of the inhibitors used. Figure 3 shows the effects of clorgyline on the metabolism of Tyr by homogenates of mesenteric blood vessels. Tyr metabolism was inhibited by 40% with 0.1 μM clorgyline. with very little further inhibition occurring at higher clorgyline concentrations. The metabolism of Tyr by preparations from MDL 72145-treated rats was completely inhibited by 0·1 μM clorgyline.

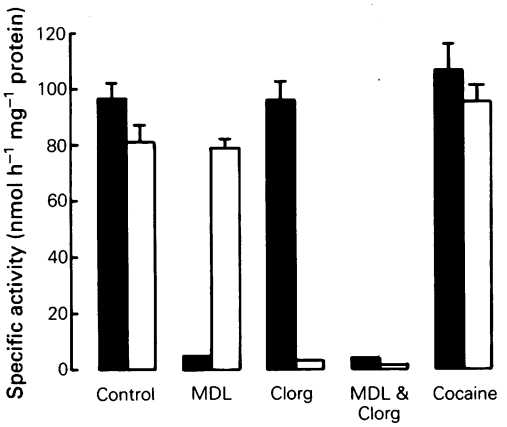


Figure 2 The specific activities of amine oxidase enzymes have been measured in homogenates of the mesenteric vascular beds following the perfusion experiments. Monoamine oxidase-A activity (open columns) was measured by incubating the homogenates with 500 μM [^{3}H]-5-hydroxytryptamine, Semicarbazide-sensitive amine oxidase activity (solid columns) was measured by the ability of the homogenates to deaminate [^{14}C]-benzylamine. The groups of mesenteric arterial beds examined in this way are the same as those given in Table 1 and the same abbreviations have been used here.

Discussion

These studies have shown that a significant amount of Bz is metabolized as it passes through the mesenteric arterial bed of the rat. The enzyme primarily responsible for this metabolism is SSAO demonstrating that this amine oxidase can function in intact blood vessels as well as in tissue homogenates. These findings complement those made with homogenates of this tissue, where almost all the metabolism of Bz could be attributed to SSAO. The lack of hydroxyl substituents on the Bz molecule make this amine extremely lipophilic (Mack & Bönisch, 1979) and able to enter and leave cells of the blood vessel wall with ease. Bz is the amine for which vascular SSAO has the highest affinity (K_m 2.06 ± 0.26 μM, $n = 4$ in mesenteric homogenates) and the one which is turned over at the fastest rate by this enzyme activity (Elliott *et al.*, 1989b).

Addition of an hydroxyl group in the *para* position of phenylethylamine results in a marked decrease in lipophilicity at physiological pH (Mack & Bönisch, 1979) and therefore in the ability to cross cell membranes. Tyr has been shown to be a substrate for both MAO-A and SSAO in tissue homogenates in rat blood vessels with little or no contribution from MAO-B (Coquil *et al.*, 1973;

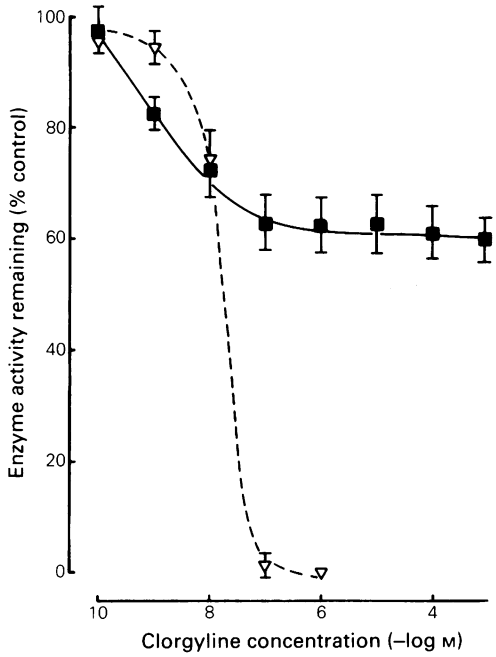


Figure 3 Shows the effect of clorgyline on the deamination of tyramine by homogenates of the mesenteric vascular bed of the rat to reveal the relative proportions of monoamine oxidase-A and semicarbazide-sensitive amine oxidase. Homogenates of this tissue were made following perfusion experiments and they were preincubated with various concentrations of clorgyline for 30 min at 37°C. The resulting metabolism of tyramine (100 μM) was measured radiochemically (see Methods). The metabolism has been measured in homogenates from control rats (■) and from rats treated with MDL 72145 (△). The metabolism found, following preincubation with clorgyline in control and MDL 72145-treated rats, has been expressed as percentages of the deamination in the absence of clorgyline and plotted against negative log clorgyline concentration. Each point is the mean value from 4 experiments; vertical bars show s.e.mean.

Clarke *et al.*, 1982). At the concentration used here (100 μM), 60% of the metabolism could be attributed to SSAO and 40% to MAO-A when tissue homogenates were examined. The metabolites of Tyr were readily released from the intact mesenteric arteries following infusion of the amine. Inhibition of SSAO reduced the metabolites appearing to 48% of control. MAO-A seemed to contribute very little to the formation of these metabolites even when SSAO had been inhibited. It has been assumed, that since MAO-B contributes very little to the metabolism of Tyr in these tissue homogenates, it is unlikely to contribute to the production of metabolites in the intact mesenteric arterial bed.

SSAO has been shown to be located in the medial part of the blood vessel wall (Lyles & Singh, 1985) and its activity is not affected by sympathectomy (Coquil *et al.*, 1973). MAO-A is an intracellular enzyme situated on the outer membrane of mitochondria (Cotzias & Dole, 1951; Hawkins, 1952; Greenawalt & Schnaitman, 1970). In tissues that have a sympathetic nerve supply, MAO has been shown to be present both intraneuronally and extraneuronally (Jarrott & Iversen, 1971) while the nerve endings are located at the adventitial-medial junction (Hodge & Robinson, 1972). Chemical sympathectomy caused a 70% decrease in MAO-A activity in rat femoral and mesenteric arteries (Coquil *et al.*, 1973). Blockade of neuronal uptake with cocaine increased the amount of metabolites formed during Tyr infusion. At the concentration used here, cocaine has been shown to potentiate the effects of directly acting sympathomimetic amines and to inhibit the responses to indirectly acting agents, such as Tyr, in this preparation (George & Leach, 1973; Elliott *et al.*, 1989c) indicating that it effectively blocked neuronal uptake. The fact that this resulted in an increase in metabolites leaving the tissue could indicate that the nerve endings in this tissue represented a significant site of accumulation of Tyr from which deaminated metabolites were not readily released. Blocking this site increased the availability of Tyr for the tyramine-metabolizing sites in the rest of the tissue. These experiments provide further evidence that neuronal MAO-A does not seem to contribute to the production of the metabolites collected from this preparation. However, the pressor response to Tyr was significantly reduced in the presence of cocaine resulting in a smaller rise in intraluminal hydrostatic pressure. It is possible that changes of this nature could have an influence on the efflux of metabolites. A possible way to exclude any such influence would be the use of an agent such as prazosin (Venning & de la Lande, 1985).

Following deamination of the amines, the aldehyde products can be metabolized to the corresponding acid or alcohol by aldehyde dehydrogenase (ALDH; EC 1.2.1.3.) or aldehyde reductase (EC 1.1.1.2) (Weissbach *et al.*, 1957; Leeper *et al.*, 1958; Goldstein *et al.*, 1961). The major metabolite extracted from the perfusate in these studies was the acidic metabolite of Tyr *p*-hydroxyphenylacetic acid (*p*-HPAA). Tacker *et al.* (1970) showed that when [^{14}C]-Tyr was given to rats by i.p. injection, 70% was excreted in the urine as *p*-HPAA. This is in contrast to the situation for noradrenaline, the metabolism of which in the whole rat and in isolated blood vessels produced alcohols as the principal metabolites (Kopin & Gordon, 1962; Venning & de la Lande, 1985). It seems that β-hydroxylated phenylethylamines are preferentially metabolized to the

corresponding alcohol by the rat, whereas phenylethylamines which lack the β-hydroxyl group are metabolized to the corresponding acid (Breese *et al.*, 1969). Mack & Bönisch (1979) demonstrated that the alcohol metabolites of hydroxylated amines were relatively lipophilic whereas the acid metabolites, at physiological pH, were present mainly in the dissociated form and were much less able to cross biological membranes. When the metabolism of noradrenaline was studied in isolated rat blood vessels, only small amounts of the acid metabolite were released from the tissue (Levin, 1974). By contrast, in these studies the acid metabolite of Tyr appeared very rapidly in the perfusing fluid. The presence of SSAO in the plasma membrane of smooth muscle cells may explain this anomaly. The subcellular location of aldehyde dehydrogenase in this tissue is not known. In brain and liver ALDH has been found in the mitochondrial and soluble fractions (Deitrich, 1966). Within the mitochondria, ALDH is to be found on the inner membrane (Pettersson & Tottmar, 1982), which is conveniently positioned to act on aldehydes formed by MAO. Microsomal ALDH has been demonstrated in the liver and the presence of this enzyme in the plasma membrane of vascular smooth muscle cells close to SSAO might be postulated from these results. Preliminary studies in which dianilino-ethane was used to precipitate the aldehyde from the metabolites collected (Wanzlick & Löchel, 1953) have confirmed that only a small proportion (less than 10%) of the metabolites collected, remain as the aldehyde.

The identity of the metabolites of Tyr and the activity responsible for their formation following inactivation of both SSAO and MAO-A (42% of the metabolism in the absence of inhibitors), remains to be determined. Some activity might be attributed to incomplete inhibition of SSAO in the intact tissue. In tissue homogenates from rats treated with MDL 72145, SSAO activity was reduced to 5% of the control value and no Tyr metabolism could be detected when MAO-A was inhibited as well. It would seem therefore, that the homogenization procedure has led to inactivation of the residual activity. It is possible that homogenization releases MDL 72145 from sites where it has been trapped in the intact preparation. It is unlikely that MAO-B is responsible for the residual metabolism of Tyr in the intact preparations from the rats treated with MDL 72145 since no Tyr metabolism due to this form of MAO could be detected in homogenates. In addition, MDL 72145 is a potent inhibitor of MAO-B (Zreika *et al.*, 1984) and the dose administered in our studies gave rise to almost complete inhibition of MAO-B in liver homogenates (data not shown).

The fact that radioactivity was extracted into the organic solvent from the acidified perfusate indicates that the amine group had been altered. An alternative way that this could be achieved is by acetylation of the amine moiety. Following inhibition of MAO and SSAO with iproniazid, the metabolism of dopamine and noradrenaline in the rat *in vivo* involved the formation of N-acetylated metabolites (Goldstein & Musacchio, 1962; Smith & Wortis, 1962). At present, this possibility remains to be explored.

In summary, SSAO can act in intact blood vessels on amines present in the perfusing fluid. The rapid release of acidic metabolites may indicate that these are formed at the cell surface and entry into the cells is not a prerequisite for metabolism.

J.E. is a Wellcome Veterinary Scholar.

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The influence of amine metabolizing enzymes on the pharmacology of tyramine in the isolated perfused mesenteric arterial bed of the rat

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1 The pressor response to the infusion of tyramine (Tyr) into the isolated perfused mesenteric arterial bed of the rat has been studied at both a low and a high dose (0.2 and 2.0 μmol) and the effect of monoamine oxidase-A (MAO-A) and semicarbazide-sensitive amine oxidase (SSAO) inhibition was examined. Very little MAO-B activity is found in homogenates of this tissue when Tyr is used as substrate.

2 Inhibition of SSAO by treating rats with 1 mg kg^{-1} (E)-2-(3',4'-dimethoxyphenyl)-3-fluoroally lamine (MDL 72145) 1 h before dissection, had no significant effect on the maximum pressure attained or the area under the curve (AUC) of the response to both low and high doses of Tyr. Inhibition of MAO-A, by inclusion of 10 μM clorgyline in the perfusing fluid, resulted in no significant potentiation at both low or high doses of Tyr. The inhibition of both these enzymes together substantially increased the AUC of the pressor response.

3 Cocaine (3 μM) significantly potentiated the responses to adrenaline (Ad). At this dose, cocaine significantly reduced the peak height and the AUC of the responses to both doses of Tyr.

4 Inhibition of extraneuronal uptake mechanisms with corticosterone (29 μM) did not potentiate the response to Ad and did not significantly alter the response to Tyr (low dose).

5 The effects of MDL 72145 and clorgyline on the directly acting amine, Ad, were studied. MDL 72145 caused a small but significant increase in the EC_{50} and in the maximum response to Ad, whilst clorgyline (10 μM) increased the EC_{50} value slightly and decreased the maximum response. When the two inhibitors were used in combination, a significant increase in the maximum response but with no change in the EC_{50} was seen.

6 These data indicate that the action of Tyr in this vascular bed is, at least, partly indirect. Inactivation of Tyr is effected by both MAO-A and SSAO in the blood vessel wall. Inhibition of both enzymes seems to be necessary to achieve a significant potentiation of the pressor response. The effects of these enzyme inhibitors on directly acting amines may mask any potentiation of the response when MAO-A or SSAO alone are inhibited.

Introduction

Tyramine (Tyr) metabolism in tissue homogenates of rat mesenteric blood vessels is catalysed by monoamine oxidase type A (MAO-A; EC 1.4.3.4) and by an amine oxidase that is resistant to inhibition by the acetylenic inhibitors of MAO, such as clorgyline (Coquil *et al.*, 1973). This enzyme activity has been called semicarbazide-sensitive amine oxidase (SSAO, EC 1.4.3.6) and its ability to metabolize Tyr added to the fluid perfusing isolated mesenteric blood vessels of the rat has been demonstrated (Elliott *et al.*, 1989). Unlike MAO, which is a mitochondrial enzyme (Greenawalt & Schnaitman, 1970), SSAO resides in the plasma membrane of smooth muscle cells (Wibo *et al.*, 1980; Lyles & Singh, 1985). This cellular location has led to speculation that this enzyme might have access to circulating amines before they have entered cells, (Callingham *et al.*, 1983) but the physiological function of SSAO remains unknown. SSAO inhibition has been shown to potentiate the contractile response of the rat aorta to tryptamaine, but only after MAO had been inhibited (Lyles & Taneja, 1987). Although Tyr is a substrate for MAO-B there is little of this enzyme present in rat mesenteric blood vessels and therefore it does not

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contribute significantly to the metabolism of this amine in homogenates of this tissue (Coquil *et al.*, 1973; Elliott *et al.*, 1989).

Tyr has been shown to release [^{3}H]-noradrenaline from labelled stores in the isolated perfused mesenteric arterial bed of the rat (George & Leach, 1975). In order to act in this way, Tyr must pass from the lumen of the vessel, across the medial layer, to the adventitial-medial junction, where the nerve endings are located (Hodge & Robinson, 1972). Tyr also causes pressor responses in the perfused mesenteric vascular bed, which can be prevented by blockade of neuronal uptake with cocaine (George & Leach, 1973). Tyr has also been shown to have some direct action on vascular smooth muscle (Toda *et al.*, 1978; Iriarte *et al.*, 1985) which may involve stimulation of α-adrenoceptors (Krishnamurty & Grollman, 1972; Miyahara & Suzuki, 1985). However, much of the evidence indicating that inhibition of MAO leads to potentiation of the actions of Tyr has been complicated by the fact that many of the agents used also inhibit SSAO (see Lyles, 1984).

In order to examine whether or not SSAO is involved in the termination of the actions of circulating Tyr, it was decided to determine the relative contributions of SSAO and MAO to Tyr inactivation in a bed of resistance blood vessels. The isolated perfused mesenteric arterial bed of the rat was chosen for this purpose. Preliminary results of this study have been described elsewhere (Elliott & Callingham, 1988).

Methods

The method of isolation and perfusion at constant flow (2 ml min^{-1}) of the mesenteric arterial bed of the rat was followed as previously described (Elliott *et al.*, 1989) and the pharmacological responses to the infusion of 20 ml of Krebs solution containing 100 μM Tyr were recorded at the same time as the metabolites appearing in the perfusate were measured. The 5 groups of preparations examined for their ability to metabolize Tyr were also evaluated for the pressor response to Tyr. These results are described here for comparison with another series of experiments where the responses to a lower dose of Tyr were also studied.

The experimental protocol for the examination of the low dose of Tyr differed slightly from that previously described (Elliott *et al.*, 1989). Once again, preparations from both control rats and rats injected with 1 mg kg^{-1} MDL 72145 1 h before dissection were examined. In some preparations clorgyline (1 or 10 μM) was added to the Krebs solution for the first 30 min of the equilibration period, after which time, perfusion with clorgyline-free Krebs solution was resumed. Following the 45 min equilibration period, a dose-response curve to adrenaline (Ad) was constructed. Doses of Ad (0.6 to 20 nmol) were administered (in 200 μl of Krebs solution) through an injection port into the perfusing fluid. Delay between injection and the beginning of the response was less than 5 s. The maximum increase in pressure was recorded. When the effects of cocaine (3 μM) and corticosterone (29 μM) were studied, these drugs were added to the Krebs solution 20 min before the infusion of Tyr. Two further doses of Ad were injected and the responses compared with those obtained in the absence of these drugs. Corticosterone was dissolved in ethanol before dilution in Krebs solution. Addition of this vehicle alone had no effect on the responses to Ad.

Three parameters of the Tyr pressor response were measured, the maximum rise in pressure, the area under the curve (AUC) of the response and the time for the response to return to within 1 mmHg of the resting perfusion pressure. The AUC of the response was measured by cutting out the chart recording and weighing the paper. A value of 1 mmHg above the resting pressure was used as the end point of the response as some preparations settled at a new resting pressure 1 mmHg or less above the original resting pressure. None of the preparations failed to return to within 1 mmHg of the baseline perfusion pressure.

The effect of corticosterone on the responses of this preparation to Ad was investigated further in some control preparations. Responses to Ad at doses around the EC_{50} value (1 and 2 nmol) were obtained in the absence of any drug. Following this, corticosterone (29 μM) or cocaine (3 μM) was added to the Krebs solution and 15 min later the doses were repeated. Finally, the second drug (cocaine or corticosterone) was added and the responses repeated in the presence of both drugs. Repetition of these doses of Ad without the addition of either cocaine or corticosterone to the Krebs solution did not result in a significant alteration in the response observed. In a small number of experiments, corticosterone was replaced with metanephrine.

Drugs and solutions

The modified Krebs-Henseleit solution had the following composition (mM):- NaCl 118, KCl 4.57, $CaCl_2$ 1.27, KH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 25 and glucose 5.55. Tyramine hydrochloride, adrenaline bitartrate, metanephrine hydrochloride and corticosterone were all obtained from Sigma Chemical Co., Poole, Dorset. MDL 72145 was a gift from Merrell Dow Research Institute, Strasbourg, France and clorgyline hydrochloride was a gift from May and Baker Ltd., (now Rhône-Poulenc), Eccles,

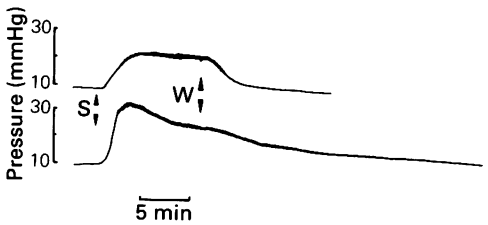


Figure 1 Typical responses of the isolated perfused mesenteric arterial bed of the rat to infusion of modified Krebs-Henseleit solution containing 10 μM tyramine (Tyr). The infusion started at 'S' and a total of 20 ml was perfused at a flow rate of 2 ml min^{-1}. At 'W' perfusion with Tyr-free Krebs solution was resumed and the Tyr was washed out of the preparations. The upper trace shows the response of a preparation from a control rat. The lower trace is a preparation from a rat that was treated with MDL 72145 1 h before use and the preparation was perfused with Krebs solution containing 1 μM clorgyline for 30 min at the start of the experiment. The baseline pressure was 9.8 mmHg for both preparations and the maximum pressure attained, the AUC of the response and the time for the response to return to within 1 mmHg of the original baseline pressure, were measured.

Manchester. Cocaine hydrochloride BP was purchased from Macarthys Medical Ltd., Romford, Essex. All other reagents were of analytical grade where possible.

Statistical analysis of the results

Statistical significance of the Tyr responses was evaluated by Student's t test (unpaired) comparing each group with the control. The significance of changes in response to Ad in the presence of cocaine or corticosterone were analysed by a paired Student's t test. The dose-response curves were fitted to the observations using a non-linear regression programme (Harwell Library non-linear programme VBO1A) which calculated the Hill slope, the EC_{50} and the maximum pressure response in each case. The equation employed was:

$$\text{Increase in pressure} = Resp_{max} \times D^n/(D^n + EC_{50}{}^n)$$

where D is the dose of Ad added, n is the Hill coefficient, EC_{50} is the dose giving the half maximal response and $Resp_{max}$ is the maximum response. Each point was weighted according to the reciprocal of its variance. The curves were also compared with the control curve using the method of De Lean *et al.* (1978), fitting the two curves simultaneously with none of the parameters constrained and then constraining each in turn (NAG library routine EO4 FDF). The F-test was applied to the sum of the squares of residuals in each situation and a change in the parameter was taken to be significant when $P < 0.05$.

Results

Responses of the preparations to infusion of Tyr (10 μM) are exemplified in Figure 1. The pressure rose within 1 min of the start of the infusion and rapidly reached its maximum. In some preparations, this level was maintained throughout the Tyr infusion but, in others, it fell gradually. This was seen in all

Table 1 Responses of isolated perfused mesenteric arteries to the infusion of tyramine (10 μM)

Group	(n)	*Max. pressure* (mmHg)	*AUC of response* (arbitrary units)	*Time to washout* (min)
Control	(8)	18.7 ± 3.7	108.0 ± 20.5	6.9 ± 0.5
Clorgyline (1 μM)	(8)	18.8 ± 2.7	118.6 ± 17.5	9.3 ± 0.7*
Clorgyline (10 μM)	(6)	16.5 ± 1.8	106.0 ± 11.8	7.9 ± 0.4
MDL	(8)	17.4 ± 3.6	109.0 ± 21.2	11.2 ± 0.7**
MDL + clorgyline	(8)	28.0 ± 5.8	204.0 ± 28.9*	21.4 ± 1.1***
Cocaine	(6)	7.6 ± 1.4*	47.8 ± 8.0*	5.0 ± 0.5*
CCS	(6)	14.5 ± 1.4	90.2 ± 8.0	7.0 ± 0.3

Tyr was infused into each preparation in modified Krebs-Henseleit solution at a flow rate of 2 ml min^{-1} and a total of 20 ml of Krebs solution containing 10 μM Tyr was infused before perfusion with Tyr-free Krebs solution was resumed. The maximum pressure attained, the AUC of the Tyr response and the time for the response to return to within 1 mmHg of the baseline following the return to perfusion with Tyr-free Krebs solution, have been measured. The groups were composed of preparations from control rats (control), from control rats perfused with clorgyline for 30 min at the start of the experiment (1 μM or 10 μM), from rats treated with 1 mg kg^{-1} MDL 72145 1 h before dissection (MDL), from MDL 72145-treated rats that have also been perfused with Krebs solution containing 1 μM clorgyline, and from control rats with 3 μM cocaine (Cocaine) or 29 μM corticosterone (CCS) added to the Krebs solution 20 min before the Tyr infusion. In each case the mean response ± s.e.mean has been given for 6 to 8 preparations in each group. The values obtained have been compared with the control values by an unpaired Student's t test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Table 2 Responses of the isolated perfused mesenteric arteries to the infusion of tyramine (100 μM)

Group	(n)	*Max. pressure* (mmHg)	*AUC of response* (arbitrary units)	*Time to washout* (min)
Control	(8)	29.3 ± 4.1	150.1 ± 13.8	13.6 ± 0.9
Clorgyline (10 μM)	(6)	39.9 ± 7.4	191.2 ± 24.2	16.6 ± 1.7
MDL	(6)	19.9 ± 5.5	169.1 ± 38.7	22.8 ± 3.5*
MDL + clorgyline	(4)	33.0 ± 4.4	302.5 ± 23.2**	44.0 ± 2.4***
Cocaine	(6)	16.5 ± 1.3*	88.4 ± 12.3**	5.8 ± 0.5***

Twenty ml of Krebs solution containing 100 μM Tyr was infused and the same response parameters were measured as described in the legend to Table 1. The 5 groups of preparations examined at this higher dose of Tyr were the same as those described in Table 1 (the same abbreviations have been used) except that no group treated with 1 μM clorgyline was examined, nor were the effects of corticosterone on the response to this dose, tested.

groups of preparations but generally the higher the peak pressure the more likely there was to be a gradual fall in the perfusion pressure during the Tyr infusion. When the perfusing fluid was changed to Tyr-free Krebs solution the pressure began to fall within 1 min. In Figure 1 the upper trace is from a control preparation and the lower trace from a preparation treated with both MDL 72145 and clorgyline (1 μM). The time for the pressure to return to the resting level was considerably longer in the second preparation than in the first. The pattern of responses to the higher dose of Tyr was very similar. All three parameters were substantially increased following the high dose of Tyr when compared with the low dose. The data for the responses to both low and high doses of Tyr are given in Tables 1 and 2 respectively. In each case the AUC of the Tyr response was only significantly increased when preparations from MDL 72145-treated rats were also treated with clorgyline. MDL 72145 treatment alone significantly increased the time for the response to return to within 1 mmHg of the resting level following wash out of both high and low doses of Tyr. Any increase in the peak response or the AUC of the response caused by clorgyline or MDL 72145 treatment alone did not reach statistical significance. Cocaine (3 μM) depressed all three parameters significantly at both high and low doses of Tyr, whereas corticosterone (tested on the low dose only), had no effect on any of these parameters.

The dose-response curves to Ad in the five groups of preparations where the low dose of Tyr was tested are shown in Figure 2 and represent the computer-generated best fit curves. The three parameters generated for each curve are shown in Table 3. When the curves were compared with the control curve by a simultaneous curve fitting method, the small increase in EC_{50}, seen in the MDL 72145-treated and clorgyline-treated (both 1 and 10 μM) groups, reached statistical significance, as did the increase in the maximum response for the MDL 72145 group and group treated with both MDL 72145 and clorgyline. There was also a significant decrease in the maximum response in the group of preparations treated with clorgyline (10 μM). No significant alteration occurred in the Hill slope in any of the groups tested.

The effects of cocaine and corticosterone on the response to Ad at doses near to the EC_{50} are shown in Figure 3. Corticosterone (29 μM) alone did not alter the response to Ad (1 or 2 nmol) whereas cocaine (3 μM) significantly increased the response at

Table 3 Effect of amine oxidase inhibitors on the sensitivity of the perfused mesenteric arterial bed to adrenaline

Group	(n)	*Hill slope*	EC_{50} (nmol)	*Max. pressure* (mmHg)
Control	(8)	1.32 ± 0.08	1.56 ± 0.08	89.3 ± 1.59
Clorgyline (1 μM)	(8)	1.48 ± 0.08	1.96 ± 0.11*	90.4 ± 1.74
Clorgyline (10 μM)	(6)	1.52 ± 0.12	1.94 ± 0.16*	81.0 ± 2.24*
MDL	(8)	1.38 ± 0.03	1.96 ± 0.04**	94.0 ± 0.60*
MDL + clorgyline (1 μM)	(8)	1.46 ± 0.11	1.72 ± 0.11	103.0 ± 2.33*

The data are derived from the 5 curves shown in Figure 2. The abbreviations for the groups of preparations used are the same as those given in Table 1. A weighted non-linear regression computer programme was used to determine the line of best fit for each curve as described in Methods and the three parameters, Hill slope, EC_{50} and maximum response were generated. A method for simultaneously fitting two curves (De Lean *et al.*, 1978) with or without one of the parameters constrained was used to test for a significant difference from the control curve. (* $P < 0.05$; ** $P < 0.01$).

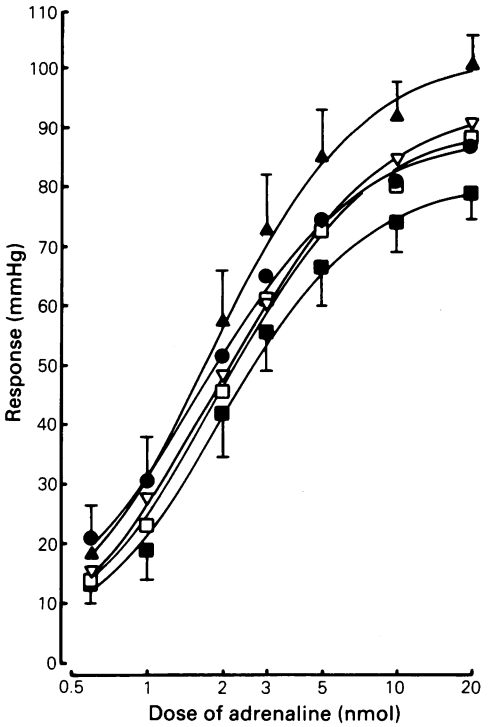


Figure 2 Dose-response curves of the isolated perfused mesenteric arterial bed of the rat to adrenaline (Ad). Doses of Ad (0.6 to 20 nmol) were injected into the perfusing fluid (in 200 μl of Krebs solution) and the maximum rise in pressure is plotted against log of the dose of Ad given. Each point represents the mean value from 6 or 8 preparations. The s.e.mean values are shown only for the two outside curves for clarity, but were of the same magnitude for the other three curves. The lines of best fit have been drawn through the points using a weighted, non-linear regression computer programme described in the methods. The values obtained for EC_{50} Hill slope and maximum response for each group of preparations are given in Table 3. The 5 curves shown are control preparations (●), preparations from rats treated with MDL 72145 (▽), preparations that have been perfused with Krebs solution containing clorgyline for 30 min (1 μM, □; 10 μM, ■) and preparations that have been perfused with Krebs solution containing 1 μM clorgyline from rats that have been treated with MDL 72145 (▲).

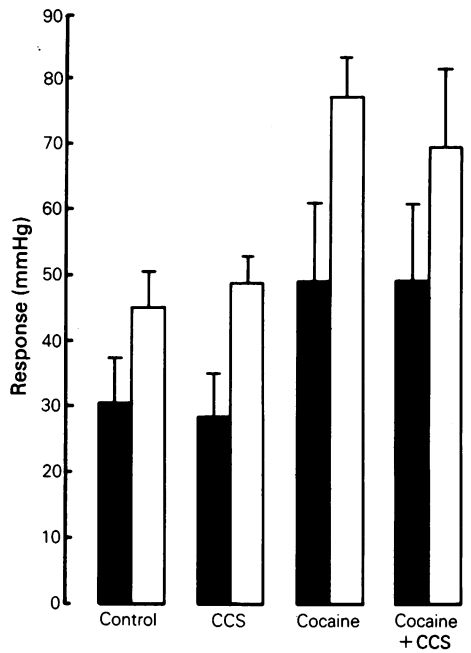


Figure 3 The effect of corticosterone and cocaine on the responses of the isolated perfused mesenteric preparation of the rat to adrenaline (Ad). The increase in pressure when 1 nmol (solid columns) or 2 nmol (open columns) of Ad was injected into the perfusing fluid (in 200 μl of Krebs solution) was recorded in the absence of either drug (Control). The responses to Ad were tested again, 15 min after the addition of either corticosterone (CCS, 29 μM) or cocaine (3 μM) to the Krebs solution. Finally the second drug was added to the Krebs solution and the responses to Ad obtained for a third time (cocaine + CCS). Each column represents the mean from at least 4 preparations with s.e.mean shown by vertical bars. The responses were compared with control by a paired t test. The addition of cocaine always resulted in a significant increase ($P < 0.05$) in the responses to both doses of Ad even when added after corticosterone (data not shown). Addition of corticosterone alone did not increase the responses significantly nor did it result in any further potentiation when added after cocaine (Cocaine + CCS).

both these doses. This increase was seen when cocaine was added first and when it was added following the addition of corticosterone. Addition of corticosterone after cocaine did not produce any further increase in the response to Ad. Metanephrine was tested in the same way as corticosterone at concentrations from 0.1 to 5 μM. No potentiation was seen at the low doses even in the presence of cocaine and at 5 μM, metanephrine produced inhibition of the responses to Ad.

Discussion

These experiments indicate, that the action of Tyr, in the perfused mesenteric arterial bed of the rat, has a substantial indirect component, confirming earlier observations (e.g., George & Leach, 1973) since pressure responses to Tyr are reduced when cocaine, at a dose which potentiates the responses to Ad, is added to the perfusing fluid. In order to act in this way, Tyr

must pass from the lumen of the vessel, across the medial layer, to the adventitial-medial junction, where the nerve endings are located (Hodge & Robinson, 1972).

When Tyr is added to the perfusing fluid, it is metabolised by SSAO and the acid metabolite, *p*-hydroxyphenylacetic acid, appears in the perfusate (Elliott *et al.*, 1989). SSAO is situated in the medial layer of the blood vessels (Lyles & Singh, 1985) and so inhibition of this enzyme might be expected to allow easier access of Tyr to the nerve endings. In the present experiments, inhibition of SSAO by MDL 72145 did not significantly increase the pressure or the AUC of the response at either of the two doses of Tyr tested. It did, however, delay the return of the pressure to the resting level. For this to occur without an increase in the AUC of the response the shape of the response must have been altered. Metabolism of Tyr by SSAO as it leaves the cells of the tissue seems to contribute to the rapid restoration of the normal resting pressure. The response may be altered because the drug treatment also reduced the postsynaptic response to α-adrenoceptor stimulation as shown by the increase in the EC_{50} value for Ad. This effect may have masked the effect of SSAO inhibition on the Tyr response. MDL 72145 when administered to rats *in vivo* at the dose used in these studies also inhibits MAO-B in tissues such as the brain and intestine (Fozard *et al.*, 1985). However, homogenates of rat mesenteric arteries possess very little MAO-B activity capable of metabolizing Tyr (Coquil *et al.*, 1973). At both the concentrations of Tyr used in these studies (100 and 10 μM) we have been unable to detect a contribution of MAO-B activity to the deamination of this amine (Elliott *et al.*, 1989, unpublished data) and so have attributed the effects seen with MDL 72145 to inhibition of SSAO in this tissue.

The other Tyr-metabolizing activity known to be present in this tissue is MAO-A, a mitochondrial enzyme present both in the nerve endings and extraneuronally (Jarrott & Iversen, 1971; Coquil *et al.*, 1973). Metabolites attributable to this activity did not appear in the perfusate, possibly because they were not readily released following their formation by the mitochondrial enzyme (Elliott *et al.*, 1989). Inhibition of this enzyme alone did not produce statistically significant increases in perfusion pressure or in the area under the response at either high or low doses of Tyr. Clorgyline has been shown to inhibit the action of directly acting sympathomimetic amines on smooth muscle preparations by blocking α-adrenoceptors (Finberg & Tenne, 1982), an effect which was thought to be reversible on wash out of the drug. In the rat mesenteric bed substantial inhibition of the response to Ad occurred in the presence of clorgyline and this effect was also reversed on washing. Nevertheless, clorgyline (10 μM) appeared to have some effect that was difficult to wash out as the EC_{50} of the dose-response curve to Ad increased and the maximum response decreased. When a lower dose of clorgyline (1 μM) was used in an attempt to overcome these problems, the EC_{50} was still increased. At the same time, the MAO-A activity was only reduced to 20% of the control value (data not shown). This shows the importance of not extrapolating from inhibition found in tissue homogenates, (where 1 μM clorgyline completely inactivates MAO-A) to the whole tissue. Potentiation of the Tyr response in other tissues was only observed following greater than 90% inhibition of MAO-A by clorgyline (Finberg & Tenne, 1982).

The evidence that MAO contributes to inactivation of Tyr can be seen from the effect of inhibiting both MAO-A and SSAO together. The AUC of the Tyr response was substantially increased at both doses due to a 3 to 4 fold increase in the time for the response to return to the baseline. It would seem, therefore, that these two enzymes work together to activate Tyr as it diffuses from the lumen of the blood vessel to its site of action. Inhibition of one of them alone has much less effect than inhibiting both together. De la Lande *et al.* (1970) found that iproniazid potentiated the action of intraluminally applied Tyr much more than extraluminal application of this amine to the isolated perfused ear artery of the rabbit. Iproniazid inhibits all the forms of amine oxidase considered here (Lyles, 1984). Similar results were obtained by Iriarte *et al.* (1985) in the rabbit aorta when both pargyline and semicarbazide were used. Rabbit blood vessels have been shown to possess SSAO as well as MAO (Rucker & Goettlich-Riemann, 1972).

The extraneuronal uptake process was considered as another possible mechanism whereby the tissue could be protected from the pressor or other effects of Tyr. Corticosterone, at a dose which gave 95% inhibition of extraneuronal uptake of noradrenaline in the rat heart (Iversen & Salt, 1970) did not alter the response to Tyr significantly. No potentiation of the tissue responses to Ad, even in the presence of cocaine to block neuronal uptake, could be demonstrated. Metanephrine, a potent and selective extraneuronal uptake blocker (Burgen & Iversen, 1965), was also tested in these present experiments, and failed to potentiate the responses to Ad. These data support those of Venning & de la Lande (1984) who concluded that rat blood vessels, unlike those of the rabbit, had a poorly developed extraneuronal uptake process. Even in rabbit blood vessels, corticosterone failed to show potentiation of Tyr applied to the intimal surface, suggesting that Tyr is not transported into cells via a corticosterone-sensitive mechanism (Iriarte *et al.*, 1985).

In conclusion, rat blood vessels contain MAO-A and SSAO, both of which appear to be important in the inactivation of Tyr in the blood vessel wall. The role of these blood vessel enzymes in protecting against ingested Tyr remains uncertain as the first enzyme activity to be encountered will be MAO in the intestinal villus. This has been shown to be responsible for a considerable, first pass metabolism of Tyr (Davis *et al.*, 1984; Hassan *et al.*, 1988) and Tyr absorbed would be exposed to MAO in the liver en route to the systemic circulation. Nevertheless, in rat *in vitro* models where MAO inhibitors are tested for their ability to potentiate the actions of Tyr, the influence of SSAO should be considered when interpreting the results. It should also be noted that, although human blood vessels possess a similar SSAO activity (Lewinsohn, 1981), it has a very low affinity for Tyr (Precious & Lyles, 1988). Thus it would be unwise to suggest, from experiments of this nature, that inhibition of SSAO in the human subject, is involved in the 'cheese reaction' (Blackwell, 1963).

J.E. is a Wellcome Veterinary Scholar.

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Spinal antinociceptive actions of μ- and κ-opioids: the importance of stimulus intensity in determining 'selectivity' between reflexes to different modalities of noxious stimulus

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1 In electrophysiological experiments in spinalized rats, μ- and κ-opioids were tested intravenously on the responses of single motoneurones to electronically controlled, alternating noxious heat and noxious pinch stimuli. The effects of μ- and κ-opioids were compared with those of the general anaesthetic α-chloralose and the dissociative anaesthetic/PCP ligand ketamine.

2 The κ-opioids U-50,488 (0.5–16 mg kg^{-1} i.v.) and tifluadom (0.05–1.6 mg kg^{-1} i.v.) had very similar actions to the μ-opioid fentanyl (0.5–16 μg kg^{-1} i.v.). Thus all three agonists reduced thermal and mechanical nociceptive reflexes in parallel and in a dose-dependent manner, but only so long as neuronal responses to the alternating stimuli elicited similar excitability levels in the neurone under study. Ketamine (0.5–16 mg kg^{-1} i.v.) had similar actions to the opioids whereas α-chloralose (20 mg kg^{-1} i.v.) had very little effect on neuronal responsiveness.

3 Apparently 'selective' depressions by both μ- and κ-opioids could be orchestrated by a deliberate mismatch of the intensities of alternating noxious heat and pinch stimuli; as measured by neuronal firing rate, the weaker of the responses to either type of stimulus was invariably reduced to a greater degree.

4 Similar 'selectivity' could be demonstrated for both μ- and κ-ligands when the weaker and stronger responses were of the same modality, being applied by the same pincher device but with alternating applied force.

5 It is concluded that the 'selective' spinal actions of κ-opioids seen in non-thermal over thermal behavioural models of nociception is likely to be related to the relative intensities, rather than the modalities, of the noxious stimuli used. The validity of the interpretation of results obtained in such behavioural studies is discussed.

Introduction

For several years there has been evidence that opioids which act preferentially on the κ- subclass of opioid receptors (Martin *et al.*, 1976) are less effective in suppressing responses to thermal than responses to non-thermal noxious stimuli. Most of this evidence comes from behavioural experiments in intact animals in which the reflex output from the spinal cord is monitored. This effect of κ-agonists was investigated more formally by Tyers (1980) and Upton *et al.* (1982) who reported that with κ- (but not μ-) preferring ligands, appreciably higher doses were required to suppress responses in the rat tail flick and hotplate tests than responses in inflamed paw and writhing tests. Since in these studies drugs were administered systemically, it was not clear to what extent the effects observed were mediated at supraspinal, rather than spinal, sites. Subsequently,

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however, there have been various studies in which the intrathecal route of drug administration has been used in chronically-implanted rats (Yaksh & Rudy, 1976) and several such studies have again indicated a markedly lesser effectiveness of κ- as compared with μ-agonists on thermally evoked reflexes (e.g. Schmauss *et al.*, 1983; Bryant *et al.*, 1983; reviewed by Yaksh & Noueihed, 1985; Millan, 1987). Other studies have, however, indicated that an apparent selectivity of opioid action may be related to the stimulus intensities used (Vonvoigtlander *et al.*, 1983; Hayes *et al.*, 1987; Millan, 1989).

However, such behavioural data are not without problems of interpretation. Thus between the various animal behavioural tests used, both the intensity and the temporal synchronization of the nociceptive inputs are inevitably different. Drug potency differences may thus reflect either differential drug effectiveness on different pathways activated by different inputs, or may alternatively reflect varying effectiveness on responses of different magnitudes and/or degrees of synchrony in a common pathway. It is not clear to what degree the μ/κ differences quoted above relate to these aspects. In this context it is relevant that in the rat, much of the sensory input arising from both thermal and non-thermal noxious stimuli is thought to be mediated by a common class of primary afferent, the polymodal nociceptor (e.g. Lynn & Carpenter, 1982). There is a difficulty in explaining how activation of different sub-classes of spinal opioid receptor could have highly 'selective' effects on different modalities of nociceptive information carried to the spinal cord largely by the same class of primary afferent.

It has therefore been important to perform experiments which allow more accurate control over some of the relevant parameters. Because of variations in drug effectiveness between cells, all data in this study were obtained from cells responding alternately to noxious heat and noxious pinch stimuli. We now report that in electrophysiological experiments on spinalized rats, systemically administered κ-opioids are equally effective against thermal and mechanical nociceptive spinal reflexes so long as the stimuli are controlled such as to elicit similar excitability levels in the neurone under study. Altering the relative intensities of these alternating stimuli can, however, result in apparent, but false, 'selectivity'. The findings are discussed in relation to the intensity of the peripheral stimuli used in behavioural tests. Some of the results have been presented previously in a preliminary form (Headley *et al.*, 1984; 1987c; Parsons *et al.*, 1986). Data on the relative potencies of the agonists, and on their selectivity between nociceptive and non-nociceptive reflexes, are presented in accompanying papers (Parsons & Headley, 1989; Parsons *et al.*, 1989).

Methods

Animal preparation

Some of the experimental details have been published elsewhere (Headley *et al.*, 1987b). Rats were anaesthetized throughout experiments with α-chloralose (100 mg kg^{-1}, i.p., initially), supplemented with halothane (1 to 2% in O_2) during surgery. Respiration was spontaneous; inspired air was enriched with O_2. Tracheal, carotid and jugular cannulae were inserted and then a dorsal laminectomy was performed from lumbar 6 to thoracic 10 vertebrae. The spinal cord was transected at the level of thoracic 10 vertebra. Blood pressure was monitored continuously and experiments were terminated if systolic blood pressure fell below 100 mmHg (13 kPa) for any sustained period (i.e. other than transient drug-induced effects). In later experiments end tidal CO_2 was also monitored with an ADC FM1 fast response CO_2 analyser.

Animals were immobilized in a recording frame and core temperature was maintained at 37°C by a combination of a lamp-warmed paraffin pool over exposed dorsal tissues and by a thermistor controlled blanket. Fluid therapy (Haemaccel: Hoechst; or isotonic saline) was given intravenously at about 80 ml kg^{-1} 24 h^{-1}. In later experiments, animals received 1 mg kg^{-1} i.v. of betamethasone (a short-lasting corticosteroid) prior to surgery; in some cases they were given a blood transfusion at the end of surgery.

Peripheral somatosensory stimuli

The natural peripheral stimuli used in these experiments were noxious pinch and noxious heat of the ipsilateral hind paw and/or toes; both were controlled electronically for constancy of intensity, duration and repetition rate throughout any drug test. Pinch stimuli (15–20 s) were, in the majority of experiments, delivered by a pair of pneumatically driven Allis tissue forceps (Brown *et al.*, 1984); no attempt was made to monitor the force applied, but similar stimuli were judged to be painful when tested on the experimenters. In later experiments a different pincher device, which delivered stimuli over a 3 mm^2 area, incorporated a strain gauge which indicated that a force of 0.3–1.5 newtons (depending on site) was required to elicit sustained nociceptive responses. Noxious thermal stimuli (ramped from 37°C to 45–50°C over 10–15 s and held for a further 15–20 s) were delivered to glabrous skin of the ipsilateral plantar foot and/or toes via a copper contact thermode (1.5 cm^2) with feedback control. With all cells (except those tested as in Figure 6) noxious heat

and noxious pinch stimuli were alternated in a regular cycle of 3–4 min and their intensities were adjusted with considerable care so that they evoked matched responses in terms of firing rates of the motoneurone under study. The alternated stimuli were delivered in close spatial proximity in the neuronal receptive field.

Neuronal recording

Extracellular recordings of nociceptive responses were made with silver wire hook electrodes from single motoneurone axons in fine filaments of ventral roots L4–L6. Spike conformation was monitored on a digital storage oscilloscope so as to help ensure that the activity of only a single spike was analysed throughout any comparative series of drug tests. The receptive field properties of neurones were carefully mapped and noted.

A pen recorder displayed continuous traces of (1) neuronal firing rate in 2 second epochs, (2) integrated spike counts for stimulus-related epochs, (3) the mechanical force applied by the pincher and (4) the temperature of the contact thermode. In later experiments, integrated spike counts elicited by pinch stimuli were divided into early and late components as the initial adaptive phase of a response to a noxious mechanical stimulus presumably contains a greater non-nociceptive component than the later sustained phase. This late response was then used for further quantitative analysis of mechanical nociceptive reflexes. The integrated spike counts were sent to a microcomputer for online computations of drug effects (see Headley *et al.*, 1985); these effects were expressed as a percentage of control, where the control was the mean of the three response cycles immediately preceding the drug administration. It is these percentage figures based on spike counts (rather than on firing rates) that have been used in the analysis presented below.

Drugs, drug administration and analysis

Drugs were selected on the basis of three criteria: (1) specificity for the receptor subtype under study, (2) suitability for systemic administration and (3) half lives that are short enough to allow multiple drug testing on any one cell. Fentanyl was chosen as the μ-agonist; for its receptor selectivity in binding tests see Magnan *et al.* (1982). U-50,488H (*trans*-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide; Upjohn) and tifluadom (1-methyl-2(3-thienylcarbonyl)-aminomethyl-5-(2-fluorophenyl)-H-2,3-dihydro-1,4-benzodiazepine; Sandoz) were chosen as κ-agonists; their receptor selectivity has been demonstrated in a variety of different models (e.g. Römer *et al.*, 1982a,b; Leander, 1983; Vonvoigtlander *et al.*, 1983; Burkard, 1984; Hayes *et al.*, 1985; 1986). Drug doses refer to fentanyl and ketamine bases, tifluadom hydrochloride and U-50,488 methane sulphonate. Fentanyl (Sublimaze, Janssen) and ketamine (Ketalar; Parke-Davis) were obtained from commercial sources.

Drugs were administered intravenously in a logarithmic (base 2 for agonists; normally base 10 for naloxone) cumulative dose regime, starting with a dose which was found to be near threshold on most cells, and reaching a maximum of $16\,\mu g\,kg^{-1}$ for fentanyl, $1.6\,mg\,kg^{-1}$ for tifluadom and $16\,mg\,kg^{-1}$ for U-50,488H and ketamine. The interval between doses was 3 to 6 min depending on the time necessary for particular drugs to produce their peak effects (assessed from previous single dose tests). The effects of every drug test were quantified with three measurements on the spike counts (1) the peak effect on neuronal response amplitude, expressed as a percentage of control values, (2) the maximal recovery from this effect and (3) the time taken for 50% recovery of responses. Drug effects were only accepted for analysis if recovery of responses by at least half of the maximum drug effect occurred over a time course compatible with the known kinetics of the drug under study. In the interests of speed of recovery the cumulative dose regime was halted once a drug had reduced the evoked responses to below 25% of control. This strategy permitted both μ- and κ-agonists to be tested under closely comparable conditions on most of the cells examined.

The fact that the complete dose-response regime was not followed on all cells has the disadvantage that traditional mean dose-response analysis cannot be performed; the apparent dose-response curves are shallower than they would be because the higher doses were tested only on the more resistant cells. Nonetheless, mean percentage reductions at particular drug doses are presented graphically in Figures 2 and 4 because they do provide a valid comparison of mean drug effectiveness against responses to the two stimulus modalities. In addition, ED_{50} values were calculated for those individual cells on which it was possible to perform a linear regression analysis on the depression of responses by at least 3 different doses tested over the 'linear' region of the dose-response curve (from 25–75% of control values). On 10 cells, these estimated ED_{50}s were compared with those obtained from a curve fitting programme (written by Dr R. Barlow) and were found to agree closely. The individual ED_{50} values were then averaged to give a mean ED_{50} for each drug.

Any differences between μ- and κ-opioids on thermal and mechanical nociceptive reflexes were tested for statistical significance on pooled data with

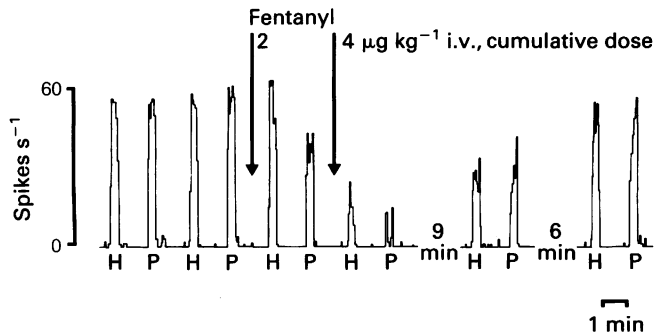


Figure 1 Effect of fentanyl on the responses of a rat motoneurone to alternating thermal and mechanical noxious stimuli. The neurone was activated by noxious heat (H; from a baseline of 37°C to 49.8°C for 30 s) of the ipsilateral hind paw and pinch (P; for 15 s) of toe 5 ('little' toe), alternating in a regular 3 min cycle. Fentanyl was administered in two divided doses of 2 $\mu g kg^{-1}$ i.v. Full recovery from the effects of fentanyl was evident after 21 min. Multireceptive motoneurone recorded in lumbar 5 ventral root (VR L5) of an α-chloralose anaesthetized, spinalized rat.

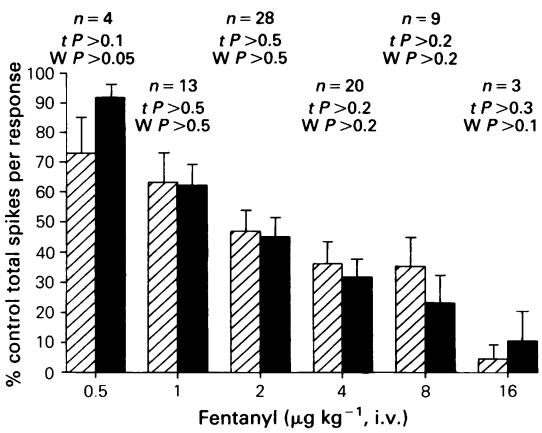


Figure 2 Pooled, paired data from tests with i.v. fentanyl on the responses of 31 motoneurones to alternating noxious heat (solid columns) and noxious pinch (hatched columns) stimuli. The mean effect of fentanyl on the total number of spikes counted in each stimulus-related epoch was determined as a percentage of control values (ordinate scale) and was plotted against dose in $\mu g kg^{-1}$, i.v. (abscissa scale). Error bars represent standard errors of the mean (s.e.mean). The number of cells tested (n) at each dose are given above each column of the histogram. Any difference in the pooled paired data at each dose was analysed for statistical significance using both parametric (paired Student's t) and non-parametric (Wilcoxon) tests. The two tests gave very similar results; two tail probabilities of the null hypothesis (HO) were greater than 0.05 for both tests at all doses. These values are presented above each column of the histogram (W P = Wilcoxon; t P = Student's t).

both parameteric (two tail paired Student's t test) and non-parametric tests (two tail Wilcoxon matched-pairs signed-ranks test; see Siegel & Castellan, 1988).

Results

The results presented below were obtained in experiments on 68 cells responding to alternating heat and pinch stimuli, recorded in 46 Wistar rats (210–600 g) of either sex. The i.v. administration of opioid agonists is presumed to have had effects mediated by a direct action within the spinal cord (see Discussion).

The μ-agonist fentanyl

As predicted, fentanyl reduced thermal and mechanical nociceptive reflexes in parallel and in a dose-dependent manner. This is illustrated in Figure 1 for a single motoneurone responding to alternating noxious heat and pinch stimuli. Figure 2 illustrates pooled data for the 31 cells tested in this way and shows that this non-selectivity of fentanyl was consistent over the whole dose-range tested and that any small differences between means of heat and pinch responses never reached statistical significance (both Wilcoxon and paired Student's t test; null hypothesis (HO) $P > 0.05$ for all doses).

Fentanyl was tested over a sufficiently wide dose range to calculate an ED_{50} for both the thermal and mechanical nociceptive responses of 8 individual cells. The mean ED_{50} on heat responses was

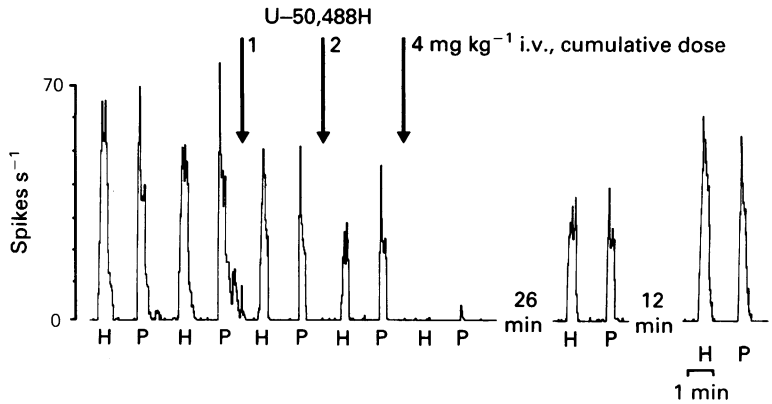


Figure 3 Effect of U-50,488 on the responses of a rat motoneurone to alternating thermal and mechanical noxious stimuli. The neurone was activated by noxious heat (H; to 48°C for 30 s) of the plantar surface of the ipsilateral hind paw and pinch (P; for 15 s) of toe 5, alternating in a regular 3 min cycle. U-50,488 was administered in divided doses ($1 + 1 + 2\,mg\,kg^{-1}$). Responses had recovered by approximately 50% some 30 min after the last dose of U-50,488 and the maximum recovery was evident 15 min later still. Multireceptive motoneurone recorded in VR L5 of an α-chloralose anaesthetized, spinalized rat.

$3.5\,\mu g\,kg^{-1}$ (± 0.8, s.e.mean) which was not significantly different to the mean ED_{50} on mechanical responses, $3.6\,\mu g\,kg^{-1}$ (± 1.0) (Wilcoxon test HO, $P = 0.76$). It is notable that the mean ED_{50} values calculated from these data on a subset of cells were not very different from the apparent mean ED_{50} values that could be predicted from all the pooled data presented in Figure 2.

Intravenous fentanyl can produce large transient reductions of blood pressure and elevations of end tidal CO_2, especially if given rapidly. Although this did not have any effect on the recorded signal it was important to test the possibility that the effects of fentanyl on nociceptive responses were secondary to its hypotensive effects (see Duggan *et al.*, 1978). Amyl nitrite vapour was therefore introduced into the oxygen line in doses sufficient to produce similar reductions in blood pressure as those seen with the largest doses of fentanyl. On 12 cells amyl nitrite transiently reduced the systolic blood pressure by 50 to 80 mmHg (6.5–10.5 kPa). However, thermal and mechanical nociceptive responses were minimally affected, being altered to means of 110% (± 4) and 115% (± 5) of control respectively.

The κ-agonists U-50,488H and tifluadom

Both of the κ-opioids reduced responses to thermal noxious stimuli, on most cells at doses at the lower end of those used in behavioural tests of nociception (e.g. Römer *et al.*, 1982a,b; Vonvoigtlander *et al.*, 1983; Hayes *et al.*, 1986; 1987; Leighton *et al.*, 1988).

A more specific question is the relative effectiveness of κ-ligands on matched reflexes to alternating heat and pinch stimuli. This aspect was studied on a total of 27 cells in 19 rats with U-50,488 H (0.5–$16\,mg\,kg^{-1}$ i.v.) and on 16 cells in 12 rats with tifluadom (0.05–$1.6\,mg\,kg^{-1}$ i.v.). Figure 3 shows an example of a cell which was activated alternately by noxious heat and noxious pinch stimuli, the intensity of which had been adjusted during the pre-drug control period so as to evoke responses with well-matched firing rates. It is readily apparent that, under these conditions, this selective κ-agonist was equally effective against responses to both forms of noxious stimulus.

The pooled data for all 27 cells is shown in Figure 4. Whilst the mean effects of the higher doses of U-50,488 are probably an underestimate of this κ-agonist's true potency (see Methods) it is nonetheless clear that, at all doses, U-50,488 was similarly effective against thermal and mechanical nociceptive responses (Wilcoxon and Student's t tests, HO, $P > 0.05$ at all doses).

The mean ED_{50} on heat responses of 10 cells was $4.5\,mg\,kg^{-1}$ (± 1.2) which was not significantly different from the mean ED_{50} on mechanical responses, $5.8\,mg\,kg^{-1}$ (± 1.4) (Wilcoxon test, HO, $P = 0.18$). As with the μ-agonist fentanyl, the calculated mean ED_{50} values for U-50,488 were not dissimilar to the apparent mean ED_{50} values estimated from the pooled data for all cells, as shown in Figure 4.

U-50,488 produced varying degrees of hypertension associated with an increase in pulse pressure and a decrease in end tidal CO_2. In several rats,

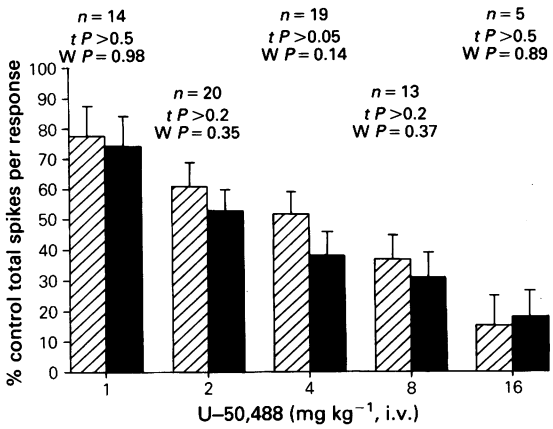


Figure 4 Pooled, paired data from tests with i.v. U-50,488 on responses of 27 motoneurones to alternating noxious heat (solid columns) and pinch (hatched columns) stimuli. Data presented as in Figure 2.

large 1.5–2 ml i.v. boluses of plasma expander were injected rapidly so as to produce a similar transient elevation of blood pressure as was produced by U-50,488. In no case did the plasma expander have a substantial effect on reflex responses.

The other κ-ligand tested was tifluadom and it produced similar parallel reductions as did U-50,488 (see Parsons *et al.*, 1989, Figure 4). When tested at 0.4 mg kg^{-1} on 9 cells, thermal and mechanical nociceptive responses were reduced similarly to 32% (±11) and 42% (±13) of control respectively. ED_{50} values could only be calculated for both types of nociceptive response on 4 of these cells; the mean ED_{50} values were 0.43 mg kg^{-1} (±0.14) and 0.24 mg kg^{-1} (±0.06) for noxious heat and noxious pinch stimuli respectively (Wilcoxon test, HO, $P = 0.28$). Tifluadom was dissolved in 5–10% ethanol for all these tests. The intravenous administration of equivalent and/or higher doses of this vehicle had no effect on nociceptive responses.

Controls with α-chloralose and ketamine

α-Chloralose was the anaesthetic of choice in these experiments on rats as it has been reported to have relatively weak effects on polysynaptic reflexes (e.g. Shimamura *et al.*, 1968). When tested at the dose which we normally gave approximately every hour to supplement anaesthesia during the recording period, i.e. 20 mg kg^{-1} i.v., α-chloralose had relatively little effect on nociceptive reflexes compared with other anaesthetics (Headley *et al.*, 1987a). Any depressant actions were often slow in onset with the peak effect occurring up to 10 min following intravenous administration (see Collins *et al.*, 1983). When tested quantitatively on 41 of the 68 rat motoneurones of this study, α-chloralose (20 mg kg^{-1}) reduced responses to noxious heat and pinch to means of 75% (±3) and 80% (±3) of control respectively. These results imply that any fluctuations of anaesthetic depth during the tests reported above are unlikely to have compromised the tests with the opioid agonists. Recent data indicate that α-chloralose does reduce spinal nociceptive reflexes in decerebrate rats (Hartell & Headley, 1989) but, importantly, fentanyl is equally effective in decerebrate and α-chloralose anaesthetized animals (unpublished).

As previously reported (Headley *et al.*, 1987b), sub-anaesthetic doses of ketamine reduced thermal and mechanical nociceptive reflexes in parallel. Thus, when compared on 16 cells tested with at least one opioid agonist, the ED_{50} values for ketamine versus noxious heat and noxious pinch were 3.4 mg kg^{-1} (±0.8) and 3.3 mg kg^{-1} (±0.7) respectively (Wilcoxon, HO, $P = 0.78$).

Effects of altered stimulus intensity

It is clear from these experiments that μ- and κ-opioids had very similar effects in depressing motoneuronal reflexes to different modalities of noxious stimulus. Under our conditions we were able to control closely the excitability levels of the motoenurone under study and, as has been stressed above, we were careful to match the firing rates during the thermal and mechanical nociceptive responses. If, however, the firing rates were not accurately matched, then apparent drug selectivity was observed. This is illustrated in Figure 5. The upper trace illustrates a test in which the stimulus intensities were adjusted correctly so as to match the neuronal firing rates evoked by the alternating heat and pinch stimuli; in this case a cumulative dose regime of U-50,488 reduced responses to both stimuli absolutely in parallel. However, a subsequent test was performed on the same neurone after the position of the thermal stimulator was adjusted such that the noxious thermal stimulus evoked a higher firing rate than the noxious mechanical stimulus (lower trace). The κ-agonist U-50,488, administered in an identical dose regime, now 'selectively' depressed the responses to noxious pinch (to 2% of control) whilst having little effect on the responses to noxious heat (79% of control).

An apparently selective depression of thermal over mechanical nociceptive responses was just as easy to orchestrate by altering the relative stimulus intensities in the appropriate fashion. Similar apparently selective actions of μ-opioids and other agonists could also be arranged at will.

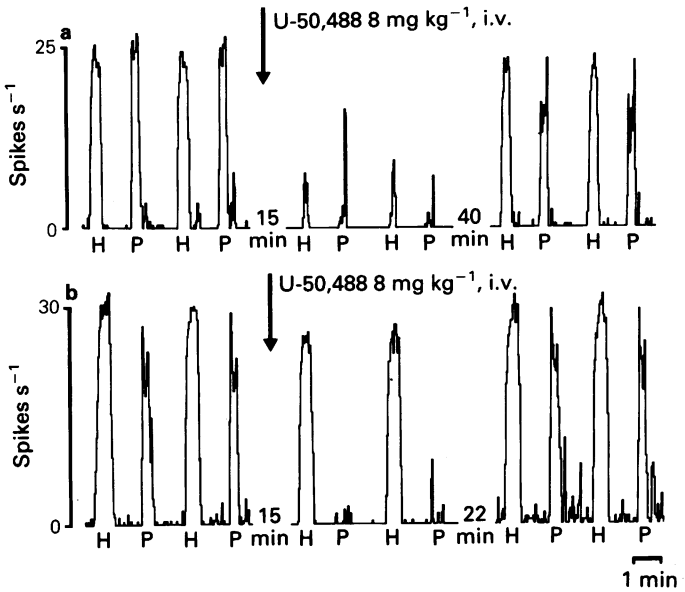


Figure 5 Effect of stimulus intensity in determining the selectivity of U-50,488 between responses to noxious pinch and those to noxious heat. The motoneurone was activated by noxious heat (H; to 49.5°C for 30 s) of the plantar foot and pinch (P; for 15 s) of toe 5 alternated in a regular 3 min cycle. Trace (a) illustrates the parallel reduction of responses to noxious heat and noxious pinch by U-50,488. The κ-agonist was administerd in divided doses (2 + 2 + 4 mg kg^{-1} i.v.) over 15 min. Trace (b) shows neuronal responses of the same cell tested later in the experiment with the same dose regime of U-50,488 but after the thermal stimulus had been altered, by a slight movement of the thermode, so as to evoke a greater neuronal firing rate. In this case U-50,488 was apparently 'selective' in depressing responses to noxious pinch over those to noxious heat. Multireceptive motoneurone in VR L4 of an α-chloralose anaesthetized, spinalized rat.

A critical test for the importance of stimulus strength was therefore to test for 'selectivity' between responses to different intensities of the *same* modality of noxious stimulus. Figure 6 illustrates one such test for a motoneurone responding to noxious pinch stimuli of two differing intensities, applied alternately by the same pincher unit to the same site. Both fentanyl and U-50,488 'selectively' reduced reflexes to the lower intensity noxious pinch stimulus whilst having less effect on the responses to the higher intensity stimulus. Thus, when quantified as the total number of spikes elicited by each stimulus, fentanyl reduced the smaller reflex responses to 12% of control whereas the larger reflex was only reduced to 70% of control values. U-50,488H had similar differential actions on this cell; the smaller and larger reflexes were reduced to 11% and 84% of control values respectively.

Discussion

It is most likely that the site of action of the i.v. administered opioids in this study is entirely within the spinal cord. Supraspinal actions should have been eliminated by the spinal section. Peripheral actions seem unlikely since, in monoarthritic rats, intraplantar naloxone (1–40 μg per paw) does not affect the antinociceptive actions of [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (DAGOL), [D-Pen^2, D-Pen^5]enkephalin (DPDPE) or U-50,488 in the uninflamed paw (Stein *et al.*, 1989). Moreover, in 'normal' rats, quaternary naltrexone (up to 40 mg kg^{-1}, s.c.) fails to antagonize the elevation of paw pressure threshold and tail flick latency produced by systemic μ- and κ-opioids (Millan *et al.*, 1989). In the present study care was taken to avoid the induction of inflammation and experiments were terminated if this did occur.

The results on non-thermal nociceptive responses presented in this paper are in broad agreement with a number of behavioural and electrophysiological studies showing that systemically administered μ and κ-opioids are all capable of reducing spinal reflexes to such stimuli.

On the other hand, the non-selectivity in our experiments of κ-opioids between thermal and mechanical nociceptive responses is in sharp contrast to the selective reduction of mechanical/visceral over

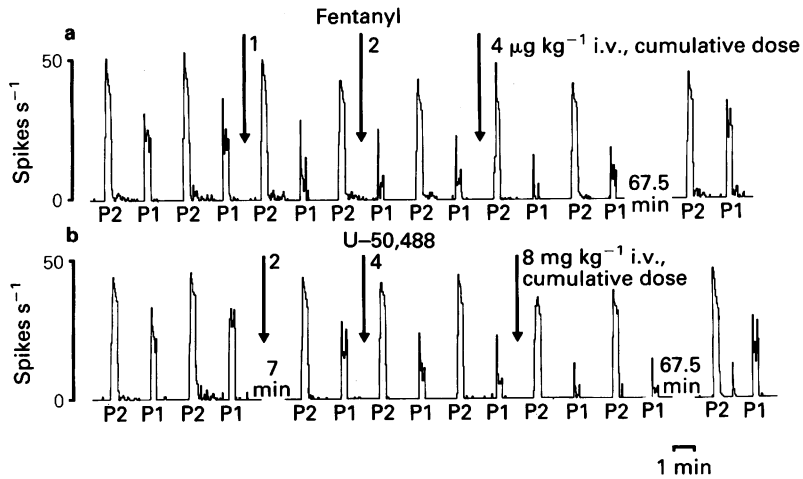


Figure 6 Differential reduction by μ- and κ-opioids of motoneuronal responses to noxious pinch stimuli alternated between different intensities. Noxious 15 s pinch stimuli to toe 5 were alternated in a regular 3.5 min cycle between P1 (0.3 newtons) and P2 (1.1 newtons). Trace (a) illustrates the greater sensitivity of responses to the lower intensity pinch stimulus to the depressant actions of fentanyl and trace (b) shows similar effects of U-50,488 on the same cell. Multireceptive motoneurone recorded in VR L4 of an α-chloralose anaesthetized, spinalized rat.

thermal nociceptive responses reported for κ-opioids in many (e.g. Tyers, 1980; Upton *et al.*, 1982; Bryant *et al.*, 1983; Schmauss *et al.*, 1983; Ward & Takemori, 1983; Schmauss & Yaksh, 1984; Schmauss 1987; see Yaksh & Noueihed, 1985) but by no means all behavioural studies (Han & Xie, 1982; Piercey *et al.*, 1982; Kaneko *et al.*, 1983; Przewłocki *et al.*, 1983a,b; Vonvoigtlander *et al.*, 1983; Herman & Goldstein, 1985; Spampinato & Candeletti, 1985).

We propose that the experimental differences can be explained, at least in part, by the limitation in nearly all behavioural tests of spinal antinociception, that the different stimuli used have not been matched in intensity so as to elicit reflex responses of similar magnitude in terms of muscular force and/or latency, and hence also in terms of spinal neuronal excitability. In addition, comparisons are often made between behavioural reflexes elicited from anatomically separate areas. In our experiments great care was taken to adjust the intensities of alternating thermal and mechanical noxious peripheral stimuli so as to match the strength of responses in terms of neuronal firing rates, and the peripheral stimuli were placed close together in the neuronal receptive field.

On those occasions when the firing rates in response to alternating noxious thermal and mechanical stimuli were not accurately matched, the weaker response was invariably reduced to a greater extent than the stronger response, regardless of whether μ- and κ-opioids were tested. The weaker response was similarly affected when different intensities of the same modality of noxious stimulus were alternated on the same cell. The weaker stimuli were still considered to be noxious in that they were aversive when tested on the experimenter. Moreover, if the less intense stimuli were not noxious then the resulting responses would, in fact, be expected to be less rather than more sensitive to the opioids tested (see also Parsons & Headley, 1989). It is clear from these results that stimulus intensity can be a crucial factor in the assessment of the selectivity of the antinociceptive effects of opioids. It is because of the importance of this that the examples illustrated are of the firing rates of the cells, although the analysis has been made on the basis of spikes elicited per response.

The relative strength of reflexes in behavioural tests cannot be assessed in terms of neuronal firing rate and therefore cannot easily be matched and quantified in this way (but see Kawakita & Funakoshi, 1987). However, judging by the reported latency and vigour of motor responses, the intensity of stimuli in the tail flick test results in considerably greater motoneuronal discharge rates than occurs in other tests such as the paw pressure or writhing tests; it would therefore be expected to be less sensitive to depression by opioids. Interpretation of the similar relative resistance of hot plate responses to κ-opioids is complicated by the probable supraspinal effects of opioids on this more complex response.

Stability of the background excitability of neurones is clearly also important in determining drug effects. In this regard, the choice of α-chloralose anaesthesia in these experiments was supported by (1) the stable baseline responses of single motoneurones recorded for up to 10 h under this anaesthetic and (2) the relative lack of effect of α-chloralose when supplementary doses were administered i.v.

In conclusion, these experiments clearly demonstrate that stimulus intensity is an important factor in determining the thermal/non-thermal selectivity of spinal opioids. Thermal/non-thermal selectivity reported in behavioural tests with κ-agonists is therefore likely to be related to differences in the stimulus intensities used. In contrast, μ-opioids with full agonist activity have more often been reported to be equally effective on thermal and non-thermal nociceptive reflexes whether following local spinal or systemic administration (but see Hayes *et al.*, 1987). It therefore seems likely that factors other than stimulus intensity are also important in determining the relative activity of opioid agonists on reflexes to different modalities of noxious stimulus. These factors may include variable access to opioid receptors following local spinal administration, and the efficacy of the agonists tested in a tissue which may have limited reserves of the opioid receptor subtype under study. These two points will be addressed in a following paper (Parsons *et al.*, 1989), as will the possibility that the spinal actions of intravenous U-50,488 and tifluadom in our experiments were mediated at μ-opioid receptors.

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Spinal antinociceptive actions and naloxone reversibility of intravenous μ- and κ-opioids in spinalized rats: potency mismatch with values reported for spinal administration

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1 The relative spinal effectiveness of μ- and κ-opioids has been assessed by their intravenous potencies on nociceptive responses (heat and/or pinch) of single motoneurones recorded in α-chloralose anaesthetized, spinalized rats.

2 The depressant actions of both μ- and κ-opioids were reversed by low intravenous doses of naloxone (10 to 100 $\mu g\,kg^{-1}$). When tested at a dose of 1 $\mu g\,kg^{-1}$ i.v., naloxone antagonized the effects of the μ-agonist morphine but had no effect on the κ-opioid U-50,488. This provides further support for the theory that the actions of μ- and κ-ligands were mediated at different subclasses of opioid receptor but highlights the difficulties in using antagonists with poor receptor selectivity to differentiate between μ- and κ-receptor-mediated effects *in vivo*.

3 The molar potency ratios of fentanyl:morphine:U-50,488:tifluadom for thermal and mechanical nociceptive responses were 620:1.0:0.74:5.7 and 520:1.0:0.56:7.7 respectively. These potency ratios, as well as the absolute potencies, agree well with those reported in several behavioural studies in which systemic administration of agonists was used in non-thermal tests.

4 The agonist potency values obtained in this study contrast with those reported for local spinal administration. By this route, the potency of lipophilic opioids (e.g. fentanyl, U-50,488 and tifluadom) relative to hydrophilic opioids (e.g. morphine) is much reduced, implying that activity of intrathecally administered opioids is more dependent on the physico-chemical properties of the agonists used than on the relative abundance in the spinal cord of functional opioid receptors of the μ- and κ-subtypes. This conclusion indicates that the results with locally applied opioids should not be used to assess spinal opioid receptor function.

Introduction

In the preceding paper (Parsons & Headley, 1989a) we have demonstrated that, following intravenous administration in spinalized rats, κ- as well as μ-opioids reduce spinal reflexes to thermal as well as to mechanical noxious stimuli. In contrast, much of the behavioural literature indicates that intrathecally administered κ-opioids are ineffective in tests of thermal nociception but are effective in models of visceral and mechanical nociception (see review by Yaksh & Noueihed, 1985 and references quoted in Parsons & Headley, 1989a).

We have shown that at least some of this difference is likely to be related to a mismatch between the different stimulus intensities used in various behavioural models. However, the fact that intrathecally administered μ-opioids have frequently been reported to be equally effective in thermal, mechanical and visceral behavioural models of nociception (see Yaksh & Noueihed, 1985) indicates that variations of stimulus intensity cannot fully explain the reported μ/κ differences; other factors must also be involved.

It has already been shown that the ability of different μ-opioids to diffuse into the cord following local

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Table 1 Mean depression of mechanical and thermal nociceptive reflex responses by four opioids

		Mechanical					*Thermal*				
Drug	*Dose* (kg^{-1})	*%Con*	n	ED_{50}	n	*Relative molar potency*	*%Con*	n	ED_{50}	n	*Relative molar potency*
Fentanyl	2 μg	44 ± 5	50				47 ± 5	42			
	0.5–16 μg			3.8 ± 0.5	40	520			3.0 ± 0.4	40	620
Morphine	2 mg	38 ± 14	8				55 ± 11	8			
	0.5–8 mg			1.9 ± 0.7	6	1.0			1.8 ± 0.6	5	1.0
U-50,488	2 mg	53 ± 6	52				56 ± 6	34			
	0.5–16 mg			4.9 ± 0.6	31	0.56			3.5 ± 0.6	28	0.74
Tifluadom	0.2 mg	40 ± 7	26				47 ± 11	12			
	0.2–1.6 mg			0.34 ± 0.04	25	7.7			0.43 ± 0.14	4	5.7

Depression of reflexes was quantified for individual motoneurones as a percentage of the mean control number of spikes per response. The mean effect of one selected dose of each opioid is shown as '% Con' in the upper row for that opioid. The lower row for each drug shows the ED_{50} calculated for the subset of cells on which at least 3 doses were tested over the linear portion of the dose response curve, and within the dose ranges shown. See text for further details of analysis. The standard errors of the means, the numbers of cells tested (*n*) and the molar potencies relative to morphine = 1 are also shown.

spinal administration can vary enormously (Durant & Yaksh, 1986; Yaksh *et al.*, 1986) but there are no equivalent data for κ-ligands. It seems possible therefore, that there is also limited access to, and activation of, spinal κ-opioid receptors following intrathecal or epidural administration of κ-selective ligands such as U-50,488 and tifluadom. To investigate this possibility we have tested selective opioid agonists intravenously in spinalized animals so as to compare their relative and absolute potencies with those reported in behavioural tests following either local spinal or systemic administration.

Although the μ/κ selectivity of naloxone is poor (Lord *et al.*, 1977; Chavkin *et al.*, 1982; Hayes & Kelly, 1985), we attempted to determine the lowest intravenous dose capable of antagonizing presumed μ- and κ-opioid receptor-mediated events. Some of these data have been presented in a preliminary form (Parsons & Headley, 1988).

Methods

Electrophysiological experiments were performed on α-chloralose anaesthetized, spinalized rats according to the protocols and analytical techniques described in the preceding papers (Parsons & Headley, 1989a). Motoneurones were activated by a variety of 'natural' peripheral stimuli, including noxious heat and pinch and innocuous tap and vibration. Drugs tested intravenously in cumulative dose regimes were the μ-agonists fentanyl and morphine, the κ-agonists U-50,488 and tifluadom and the opioid antagonist naloxone. Drug doses refer to fentanyl base, U-50, 488 methane sulphonate and tifluadom, morphine and naloxone hydrochlorides. Morphine, fentanyl and naloxone were obtained from commercial sources.

In tests with naloxone the protocols were varied as follows. When attempting to antagonize the actions of all opioids except morphine the depressant effects of the agonist and the subsequent time course for full recovery of responses were first established in the absence of naloxone. When this time course was sufficiently long, as was often the case with U-50,488, then the same dose regime of agonist was repeated prior to the subsequent i.v. administration of one or more doses of naloxone. Thereafter responses were compared with the control agonist test. When the time course for recovery was very rapid, as was normally the case with fentanyl and tifluadom, then the same dose regime of agonist was repeated but was preceded by a single dose of naloxone (see e.g. Figure 4). The agonist was then tested again at regular 30 min intervals until full recovery from the naloxone antagonism was achieved. Systemic morphine has a long half life so that recovery cannot easily be observed in electrophysiological experiments. Morphine was therefore tested as the last opioid agonist in any one experiment and its effects were always challenged with the subsequent administration of one or more doses of naloxone.

Results

Recordings were made from a total of 173 motoneurones in 99 α-chloralose anaesthetized, spinalized rats. On several neurones noxious heat and pinch stimuli were alternated; on other cells however, noxious stimuli were alternated with innocuous

cutaneous stimuli (see Parsons & Headley, 1989b) and on a few cells only a single modality of peripheral stimulus was tested.

Comparison of the relative potencies of μ- and κ-agonists on nociceptive reflexes

Depression of reflexes on any individual cell was quantified as a percentage of the control number of spikes per response (mean of 3 pre-drug responses). The relative potencies of the opioid agonists were analysed in two ways. Firstly, the percentage depression was averaged for each dose of each drug. Secondly, when 3 or more doses of opioid had been tested over the 'linear' region of the dose-response curve, the ED_{50} was calculated by linear regression analysis (see Parsons & Headley, 1989a). Since the criteria for ED_{50} estimation could not be met on all cells, both forms of analysis are presented in Table 1; the considerable differences in sensitivity between individual cells are reflected in the s.e.mean values.

Noxious pinch

Fentanyl was tested on individual cells in a log-dose regime (but over variable dose-ranges) between 0.5 and 16 $\mu g\,kg^{-1}$ i.v. Its potency on reflexes to noxious pinch was assessed in tests on 67 motoneurones recorded in 47 rats. The potency of fentanyl on mechanical nociceptive reflexes of one such cell is shown in Figure 1a. With 40 of the neurones tested with this μ-agonist a sufficient number of cumulative doses were given to calculate an ED_{50} on mechanical nociceptive reflexes for that cell. The pooled data are presented in Table 1.

Because morphine is the standard against which other opioids are frequently tested, this μ-agonist was compared with fentanyl (and U-50,488) on responses to noxious pinch of 9 motoneurones recorded in 9 rats. In the example in Figure 1 the effects of morphine were completely reversed by naloxone 20 $\mu g\,kg^{-1}$. When the ED_{50} data from these i.v. tests are pooled, fentanyl is 500 times more potent than morphine against motoneuronal responses to noxious pinch (see Table 1).

The actions of the κ-agonist U-50,488 (0.5 to 16 $mg\,kg^{-1}$ i.v.) were tested on the mechanical nociceptive responses of 63 motoneurones in 40 rats and the example in Figure 1 illustrates that when tested at a cumulative dose of 4 $mg\,kg^{-1}$, U-50,488 reduced the responses to noxious pinch to a similar degree as the subsequent administration of morphine over the same cumulative dose-range.

The κ-agonist tifluadom was tested in 24 rats at 0.1 to 1.6 $mg\,kg^{-1}$ i.v. on the nociceptive responses of 31 motoneurones to noxious pinch and showed similar actions to the other opioids. Tifluadom was of intermediate potency between U-50,488 and fentanyl (Table 1 and Figure 4).

In these tests on mechanical nociceptive reflexes the molar potency ratios of fentanyl:morphine:U-50, 488:tifluadom, based on the ED_{50} estimates, were thus, 520:1.0:0.56:7.7. The larger pool of data when all drug tests are included provide similar relative potency values (Table 1).

Noxious heat

All four opioid agonists showed a similar potency on reflexes to noxious heat as on those to noxious pinch (see also Parsons & Headley, 1989a). Fentanyl (51 cells in 39 rats) was again almost three orders of magnitude more potent than morphine (10 cells in 10 rats); this is illustrated for an individual motoneurone in Figure 1. The potencies of U-50,488 (46 cells in 32 rats) and morphine were similar when tested on thermal nociceptive responses, as is again shown in Figure 1. The relative potencies of morphine, U-50,488 and fentanyl were consistent on all 9 neurones tested with all 3 agonists. The intermediate potency of tifluadom (16 cells in 13 rats) on both thermal and mechanical nociceptive reflexes is shown for one cell in the top trace of Figure 4.

The pooled data presented in Table 1 illustrate that, when tested on thermal nociceptive reflexes, the molar potency ratios of fentanyl:morphine:U-50, 488:tifluadom (620:1.0:0.74:5.7) were very similar to those found for mechanical nociceptive reflexes.

Comparison of ED_{50} potency and neuronal firing rate

A notable feature in electrophysiological studies is the variability in the potency of agonists between cells. In view of the influence of firing rate on drug efficacy (Parsons & Headley, 1989a), the inter-cell variability in ED_{50} values might depend on variations in firing rates during sensory responses. However, linear regression analysis of the dependence of ED_{50} values on control firing rates showed only a weak correlation with U-50,488 on pinch (slope = 0.0904 $mg\,kg^{-1}\,Hz^{-1}$, $r = 0.455$, HO $P = 0.011$; Spearman rank coeff. = 0.423, HO $P = 0.088$; Kendall rank coeff. = 0.316, HO $P = 0.008$, $n = 31$). For U-50,488 on heat ($n = 28$) and fentanyl on both heat ($n = 40$) and pinch ($n = 40$) there was no correlation between ED_{50} and control firing rate (coeffs. < 0.15 and HO $P > 0.3$ for all three tests).

Actions of the opioid antagonist naloxone

Effects on nociceptive reflexes in opioid-naive animals Before using naloxone to antagonize the actions of any exogenously administered opioid

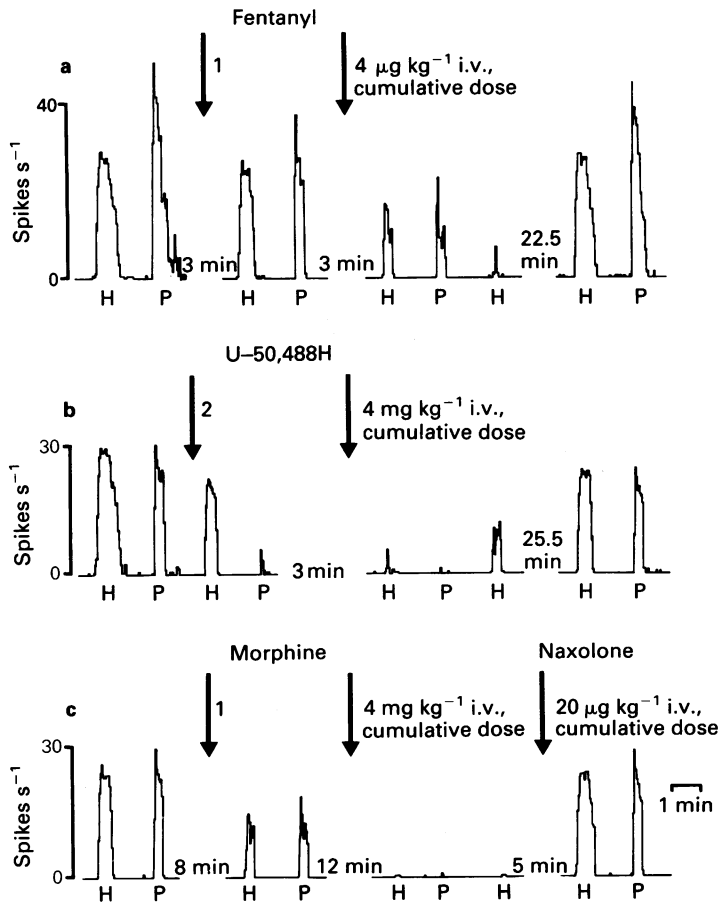


Figure 1 Comparison on a single motoneurone of the intravenous potencies of the μ-agonists fentanyl and morphine and the κ-agonist U-50,488. Responses were elicited by alternating noxious heat (H) of the ipsilateral plantar foot to 50°C for 30 s and noxious pinch (P) of toe 5 for 15 s. Trace (a) shows that fentanyl reduced nociceptive reflexes after divided doses of $1 + 1 + 2\,\mu g\,kg^{-1}$ i.v.; second $1\,\mu g\,kg^{-1}$ dose not shown. Trace (b) shows similar actions on the same cell of U-50,488 with a dose regime of $2 + 2\,mg\,kg^{-1}$ i.v. Trace (c) shows the effects of morphine administered in divided doses of $0.5 + 0.5 + 1 + 2\,mg\,kg^{-1}$ i.v.: first and third doses not shown. The depressant actions of morphine were rapidly reversed by the subsequent administration of naloxone $20\,\mu g\,kg^{-1}$ i.v. Recording in VR L5 of an α-chloralose anaesthetized, spinalized rat.

ligand it was important to test whether it had any actions in opioid-naive rats, as would be expected if there were any tonically released opioid peptides in these preparations. Naloxone was therefore tested on the responses to noxious pinch of 14 motoneurones in 14 opioid-naive rats and on the responses to noxious heat with 10 of these cells. Neither response was affected to any discernible degree by doses of naloxone of $100\,\mu g\,kg^{-1}$ i.v. or higher. This is illustrated for a single motoneurone in Figure 2; naloxone was administered i.v. in divided doses to a cumulative total of $400\,\mu g\,kg^{-1}$ and had no effect on either the thermal or the mechanical nociceptive responses. This lack of effect was consistent; in pooled data from tests at $100\,\mu g\,kg^{-1}$ i.v., responses to heat were 115% (± 7, $n = 10$) and those to pinch were 97% (± 4, $n = 14$) of control values. Naloxone was tested at ten times this dose ($1\,mg\,kg^{-1}$) in 6 cells but still had no discernible effect; responses to heat were 111% (± 18, $n = 3$) and to pinch were 90% (± 17, $n = 6$) of control.

Antagonism of μ- and κ-opioids The above control studies indicate that any reversal of exogenous opioid depressions by low doses of naloxone in our preparations should be mediated by direct antagonism of the exogenous drug rather than by other

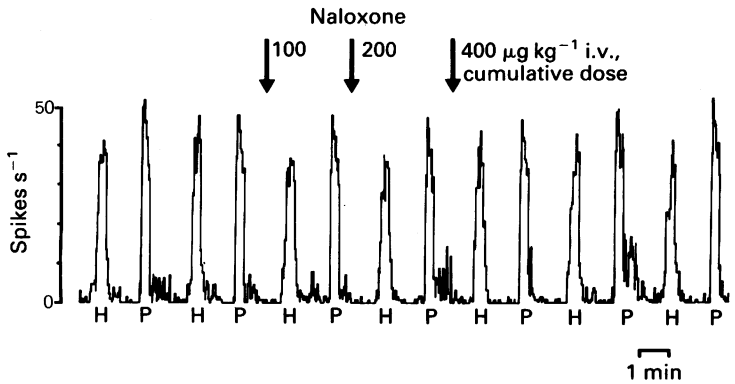


Figure 2 Lack of effect of naloxone on motoneuronal nociceptive responses in an opioid-naive rat. The multireceptive motoneurone was recorded in VRL5 and was activated by noxious heat (H) of the ipsilateral plantar foot to 48°C for 30 s and pinch (P) of toe 5 for 15 s alternating in a regular 3 min cycle. α-Chloralose anaesthetized, spinalized rat.

mechanisms. Naloxone was therefore tested for antagonism of the actions of μ- and κ-agonists. The results are summarised in Table 2.

Due to inter-cell variability, the cumulative doses of fentanyl required to reduce responses to noxious pinch (to a mean of 23% (±13) of control; $n = 6$) varied from 4 to 16 μg kg^{-1} i.v. The actions of this μ-agonist were consistently and fully reversed (by 105% ±5) by naloxone 20–200 μg kg^{-1} (see Table 2).

A dose of naloxone generally considered low (100 μg kg^{-1} i.v.) was tested for antagonism of the κ-opioid U-50,488 (2 to 16 mg kg^{-1} i.v.) on 10 motoneurones responding to alternating noxious heat and pinch, and on 3 responding to alternating noxious and non-noxious stimuli. Figure 3 illustrates the reversal by naloxone 100 μg kg^{-1} i.v. of the depression of nociceptive reflexes by U-50,488 2 mg kg^{-1} i.v. The antagonism of U-50,488 by this dose of naloxone was consistent for both thermal and mechanical nociceptive responses of all the cells tested (see pooled data in Table 2.)

Naloxone 100 μg kg^{-1} i.v. was also tested for antagonism of tifluadom on 8 motoneurones. As with U-50,488, this dose was sufficient to antagonize substantially the actions of the κ-agonist. Figure 4 illustrates for a single neurone the antagonism of

Table 2 Antagonism by various doses of naloxone of the spinal actions of μ- and κ-opioids on nociceptive reflex responses of single motoneurones

		Pinch			*Heat*		
Agonist	*Naloxone dose*	*Pre-naloxone %Con*	*Reversal %Rev*	n	*Pre-naloxone %Con*	*Reversal %Rev*	n
Fentanyl	20–200	23 ± 13	105 ± 5	6	8 ± 6	127 ± 12	4
Morphine	100	6 ± 6	103 ± 15	5	0 ± 0	117 ± 27	3
	50	20 ± 10	88 ± 11	3	2 ± 7	96 ± 27	3
	20	15 ± 9	74 ± 8	4	3 ± 2	81 ± 17	5
	10	15 ± 6	58 ± 14	5	16 ± 9	76 ± 19	9
	1				13 ± 3	57 ± 24	5
U-50,488	100	14 ± 5	79 ± 14	10	14 ± 4	89 ± 13	13
	50				17 ± 9	90 ± 9	5
	10				7 ± 3	60 ± 11	8
	1				10 ± 4	6 ± 4	5
Tifluadom	100	12 ± 6	81 ± 10	8	5 ± 3	71 ± 5	5

See 'Methods' for protocol. The range of cumulative i.v. doses of the μ- and κ-agonists giving the pre-naloxone reductions of reflex responses (% Con) were as follows: fentanyl 4–16 μg kg^{-1}; morphine 0.5–8 mg kg^{-1}; U-50,488 0.5–16 mg kg^{-1}; tifluadom 0.2–1.6 mg kg^{-1}. The effects of naloxone (1–200 μg kg^{-1} i.v., as indicated) were quantified as the percentage reversal of the agonist-induced depression (%Rev), such that 100% indicates complete reversal of the effect of the agonist. Means (% Con, % Rev), standard errors of these means and numbers of cells tested (n) are indicated.

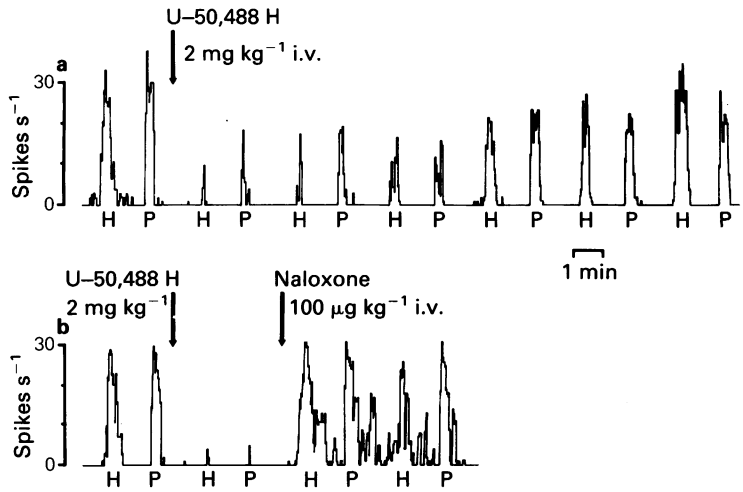


Figure 3 Naloxone antagonism of the depressant actions of U-50,488 on motoneuronal thermal and mechanical nociceptive responses. The motoneurone was recorded in VRL5 and was activated by noxious heat (H) of the ipsilateral plantar foot to 48.8°C for 30 s and pinch (P) of toe 5 for 15 s alternating in a regular 3 min cycle. Trace (a) shows the depressant actions of U-50,488 2 mg kg^{-1} i.v. on nociceptive responses and the subsequent time course for recovery of responses from this effect. Trace (b) shows data recorded from the same cell later in the experiment. U-50,488 2 mg kg^{-1} i.v. reduced responses to the same degree as previously and this effect was rapidly reversed by the subsequent administration of naloxone 100 μg kg^{-1} i.v. α-Chloralose anaesthetized, spinalized rat.

tifluadom by naloxone 100 μg kg^{-1}; full recovery from this antagonism was not seen with subsequent tifluadom tests until 153 min post naloxone (see 'Methods'). This implies that a lower dose of naloxone might have been sufficient to antagonize the effects of tifluadom. The mean depressions by tifluadom and reversals by naloxone are presented in Table 2.

The effectiveness of a dose of 100 μg kg^{-1} i.v. naloxone in antagonizing κ- as well as μ-effects prompted us to test even lower doses of i.v. naloxone. Despite the poor μ/κ selectivity of naloxone, morphine and U-50,488 were differentially sensitive to antagonism by naloxone. This is illustrated in Figures 5 and 6. The top trace of Figure 6 shows the selective actions of U-50,488 1 mg kg^{-1} i.v. in depressing responses to noxious heat to a greater degree than those to innocuous vibration, and the subsequent time course for recovery from this effect. (The selectivity of both μ- and κ-ligands between reflexes to noxious and non-noxious stimuli will be discussed in greater detail in the subsequent paper: Parsons & Headley, 1989b). The bottom trace shows data recorded from the same cell later in the experiment. The prior administration of naloxone 1 μg kg^{-1} i.v. had no effect on the antinociceptive actions of U-50,488 but a subsequent dose of 9 μg kg^{-1} i.v. (cumulative 10 μg kg^{-1} i.v.) completely reversed the effects of the κ-ligand. Table 2 shows pooled dose-response data for naloxone antagonism of the actions of μ- and κ-ligands. These data show both the dose-dependency of naloxone and the greater effectiveness of naloxone on μ compared with κ-ligands.

Discussion

The antinociceptive actions of μ- and κ-opioids in our experiments were evident at doses similar to, or slightly lower than, those found to be effective following systemic administration in spinally intact rats in behavioural tests of thermal, mechanical and visceral nociception. However, it should be noted that in the present study all drugs were administered intravenously and, as a result, at any given dose, higher concentrations may have reached the opioid receptors under study than if, as in most behavioural studies, they had been injected via an intraperitoneal or subcutaneous route.

The variation in the potency of each opioid agonist between cells was not strongly dependent on differences in control firing rates. This initially surprising result does not, however, contradict our previous data (Parsons & Headley, 1989a). Firstly, although there were differences in the firing rates attained during pre-drug control responses, this reflected variation in the peak firing rates obtainable from individual cells rather than variation in the stimulus intensities used, this being particularly so

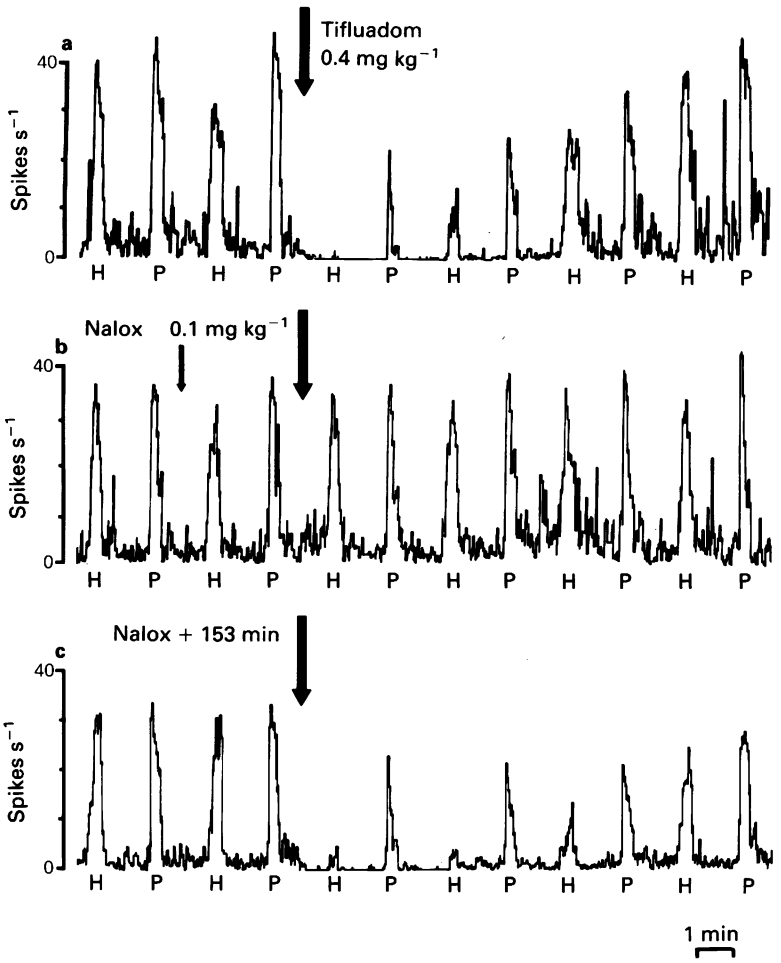


Figure 4 Naloxone (Nalox) antagonism of the depressant actions of tifluadom on motoneuronal thermal and mechanical nociceptive responses. The cell was activated by noxious heat (H) and pinch (P) stimuli alternating in a regular 3 min cycle. See text for details of protocol. Multireceptive motoneurone recorded in VRL5 of α-chloralose anaesthetized, spinalized rat.

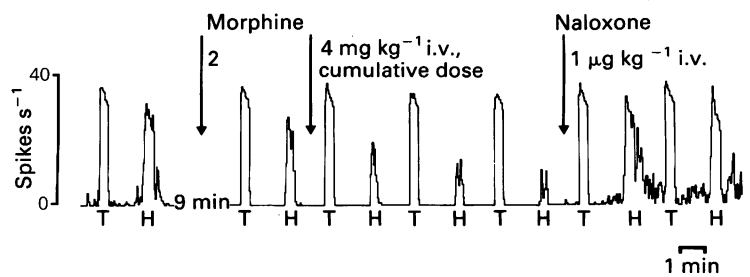


Figure 5 Antagonism of the antinociceptive actions of morphine by very low doses of naloxone. This single VRL4 motoneurone was activated by noxious heat (H) of the ipsilateral plantar foot to 48.5°C for 30 s and by non-noxious tap (T) of toe 5 at 8 Hz for 20 s, alternating in a regular 190 s cycle. Morphine 4 mg kg^{-1} i.v. cumulative dose selectively depressed responses to noxious heat and this effect was rapidly reversed by the intravenous administration of naloxone at 1 μg kg^{-1}. α-Chloralose anaesthetized, spinalized rat.

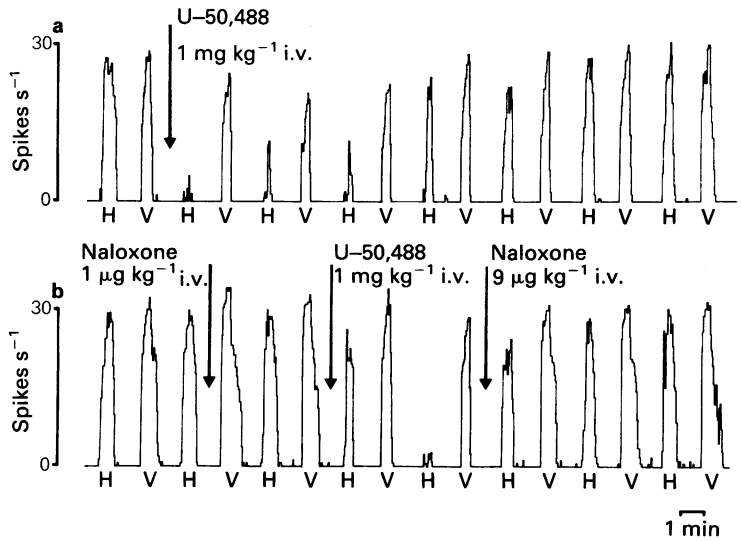


Figure 6 Dose-dependency of naloxone in antagonizing the selective antinociceptive actions of U-50,488 tested on motoneuronal responses to noxious and non-noxious stimuli. The cell was activated by noxious heat (H) of the ipsilateral plantar foot to 48°C for 30 s and non-noxious vibration (V) of the hock at 8 Hz for 20 s (stimulus similar to top stimulus in Figure 5 except that the probe was in constant contact with the skin). Stimulus cycle 190 s. Trace (a) shows the selective antinociceptive effects of U-50,488 1 mg kg^{-1} i.v. and the subsequent time course for recovery of nociceptive responses from this effect. Trace (b) shows data recorded from the same cell later in the experiment. U-50,488 was administered at the same dose of 1 mg kg^{-1} i.v. but this time was preceded by a dose of naloxone 1 μg kg^{-1} i.v. The effects of U-50,488 were unaltered by this dose of naloxone. However, the subsequent administration of naloxone 9 μg kg^{-1} i.v. rapidly reversed the effects of U-50,488. α-Chloralose anaesthetized, spinalized rat.

with heat, which was usually held at noxious levels of between 46.5°C and 48.5°C to avoid frank tissue damage. Secondly, some of the data used for the above analysis were recorded from neurones responding to a single modality of noxious stimulus. In contrast, any difference in the firing rates evoked in single neurones by alternating noxious heat and noxious pinch should reflect genuine differences in the intensities of the noxious stimuli used; in this case we have clearly shown the importance of stimulus intensity in determining apparent drug 'selectivity' (Parsons & Headley, 1989a).

Relative potencies of μ- and κ-agonists

The potency ratios for fentanyl:morphine:U-50,488:tifluadom were similar to those reported in many behavioural studies (e.g. Irwin *et al.*, 1951; Janssen *et al.*, 1963; see Yaksh & Noueihed, 1985 and references in Parsons & Headley, 1989a). In contrast, following intra-cerebroventricular (i.c.v.), intrathecal or epidural administration, the relative potencies of these ligands have been found to be very different. Thus, when tested in both thermal and non-thermal models of nociception, the potency ratio for fentanyl:morphine is reduced to less than 5:1 (Herz & Teschemacher, 1971; Durant & Yaksh, 1986; Yaksh *et al.*, 1986; see Yaksh & Noueihed, 1985). When tested on non-thermal nociceptive reflexes, the potency of intrathecal U-50,488 relative to morphine is markedly reduced to less than 0.03:1; the ratio is even wider for thermal nociceptive reflexes (Schmauss *et al.*, 1983b; Durant & Yaksh, 1986; Yaksh *et al.*, 1986; Schmauss, 1987; see Yaksh & Noueihed, 1985) and for some other synthetic κ-ligands e.g. buprenorphine (Bryant *et al.*, 1983). The same is the case when U-50,488 is applied topically in electrophysiological studies of C-fibre evoked activity of single dorsal horn cells (Dickenson & Sullivan, 1986; Knox & Dickenson, 1987).

The different potency ratios found between those studies using systemic as compared to those using topical administration seems to be related to the relative lipid partition coefficients of the compounds studied. Thus, following local spinal or i.c.v. administration, the more lipophilic drugs quickly gain access to the CNS but are also rapidly redistributed by the systemic circulation (see Cousins & Mather, 1984).

This highlights a disadvantage of using local administration of agonists or antagonists when assessing the relative roles of opioid receptors; namely that this technique does not allow any esti-

mate to be made of the relative drug access to the receptors under study. For most opioid drugs, diffusion from intrathecal or epidural sites into the cord has not been assessed. For the μ-agonists fentanyl and sufentanyl it is apparently very poor, for the effective dose intrathecally can exceed the effective systemic dose (Colpaert *et al.*, 1986; Durant & Yaksh, 1986; Yaksh *et al.*, 1986). That this is due to inadequate access to receptors rather than a lack of spinal μ-receptors is indicated by the generally accepted efficiency of the hydrophilic μ-agonist morphine given by the same route.

Despite these problems, and despite the absence of specific data for U-50,488 and tifluadom, the potencies of spinally administered κ-opioids relative to morphine are frequently taken as a measure of the effectiveness of spinal receptors for these agonists. Of particular relevance to this point are (1) reports of high lipid/water partition coefficients of drugs with very similar structures to U-50,488 (see Cheney *et al.*, 1985) and (2) the rapid time to peak effect and subsequent rapid redistribution to the systemic circulation of the selective κ-ligand PD117302 administered i.c.v. (Leighton *et al.*, 1988).

Differential receptor access by i.t. drugs would be heightened if spinal κ-receptors are located in deeper laminae than are μ-receptors, as is suggested by reports that microelectrophoretic administration of κ-agonists was more effective in deeper laminae than in superficial laminae of the spinal cord, whereas μ-agonists were effective in all laminae (Fleetwood-Walker *et al.*, 1988). A diffuse distribution of spinal κ-receptors is also supported by some (Członskowski *et al.*, 1983; Slater & Patel, 1983) but not by all (Morris & Herz, 1987) studies on the regional localisation of spinal κ-opioid binding sites. In contrast, spinal levels of the presumed endogenous κ-ligand dynorphin appear to be highest in the superficial dorsal horn (Vincent *et al.*, 1982).

Hayes *et al.* (1987) have recently reported that, following systemic administration in intact rats, μ- as well as κ-selective ligands are most effective in visceral models and are least effective in thermal models of nociception. Furthermore, this difference in sensitivity of visceral and thermal tests of nociception was greatest when used to test ligands with low intrinsic activity at the opioid receptors under study. Similar observations have been reported following intrathecal administration of partial agonists (Schmauss *et al.*, 1983a). This effect would be compounded in tissues with a low effective receptor reserve (see Miller *et al.*, 1986) and presumably also in tissues where there is poor receptor access by the drugs under study.

If this were the case for κ-receptors, then following intrathecal administration, the low concentrations of κ-opioids that might penetrate to deeper laminae of the dorsal horn might activate κ-receptors sufficiently to reduce non-thermal but not the more vigorous thermal nociceptive reflexes. In contrast, the μ-opioids tested intrathecally may have a higher intrinsic activity at their receptors and/or there may be a higher μ-receptor reserve in more accessible superficial laminae of the spinal dorsal horn.

Potency of naloxone against μ- and κ-agonists

The fact that, in these experiments, naloxone administered to opioid-naive animals did not potentiate nociceptive reflexes to natural peripheral stimulation is in contrast to several reports that low doses of naloxone can potentiate electrically-evoked reflexes (see Duggan & North, 1984); this will be discussed in further detail elsewhere. This lack of effect does however allow us to interpret the reversal of exogenous opioid agonists by naloxone as an indication of competitive rather than physiological antagonism.

The results from our studies on the naloxone reversibility of the agonists tested therefore indicate that the antinociceptive actions of all the agonists were mediated via opioid receptors. The very low intravenous doses of naloxone required to reverse the effects of the κ-opioids U-50,488 and tifluadom illustrate the danger of using naloxone *in vivo* in attempts to distinguish the relative roles of opioid receptor subtypes. However, the fact that the depression of nociceptive responses by morphine was substantially antagonized by naloxone $1\,\mu g\,kg^{-1}$ i.v. whereas $10\,\mu g\,kg^{-1}$ i.v. or more was necessary to affect U-50,488 to a comparable degree, is compatible with the μ/κ selectivity of naloxone reported in many systems. This result therefore provides additional evidence that the actions of U-50,488 and tifluadom were mediated at κ-receptors. Further support for this conclusion is provided by the fact that μ- and κ-ligands had contrasting effects on cardiovascular and respiratory systems in our experiments (see Parsons & Headley, 1989a).

The potency ratios of morphine, fentanyl, U-50, 488 and tifluadom in these electrophysiological tests on spinal nociceptive reflexes are very close to those reported in the literature following systemic administration in intact animals. This implies that spinal sites mediate a major contribution of the effects of systemic opioids on spinal reflexes, a finding not incompatible with a greater contribution of supraspinal than spinal sites to true analgesia. These potency ratios do, however, contrast sharply with those reported for epidural or intrathecal tests. This is likely to reflect differential spinal penetration following topical administration. In the previous paper (Parsons & Headley, 1989a) we have highlighted a further problem associated with many behavioural tests of nociception, namely that any differences of

stimulus intensities will result in apparent but false modality selectivity by the test drugs. Neither problem applies to our electrophysiological experiments and, despite the undoubted caveat of interpreting recordings obtained from acutely prepared animals, these electrophysiological results do have considerable credence. The generally held view, based primarily on behavioural data, that spinal μ- and κ-opioids have differential effects on reflexes to thermal versus non-thermal stimuli, therefore needs radical reassessment.

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On the selectivity of intravenous μ- and κ-opioids between nociceptive and non-nociceptive reflexes in the spinalized rat

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1 In electrophysiological experiments in spinalized, α-chloralose anaesthetized rats, opioids and anaesthetics were tested intravenously (i.v.) on the responses of individual motoneurones to alternating noxious (heat or pinch) and non-noxious (tap or vibration) stimuli.

2 On cells that were sensitive to low doses of μ-opioids, both fentanyl (0.5–4 $\mu g\,kg^{-1}$ i.v.) and morphine (0.5 $mg\,kg^{-1}$ i.v.) selectivity reduced reflexes to noxious stimuli to a greater degree than reflexes to non-noxious stimuli. In contrast, on cells that were relatively resistant to these agonists, the higher doses required to reduce nociceptive reflexes (fentanyl 8 $\mu g\,kg^{-1}$ i.v.; morphine 1–8 $mg\,kg^{-1}$ i.v.) depressed non-nociceptive reflexes to a similar degree.

3 A similar spectrum of selectivity was seen with U-50,488 (0.5–16 $mg\,kg^{-1}$ i.v.) although statistically significant selective depression of reflexes was only evident at the lowest dose tested (0.5 $mg\,kg^{-1}$ i.v.). All effects of U-50,488 were readily reversed by low doses of the opioid antagonist, naloxone (10–100 $\mu g\,kg^{-1}$ i.v.).

4 The dissociative anaesthetic/PCP ligand ketamine (0.5–4 $mg\,kg^{-1}$ i.v.) was similar in having selective actions at low doses on sensitive cells but non-selective actions when higher doses were required. In contrast, the general anaesthetics methohexitone (4 $mg\,kg^{-1}$ i.v.) and alphadolone/alphaxalone (1 $mg\,kg^{-1}$ i.v.) were consistently non-selective between reflexes to noxious and non-noxious stimuli. α-Chloralose (20–40 $mg\,kg^{-1}$ i.v.) had very little effect on reflexes to any of the synaptic inputs tested.

5 The sensitivity of nociceptive reflexes to depression by opioids and dissociative anaesthetics thus determines the selectivity of these agents between nociceptive and non-nociceptive reflexes. The same is not true for the general anaesthetics tested.

6 Taken together with our previous findings that κ- as well as μ-agonists reduce thermal and mechanical nociceptive reflexes in parallel, these results indicate a general similarity of spinal μ- and κ-opioid actions in the spinal cord of the rat.

Introduction

We have demonstrated in accompanying papers (Parsons & Headley, 1989; Parsons *et al.*, 1989) that both μ- and κ-opioid agonists reduce thermal and mechanical nociceptive spinal reflexes in parallel when administered intravenously in α-chloralose anaesthetized, spinalized rats. These actions were mediated at separate subclasses of opiate receptor.

However, the κ-selective, endogenous opioid peptide dynorphin A and related peptides have been shown to produce non-opioidergic motor paralysis following local spinal administration (Przewłocki *et al.*, 1983; Herman & Goldstein, 1985; Spampinato & Candeletti, 1985; Stevens & Yaksh, 1986). Similar effects on motor function are seen following intrathecal (i.t.) administration of some (e.g. Leighton *et al.*, 1988) but not all synthetic κ-ligands (e.g. Schmauss & Yaksh, 1984; Stevens & Yaksh, 1986; Leighton *et al.*, 1988; see Yaksh & Noueihed, 1985). This raises the possibility that the spinal actions of U-50,488 and trifluadom in our preparations reflect non-selective motor paralysis rather than true anti-nociceptive actions of these κ-opioids. There is a substantial body of electrophysiological data

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showing that the spinal antinociceptive actions of μ-opioids on the responses of dorsal horn neurones to electrical and natural peripheral stimuli are selective for those responses mediated by activity on Aδ and C primary afferent fibres over activity in larger diameter fibres (see Duggan & North, 1984, but c.f. Einspahr & Piercey, 1980).

There is some evidence that microelectrophoretically-administered κ-opioids can have similarly selective effects on the responses of dorsal horn neurones (Fleetwood-Walker *et al.*, 1988) although Knox & Dickenson (1987) have reported that the effects of topically administered κ-opioids are variable and are not reversed by naloxone. Equivalent studies on spinal reflexes, whether to noxious or non-noxious stimuli have, however, only been performed with μ-opioids on responses evoked by electrical stimulation of primary afferents (see Duggan & North, 1984).

For these reasons it was important to test the relative selectivity of μ- and κ-ligands between spinal reflex responses to noxious and non-noxious natural peripheral stimuli. We now show that both μ- and κ-ligands act similarly in depressing nociceptive reflexes more than reflexes to low threshold tactile stimuli, but that the degree of selectivity varies with the absolute doses required to cause a reduction of the nociceptive reflexes.

Methods

Experiments on 33 motoneurones in 27 α-chloralose anaesthetized, spinalized rats were performed as described in accompanying papers (Parsons & Headley, 1989; Parsons *et al.*, 1989). In this study, all motoneurones were activated by alternating stimuli, one noxious (heat or pinch) and one non-noxious (light tap or slightly more vigorous vibration). As there were no apparent differences in the effects of the various test agents between responses to noxious heat and pinch, or between responses to non-noxious tapping and vibration, the data for each class of stimulus were pooled when comparing the selectivity of ligands between responses to noxious and non-noxious stimuli.

In addition to the μ- (fentanyl and morphine) and κ- (U-50,488) opioids (see Parsons & Headley, 1989), the following anaesthetics were tested, usually at several doses in a $\log_2$-dose progression: the short-acting barbiturate methohexitone sodium (Lilly), the steroid mixture alphaxalone acetate/alphadolone acetate (Saffan, Glaxo), the dissociative anaesthetic/PCP ligand ketamine hydrochloride (Ketalar, Parke Davis) and the background anaesthetic used in this study, α-chloralose (Sigma).

Drug actions were quantified by their effects on the number of spikes elicited by each stimulus. Data pooled from each test dose of the ligands under study were analysed for statistical significance using the one-tail probabilities given by the Wilcoxon matched-pairs signed ranks test (see Siegel & Castellan, 1988).

Results

μ-Agonists

Fentanyl was tested at 0.5–8 $\mu g\,kg^{-1}$ i.v. on the responses of 24 neurones in 17 rats to alternating noxious and non-noxious stimuli. In 19 of these cells the noxious stimulus was heat; in the remaining 5 neurones noxious pinch was used. A range of degrees of selectivity was observed in these tests. On most cells low doses of fentanyl were selective in depressing nociceptive reflexes to a considerably greater degree than reflexes to non-noxious stimuli. Clear selectivity is illustrated in Figure 1 but the examples in Figures 2 and 3 show progressively less selective actions of fentanyl.

The effects of morphine (0.5–8 $mg\,kg^{-1}$ i.v.) were compared with those of fentanyl on the responses to alternating noxious and non-noxious stimuli of 5 cells in 5 rats. On any one neurone the two μ-agonists had very similar effects, although the degree of selectivity varied between the 5 cells tested. An example of selective antinociceptive effects of low doses of both μ-agonists is illustrated in Figure 1. The effects of morphine were rapidly reversed by the subsequent administration of a low dose of intravenous naloxone (Figure 1; see also Figure 5 in Parsons *et al.*, 1989).

In contrast, in those cells requiring higher doses of the opioid agonists to effect reduction of nociceptive reflexes, both of the μ-agonists were notably less selective for the nociceptive responses. Figure 3 illustrates a case of such non-selective reductions by relatively high doses of fentanyl and morphine.

Thus whilst nociceptive responses were reduced significantly more than non-nociceptive responses (HO, $P < 0.05$) at fentanyl doses of 0.5–4 $\mu g\,kg^{-1}$ and morphine at 0.5 $mg\,kg^{-1}$, the degree of selectivity was less than that generally reported for dorsal horn neurones with μ-agonists (for review see Duggan & North, 1984). At a dose of 8 $\mu g\,kg^{-1}$ fentanyl, which was only tested on the more resistant neurones (see Parsons & Headley, 1989), and with doses of morphine above 1 $mg\,kg^{-1}$, the selectivity was even poorer and did not achieve statistical significance (see Table 1).

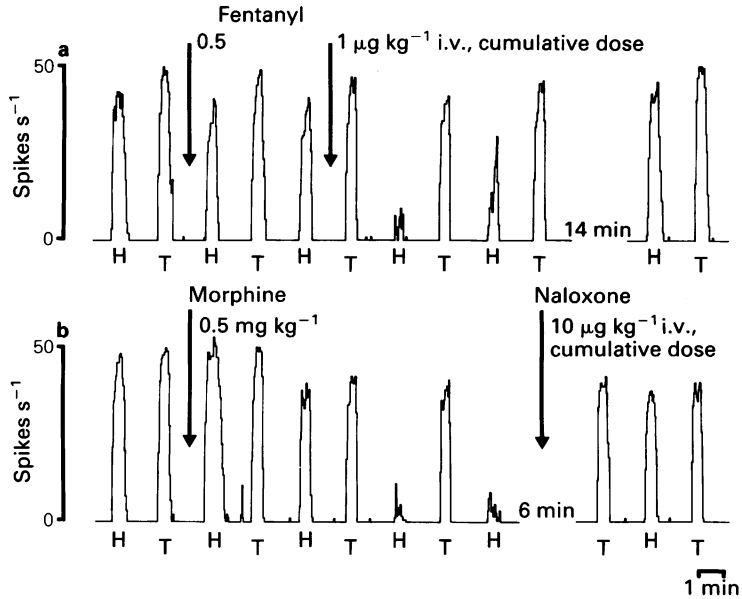


Figure 1 Comparison of the selectivity of two μ-agonists on reflexes of the same motoneurone to alternating noxious and non-noxious stimuli. Responses were elicited by noxious heat (H; 46.9°C) to the lateral plantar foot for 30 s and non-noxious tap (T; 8 Hz) of toe 5 for 20 s, alternating in a regular 200 s cycle. Panel (a) shows the selective depression of thermal nociceptive responses by fentanyl administered in divided doses of 0.5 + 0.5 μg kg^{-1} i.v. Panel (b) shows a similar selective depression by morphine 0.5 mg kg^{-1} i.v. Note the fentanyl: morphine potency ratio of 500:1. The effects of morphine were reversed by the subsequent i.v. administration of naloxone 10 μg kg^{-1}. Recording from lumbar 4 ventral root (VRL4). In this and in all following figures the data were recorded in spinalized, α-chloralose anaethetized rats.

κ-Agonists

The κ-agonist U-50,488 (0.5–16 mg kg^{-1}) was tested in 16 rats on 22 neurones activated by alternating noxious (heat $n = 17$, pinch $n = 5$) and non-noxious stimuli and, like the μ-agonists, showed preferential antinociceptive effects at the lower end of the dose-range examined. Figure 2 illustrates that the selectivity of low doses of this κ-ligand was very similar to that of the μ-agonist fentanyl when compared on the same neurone responding to the same alternating noxious and non-noxious stimuli. This selectivity at low doses of U-50,488 is also reflected in the pooled data presented in Table 1. These selective actions were reversed by low doses of naloxone (see Figure 6 in Parsons *et al.*, 1989).

As with the μ-agonists, when higher doses of U-50,488 were needed to reduce synaptic responses, the drug was non-selective between responses to noxious and non-noxious stimuli (Figure 3). In general, however, U-50,488 displayed poorer selectivity than the μ-agonists at any given level of reduction of reflexes. The middle panel of Figure 4 illustrates an example of total lack of selectivity by U-50,488 at 8 mg kg^{-1} i.v. whilst fentanyl at 4 μg kg^{-1} (Figure 4a) showed some selectivity. This trend is shown in the pooled data of Table 1 from which it can be seen that when tested at doses of 2 mg kg^{-1} or more, U-50,488 was totally non-selective.

The non-selective actions of U-50,488 were nonetheless opioid-receptor-mediated since the effects were reversed by low i.v. doses of naloxone on all 5 cells tested. On these cells, doses of U-50,488 of 4–16 mg kg^{-1} were required and depressed the nociceptive and non-nociceptive responses to means of 12% ($\pm$4) and 8% ($\pm$5) of control respectively. Naloxone (10–100 μg kg^{-1}) fully reversed these depressions by U-50,488 to means of 104% ($\pm$7) and 93% ($\pm$8) of control (Figure 4).

Anaesthetics

The selectivity of several classes of anaesthetic was compared with that of opioids in order to ascertain to what degree the selectivity of the opioid drugs on spinal reflexes might be a reflection of true antinociception as opposed simply to a greater sensitivity of nociceptive over non-nociceptive pathways to any central depressant.

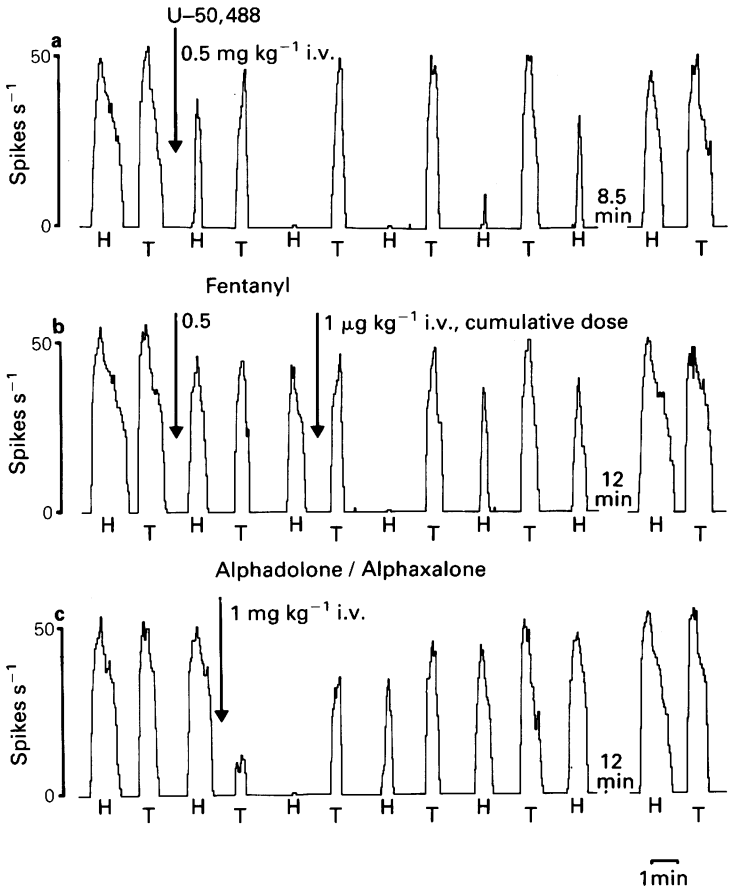


Figure 2 Comparison of the selectivity of two opioids and a general anaesthetic on reflexes of the same motoneurone to alternating noxious and non-noxious stimuli. Responses were elicited throughout by noxious heat (H; 46.9°C) to the lateral plantar foot for 30 s and non-noxious tap (T; 8 Hz) of toe 5 for 20 s alternating in a regular 200 s cycle. Panel (a) shows the relatively greater depression of nociceptive than non-nociceptive responses by the κ-agonist U-50,488 0.5 mg kg^{-1} i.v. Panel (b) shows a similar degree of selectivity by the μ-agonist fentanyl tested in divided doses of 0.5 + 0.5 μg kg^{-1}. Panel (c) shows that the steroid mixture alphaxalone/alphadolone 1 mg kg^{-1} was non-selective. Recording in VRL4.

As with the opioids, low doses of the dissociative anaesthetic ketamine showed selective antinociceptive effects when compared with fentanyl and/or U-50,488 on 11 cells responding to alternating noxious heat and non-noxious tap stimuli. However, this selectivity was again dependent on the sensitivity of the nociceptive responses, attaining statistical significance, in this group of cells, only at low doses of 0.5 and 1 mg kg^{-1} ketamine (see Table 1).

Figure 2 shows a test with the steroid mixture alphaxalone/alphadolone (1 mg kg^{-1} i.v.) on a cell on which both μ- and κ-opioids were selective in reducing the nociceptive reflexes. The steroid was far less selective between responses to noxious over responses to non-noxious stimuli than were the opioids ($n = 3$). The barbiturate methohexitone tested at 4 mg kg^{-1} was slightly more effective against responses to tactile stimuli than responses to noxious stimuli, but this selectivity did not reach statistical significance ('reversed' Wilcoxon one-tailed test for depression of tactile responses to a greater degree than nociceptive responses, HO, $P = 0.17$, $n = 6$).

α-Chloralose 20 mg kg^{-1} i.v. was tested on the responses of 11 cells and showed no clear effects on alternating reflexes to noxious heat (91% ± 8) and non-noxious tap (104% ± 4). A higher dose of 40 mg kg^{-1} i.v. was tested on 7 cells and had a slight-

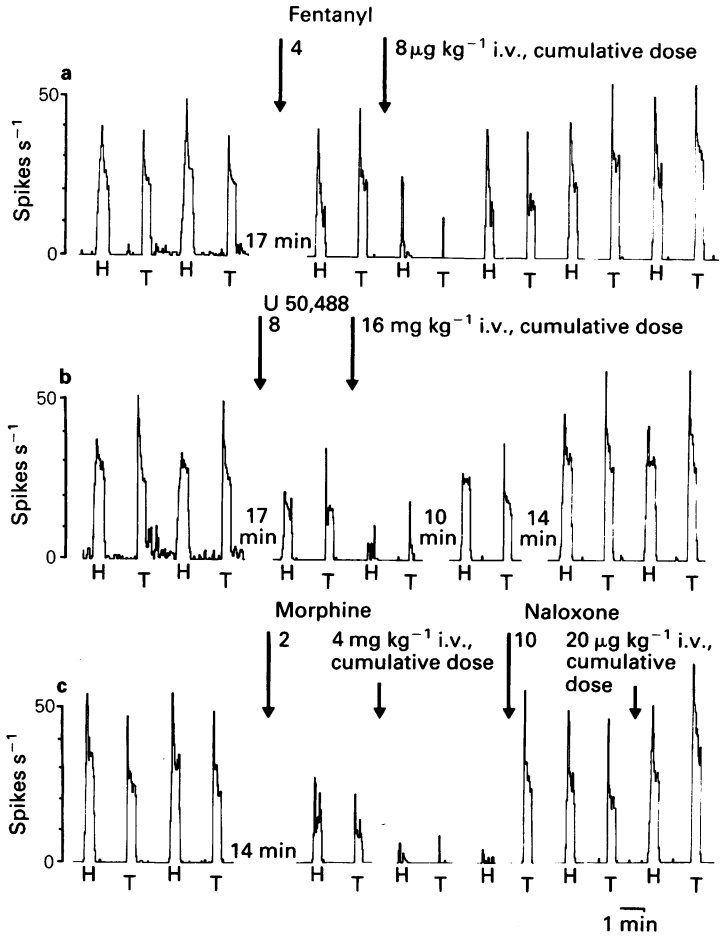


Figure 3 Comparison of the selectivity of high doses of μ- and κ-opioids on reflexes of the same motoneurone to alternating noxious and non-noxious stimuli. Responses were elicited by noxious heat (H; 49°C) to the plantar foot for 30 s and non-noxious tap (T; 9 Hz) of toe 5 for 20 s, alternating in a regular 210 s cycle. Panel (a) shows that fentanyl non-selectively depressed reflexes to the alternating stimuli following divided doses of $0.5 + 0.5 + 1 + 2 + 4\,\mu g\,kg^{-1}$ i.v. (first 3 doses not shown). Panel (b) shows a similar lack of selectivity by the κ-agonist U-50,488 administered in divided doses of $1 + 1 + 2 + 4 + 8\,mg\,kg^{-1}$ i.v. (first 3 doses not shown). Morphine was similarly non-selective (panel (c)) following divided doses of $1 + 1 + 2\,mg\,kg^{-1}$ i.v. (first dose not shown). The effects of morphine were rapidly reversed by the subsequent i.v. injection of naloxone $10 + 10\,\mu g\,kg^{-1}$ i.v. Recording in VRL4.

ly greater, but still non-selective effect on nociceptive (83% ± 11) and non-nociceptive (86% ± 6) reflexes (see also Headley *et al.*, 1987; Parsons & Headley, 1989).

Discussion

These results in the rat show that activation of spinal μ- and κ-opioid receptors can cause selective antinociceptive actions on reflexes. Moreover, the selective depression of nociceptive over non-nociceptive reflexes by opioids was not mimicked by the barbiturate or steroid anaesthetics tested and therefore probably reflects the antinociceptive/analgesic effects of spinal opioids widely reported in animals and man (see reviews by Cousins & Mather, 1984; Duggan & North, 1984; Yaksh & Noueihed, 1985). In contrast to the other anaesthetics tested, low doses of the dissociative anaesthetic ketamine did produce selective antinociceptive effects comparable with those seen with μ- and κ-opioids.

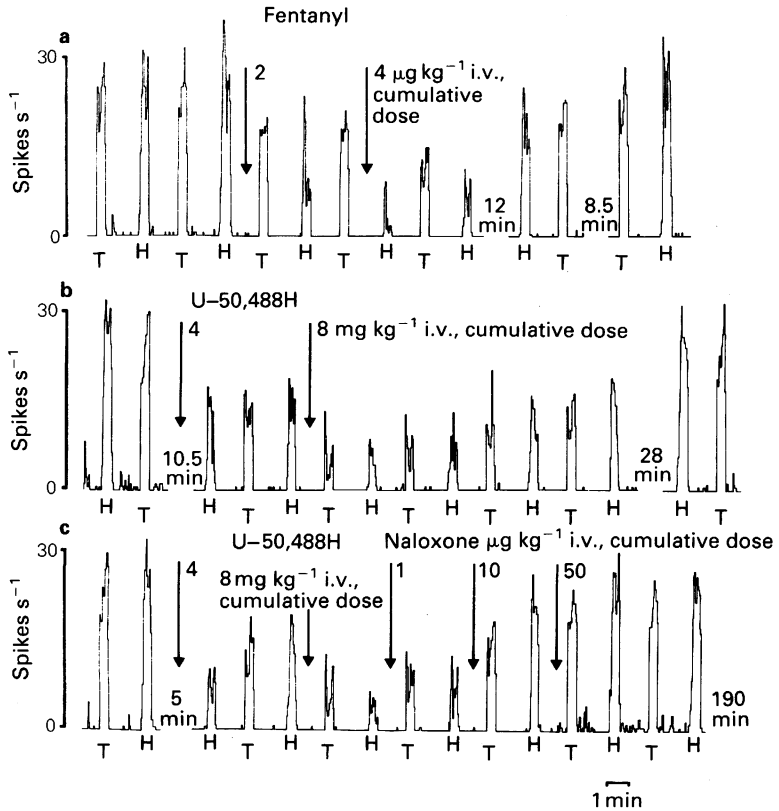


Figure 4 Different nociceptive versus non-nociceptive selectivity between μ- and κ-opioids and the reversibility of non-selective κ-effects by naloxone. Responses of a motoneurone in L4 segment were elicited by noxious heat (H; 48.5°C) to the lateral plantar foot for 30 s and non-noxious tap (T; 8 Hz) of toe 2 for 20 s, alternating in a regular 200 s cycle. Panel (a) shows moderate selectivity for nociceptive over non-nociceptive reflexes by fentanyl administered in divided doses of $2 + 2\,\mu g\,kg^{-1}$ i.v. Panel (b) shows that U-50,488 was non-selective following divided doses of $1 + 1 + 2 + 4\,mg\,kg^{-1}$ i.v. (first two doses not shown). Panel (c) shows a similar reduction of responses by a repeat test with U-50,488 and the subsequent reversal of this effect by naloxone $1–50\,\mu g\,kg^{-1}$ i.v. Compare the time courses of recovery/reversal of the actions of U-50,488 in the two tests.

The degree of nociceptive selectivity displayed by the opioids on each neurone was clearly related to the dose of the agents required to cause a reduction of the nociceptive reflex. In turn, neuronal sensitivity to the μ- and κ-opioids always went approximately in parallel, thus explaining the similar degrees of selectivity displayed by the ligands for μ- and κ-receptors. It remains to be established why different motoneurones should display such variable sensitivity (see also Parsons *et al.*, 1989).

In these studies low doses of naloxone consistently reversed the actions of both μ- and κ-opioids whether these were selective (Figure 1; Figures 5 and 6 in Parsons *et al.*, 1989) or non-selective (Figures 3 and 4) between nociceptive and non-nociceptive reflexes. This provides direct evidence that the relatively weaker selectivity seen in these studies on spinal reflexes, compared with that seen in most studies of dorsal horn nerones, is not related to the flaccidity reported for some tests with intrathecal κ-ligands, for such motor effects are not reversible by naloxone.

A further depressant action of κ-opioids is the sedation they cause following systemic administration (see Millan, 1987). Such actions will not have affected the spinal reflexes studied here since, in all animals, the spinal cord was sectioned so as to prevent any supraspinally-mediated effects influencing activity at a spinal level.

In conclusion, the results presented in this paper demonstrate that the selectivity shown by μ- and κ-opioids between nociceptive and non-nociceptive

Table 1 Selectivity of opioids and anaesthetics in depressing reflexes to noxious and non-noxious stimuli

Drug	*Dose* (*i.v.* kg^{-1})	*Nociceptive* %*con*	*Non-nociceptive* %*con*	n	*Probability HO*
Fentanyl	0.5 μg	66 ± 8	90 ± 3	13	0.0007
	1.0 μg	43 ± 9	78 ± 6	18	0.0005
	2.0 μg	54 ± 7	77 ± 6	16	0.0006
	4.0 μg	26 ± 9	50 ± 9	14	0.025
	8.0 μg	31 ± 18	29 ± 15	4	0.356
Morphine	0.5 mg	55 ± 20	80 ± 8	4	0.034
	1.0 mg	50 ± 22	72 ± 11	4	0.134
	2.0 mg	37 ± 19	47 ± 29	3	0.298
	4.0 mg	3,19	4,96	2	
U-50,488	0.5 mg	52 ± 15	81 ± 5	10	0.038
	1.0 mg	58 ± 12	72 ± 6	13	0.071
	2.0 mg	59 ± 12	54 ± 10	13	0.405
	4.0 mg	53 ± 15	40 ± 14	7	0.412
	8.0 mg	39 ± 9	29 ± 11	6	0.370
	16.0 mg	9 ± 1	6 ± 2	4	0.358
Ketamine	0.5 mg	51 ± 14	83 ± 4	8	0.034
	1.0 mg	46 ± 13	74 ± 5	9	0.026
	2.0 mg	47 ± 13	65 ± 10	8	0.117
Methohexitone	4.0 mg	29 ± 12	20 ± 9	6	0.330

Depression of reflexes on any individual cell was quantified as a percentage of the mean ($n = 3$) control total number of spikes per response. The mean effect at the agonist doses indicated is given under '%con'. The standard error of this mean and the number of cells tested (n) are also shown. One tailed probabilities that the nociceptive responses were depressed to a greater degree than the non-nociceptive responses were calculated using the Wilcoxon matched-pairs signed-ranks test and the probability of the null hypothesis (HO) is shown. Individual values have been quoted for morphine 4 mg kg^{-1} i.v. Reflex responses were recorded from the axons of single motoneurones in spinalized, α-chloralose anaesthetized rats.

reflexes is directly related to the sensitivity of reflex pathways to these agents and, in turn, that the relative sensitivities to μ- and κ-opioids is similar for each individual motoneurone tested. Similar dose-dependent selectivity was seen with dissociative but not with general anaesthetics. In accompanying papers (Parsons & Headley, 1989; Parsons *et al.*, 1989) we have shown that, in this same preparation, κ- and μ-opioids have indistinguishable actions on reflexes to thermal and mechanical noxious stimuli. However, despite these similarities, the differential sensitivity of μ- and κ-ligands to naloxone indicates that the actions of these ligands were indeed mediated at different subtypes of opioid receptor. It is therefore apparent that, when tested on reflexes to various modalities of natural peripheral stimulus, the activation either of spinal μ- or of spinal κ-opioid receptors by systemic agonists produces a very similar spectrum of effects when tested in spinalized rats.

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Demonstration in *Tupaia* papillary muscle preparations of α-adrenoceptors mediating positive inotropic effects: comparison with guinea-pigs

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1 Positive inotropic responses to α- and β-adrenoceptor agonists of isolated papillary muscles from the tree shrew (*Tupaia*), were compared with those from guinea-pigs.

2 In *Tupaia*, the concentration-response curve for phenylephrine, unlike that for isoprenaline, was not affected by pindolol in a concentration (10^{-8} M) sufficient to block β-adrenoceptor-mediated responses, but it was significantly shifted to the right by phentolamine (10^{-6} M). In guinea-pig papillary muscles, however, the concentration-response curve for phenylephrine, like that for isoprenaline, was shifted to the right by pindolol (10^{-8} M) but was unaltered by phentolamine (10^{-6} M). Furthermore, when the mean concentrations of agonists inducing maximal positive inotropic responses were compared (relative to that of isoprenaline = 1.0), phenylephrine was found to be only slightly less potent (0.84 ± 0.04; $n = 5$) in *Tupaia* and much less potent (0.33 ± 0.06; $n = 5$) in the guinea-pig.

3 Although in *Tupaia* papillary muscles the increase in developed tension induced by a combination of phenylephrine and isoprenaline did not significantly differ from that by phenylephrine alone, it was approximately 3 times larger than that produced by phenylephrine alone in guinea-pigs.

4 These results indicate that in papillary muscles from *Tupaia*, unlike the guinea-pig, the positive inotropic effects of phenylephrine can be mediated by α-adrenoceptors.

Introduction

Since Wenzel & Su (1966) first reported the presence of α-adrenoceptors in the myocardium of rats, there has been no doubt about the presence of α-adrenoceptors as possible mediators of positive inotropic effects in the heart of common laboratory mammals and also of primates and man (Wagner & Brodde, 1978; Schümann, 1980; Davey, 1986). Current literature indicates that the positive inotropic effects of sympathomimetic agents arise from combined activation of α- and β-adrenoceptor populations, even though the functional significance of the mammalian myocardial α-adrenoceptors remains uncertain (Benfey, 1982; Endoh, 1986).

Tree shrews (e.g. *Tupaia glis*) have some anatomical (Le Gros Clark, 1971) and serological (Goodman, 1966; Moore & Goodman, 1968) analogies to primitive primates and are characterized by rapid reproduction (Schwaier, 1973; 1975). Although *Tupaia* may be a promising experimental animal in various research fields (Noyes, 1968; McClure *et al.*, 1972; Sakai *et al.*, 1979), little attention has been paid to their possible usefulness in applied medical research. Therefore, it was of interest to examine whether or not α-adrenoceptors mediating the positive inotropic response to sympathomimetic agents exists in the heart of *Tupaia*.

The effect of α- and β-adrenoceptor agonists on isolated papillary muscles of *Tupaia*, were compared with effects in the guinea-pig.

Methods

Male adult Hartley guinea-pigs and *Tupaia glis* of either sex (bred in the Chugai Farm) were killed by a blow to the back of the neck under light ether anaesthesia. Papillary muscles with a diameter of 1.5 mm or less were excised from the right ventricle of guinea-pigs, or from the left ventricle (the muscle from the right ventricle is too small) of *Tupaia*. The muscles were mounted in a 20 ml bath maintained at 37°C containing a modified Krebs-Henseleit solution

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Table 1 Basal tension, maximal increase in the developed tension (Max Δ T) induced by phenylephrine or isoprenaline, and $-\log EC_{50}$ values for each agonist after repeated determination of the concentration-response curves (CRC) in the papillary muscles of *Tupaia* or guinea-pig

		Phenylephrine			*Isoprenaline*		
		$-\log EC_{50}$ *values*	*Developed tension* (mg)		$-\log EC_{50}$ *values*	*Developed tension* (mg)	
			Basal	Max Δ *T*		*Basal*	Max Δ *T*
Tupaia	1st CRC	4.62 ± 0.06	155 ± 41	91 ± 21	7.26 ± 0.17	207 ± 47	106 ± 26
	2nd CRC	4.56 ± 0.08	134 ± 31	67 ± 16	7.10 ± 0.24	168 ± 37	88 ± 14
	3rd CRC	4.69 ± 0.07	135 ± 26	57 ± 14	7.13 ± 0.20	153 ± 34*	80 ± 13
	4th CRC	4.68 ± 0.09	150 ± 29	51 ± 16*	6.95 ± 0.24	153 ± 34*	79 ± 17
Guinea-pig	1st CRC	4.33 ± 0.11	176 ± 49	154 ± 36	7.80 ± 0.10	179 ± 64	560 ± 193
	2nd CRC	4.06 ± 0.07	155 ± 35	98 ± 32	7.80 ± 0.11	164 ± 53	529 ± 162
	3rd CRC	4.21 ± 0.08	135 ± 29	89 ± 18*	7.79 ± 0.09	145 ± 44	551 ± 175
	4th CRC	4.18 ± 0.10	134 ± 30	82 ± 18*	7.81 ± 0.09	137 ± 39	557 ± 174

Given are means ± s.e. mean of 5 preparations. *$P < 0.05$ compared with the 1st CRC value.

of the following composition (mM): NaCl 119.0, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 24.8, KH_2PO_4 1.2 and glucose, 10.0; the bathing solution was continuously aerated with 95% O_2 and 5% CO_2. A tension of 0.5 g was applied to the muscles; they were stimulated by two platinum electrodes using field stimulation from a Nihon Kohden electric stimulator (model SEN-7103) (frequency, 0.5 Hz; intensity, 1.2–1.5 times threshold; duration, 5 ms).

The developed tension of the muscle was measured isometrically with a Nihon Kohden force transducer (TB-611T) attached to a Yokogawa pen recorder (Type 3066; frequency response characteristics: 0.7 Hz). The muscle segments at 0.5 Hz stimulation were first equilibrated for at least 30 min, with changes of the bathing medium every 10–15 min before drug challenge. Then, to assess its responsiveness and stability, the preparation was exposed to a test concentration of phenylephrine or isoprenaline likely to be submaximally effective. When these responses had reached a plateau, the drug was washed out and the preparation allowed to recover for at least 60 min. The relationship between concentration of agonists and inotropic responses was subsequently examined by means of cumulative additions of either phenylephrine or isoprenaline, the final bath concentration being increased in three fold increments. Maximum inotropic responses were obtained for each tissue.

Experimental protocols

After equilibration of the preparation, one of 3 different experimental procedures was then started.

(1) Concentration-response curves to phenylephrine or isoprenaline were constructed 4 times in the absence of any antagonists. After completion of the first curve the bathing fluid was renewed every 10–15 min for 60 min until the developed tension of the papillary muscle returned to control values; the next concentration-response curve was then constructed.

(2) Concentration-response curves to phenylephrine or isoprenaline were constructed in the absence of antagonists. It was confirmed that there were no significant differences between the corresponding values obtained from the second and third curves (Table 1). After the second concentration-response curve was constructed, the tissue was washed out repeatedly in the absence of antagonists and equilibrated for at least 60 min. An antagonist was added to the organ bath and, 30 min later, the cumulative concentration-response curve to an agonist drug was constructed again for each tissue.

(3) Papillary muscles were exposed to a high concentration of phenylephrine, which was incubated with the tissue for 5–10 min before successive applications of isoprenaline.

Drugs

Drugs used were phentolamine mesylate (Ciba-Geigy), (−)-isoprenaline hydrochloride (Nikken Kagaku), (−)-phenylephrine hydrochloride (Sigma), and (±)-pindolol base (Sandoz). All drugs were dissolved in distilled water, and/or diluted with 0.9% saline solution. Drug solutions were usually added to the bath in a volume of 0.1 ml in individual syringes (Terumo Co.). All drug concentrations are expressed as final molar (M) concentrations in the bathing solution.

Statistical analysis

Values in the text are means ± s.e. mean. The concentrations causing 50% of the maximum response to each agonist (EC_{50} value) for phenylephrine or isoprenaline were determined from each concentration-response curve. The statistical significance of the differences between mean values was

estimated by means of Student's t test and expressed as a P value; a value of 0.05 or less was considered significant.

Results

Repeated determination of concentration-response curves for phenylephrine or isoprenaline

Summarized data are shown in Table 1. Phenylephrine over the concentration-range 10^{-7} to 10^{-3} M elicited a concentration-dependent increase in developed tension in papillary muscle preparations of *Tupaia* and guinea-pig, and at 3×10^{-4} M the concentration-response curve reached a maximum (Figures 1 and 2). Although the curve for phenylephrine was constructed 4 times in the absence of any antagonists, the basal developed tension remained unchanged by this procedure. Likewise, the negative logarithm of the EC_{50} ($-\log EC_{50}$), values for phenylephrine calculated from four consecutive concentration-response curves, were nearly identical. Even though there were no significant differences between the corresponding values obtained from the second, third and fourth curves, the maximum increases in the developed tension in the three consecutive curves, compared with those in the first curve, were considerably diminished. The degree of attenuation was larger in the guinea-pig than in *Tupaia*.

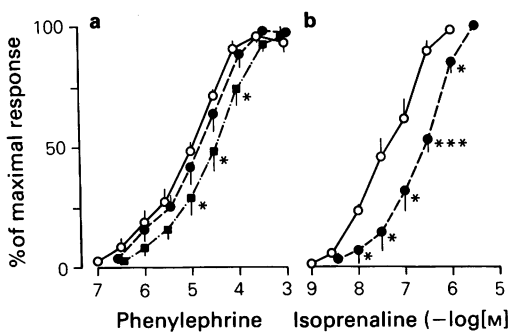


Figure 1 Effects of pindolol and/or phentolamine on the positive inotropic responses to phenylephrine or isoprenaline in *Tupaia* papillary muscles. The concentration-response curves for phenylephrine (a) or isoprenaline (b) were constructed in the absence or presence of either phentolamine or pindolol. Increases in tension are plotted as a percentage of the maximum response. Each point represents mean of 10 (phenylephrine control) or 5 (the others) experiments; vertical lines show s.e.mean: (○) Control; (●) pindolol 10^{-8} M; (■) phentolamine 10^{-6} M. $^{*}P < 0.05$ $^{***}P < 0.001$ compared to the corresponding value from the group not treated with any antagonists (control).

Isoprenaline (10^{-9} to 10^{-6} M) caused a concentration-dependent positive inotropic effect in papillary muscle preparations of *Tupaia* and guinea-pig (Figures 1 and 2). In guinea-pigs, neither the basal developed tension nor the maximum positive inotropic effect of isoprenaline was significantly changed by this procedure. Additionally, the $-\log EC_{50}$ values for isoprenaline calculated from four successive concentration-response curves were also identical, suggesting that no spontaneous decrease in sensitivity to isoprenaline had taken place. A consistent finding in *Tupaia*, was a significant reduction in basal tension following the construction of the first concentration-response curve for isoprenaline; the basal tensions for the third and fourth curves were significantly lower than that for the first curve but there were no significant differences between the relevant values for second, third and fourth curves.

The mean molar concentrations of agonists inducing maximal positive inotropic responses were compared by assigning the value 1.0 to the values of isoprenaline. In *Tupaia* phenylephrine (0.84 ± 0.04; $n = 5$) was only slightly less active than isoprenaline, whereas in the guinea-pig, it had only one-third (0.33 ± 0.06; $n = 5$) of the potency of isoprenaline.

Effects of β- and α-adrenoceptor antagonists on the positive inotropic response to isoprenaline and phenylephrine

In *Tupaia* papillary muscles, pindolol (10^{-8} M) failed to affect the concentration-response curve for the papillary muscle tension responses to phenylephrine (Figure 1). The $-\log EC_{50}$ value for phenylephrine after blockade of β-adrenoceptors was 4.86 ± 0.15 ($n = 5$) and did not differ significantly from that of the control (4.99 ± 0.09; $n = 10$). The concentration of pindolol (10^{-8} M) used was sufficient to block β-adrenoceptors, as shown by a rightward shift of the concentration-response curve for isoprenaline (Figure 1). A significant difference ($P < 0.05$) was observed with the $-\log EC_{50}$ value for isoprenaline between groups treated (6.67 ± 0.18; $n = 5$) and untreated (7.16 ± 0.11; $n = 5$) with pindolol (10^{-8} M), and the concentration-ratio (CR; EC_{50} value of agonist in the presence of antagonist divided by the control EC_{50} value) was 3.57 ± 1.03 ($n = 5$). On the other hand, the α-adrenoceptor antagonist, phentolamine, significantly blocked the positive inotropic effect of phenylephrine. The concentration-response curve for phenylephrine was shifted to the right in the presence of phentolamine (10^{-6} M) (Figure 1). The $-\log EC_{50}$ value for phenylephrine was significantly ($P < 0.05$) reduced from 4.99 ± 0.09 ($n = 10$) to 4.52 ± 0.16 ($n = 5$) in the presence of phentolamine (CR = 3.47 ± 0.58; $n = 5$).

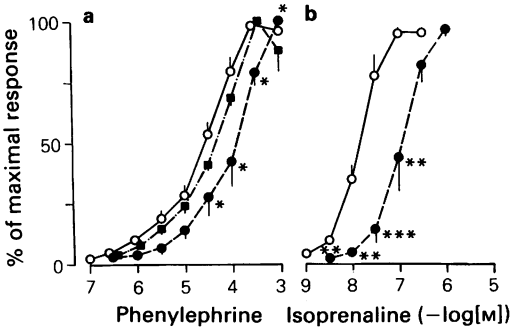


Figure 2 Effects of pindolol and/or phentolamine on the positive inotropic responses to phenylephrine or isoprenaline in guinea-pig papillary muscles. The concentration-response curves for phenylephrine (a) or isoprenaline (b) were constructed in the absence or presence of either phentolamine or pindolol. Increases in tension are plotted as a percentage of the maximum response. Each point represents mean of 10 (phenylephrine control) or 5 (the others) experiments; vertical lines show s.e.mean. (○) Control; (●) pindolol 10^{-8} M; (■) phentolamine 10^{-6} M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the corresponding value from the group not treated with any antagonists (control).

In guinea-pig papillary muscles, pindolol (10^{-8} M) caused a rightward shift of the concentration-response curve for phenylephrine as well as for isoprenaline (Figure 2). The $-\log EC_{50}$ values for phenylephrine and isoprenaline were significantly reduced from 4.56 ± 0.10 ($n = 10$) to 3.99 ± 0.18 ($n = 5$) ($P < 0.05$, CR = 3.18 ± 0.71; $n = 5$) and from 7.80 ± 0.10 to 6.92 ± 0.13 ($P < 0.001$, each $n = 5$, CR = 9.86 ± 3.64; $n = 5$) in the presence of pindolol (10^{-8} M), respectively. The treatment with phentolamine (10^{-6} M) did not affect the concentration-response curve for phenylephrine. No significant difference was observed in the $-\log EC_{50}$ value for phenylephrine between treated (4.36 ± 0.02; $n = 5$) and untreated groups (4.56 ± 0.10; $n = 10$) with phentolamine (10^{-6} M).

When compared with the $-\log EC_{50}$ values for phenylephrine and isoprenaline in papillary muscles from *Tupaia* and the guinea-pig, *Tupaia* possessed a significantly higher sensitivity to phenylephrine (*Tupaia*, 4.99 ± 0.09; guinea-pig, 4.56 ± 0.10; $P < 0.05$, each $n = 10$), whereas the guinea-pig had significantly higher sensitivity to isoprenaline (*Tupaia*, 7.16 ± 0.11; guinea-pig, 7.80 ± 0.10; $P < 0.05$, each $n = 5$). There were no significant changes in maximum developed tension between treated and untreated groups with each antagonist (not shown).

Positive inotropic responses to simultaneous muscle stimulation by α- and β-adrenoceptor agonists

Papillary muscles from *Tupaia* and guinea-pig were exposed to a maximal concentration of phenylephrine (10^{-3} M). In the presence of phenylephrine, isoprenaline in a concentration (10^{-6} M) sufficient to cause a maximum response was added to the bath. In *Tupaia* papillary muscles, the increase in developed tension induced by a combination of phenylephrine and isoprenaline (95 ± 19 mg; $n = 5$) did not significantly differ from that induced by phenylephrine alone (73 ± 16 mg; $n = 5$). On the other hand, in guinea-pig papillary muscle preparations, the combination of phenylephrine and isoprenaline produced a developed tension (365 ± 70 mg; $n = 5$), which was approximately 3 times higher than that with phenylephrine alone (123 ± 29 mg; $n = 5$).

Discussion

The present experiment revealed that in *Tupaia* papillary muscles the concentration-response curve for phenylephrine was shifted to the right by phentolamine, whereas it was not affected by pindolol in a concentration sufficient to block the β-adrenoceptors. This finding is consistent with results of previous studies on rat cardiac tissues which showed that positive inotropic responses to phenylephrine were mainly due to α-adrenoceptor stimulation (Wagner & Brodde, 1978; Shibata *et al.*, 1980; Williamson & Broadley, 1987). On the other hand, it was noted that in guinea-pig papillary muscles the concentration-response curve for phenylephrine remained unaffected by phentolamine, but like isoprenaline, was significantly shifted to the right by pindolol. Thus, in papillary muscles of the guinea-pig, unlike *Tupaia*, we could not demonstrate the presence of any functionally active α-adrenoceptors under the present conditions.

The question whether phenylephrine causes positive inotropic responses of the myocardium through stimulation of α- and β- adrenoceptors is still under investigation. The α-adrenoceptor-mediated positive inotropic response to phenylephrine appears to be concentration-dependent, species-dependent and varies between different regions of the heart (Schümann *et al.*, 1974; Wagner & Brodde, 1978; Shibata *et al.*, 1980). Nevertheless, there has been a substantial amount of evidence from both functional and binding studies supporting the existence of post-junctional α-adrenoceptors in the mammalian heart, including human heart (Wagner & Brodde, 1978; Benfey, 1982; Endoh, 1986).

In the present experiments, the potency of phenylephrine, relative to that of isoprenaline, probably indicates that in *Tupaia* papillary muscles the posi-

tive inotropic effect of phenylephrine is mediated almost exclusively by α-adrenoceptors, but in guinea-pig predominantly via β-adrenoceptors. Moreover, it should be noted that in *Tupaia* papillary muscles, the increase in developed tension caused by a combination of phenylephrine and isoprenaline did not significantly differ from that caused by phenylephrine alone, whereas in guinea-pig papillary muscles the response to a combined use of phenylephrine and isoprenaline, compared with phenylephrine alone, was approximately three times greater with respect to the developed tension. This finding lends further support to the idea that α-adrenoceptors may play a functionally significant role in the regulation of *Tupaia* myocardial contraction and provides further evidence for the existence of α-adrenoceptors mediating the positive inotropic effect in *Tupaia* papillary muscles.

For the present study, the α-adrenoceptor subtypes in the papillary muscle preparations could not be classified because phentolamine, a non-selective α-adrenoceptor antagonist (Davey, 1986), was used. However, it is known that stimulation of α_1-adrenoceptors produces a positive inotropic effect in cardiac tissues from cat, guinea-pig, rabbit, rat and man (Davey, 1986; Ask *et al.*, 1987; Williamson & Broadley, 1987). Taken together, these findings suggest that the α-adrenoceptor subtypes in *Tupaia* papillary muscles also may be of the α_1-type, but this requires further investigation.

In conclusion, the present study demonstrated the existence of functionally active α-adrenoceptors in *Tupaia* papillary muscles, which clearly mediated the positive inotropic effect in the presence and absence of a β-adrenoceptor antagonist. Although myocardial β-adrenoceptors are well known to mediate effects of sympathetic nerve stimulation and sympathomimetic agents, it is still doubtful whether myocardial α-adrenoceptors have a clinically physiological significance in man. In view of this, the papillary muscle preparation from *Tupaia*, a primitive primate (Schwaier, 1973; 1975) may provide a useful alternative to the other species for studying the physiological role of α-adrenoceptors in the myocardium.

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Muscarinic suppression of the M-current in the rat sympathetic ganglion is mediated by receptors of the M_1-subtype

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1 Under voltage-clamp dissociated adult and foetal rat superior cervical ganglion (s.c.g.) cells exhibited a non-inactivating voltage- and time-dependent component of K^+ current termed the M-current (I_M). I_M was detected and measured from the current decay during hyperpolarizing voltage steps applied from potentials where I_M was pre-activated.

2 Neither the resting membrane current nor the amplitude of these current decay relaxations were reduced by omitting Ca from the bathing fluid, showing that the M-current was not a 'Ca-activated' K-current dependent on a primary Ca-influx. Concentrations of (+)-tubocurarine sufficient to block the slow Ca-activated K-current I_{AHP} did not inhibit I_M or antagonize the effect of muscarinic agonists on I_M, showing that I_M was not contaminated by I_{AHP}. Tetraethylammonium (1 mM), which blocks the fast Ca-activated K-current I_C, produced a small inhibition of I_M. This was not due to contamination of I_M by I_C since muscarinic agonists did not consistently block I_C.

3 The muscarinic agonists muscarine, oxotremorine, McN-A-343 and methacholine reversibly suppressed I_M, resulting in an inward (depolarizing) current. The rank order of potency was: oxotremorine $\geqslant$ muscarine > McN-A-343 > methacholine.

4 The suppression of I_M by muscarine was similar in cultured cells derived from adult and foetal tissue to that seen in the intact ganglia.

5 I_M-suppression by muscarine was inhibited by pirenzepine (Pz) and AF-DX 116 with mean pK_B values of 7.53 ± 0.13 ($n = 3$) and 6.02 ± 0.13 ($n = 4$) respectively.

6 The suppression of I_M by muscarinic agonists was not affected by gallamine (10–30 μM). 4-Diphenylacetoxy-N-methylpiperidine methiodide inhibited the response at 300 nM.

7 Pirenzepine inhibited the contractions of the guinea-pig isolated ileum produced by muscarine with a mean pK_B of 6.37 ± 0.03 ($n = 8$).

8 These results suggest that the receptors mediating suppression of the M-current accord with those designated pharmacologically as M_1 and that these receptors reach maturity at a very early stage in the development of the rat s.c.g.

Introduction

Muscarinic receptors have been classified into at least two types, M_1 and M_2 (Hammer & Giachetti, 1982). This classification was based initially on the relative affinities of the antagonist pirenzepine (Pz) for the two receptor-types, as measured by competition with [^{3}H]-N-methylscopolamine binding (Hammer *et al.*, 1980). M_1 receptors are those for which Pz has a high affinity (K_I 20–30 nM) and M_2 receptors are those for which Pz has a low affinity (K_I 0.3–1.2 μM) (Hammer & Giachetti, 1982). The high-affinity M_1 receptors are particularly abundant in nervous tissue (Hammer *et al.*, 1980).

Evidence supporting the presence of M_1 receptors in the nervous system was previously obtained by measuring the effectiveness of pirenzepine in antagonizing the depolarizing action of muscarine on rat isolated superior cervical ganglia (Brown *et al.*,

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1980b). These experiments yielded a pA_2 of 8.36 (K_B 4.4 nM), as opposed to a pA_2 of 6.99 (K_B 0.1 μM) against the muscarine-induced contractile response of the rat ileum.

The depolarization of the ganglion produced by muscarine is due, in part at least, to the inhibition of a voltage-dependent K-current, the M-current (Constanti & Brown, 1981). Subsequent measurement of the effect of pirenzepine on the depolarizing response in guinea-pig submucous and myenteric neurones (also due to a reduced K-conductance, but of a different, voltage-independent type), yielded an identical pA_2 of 8.4 (North *et al.*, 1985), and has led to the proposition (North, 1986) that muscarinic receptors inducing K-channel closure might generally belong to the M_1 category in terms of their pharmacological classification.

However, there are some difficulties both with this general proposition and with the interpretation of the original experiments of Brown *et al.* (1980b) on the rat ganglion. Firstly, while the experiments of Constanti & Brown (1981), together with the parallel experiments of Adams *et al.* (1982b) on frog ganglion cells, showed unambiguously that inhibition of the M-current accounted for the inward (depolarizing) current in the majority of neurones in these ganglia, other studies have indicated that additional conductance changes can contribute to the depolarization in at least some ganglion cells (e.g., Kuba & Koketsu, 1976; Hashiguchi *et al.*, 1982; Jones, 1985; Brown & Selyanko, 1985; Mochida & Kobayashi, 1986; Cassell & McLachlan, 1987). Secondly, in two other types of cell exhibiting M-currents, toad stomach smooth muscle cells (Sims *et al.*, 1986) and pyramidal cells in the guinea-pig olfactory cortex (Constanti & Sim, 1987), the inhibition of the M-current by muscarinic agonists proved insensitive to pirenzepine at concentrations up to 1 μM. Thirdly, ligand binding studies on homogenates of calf sympathetic ganglia have revealed a mixture of high- and low-affinity binding sites for pirenzepine (Hammer & Giachetti, 1982). These observations raise the question whether the ability of pirenzepine to inhibit the extracellularly-recorded depolarization of the rat superior cervical ganglion truly reflects antagonism of M-current inhibition in the individual neurones or whether it might have resulted from some other action.

For these reasons, we have directly assessed the pharmacological classification of the muscarinic receptors responsible for M-current inhibition in single neurones of the rat superior cervical ganglion, using voltage-clamp methods. For this purpose, we have used not only pirenzepine but also AF-DX116 (11-[2-[(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one), a cardioselective muscarinic antagonist: this compound competes against radiolabelled N-methylscopolamine or pirenzepine binding with high affinity to M_2-muscarinic receptors in the heart (K_I 115 nM), intermediate affinity to neuronal M_1-receptors (K_I 760 nM) and low affinity to glandular muscarinic receptors (K_I 3.2 μM) (Giachetti *et al.*, 1986; Hammer *et al.*, 1986; Giraldo *et al.*, 1987). We also provide data on the relative activity of several different muscarinic agonists on the M-current recorded under these conditions.

Methods

Intact superior cervical ganglia (s.c.g.) were isolated from urethane anaesthetized (1.5 mg kg^{-1}) Sprague-Dawley rats (body weight 170–250 g). For the dissociation and maintenance of sympathetic neurones in culture, s.c.g. were isolated from anaesthetized rats (20–30 days postnatal, 50–70 g body weight) or from embryonic rats (E17–E21) removed from adult females previously anaesthetized with ether and killed by cervical dislocation. Cells were prepared from these ganglia as described previously (Marrion *et al.*, 1987; Smart, 1987).

Recording

Intact rat s.c.g. were stripped of their connective tissue capsule, mounted in a chamber with a Sylgard (Dow-Corning) base and superfused (at approximately 12 ml min^{-1}), at 20–23°C, with a modified bicarbonate-buffered Krebs solution of composition (mM): NaCl 106.2, Na isethionate 11.8, KCl 5.85, $MgCl_2$ 1.2, $CaCl_2$ 2.52, $NaHCO_3$ 25 and D-glucose 11 (saturated with 95% O_2:5% CO_2; pH 7.4). Dissociated neurones were perfused at 7–9 ml min^{-1}, at 25°C, with a modified Krebs solution of the following composition (mM): NaCl 118, KCl 5.85, $MgCl_2$ 1.2, $CaCl_2$ 2.52, $NaHCO_3$ 25 and D-glucose, 11. The solution was bubbled with 95% O_2:5% CO_2 (pH 7.4). Individual neurones were visualized by inverted phase-contrast optics (Nikon TMS). Intact s.c.g. neurones were impaled with 4 M K acetate-filled microelectrodes (d.c. resistance 60–80 MΩ) and voltage-clamped with a single-electrode discontinuous amplifier (Axoclamp 2, sampling rate 2–4 kHz, 30% duty cycle). Cultured cells were impaled with 1 M KCl-containing microelectrodes (d.c. resistance 50–80 MΩ) and voltage-clamped with a switching amplifier having a 50% duty cycle (sampling rate 3 kHz). Membrane currents were recorded on a Gould Brush 2400S pen recorder.

Measurement of drug action

The experimental protocol for measuring M-current inhibition by muscarinic agonists is described in the Results.

To determine K_B values for the antagonists, approximate dose-response relationships were deduced from two-point cumulative applications of muscarine in the absence and presence of increasing concentrations of antagonist. Steady-state responses to muscarine were attained in approximately 2–3 min, so cumulative addition of two or more concentrations of agonist meant contact times in excess of 5 min. It was noted that the cumulative addition of any more than three concentrations of agonist yielded smaller responses than expected from sequential dosing. This, together with the observation that a recovery time from agonist application of 15–20 min was required to produce reproducible effects, implied a slow desensitization process for these responses (c.f. Brown & Constanti, 1980; Akasu & Koketsu, 1981; Adams *et al.*, 1982b). Thus, to prevent this, only two cumulative additions were used and >20 min recovery time was allowed. The concentrations of muscarine used were chosen to fall approximately on the linear portion of the concentration-response curve (see Figure 7b). Maximal concentrations of muscarine were avoided in antagonist studies because of the difficulty in obtaining full recovery of I_M after removing the agonist. The control concentration-response relationship was repeated at least twice before adding an antagonist. Each concentration of antagonist was added for 30 min before adding muscarine and left in contact during muscarine application. To complete an adequate number of agonist concentration curves in the presence of various antagonist concentrations (usually 3–6 concentrations of antagonist), cell impalements had to be maintained for 3–6 h, with constant I_M and total holding current. To avoid deleterious effects of constant ionic current injection on both cell and electrode properties, the clamp was released and returned to the initial resting membrane potential (using d.c. current injection if necessary), during recovery from agonist application and incubation with the next antagonist concentration. To minimize d.c. potential drifts during the impalement the reversal potential for I_M (E_K), together with the spike threshold and overshoot were monitored throughout. Any cell which showed apparent drifts in E_K or spike threshold was discarded.

For comparison, data are presented on antagonism of the muscarinic contractile response in the guinea-pig ileum. Ileal segments were removed from guinea-pigs killed by a blow to the head and exsanguination, and mounted in 25 ml organ baths under 1 g tension. They were bathed in 95% O_2:5% CO_2-aerated Krebs solution of identical composition to that used with dissociated neurones (see above). Contractions were recorded, at 32°C, by isotonic transducers onto flat-bed chart recorders. Full antagonism determinations were carried out on each preparation, the antagonist being added once the control muscarine concentration-response relationship had stabilized. Each antagonist was present for 30 min before starting full concentration-response curves to muscarine.

All antagonist affinity determinations were analysed by the method of Arunlakshana & Schild (1959). Schild plots were obtained by measuring dose-ratios from the displacements of the concentration-response relationships for muscarine by increasing antagonist concentrations. The line of best fit was obtained by the method of least squares, using unweighted data (Colquhoun, 1971). In all cases the slope of this line was not significantly different from unity ($P < 0.02$; two-tailed t test). Since the use of the Schild analysis presupposes competitive antagonism, all slopes were then constrained to unity, such that $pA_2 = pK_B$, and individual pK_B values determined for each experiment by the method of Tallarida *et al.* (1979). These pK_B values were averaged and the standard error of the mean (s.e.mean) calculated. Individual values for the antagonist dissociation constant K_B were calculated as $-(\text{antilog}/pK_B)$.

DL-Muscarine chloride, oxotremorine sesquifumarate and methacholine chloride were obtained from Sigma. McN-A-343 ((3-*m*-chlorophenylcarbamoyloxy)-2-butynyltrimethylammonium) was supplied from Research Biochemicals Industries, MA, U.S.A. Gallamine triethiodide was obtained from May and Baker. Both pirenzepine and AF-DX 116 (see Introduction) were a gift from Dr Karl Thomae, GmbH, Biberach an der Riss, F.R.G. 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methiodide) was a gift from Dr R.B. Barlow, Bristol.

Results

Characterization of M-current

The M-current, as originally described in frog sympathetic neurones (Adams *et al.*, 1982a), may be defined as a subthreshold voltage-gated K-current which is activated over the membrane potential range −70 to 0 mV and which does not inactivate with time. When the membrane potential is suddenly changed, the current shows an exponential growth or decay with a relatively long time-constant of about 150 ms at the half-activation potential (−35 mV). This time-constant accelerates as the potential deviates from the half-activation point according to a simple Boltzmann expression with an e-fold slope factor of about 20 mV. Rat superior cervical sympathetic neurones possess a current with the same kinetic features but minor quantitative dif-

ferences (Constanti & Brown, 1981; Freschi, 1983; Brown & Selyanko, 1985). This section ascertains whether the current investigated in the present experiments accords with these previous descriptions of the M-current and can be differentiated from other K-currents such as those activated by Ca ions.

The most convenient way of recording the behaviour of the M-current is to hold the cell membrane potential at a value depolarized to the normal resting potential, such that a substantial fraction of the current is tonically activated, and then to hyperpolarize the cell so as to deactivate the current (Figure 1a). Under these conditions the decay of the current ('deactivation') is seen as a slow 'tail', which is outward in direction when the command potential is positive to the K equilibrium (E_K) potential but which reverses to an inward tail at command potentials negative to E_K. When the membrane potential is restored to the original holding potential, the reactivation of the M-current is seen as a slowly-increasing current; on repolarizing from command potentials negative to −60 mV, the onset of this outward M-current is masked by a superimposed transient outward K-current, the A-current (see Adams *et al.*, 1982a; Galvan & Sedlmeir, 1984; Belluzzi *et al.*, 1985a). The range of membrane potential over which the M-current is activated can easily be found by plotting the inward current generated at the end of each voltage step against the recorded command potential (Figure 1b). M-current activation is then manifest by the appearance of a component of outward rectification positive to about −70 mV: below this potential, the curve is linear, representing current-flow through a voltage-insensitive 'leak' conductance (or conductances).

The speed with which the M-current decays when a cell is hyperpolarized increases with increasing hyperpolarization. This is shown by the accelerating time-constants for the inward relaxations during step-hyperpolarizations of increasing amplitude in Figure 2. The time-constant is longest (about 120 ms) at −45 mV (see Adams *et al.*, 1982a) and then shortens e-fold for each 20 mV hyperpolarization, in agreement with previous descriptions in this tissue (Constanti & Brown, 1981; Brown, 1988). This is to be expected for an ionic current gated by voltage, but contrasts sharply with the deactivation kinetics of the sustained Ca-dependent K-current described by North & Tokimasa (1987) in myenteric neurones: here, the current decay during a hyperpolarizing step is an order of magnitude slower than the decay of M-current and does *not* accelerate with increasing hyperpolarization, implying that it is governed by some other rate-limiting process than voltage-gating of channels, such as the decay of intracellular Ca.

Muscarine reduced the amplitude of these deactivation relaxations without changing their time-course (see Figures 7a, 10 and 11) (cf. Adams *et al.*, 1982b). Hence, muscarine appears to inactivate a proportion of available channels without changing their kinetics. This is contrary to expectations were these deactivation relaxations to reflect decay of Ca-gating, since in myenteric neurones muscarinic agonists accelerate the decay of the Ca-dependent K-

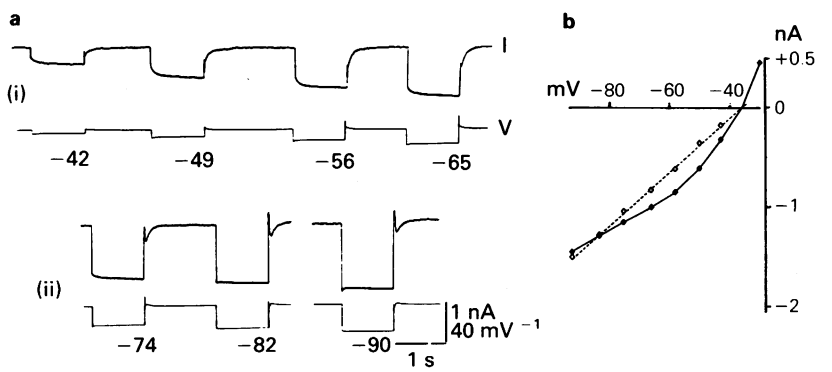


Figure 1 M-current deactivation: (a) (records (i) and (ii) continuous): membrane currents (upper records, I) induced by 1 s hyperpolarizing voltage commands (lower records, V) from a constant holding potential of −36 mV to the potentials indicated. Inward current downwards. Slow current relaxations after the initial current step during the hyperpolarization show the slow decay ('deactivation') of that component of M-current which had been activated at −36 mV (see text). This deactivation relaxation reverses in direction at command potentials negative to −80 mV. (b) Membrane current changes in (a), measured as deflections from the holding current (ordinate), plotted against command potential (abscissae): (◇- - - -◇) 'instantaneous' current at the beginning of the voltage step; (◆———◆) 'steady-state' current at the end of the 1 s voltage step. (An additional steady-state response to a small depolarizing step to −30 mV is also plotted.) The two curves intersect at the reversal potential (−84 mV) for the M-current deactivation relaxation in (a).

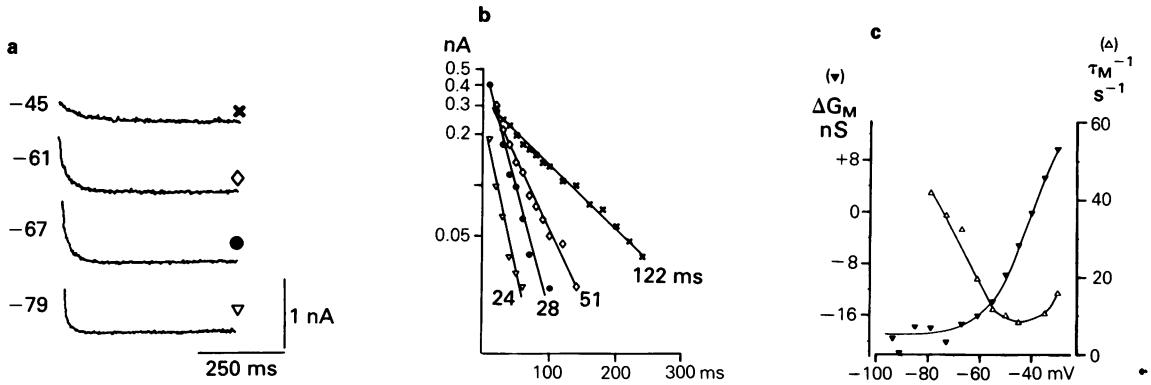


Figure 2 Kinetics of M-current deactivation. Records in (a) show M-current decay tails (after subtracting instantaneous currents, see Figure 1) during hyperpolarizing steps to the potentials shown from a holding potential of −40 mV. (b) Time-course of M-current decay tails. Ordinate scale: amplitude of current tail (nA, logarithmic scale); abscissa scale, elapsed time (ms) from the beginning of the hyperpolarizing step. Numbers on graph give calculated time-constant in ms. (c) reciprocal time-constant of M-current decay (△, $\tau_M^{-1} s^{-1}$) and change in peak M-conductance during the voltage command (▼, ΔG_M nS) plotted against command potential (abscissa scale). ΔG_M was measured by dividing the initial amplitude of the M-current tail (at $t = 0$ in b) by the difference between the command potential V_c and the reversal potential of the M-current tail (−85 mV). Curves drawn by eye.

current (North & Tokimasa, 1983; Galligan *et al.*, 1989).

These tests provide evidence that the membrane current studied in the present experiments corresponds to that current designated the 'M-current' in frog ganglia and to that we have previously described in rat ganglion cells, and that it clearly differs in its kinetic behaviour from the tonic Ca-activated K-current recorded in myenteric neurones (North & Tokimasa, 1987).

Ca-dependence? The M-current as originally described in frog sympathetic neurones was not classified as a 'Ca-activated' K-current in the sense of being dependent upon the entry of Ca into the cells for its generation, since it was not reduced by removing extracellular Ca or by adding divalent cations to block Ca entry through voltage-gated Ca-channels (Adams *et al.*, 1982a,b). Likewise, in rat ganglion cells, both the M-current and the inhibitory effect of muscarine persist when Ca entry is sufficiently blocked by Cd or noradrenaline to inhibit Ca-activated after-currents (Brown & Selyanko, 1985; Brown, 1986; 1988). However, Belluzzi *et al.* (1985b) have reported that muscarine can inhibit a large, Ca-dependent K-current activated at potentials positive to −30 mV in rat sympathetic neurones (probably by inhibiting the priming Ca-current: see also Wanke *et al.*, 1987; Marrion *et al.*, 1987); and it can also partly reduce the smaller, slower Ca-dependent after-current in these cells, perhaps through the same mechanism (Brown, 1988). Hence, we have rechecked the 'Ca-dependence' of the M-like currents recorded from rat sympathetic neurones, and of the effect of muscarine thereon, to test whether they may be attributable to, or contaminated by, Ca-activated currents.

As shown in Figure 3, removal of Ca from the bathing solution (with the addition of twice the concentration of Mg, to limit changes in surface potential: see Frankenhaeuser & Hodgkin, 1957) effectively inhibited the inward Ca current produced by brief depolarizing pulse (trace b), but did not reduce the slow current-relaxations accompanying applied voltage steps which we have attributed above to the deactivation and reactivation of the M-current (trace a); nor did it reduce the outward rectification of the current voltage curve associated with the development of the M-current (Figure 3, graph c(i)). Further, when applied in the absence of Ca, muscarine suppressed the M-current relaxations and induced a (reversible) inward current at the holding potential (trace a(ii)) in the manner reported previously (Constanti & Brown, 1981; Brown & Selyanko, 1985) and subsequently in this paper. The principal effect of removing Ca was to increase the 'leak' current, which probably accounts for the slowly-developing, small inward shift in the holding current. These results were confirmed in 5 other cells, in none of which did Ca-free solution inhibit the M-current even though it effectively inhibited the Ca-current and the slow Ca-activated after-current. Further, and in contrast to myenteric AH-neurones (Grafe *et al.*, 1980; North & Tokimasa, 1987), removal of Ca did not induce any consistent inward current at holding potentials between −60 and −30 mV, at temperatures from 22°C to 35°C.

These results agree with the negative effect of

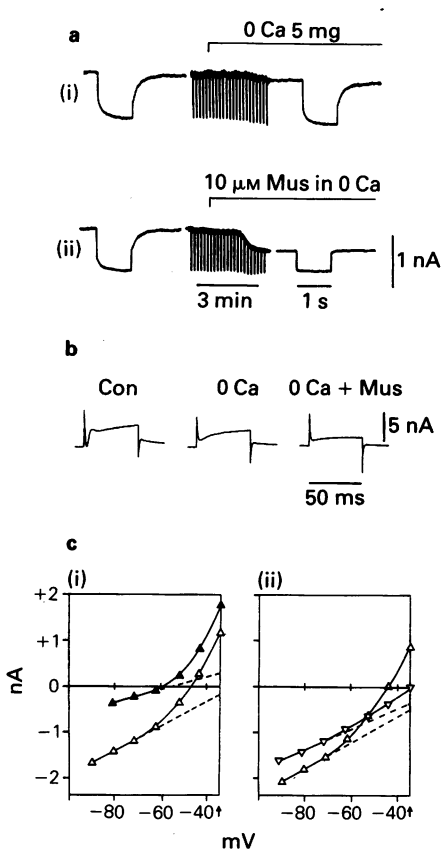


Figure 3 Removing extracellular Ca^{2+} does not change M-current or prevent its inhibition by muscarine. (a) Effects of (i) replacing normal Krebs solution (containing 2.5 mM Ca^{2+} and 1.2 mM Mg^{2+}) with a Ca^{2+}-free solution containing 5 mM Mg^{2+} and (ii) subsequent addition of 10 μM muscarine (Mus) to the 0 Ca/5 Mg solution on membrane current responses (upper records) to 1 s hyperpolarizing steps to −52 mV from a holding potential of −34 mV. Tetrodotoxin (1 μM) present throughout. During addition of 0 Ca/5 Mg solution or muscarine, the voltage step were applied each 10 s and responses recorded at a slower speed. (b) Current responses in the same cell to 50 ms, +40 mV depolarizing steps from −34 mV recorded (i) in normal Krebs solution, (ii) in 0 Ca/5 Mg solution and (iii) after subsequent addition of muscarine (Mus). Control response shows an inward Ca^{2+}-current which is inhibited in 0 Ca/5 Mg solution. (c) 'Steady-state' current-voltage curves (plotted as in Figure 1b) determined from varying-amplitude hyperpolarizing voltage steps (i) before (▲) and after (△) switching to the 0 Ca/5 Mg solution and (ii) still in 0 Ca/5 Mg solution) before (△) and after (▽) adding 10 μM muscarine. Currents are plotted with reference to the zero current level at the initial resting potential (−59 mV). The arrow ↑ shows holding potential (initial holding current, +1.78 nA). Subtraction of the extrapolated leak current (dashed line) from total current provided the following measures of voltage-dependent M-conductance (G_M) at the holding potential (reversal potential −80 mV); control, 16.4 nS; 0 Ca/5 Mg, 14.2 nS; muscarine in 0 Ca/5 Mg, 3.8 nS. Leak conductances G_L (measured from the slope of the current-voltage line negative to −60 mV) were: control, 7.0 nS; 0 Ca/5 Mg, 13.3 nS in *a*, 14.0 nS in *b*; muscarine in 0 Ca/5 Mg, 10.5 nS. Thus 0 Ca/5 Mg did not materially affect G_M but doubled G_L. In contrast muscarine reduced G_M by ~75% and also reduced G_L by ~25%. (This leak conductance is a mixed conductance, not solely a K^+ conductance, so its reduction by muscarine produces an outward current whose effect subtracts from that resulting from I_M-inhibition: see Brown & Selyanko, 1985. This effect on leak conductance was variable and not apparent in all cells.)

added Cd ions on both total resting current and M-current reported previously (Brown & Selyanko, 1985; Brown, 1988). They imply that neither the M-current nor any other component of resting membrane current between −60 and −30 mV recorded from adult rat s.c.g. cells under these experimental conditions was directly activated by an influx of Ca ions. However, they do not exclude possible contributions by Ca-activated K-currents maintained by resting levels of intracellular Ca to the recorded membrane currents. To test this, we assessed the effects of drugs which directly block known Ca-activated K-currents in these cells. As in frog ganglion cells, two such currents appear to co-exist in rat s.c.g. neurones, which have been termed I_C and I_{AHP} (see Pennefather *et al.*, 1985). I_C deactivates rapidly (in a few ms) following its activation by a priming Ca-pulse, is carried by high-conductance channels (200 pS in symmetrical K solution) and is blocked by a low concentration (1 mM) of tetraethylammonium (Smart, 1987; cf. Adams *et al.*, 1982b). I_{AHP} is deactivated over several hundred ms after a Ca pulse, is insensitive to tetraethylammonium up to 5 mM and instead is blocked by apamin or (+)-tubocurarine (Kawai & Watanabe, 1986; Gurney *et al.*, 1987; Marrion *et al.*, 1987; Brown, 1988; see also Nohmi & Kuba (1984), Pennefather *et al.* (1985) and Goh & Pennefather (1987) for comparable information on frog ganglion cells). Accordingly, we tested the effects of (+)-tubocurarine (Tc) and tetraethylammonium (TEA) on recorded membrane currents.

Figure 4 shows the effect of incremental concentrations of muscarine and Tc on the M-current (upper traces) and on the slowly-deactivating Ca-dependent K-current I_{AHP} (lower traces). Tc progressively reduced the magnitude of I_{AHP} but had no clear effect on either the amplitude of the M-current tails or total resting current or conductance, even when the AHP-current was virtually eliminated. This was confirmed in 16 out of 18 other adult s.c.g. cells using holding potentials between −50 and −30 mV. (In 2 cells, 100 μM Tc produced a small inward

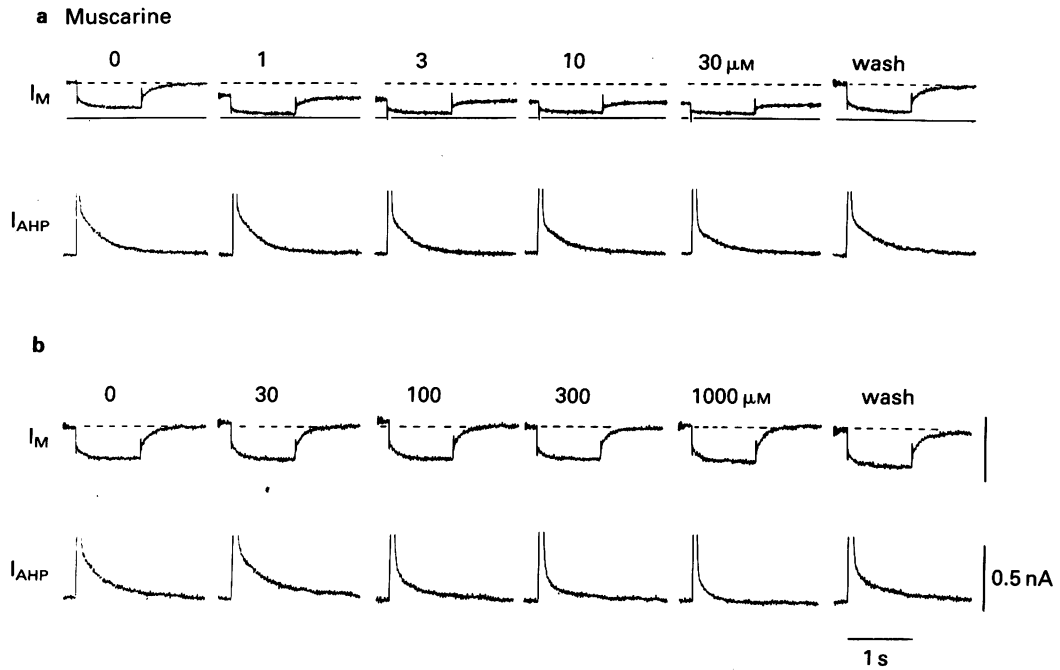


Figure 4 Comparative effects of (a) muscarine and (b) (+)-tubocurarine (Tc) on the deactivation of I_M (upper records) and the activation of the Ca-dependent K-current I_{AHP} (lower trace) in the same neurone. The cell was held at −43 mV and subjected to 1 s, −20 mV hyperpolarizing steps to deactivate I_M or 50 ms, +40 mV steps to activate I_{AHP}. (The latter is revealed as a slowly-declining outward tail-current after the command: currents during the command are off-scale.) Drugs were applied cumulatively over 15–20 min to give the concentrations indicated, then washed out for 15 min ('wash'). The horizontal line below the upper record of (a) shows the zero-current reading (initial resting potential −51 mV); dashed lines = initial holding current. Note that muscarine produced an incrementing inward current and reduced I_M deactivation/reactivation relaxations at concentrations less than those which inhibited I_{AHP}, whereas Tc reduced I_{AHP} without producing an inward current or inhibiting I_M deactivation/reactivation relaxations. Tetrodotoxin (1 μM) present throughout.

current without change in amplitude of the M-current relaxations.) In contrast, muscarine produced a clear inward current and a substantial inhibition of the M-current tails at a concentration which did not materially reduce the Ca-activated AHP-current. Some inhibition of I_{AHP} was observed at higher concentrations of muscarine, as previously reported in both frog (Tokimasa, 1984; Pennefather *et al.*, 1985) and rat (Brown, 1988) ganglia, but this was less complete and, in different experiments, less consistent than that produced by Tc. (It may have been secondary to inhibition of the priming Ca-current used to generate I_{AHP}: see Wanke *et al.*, 1987; Marrion *et al.*, 1987). Further, muscarinic agonists were fully capable of inhibiting I_M after prior blockade of I_{AHP} with Tc (see Figure 6 below).

TEA did not inhibit I_{AHP} but instead reduced a component of outward current which activated rapidly following an inward Ca-current and which deactivated within a few ms at the end of the depolarizing step (Figure 5). This current was also inhibited in Cd solution (figure 5b) and hence represents I_C, the TEA-sensitive component of Ca-activated K-current. Its rather 'notchy' appearance at command potentials around −10 mV accords with some earlier observations in frog ganglia (MacDermott & Weight, 1982; Brown *et al.*, 1983). A concentration of TEA (1 mM) that blocked this current also produced a small inward current (Figure 6), but this was probably due to a secondary and independent blocking action of TEA on I_M (rather than indicating a contribution of I_C to resting current) since it also occurred in Ca-free or Cd solution. Inspection of M-current deactivation tails in the presence and absence of TEA did not reveal any component of the relaxation which might have been due to a contribution by I_C: the time-constant for I_C channel closure is only about 1 ms at the relevant potentials (Smart, 1987) so would be unlikely to affect the M-current tail. In contrast to TEA, muscarinic agonists did not consistently reduce I_C in these experiments (Figure 5a; cf. Belluzzi *et al.*, 1985b) and does not inhibit activation

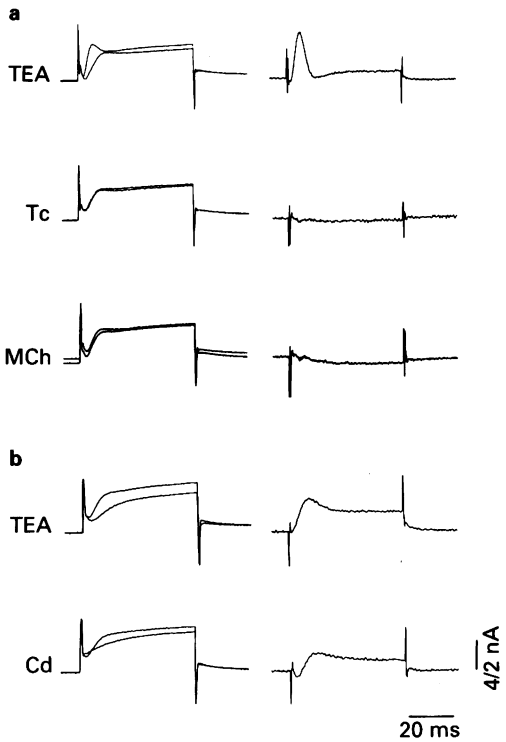


Figure 5 Effects of 1 mM tetraethylammonium (TEA), 0.5 mM (+)-tubocurarine (Tc), 3 mM methacholine (MCh) and 0.2 mM $CdCl_2$ (Cd) on the rapid Ca-activated K-current I_C evoked by 50 ms, +40 mV commands from −40 mV: (a) and (b) show records from two separate cells (a refers to the same cell as that illustrated in Figure 6). Records on the left show superimposed currents (average of 3 pulses) recorded before and after drug application at 4 nA calibration; records on the right show subtracted currents (i.e. drug-sensitive component) at 2 nA calibration. Note that TEA reduced a transient component of outward current which was also inhibited by Cd (which reduced the initial inward Ca-current recorded in 1 μM tetrodotoxin). This current was not inhibited by MCh or Tc.

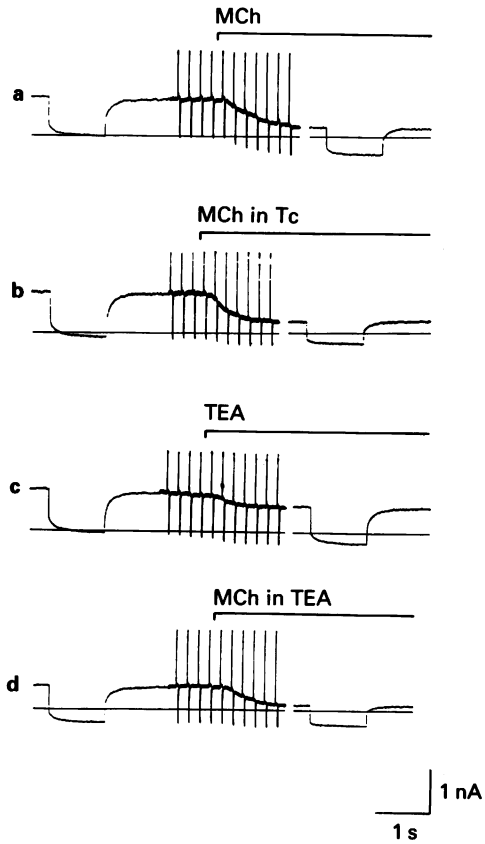


Figure 6 Block of Ca-activated K-channels by (+)-tubocurarine and tetraethylammonium does not prevent M-current inhibition by methacholine. Records show responses to 3 mM methacholine (MCh) recorded (a) before and (b) and (d) after adding 500 μM (+)-tubocurarine (Tc) or 1 mM tetraethylammonium (TEA) respectively to the bathing fluid. Tc itself did not affect the resting current or M-current relaxations (see Figure 4). Addition of TEA produced a small inward current and partial inhibition of I_M as shown in record (c). The cell was held at −40 mV throughout. Initial resting potential −57 mV. Horizontal line shows the zero current level. Downward current deflections are responses to 1 s, −20 mV hyperpolarizing commands. Upward deflections are outward currents (off-scale) evoked by the +40 mV, 50 ms commands shown in Figure 5. Tetrodotoxin (1 μM) present.

of single I_C-channels (Smart, 1987). The residual M-current in TEA solution remained fully sensitive to muscarinic agonists (Figure 6).

These tests suggest that tonic activation of neither of the two known Ca-activated K-currents by resting levels of intracellular Ca contribute significantly to the M-current or other resting currents recorded in the present experiments. They do not, of course, exclude the possibility that I_M might itself be a separate species of Ca-activated K-current maintained in the activated state by resting intracellular Ca concentrations. However, this is rendered most unlikely by recent observations in internally-dialyzed frog ganglion cells (Pfaffinger *et al.*, 1988) that I_M was maintained when intracellular Ca was buffered to less than 10 nM with BAPTA. (Contrarily, there is some evidence that I_M in frog ganglia might be inhibited rather than activated by raising intracellular Ca: Tokimasa, 1985; Pfaffinger *et al.*, 1988; N.V. Marrion, S.J. Marsh & P.R. Adams, unpublished). Rat s.c.g. neurones are probably no different in this respect since M-currents were well-preserved, with

normal sensitivity to muscarine, in rat s.c.g. cells impaled with microelectrodes filled with 1.5 M K citrate with 200 mM added EGTA (Marrion, 1988).

Agonist-induced suppression of I_M

The method used to measure the inhibition of I_M by muscarinic agonists in the remaining experiments described in this paper was essentially that illustrated in Figure 3. The cell was held at a depolarized potential to activate I_M and subjected to repeated hyperpolarizing steps to deactivate it. The amplitude of the slow inward current relaxation tail induced by decay of the M-current was used as the measure of I_M for calculation purposes, as shown in Figure 7a. This procedure has two advantages over the converse procedure of measuring the amount of M-current activated by depolarizing steps: outward current accompanying activation is frequently contaminated by superimposed transient K currents (as in Figure 1) or spike currents; and inhibition of a previously-activated M-current generates a large inward holding current which provides a useful index of the time-course of drug action, to ensure that measurements are made at steady-state.

Figure 7b shows the concentration-response relationship for the suppression of the M-current by muscarine in impaled intact and cultured rat s.c.g. neurones. In all of these neurones the response to muscarine followed a similar concentration-dependence, with a threshold near to 100 nM and a maximum close to 20 μM. This result suggests that the age of the neurones and the techniques used for dissociating and maintaining them in culture has little, if any, effect on muscarinic suppression of the M-current.

Figure 8 shows concentration-response relationships for 3 different agonists on foetal s.c.g. neurones.

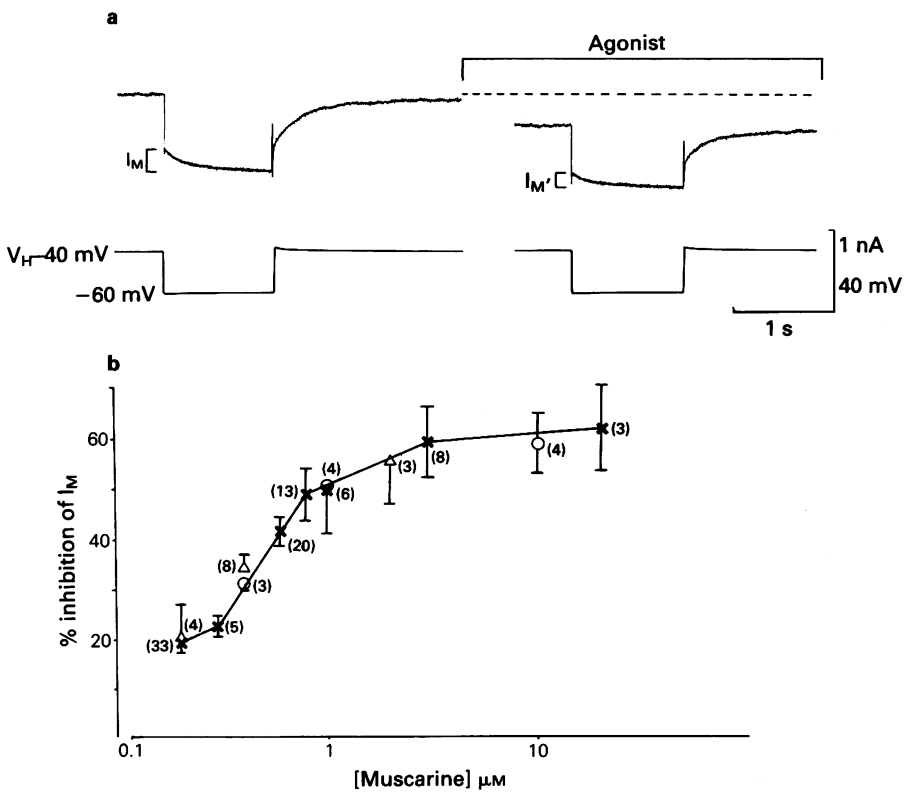


Figure 7 (a) Method for measuring I_M-inhibition. Records show inward currents (upper traces) produced by 1 s, 20 mV hyperpolarizing steps (lower trace) from a holding potential of −40 mV. The slow component of the inward current reflects the deactivation of I_M. Addition of agonist produced an inward current at the holding potential and reduced the amplitude of the slow component of inward current to $I_{M'}$. (b) Shows % inhibition of I_M (= $[(I_M - I_{M'})/I_M] \times 100\%$; ordinate scale) plotted against concentrations of muscarine (μM; abscissa scale). Points marked (X) refer to intact adult rat s.c.g. cells; (○) and (△) refer to dissociated adult and foetal cells respectively. Each point gives the mean; bars = s.e.mean (n in parentheses = number of cells tested).

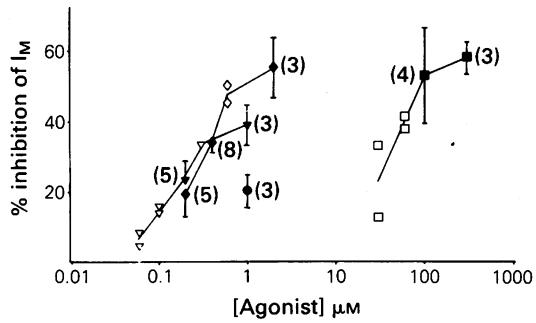


Figure 8 Effect of some agonists on I_M in dissociated foetal s.c.g. neurones. Ordinate scale: % inhibition of I_M (see Figure 7). Abscissa scale: agonist concentration (μM). Agonists: (▽) oxotremorine; (◇) muscarine; (●) McN-A-343; (□) methacholine. Closed symbols: means (bars show s.e.mean, *n* in parentheses); open symbols: single tests.

The apparent rank order of potency was: oxotremorine ⩾ muscarine > McN-A-343 > methacholine. The threshold concentrations for oxotremorine and muscarine were similar (~0.1 μM) but muscarine produced a larger maximal depression, suggesting that oxotremorine may behave as a 'partial agonist' by reference to muscarine (see also Brown *et al.*, 1980a). In contrast, methacholine was about 200 times less potent than muscarine but produced a comparable maximal (~60%) inhibition, implying that it was a weak 'full' agonist.

Antagonism of I_M-suppression

Pirenzepine Initial experiments on foetal s.c.g. neurones revealed that inhibition of I_M by muscarinic agonists was readily antagonized by low concentrations (< 100 nM) of pirenzepine (Figure 9). Measurements of the K_B for pirenzepine were obtained from experiments on intact ganglia since these allowed long stable impalements over the several hours necessary (see Methods). Cumulative addition of muscarine (0.3 and 3 μM) produced reversible inward currents concomitant with a reduction in the amplitude of the I_M deactivation and reactivation relaxations (Figure 10). These effects were progressively reduced, and required higher concentrations of muscarine for their appearance, after applying increasing concentrations of pirenzepine for 30 min over the range 60 nM to 1 μM. Figure 10b shows the % inhibition of I_M by muscarine before and after addition of pirenzepine. The suppression was shifted rightward in an approximately parallel manner, compatible with competitive antagonism. Schild plots (see Methods) were not significantly different from unity-slope. Constrained unity-slope plots for 3 experiments (Figure 12) yielded pK_B values of 7.37,

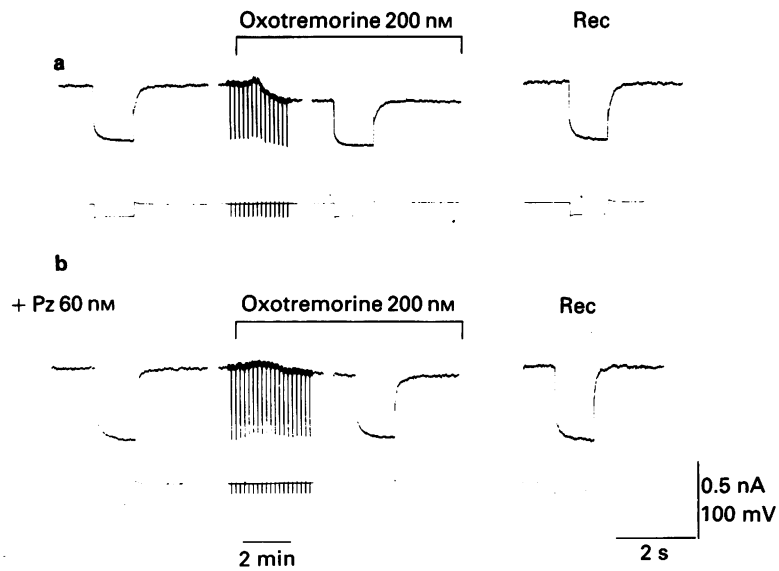


Figure 9 Effects of oxotremorine (200 nM) on a dissociated foetal s.c.g. cell recorded (a) before and (b) 30 min after adding 60 nM pirenzepine (Pz) to the bathing fluid. Upper trace, current; lower trace, voltage. The cell was clamped at −35 mV and commanded to −55 mV for 1 s at 10 s intervals. The recorder was slowed prior to adding oxotremorine. Addition of oxotremorine (bars) produced an initial, brief outward current followed by an inward current during which I_M was reduced (see Figure 1). Currents recovered after 10 min washing (record marked 'Rec'). The effect of oxotremorine was reduced in pirenzepine solution.

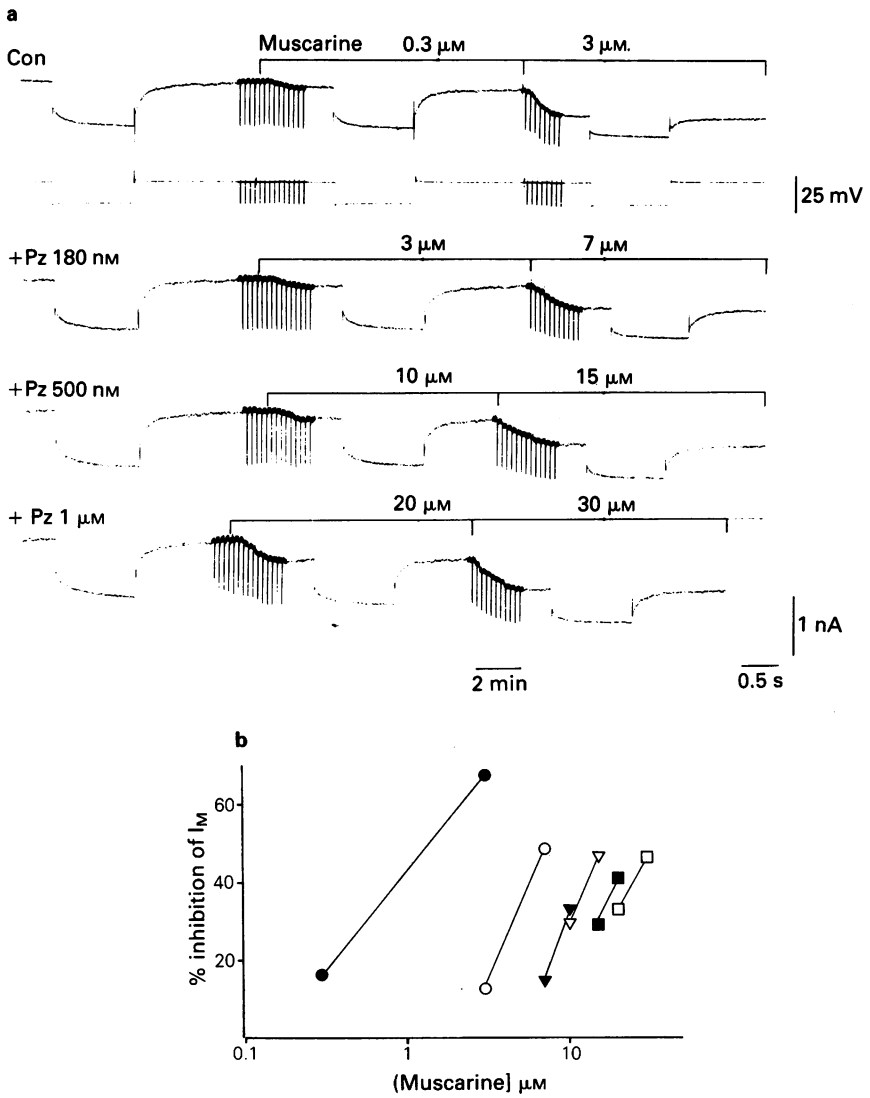


Figure 10 Antagonism of muscarine-induced I_M-inhibition by pirenzepine. Records in (a) show effects of cumulative additions of muscarine on clamp-currents recorded from an *in situ* adult rat s.c.g. cell in normal Krebs solution (Con) and in sequentially-increasing concentrations of pirenzepine (Pz). (Note that the concentrations of muscarine were increased in pirenzepine.) The cell was clamped at −27 mV and commanded to −47 mV for 1 s at 10 s intervals. Records show membrane current (voltage omitted for clarity). (b) Relation between % inhibition of I_M (ordinates, see Methods and Figure 7 for calculation) and concentration of muscarine, measured in the absence (●) and presence of 180 nM (○), 300 nM (▼), 500 nM (▽), 800 nM (■) and 1000 nM (□) pirenzepine. (Data from cell illustrated in a).

7.72 and 7.50, giving a mean of 7.53 ± 0.13 ($n = 3$) (geometric mean $K_B = 29.5$ nM); the arithmetic mean of the individual K_B values was 31.1 ± 6.8 nM.

AF-DX 116 Affinity determination for the cardioselective muscarinic receptor antagonist AF-DX 116 was undertaken; the same protocol was used as for pirenzepine. Cumulative addition of muscarine (0.2 and 0.6 μM) evoked reversible, concentration-dependent inward currents and reductions in the amplitude of I_M. AF-DX 116 (1–4 μM) antagonized the response to muscarine in a concentration-

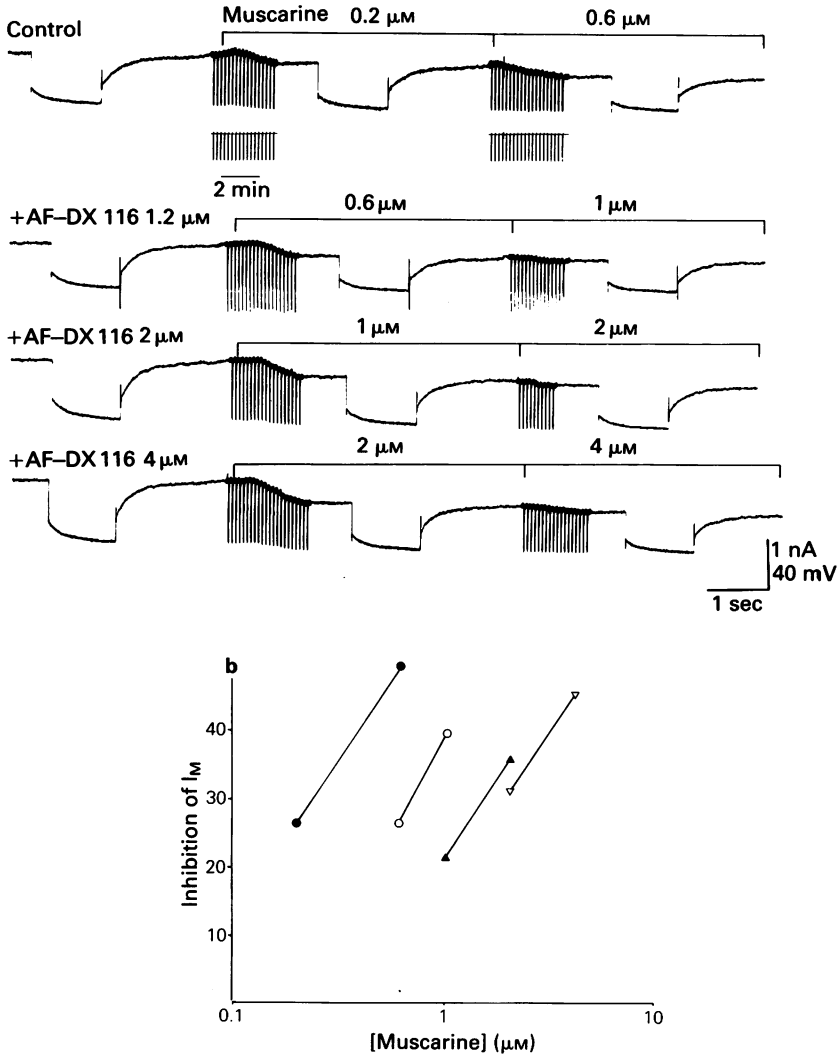


Figure 11 Antagonism of muscarine-induced inhibition of I_M by AF-DX 116. (a) Current records as in Figure 10a. Holding potential −40 mV. (b) Dose-response relation for I_M inhibition by muscarine measured in 0 (●), 1.2 (○), 2 (▲) and 4 (▽) μM AF-DX 116.

dependent manner (Figure 11). The parallel rightward displacement of the concentration-response relationship for muscarine by AF-DX 116 suggested competitive antagonism. Schild plots of four individual experiments (Figure 12) yielded pK_B values of 6.24, 5.86, 6.17, 5.81 (slopes constrained to unity). The calculated mean pK_B value was 6.02 ± 0.13 ($n = 4$, geometric mean K_B 955 nM); arithmetic mean K_B was 1.05 ± 0.25 μM.

Ileum The two antagonists studied above (pirenzepine and AF-DX 116) were also evaluated for their potency in antagonizing muscarine-induced contractile responses in the guinea-pig ileum. Both antagonists yielded parallel rightward shifts of the muscarine concentration-response relationship, providing pK_B values for pirenzepine and AF-DX 116 of 6.37 ± 0.03 ($n = 8$; K_B 0.43 μM) and 5.98 ± 0.03 ($n = 8$; K_B 1.05 μM) respectively.

Pooled data Figure 13 shows Schild plots and pK_B for pooled data on both the s.c.g. and ileum and derived pA_2 values with 95% confidence limits are given in Table 1. The mean pA_2 values for pirenze-

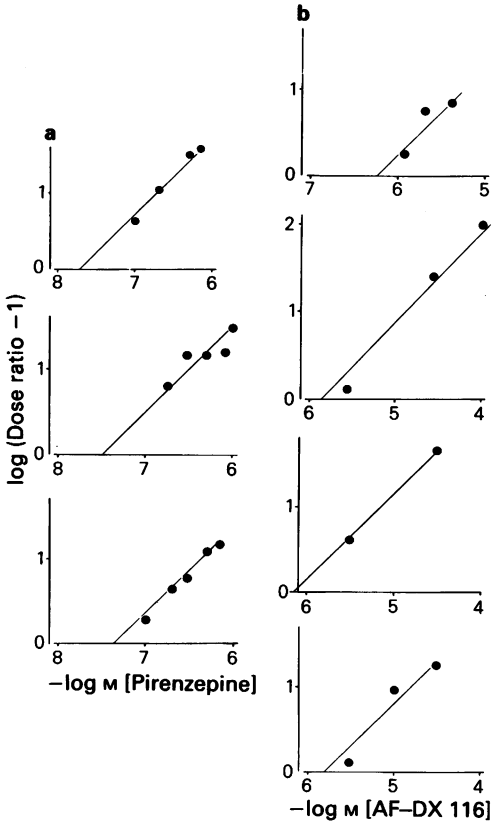


Figure 12 Schild plots of log (dose ratio − 1) against − (log molar) antagonist concentration for antagonism of muscarine-induced inhibition of M-current (see Figures 10 and 11) by (a) pirenzepine (3 experiments) and (b) AF-DX 116 (4 experiments). Regression lines are least-squares fits to data points with slopes constrained to unity (see Methods).

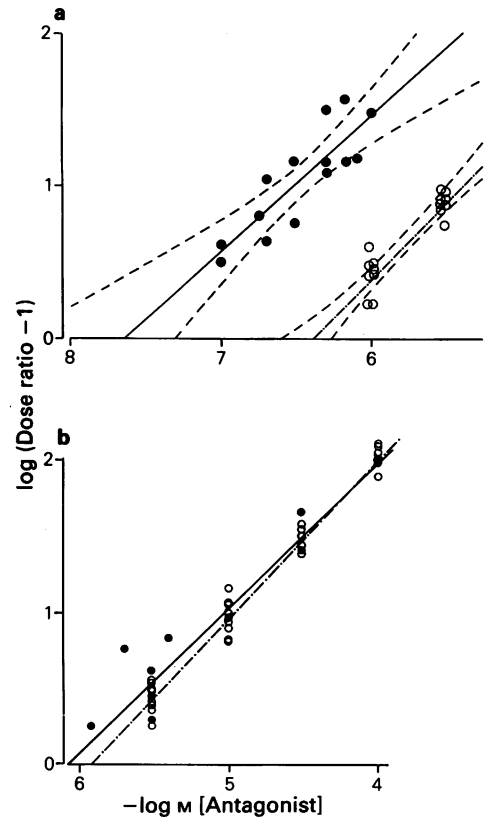


Figure 13 Schild plots for antagonism of muscarine-induced inhibition of I_M in the rat s.c.g. (●) and contraction of guinea-pig ileum (○) by (a) pirenzepine and (b) AF-DX 116, using pooled data for dose-ratios. Lines are least squares regression lines for unweighted data (without slope constraint), with 95% confidence limits of the calculated regressions, determined as in Colquhoun (1971). Calculated data from the regressions are given in Table 1.

pine on the s.c.g. and ileum were significantly different from each other but those for AF-DX 116 were not.

Gallamine This behaves as an allosteric inhibitor at cardiac M_2 receptors (Clark & Mitchelson, 1976; Stockton *et al.*, 1983). Constanti & Sim (1987) and Dutar & Nicoll (1988) have reported that muscarinic inhibition of I_M in guinea-pig olfactory cortical neurones and rat hippocampal pyramidal neurones respectively are antagonized by 20–30 μM gallamine. In contrast, 20 to 30 μM gallamine produced no obvious antagonism to muscarinic inhibition of I_M in either foetal (Figure 14) or adult ($n = 5$) rat s.c.g. neurones.

4-DAMP It had previously been reported that this compound (see Barlow *et al.*, 1976) inhibited muscarine-induced depolarization of the intact rat s.c.g. with a high affinity (pA_2 8.61; Brown *et al.*, 1980b). In accordance with this, incubation of voltage-clamped foetal s.c.g. neurones with 300 nM 4-DAMP reversibly abolished the suppression of I_M by 0.4 μM muscarine.

Discussion

The present observations clearly point to an M_1 subtype of muscarinic receptor as the receptor responsible for M-current inhibition in rat sympathetic neurones. The K_B for pirenzepine (mean, 31 nM) accords reasonably well with the K_I values for competition with N-methylscopolamine binding to

Table 1 Constants for pirenzepine and AF-DX 116 antagonism of muscarine-induced I_M-inhibition in rat superior cervical ganglion cells (scg) and contraction of guinea-pig isolated ileum (ileum)

	Pirenzepine		*AF-DX 116*	
	scg	ileum	scg	ileum
No. of tissues	3	8	4	10
No. of determinations	14	16	10	30
Unconstrained slope				
Slope	0.885	1.018	0.960	1.050
Upper C.L.	1.217	1.229	1.300	1.113
Lower C.L.	0.552	0.808	0.620	0.986
pA_2	7.65	6.39	6.08	5.93
Upper C.L.	8.09	6.59	6.69	5.74
Lower C.L.	7.21	6.20	5.46	6.11
Constrained slope				
pK_B	7.51	6.40	6.04	5.99
Upper C.L.	7.62	6.45	6.22	6.02
Lower C.L.	7.41	6.35	5.85	5.95

Values are derived from the Schild plots of pooled data shown in Figure 13. Data were subjected to linear regression analysis according to Colquhoun (1971) and after constraining the slope to unity by the method of Tallarida *et al.* (1979). 95% confidence limits (C.L.) for derived pA_2 values were checked using equation 6.22 of Kenakin (1987) and equation (18) of Lee & Lee (1982). Computations were performed by methods described by Lee & Lee (1982) to improve accuracy.

calf (Hammer & Giachetti, 1982) and human (Watson *et al.*, 1984) ganglion homogenates (23 and 14 nM respectively) and for the high-affinity site in other neural tissue ($K_I = 25$ nM: Hammer *et al.*, 1980). This was significantly lower than the K_B (427 nM) for inhibition of ileal contractions, which presumably measures M_2 receptors (see Eglen & Whiting, 1987). A primary role for M_1 receptors in inhibiting I_M is also supported by the high K_B for AF-DX 116 on the ganglion (1.05 μM), which is much higher than the K_I for cardiac M_2 receptors (115 nM) but accords better with that for neuronal M_1 receptors (760 nM) as determined from radiolabelled ligand-binding competition (Hammer *et al.*, 1986).

The dissociation constants for pirenzepine determined in the present study against M-current inhibition and ileal contraction are both somewhat higher than those (4.4 and 102 nM) previously measured against rat ganglion depolarization and ileal contraction (Brown *et al.*, 1980b). We can offer no specific explanation for this difference. However, the differences between the K_B values on ganglion and ileum in the two sets of experiments are quite similar, suggesting some consistent variation in technique or conditions.

In agreement with the present data, pirenzepine (at 200 nM) antagonized the inhibition of I_M in frog sympathetic neurones by muscarine (Brown, 1988). In contrast, M-current inhibition in toad gastric smooth muscle and guinea-pig olfactory cortex neurones was reported to be resistant to antagonism by pirenzepine at concentrations up to 0.3–1 μM (Sims *et al.*, 1986; Constanti & Sim, 1987). While it is difficult to make comparisons in the absence of K_B measurements on these latter tissues, our results suggest that some antagonism at least should have been detectable at the concentrations used were the receptors equally sensitive to pirenzepine as those in the rat ganglion. The contrast with the olfactory cortex receptor is emphasized by the fact that the action of muscarine on the latter was inhibited by 20–30 μM gallamine (Constanti & Sim, 1987), and a similar effect of gallamine on hippocampal pyramidal cells has recently been reported (Dutar & Nicoll, 1988); whereas we could detect no antagonism of the action of muscarine or oxotremorine on the M-current in rat ganglion cells with 30 μM gallamine. Since gallamine acts allosterically rather than competitively (Clark & Mitchelson, 1976; Stockton *et al.*, 1983) this difference may, of itself, be insufficient to characterize the receptors fully. Nevertheless, the differential effects of gallamine and pirenzepine do suggest a clear pharmacological difference in the muscarinic receptors responsible for M-current inhibition in central and peripheral neurones.

Recent experiments with cDNA and genomic DNA transfection methods (Fukuda *et al.*, 1988) have established that activation of the genetic m_1-subtype of the pig brain muscarinic receptor (which corresponds most closely to the pharmacological M_1-subtype: Fukuda *et al.*, 1987) can inhibit the M-like K-current in recipient NG108-15 neuroblastoma/glioma hybrid cells, whereas activation of transfected pig heart m_2 (Kubo *et al.*, 1986) and rat brain m_4 receptors (Bonner *et al.*, 1987) was ineffective. However, these experiments further revealed that activation of transfected brain m_3 receptors (Bonner *et al.*, 1987) could also inhibit the NG108-15 cell M-like current. These receptors show an intermediate affinity for pirenzepine (IC_{50} 156 nM for competition against ^{3}H-QNB binding, versus 35 nM for rat brain m_1 receptors: Bonner *et al.*, 1987). The equivalent human HM4 receptor also shows a two fold lower affinity for pirenzepine than the human HM1 receptor, and a six fold lower affinity for AF-DX 116 (Peralta *et al.*, 1987). mRNA hybridization studies suggest that both m_1 and m_3 receptors are expressed in rat sympathetic neurones (N.J. Buckley, personal communication). However, it is not yet clear whether their expression or their

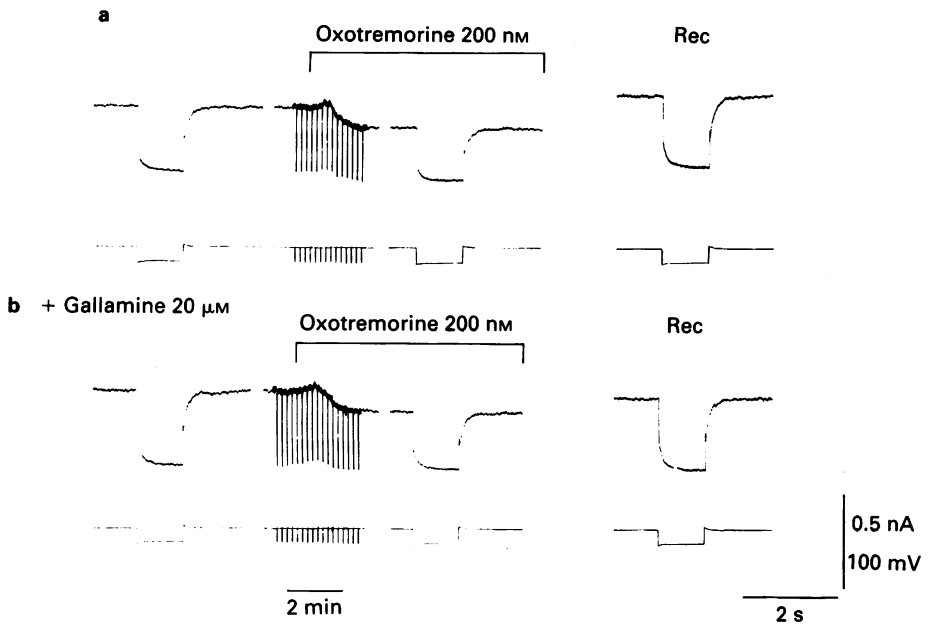


Figure 14 Effects of 200 nM oxotremorine on membrane current in a dissociated foetal s.c.g. cell recorded before and 30 min after adding 20 μM gallamine. (Compare with Figure 9, q.v. for details).

capacity to couple to M-currents are quantitatively equivalent; nor is it clear whether their dissociation constants for pirenzepine and AF-DX 116 are sufficiently different to be resolved by the experiments described in the present paper. All that we can conclude at this stage is that the inhibition of the M-current in the rat superior cervical ganglion is mediated by a single type of receptor (or two or more very similar types of receptor), which most closely resemble the pharmacological M_1-subtype as opposed to the M_2-subtype; and that this much at least is in accord with current knowledge regarding the activity of the genetic subtypes so far identified.

One final point worth noting is that the comparability of both agonist and antagonist action on cultured foetal cells on the one hand and cultured or intact adult cells on the other implies that there is little change in the nature or effectiveness of the muscarinic receptors mediating M-current inhibition during development of the s.c.g.

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Antagonism of platelet activating factor-induced chemiluminescence in guinea-pig peritoneal macrophages in differing states of activation

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1 The effects of the platelet activating factor (Paf) antagonists alprazolam, BN 52021, kadsurenone, L 652,731 and SRI 63119 have been studied on Paf-induced chemiluminescence (CL) of guinea-pig, *C. parvum*-activated peritoneal macrophages *in vitro*.

2 All antagonists produced a shift to the right in the dose-response curve to Paf (0.001–10 μmol l^{-1}). Schild plots for BN 52021, L 652,731, kadsurenone and SRI 63119 were linear, but only for BN 52021 and kadsurenone did the mean slope not differ significantly from unity. Mean pA_2 values for BN 52021 and kadsurenone were 6.60 ± 0.05 and 6.41 ± 0.14 (mean + s.e.mean) respectively. Calculation of IC_{50} values for all antagonists (at 0.1 μmol l^{-1} Paf) gave an order of potency: L 652731 > kadsurenone $\geqslant$ BN 52021 > alprazolam > SRI 63119.

3 When individual pA_2 values for BN 52021 and kadsurenone were plotted against the maximal CL response to Paf of cell suspensions in the absence of antagonist (reflecting the degree of activation of the macrophages by the *C. parvum*), it was found that the affinity of both antagonists for macrophage Paf receptors remained relatively constant irrespective of the activation state of the cells.

4 We conclude that activation of guinea-pig peritoneal macrophages does not account for the increased affinity for macrophage Paf receptors previously observed for kadsurenone. Kadsurenone and BN 52021 presumably bind to a site on Paf receptors which is not affected by the activation process, while alprazolam and SRI 63119 are non-specific antagonists. The reason for the difference between the competitive nature of kadsurenone and its structural analogue L 652,731 is unclear.

Introduction

Platelet activating factor (Paf) is formed on stimulation of a variety of inflammatory and immunocompetent cells and stimulates neutrophil, eosinophil, lymphocyte and macrophage functions (Braquet *et al.*, 1987). Among the responses of macrophages to stimulation by Paf are increased glucose consumption and the generation of reactive oxygen species (the 'oxidative burst'), increased interleukin 1 formation and release of arachidonic acid metabolites (Hartung *et al.*, 1983; Hartung, 1983; Hayashi *et al.*, 1985; Barrett *et al.*, 1987; Huang *et al.*, 1988; Stewart & Dusting, 1988). The ability of Paf to stimulate the macrophage oxidative burst is closely related to the presence of specific binding sites on the cell membrane, since rat and mouse peritoneal macrophages barely respond to Paf and cells from these species do not exhibit specific Paf-binding (Parnham & Leyck, 1983; Kudo *et al.*, 1986; Parnham, 1988). While resident, non-activated peritoneal macrophages from guinea-pigs also weakly respond with an oxidative burst to Paf and do not exhibit specific [^{3}H]-Paf binding sites, immunologically activated or elicited macrophages from this species are particularly sensitive with regard to both induction of the oxidative burst and specific binding (Hartung *et al.*, 1983; Kudo *et al.*, 1986).

Recently, we have shown that the affinity (pA_2) of the Paf receptor antagonist, kadsurenone, for receptors on guinea-pig *Corynebacterium parvum*-activated, peritoneal macrophages (determined by measuring the oxidative burst to Paf in terms of chemiluminescence) is approximately 100 fold greater than that for Paf receptors on pig peripheral blood leucocytes (Lambrecht & Parnham, 1986). This led us to propose the existence of a high affinity Paf receptor subtype, Paf_2, on guinea-pig activated

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macrophages, a proposal supported by recent studies by other authors (Stewart & Dusting, 1988). Subsequent to our original finding we have studied the affinities of a variety of Paf receptor antagonists for Paf_2 receptors on guinea-pig activated macrophages, using kadsurenone as a reference. During these experiments it became apparent that the previously published high pA_2 value for the action of kadsurenone on this cell type in many cases could not be achieved. Postulating that the high affinity of kadsurenone previously observed (Lambrecht & Parnham, 1986) was related to the high degree of activation of the macrophages by the heat-killed *C. parvum*, we compared the calculated affinities of the various Paf antagonists for macrophage Paf receptors with the activation state (determined as the maximal chemiluminescence responses of individual cell suspensions) of the cells. A preliminary account of some of these data has been published (Bittner *et al.*, 1988).

Methods

Male Pirbright white guinea-pigs (200–300 g, Süddeutsche Versuchstierfarm, W. Germany), kept under constant light and temperature with food and water *ad libitum*, each received an i.p. injection of 1.5 ml heat-killed *Corynebacterium parvum* (7 mg ml^{-1}, Deutsche Wellcome, Burgwedel) and were killed with CO_2 4, 8 or 12 days later. Three different time points were chosen for harvest of the cells to obtain a broad distribution of macrophage activation states in order to facilitate the determination of correlations with antagonist affinities. Harvesting of cells by peritoneal lavage, resuspension in buffer and stimulation with synthetic hexadecyl-*rac*-Paf (Nattermann, Cologne) in the presence of the chemiluminescence (CL)-enhancer, luminol, in a six-channel Berthold Biolumat LB 9505 were carried out as described previously (Lambrecht & Parnham, 1986). Dose-response curves to Paf in the presence and absence of increasing concentrations of individual receptor antagonists (alprazolam, 1–10 μmol l^{-1}, BN 52021, 0.32–10 μmol l^{-1}; L 652,731 0.1–10 μmol l^{-1}; kadsurenone, 1–10 μmol l^{-1}; SRI 63119, 0.32–10 μmol l^{-1}) were performed on the same cell suspension, each cell suspension being prepared from a single guinea-pig. Each antagonist concentration was tested against the complete Paf dose-response curve once for each cell suspension. Because of the limitation of the number of cells, only one antagonist was tested per cell suspension. Dose-ratios (DR) for antagonist-induced shifts in the dose-response curves to Paf were estimated, Schild plots constructed and pA_2 values calculated as described previously (Lambrecht & Parnham, 1986). In addition, because the Schild plots were not all linear, antagonist potencies were also compared by calculating for each antagonist concentration percentage inhibition of CL responses to 0.1 μmol l^{-1} Paf. This concentration was chosen as it was a sub-optimal concentration lying on the linear part of the dose-response curve to Paf. IC_{50} values were determined for each antagonist from regression lines fitted for log concentration of antagonist against percentage inhibition.

In a further series of experiments the maximal activation state (MAS) of cell suspensions from individual animals was determined as the absolute CL (c.p.m.) generated at the peak of the Paf dose-response curve in the absence of antagonist. The MAS for the cell suspension was plotted against the pA_2 for the antagonist determined on that cell suspension (at one antagonist concentration per suspension) and the regression analysis for all such plots obtained for each antagonist carried out using an IBM personal computer programme (EPISTAT).

Drugs

Antagonists used were kadsurenone and L 652,731 ((±)-2,5-*bis*(3,4,5-trimethoxyphenoxy)tetrahydrofuran)- (kindly provided by Dr T.Y. Shen, Merck, Sharpe & Dohme, Rahway, U.S.A.), BN 52021 (ginkgolide B) (kindly provided by Dr P. Braquet, IPSEN-Beaufort, Paris, France), SRI 63119 (**R,S**)-3-[4-[(3-octadecylaminocarbonyloxy-2-methoxy)-propoxy]butyl]thiazolium bromide (kindly provided by Dr C. Winslow, Sandoz, Hanover, U.S.A.) and alprazolam (Upjohn, W. Germany).

Results

Antagonism of Paf-induced chemiluminescence

All five Paf receptor antagonists tested produced a shift to the right in the dose-response curve to Paf on guinea-pig activated macrophages (Figures 1 and 2). At the lowest concentration, BN 52021 appeared to reduce the maximal response to Paf, while alprazolam failed to produce a parallel shift in the dose-response curve, rather exhibiting greater antagonism at lower Paf concentrations (Figure 2). The remaining antagonists all produced parallel shifts in the Paf dose-response curve.

Only kadsurenone and BN 52021 yielded Schild plots which did not differ significantly from unity (Figure 3; Table 1). For the remaining three antagonists, the lack of a slope of unity and for alprazolam, the lack of linearity of the Schild plot (data not shown), meant that the conditions for competitive antagonism were not fulfilled and pA_2 values could not be used. Comparative antagonist potency was therefore determined on the basis of IC_{50} values for

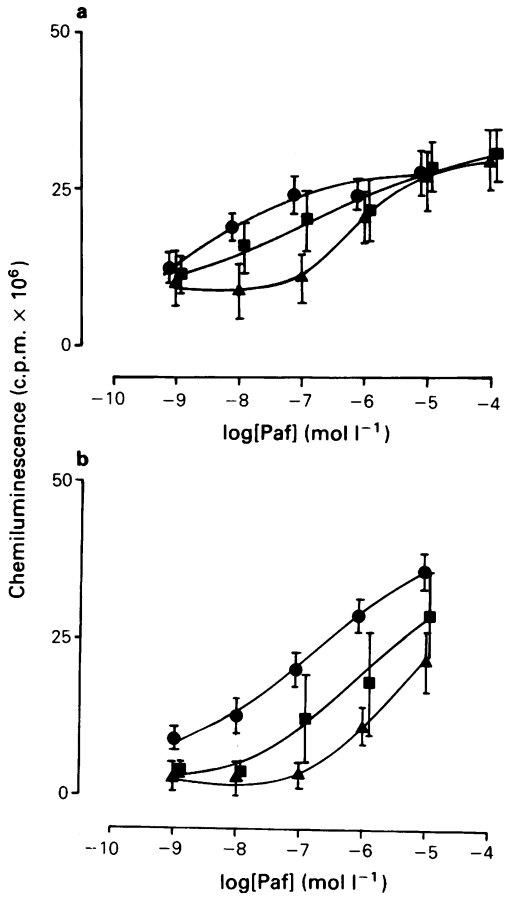


Figure 1 Inhibition by kadsurenone (a) and L 652,731 (b) of the chemiluminescence (CL) responses of guinea-pig, *C. parvum*-activated peritoneal macrophages to Paf. Complete dose-response curves were obtained on individual cell suspensions from single guinea-pigs in the absence (●) and in the presence of antagonists at concentrations of 1.0 (■) and 10.0 μmol l^{-1} (▲). Each point is the mean of 3–6 observations obtained from 6 animals; vertical lines show s.e.mean. For reasons of clarity, all antagonist concentrations used are not given.

inhibition of CL responses to 0.1 μmol l^{-1} Paf. As shown in Table 1, the order of potency was: L 652,731 > kadsurenone ≥ BN 52021 > alprazolam > SRI 63119, kadsurenone and BN 52021 exhibiting the clearest concentration-response relationship, as indicated by the regression coefficients. Since the Schild plots for kadsurenone and BN 52021 have properties consistent with competitive antagonism of Paf, their calculated pA_2 values (Table 2) apparently reflect their affinities for Paf receptors on guinea-pig activated macrophages and confirm the IC_{50} data showing identical affinities.

Relationships between antagonist affinity and macrophage activation state

The similarity in the apparent affinities of BN 52021 and kadsurenone for macrophage Paf receptors was

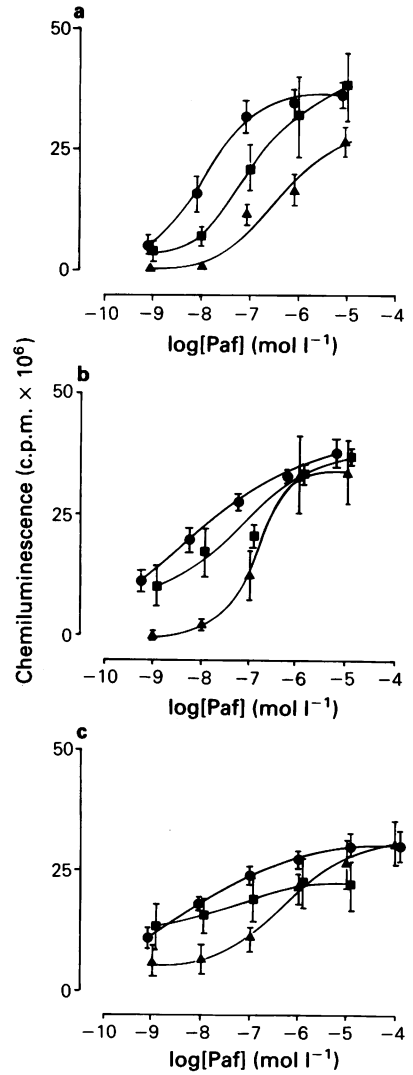


Figure 2 Inhibition by SRI 63119 (a), alprazolam (b) and BN 52021 (c) of the CL responses of guinea-pig, *C. parvum*-activated peritoneal macrophages to Paf. Complete dose-response curves were obtained on individual cell suspensions from single guinea-pigs in the absence (●) and in the presence of antagonists at concentrations of 1.0 (■) and 10.0 μmol l^{-1} (▲) for BN 52021 and SRI 63119 and 3.0 (■) and 30.0 μmol l^{-1} (▲) for alprazolam. Each point is the mean of 2–6 observations from 6 animals; vertical bars show s.e.mean. For reasons of clarity, all antagonist concentrations used are not given.

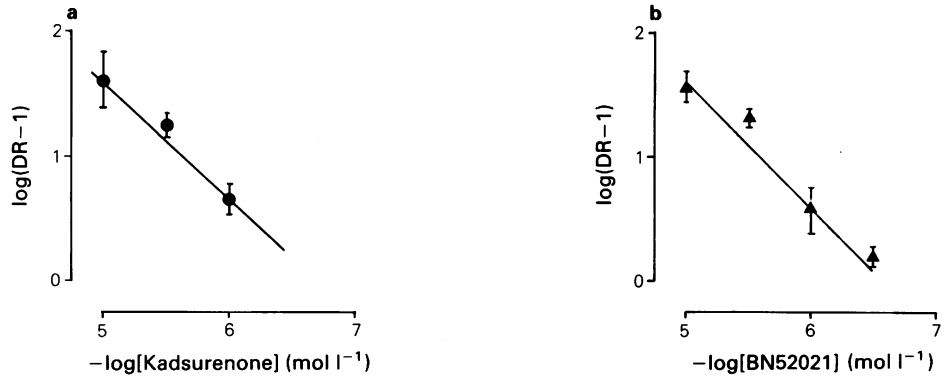


Figure 3 Least squares fit Schild plots for antagonism by kadsurenone (a) and BN 52021 (b) of Paf (10^{-10}–10^{-4} mol l^{-1})-induced CL responses of guinea-pig activated peritoneal macrophages. Each point is the mean value obtained from 3–6 separate observations each made on a different cell suspension, each cell suspension harvested from a different animal; s.e.mean shown by vertical bars. Ordinate scale: log (DR − 1) where DR = dose-ratio measured from the displacement of the Paf dose-response curve by the respective antagonist. Abscissa scale: negative logarithm of the molar concentration of the respective antagonist.

supported when their affinities (pA_2 values) were correlated with the maximal activation state (MAS) of individual cell populations (Figure 4). Both compounds exhibited a similar affinity, irrespective of the MAS of the macrophages (Table 2).

Discussion

The data presented in this paper indicate that responses of guinea-pig activated macrophages to Paf can be antagonized by a variety of structurally different antagonists, and that the affinities of at least two compounds which exert competitive antagonism of the macrophage Paf receptors do not vary according to the activation state of the macrophages.

A number of reports have appeared in the literature recently suggesting that subtypes of Paf receptors can be detected on different cell membranes. For instance, Hwang & Lam (1986), using [^{3}H]-Paf-binding, reported that the order of potency of seven different Paf receptor antagonists differs between human and rabbit platelets, while Hwang (1988) found different orders of potency when measuring antagonism of Paf binding and signal transduction parameters in human leukocytes and platelets. Hwang (1988) suggested that the differences in affinity which we observed (Lambrecht & Parnham, 1986) for kadsurenone between guinea-pig activated peritoneal macrophages and pig peripheral blood leukocytes stimulated with Paf may have been due to such species and/or intercellular differences in Paf

Table 1 Characteristics of the antagonism by various Paf receptor antagonists of the chemiluminescence response to Paf in guinea-pig activated peritoneal macrophages

	Inhibitory-potency			*Schild plot*
	IC_{50} (μmol l^{-1})	*Relative potency*	*Regression coeff. (r)*	*regression slopes*
Alprazolam	161.00 (21)	383.3	0.505†	−0.365 ± 0.115 (10)**
BN 52021	6.08 (23)	14.3	0.662††	−0.925 ± 0.117 (24)
L 652731	0.42 (24)	1.0	0.426†	−0.625 ± 0.024 (24)*
Kadsurenone	5.25 (16)	12.3	0.773††	−0.960 ± 0.064 (15)
SRI 63119	311.00 (17)	731.0	0.535†	−0.574 ± 0.056 (13)*

Values are given as means ± s.e.mean; numbers in parentheses indicate the number of experiments.
Significance of difference of correlation from zero: †$P < 0.05$, ††$P < 0.001$. Significance of difference from unity: *$P < 0.05$, **$P < 0.01$.
The no. of observations for IC_{50} determinations differ from those for Schild plots, because in some experiments only one or two antagonist concentrations were used. For kadsurenone, in 3 experiments, the dose-ratio at 1 μmol l^{-1} antagonist was too small to permit accurate calculation for the Schild plot.

Table 2 Relationship between antagonist affinity and macrophage activation state (MAS) for kadsurenone and BN 52021

	Mean pA_2	n	*Correlation coefficient (r) for MAS vs* pA_2
Kadsurenone	6.41 ± 0.14	14	0.461
BN 52021	6.60 ± 0.05	13	−0.020

Values are given as means ± s.e.mean.
Significance of difference of correlation from zero: $P > 0.05$ in each case.

receptor characteristics. In any case, our present data demonstrate that the affinity of kadsurenone, like that of BN 52021, for Paf receptors on the total population of guinea-pig peritoneal macrophages appears to be independent of the state of cellular activation.

Activation of mouse peritoneal macrophages by *C. parvum* has been shown to enhance markedly the expression of surface Fc (IgG_{2b}) receptors and Ia antigens and to decrease slightly the expression of surface C3 receptors for complement (Glass *et al.*, 1984). Recently, Walker & Beelen (1988) have reported that in rat peritoneal macrophages, fractionation reveals discrete groups of cells with markedly increased expression of Fc receptors and phagocytic capacity. At present, we cannot rule out the possibility that in discrete groups of cells in our study the affinity of kadsurenone for Paf receptors may have been increased, but this was not detectable in the total peritoneal cell population. Different proportions of such discrete groups of cells may have accounted for the high affinity for kadsurenone observed in our previous study (Lambrecht & Parnham, 1986) as well as for our failure to confirm preliminary findings on the relation between cell activation and Paf receptor antagonist affinity (Bittner *et al.*, 1988). The fact that BN 52021 exhibited similar mean pA_2 values to kadsurenone on Paf-stimulated guinea-pig macrophages and did not show a change in affinity with increasing cell activation suggests that BN 52021 binds to a similar site on the Paf receptor to kadsurenone.

Recently, Stewart & Dusting (1988) have reported that BN 52021, L 652,731 and another Paf receptor antagonist WEB 2086 all exhibit lower affinities (pA_2 values) for guinea-pig resident macrophage receptors than for platelet and leukocyte Paf receptors. These authors used Paf-stimulated macrophage prostacyclin production as their parameter. Guinea-pig resident macrophages barely respond to Paf with an oxidative burst, in contrast to *C. parvum*-activated cells (Hartung *et al.*, 1983), while specific ^{3}H-Paf

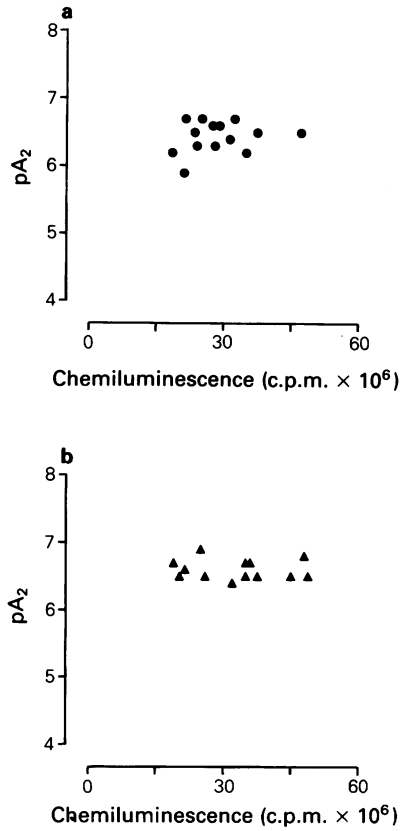


Figure 4 Correlations for (a) kadsurenone and (b) BN 52021 between pA_2 values determined on different guinea-pig activated macrophage suspensions and the maximal activation state (MAS; measured as the peak CL response to Paf in the absence of antagonist) of the individual cell suspension from which the pA_2 value was calculated. Each point is the mean of 1–3 determinations of pA_2 values calculated for different antagonist concentrations on the same cell suspension.

binding sites could not be detected on resident cells (Kudo *et al.*, 1986). The possibility thus arises, as suggested by Stewart & Dusting (1988), that cell membrane-associated Paf may exert biological effects, independently of receptor binding, for instance on resident macrophages. Certainly, Paf exhibits a high degree of non-specific binding to a number of cells but no report has appeared in which this non-specific binding has been correlated with a biological effect.

In a study on permanent macrophage cell lines, Valone (1988) reported that HEL, U-937 and HL-60 cells bound 'substantial quantities' of Paf, but he discussed this binding as being non-receptor-mediated

on the basis of antagonist effects. Interestingly, he observed that kadsurenone did inhibit [^{3}H]-Paf binding to these cells, but BN 52021 did not. Also in P388D$_1$ mouse macrophage-derived cells BN 52021 was 14 fold less potent as an inhibitor of Paf binding in comparison to kadsurenone (Valone, 1988). Such a distinction does not appear to hold for guinea-pig peritoneal macrophages.

The fact that in our experiments the Schild plot for kadsurenone was linear with a slope which was not significantly different from unity, while its analogue L 652,731 produced a plot with a slope different from unity requires explanation. It seems likely that L 652,731, while more potent than kadsurenone, may require a longer period of time to achieve thermodynamic equilibrium with the Paf receptors as has been proposed for other receptor types (Kenakin, 1987). Alternatively, the specificity of action of the two antagonists may differ. Stewart & Dusting (1988) were also unable to calculate a pA_2 value for L 652,731 with guinea-pig resident macrophages, though, in their case, this was because of the low potency of the compound on these cells. The apparent non-competitive nature of the antagonism by alprazolam as reflected by the non-linear Schild plot for this compound, may be due to more than one mechanism of antagonism of the Paf-induced CL response as has been proposed for other receptor types (Kenakin, 1987). Alprazolam is a triazolobenzodiazepine (Kornecki *et al.*, 1984) and is used clinically as an anxiolytic and hypnotic agent. Recently, mouse peritoneal macrophages have been shown to exhibit saturable binding sites for the benzodiazepine, flunitrazepam (Zavala *et al.*, 1985), while a benzodiazepine (diazepam) has been reported to inhibit immune responses in mice *in vivo* (Descotes *et al.*, 1982). Consequently, the antagonism by alprazolam of Paf-induced CL responses of guinea-pig peritoneal macrophages may have been due at least to interaction of the antagonist with both Paf and benzodiazepine receptors. Similarly, SRI 63119, a compound structurally related to Paf (Handley *et al.*, 1986), probably exerted a non-specific action on macrophage CL. In fact, in another recent study, SRI 63119 also failed to produce a Schild plot with a slope of unity, when tested on rabbit platelet aggregation (O'Donnell & Barnett, 1988).

Lack of linearity and of slopes of unity of Schild plots complicate direct comparison of antagonist potencies between different studies. O'Donnell & Barnett (1988) have drawn attention to the fact that only with pA_2 values can direct comparisons of antagonist potencies be made, IC_{50} values obtained under different conditions only permitting orders of potency to be assessed. In this respect, the pA_2 value we observed for BN 52021 (6.6) corresponds closely to the value of 6.85 obtained by Stewart & Dusting (1988) on rabbit washed platelets and of 6.38 obtained by O'Donnell & Barnett (1988) on rabbit platelet-rich plasma. The binding sites for BN 52021 and the Paf receptors of these cells, therefore would appear to be similar. The mean pA_2 of 6.41 we observed in the present study for kadsurenone correlates closely with the values obtained by Shen *et al.* (1985) of 6.28 for rabbit platelet aggregation and 6.32 for human leukocyte aggregation and the value of 6.16 obtained by Lambrecht & Parnham (1986) for pig leukocyte aggregation.

Comparison of orders of potency of four of the antagonists we studied reveals that in most cell systems L 652,731 is more potent than kadsurenone (Table 1; Hwang & Lam, 1986), which is as potent or more potent than BN 52021 (Hwang & Lam, 1986; O'Donnell & Barnett, 1988; Stewart & Dusting, 1988; Ward & Westwick, 1988). The exception is guinea-pig resident macrophages in which BN 52021 appeared to be more potent than L 652,731, but did not fulfil the criteria for a competitive inhibitor (Stewart & Dusting, 1988). Although we observed that SRI 63119 was a weak antagonist on activated macrophages, O'Donnell & Barnett (1988) found it to be a potent Paf antagonist on rabbit platelets. However, the pA_2 value given is dubious since the compound did not give a Schild plot with a slope of unity. In addition, the authors used platelet-rich plasma in which endogenous Paf may have interfered with the activity of the structurally-related antagonist.

In conclusion, we have shown that structurally different Paf receptor antagonists can be differentiated on the basis of their effects on Paf receptors on guinea-pig activated macrophages. The finding that kadsurenone and BN 52021 do not exhibit increasing affinity for Paf receptors, with increasing macrophage activation, would appear to rule out cell activation as an explanation for our previously reported finding on the differential affinity of kadsurenone for Paf receptors on macrophages and neutrophils. Cell fractionation studies are required to determine whether discrete fractions of peritoneal macrophages differ with regard to Paf antagonist affinity.

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Inhibition of agonist-stimulated inositol lipid metabolism by the anticonvulsant carbamazepine in rat hippocampus

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1 The effect of the anticonvulsant, anti-manic drug carbamazepine was examined on inositol lipid signalling in rat hippocampus *in vitro*.

2 Hippocampal miniprisms were labelled with [^{3}H]-inositol before stimulation with a variety of neuroactive agents that increase phosphoinositide turnover.

3 The presence of carbamazepine (0.1–100 μM) during labelling caused a dose-related reduction of basal and carbachol-evoked [^{3}H]-inositol phosphate accumulations. The effect of the drug on basal inositol phosphate levels was lost when slices were labelled with [^{3}H]-inositol before incubation with carbamazepine.

4 Incubation of slices with carbamazepine after labelling with [^{3}H]-inositol and before stimulation showed the inhibitory effect of the drug to be selective according to the agonist used. Responses to carbachol, histamine and the sodium-channel agent veratrin were reduced by carbamazepine whilst the responses to 5-hydroxytryptamine, noradrenaline and substance P were unaffected.

5 Inhibition of carbachol, histamine and veratrin-induced stimulation by carbamazepine share a similar dependence on length of pre-incubation time with the drug. However, the effect of carbamazepine (100 μM) on the respective dose-response curves suggests that the mechanism of inhibition of the carbachol response differs from the inhibition of the histamine and veratrin responses. These effects may be significant in the mechanism of action of carbamazepine as an anticonvulsant and in its effectiveness against manic depression.

Introduction

Carbamazepine (5H-dibenz[b,f]azepine-5-carboxamide, Tegretol, Ciba-Geigy) is a first-line anticonvulsant used in the treatment of generalised (tonic-clonic) and partial seizures. In addition, it is effective against trigeminal neuralgia (Bonduelle, 1976), arrhythmia and manic depression (Post *et al.*, 1983). Electrophysiological experiments (Willow *et al.*, 1983), receptor binding studies (Willow & Catterall, 1985) and release studies (Crowder & Bradford, 1987; E.E. McDermott, J.D. Turner & S.D. Logan, unpublished results) have shown carbamazepine (Cbz) to inhibit Na^+ and Ca^{2+} influx by an interaction with voltage-dependent sodium channels (Willow, 1986). At therapeutic levels of the drug (Morselli, 1976), Cbz has been found to affect binding (Skerritt *et al.*, 1982; 1983; Marangos *et al.*, 1983; 1985; 1987) and functionality (Lewin & Bleck, 1977; Phillis, 1984) of purinoceptors. At higher concentrations it has been shown to bind to the 'micromolar benzodiazepine receptor' (Weiss *et al.*, 1985) and inhibit Ca^{2+}-calmodulin-dependent protein phosphorylation (De Lorenzo, 1984).

The phosphoinositide hydrolysis signalling pathway involves the metabolism of the membrane lipid phosphatidylinositol-4,5 bisphosphate (PtdIns4,5P_2) to produce the second messengers inositol 1,4,5-trisphosphate (which mobilises intracellular calcium) and diacylglycerol (which activates protein kinase C) (for reviews see Berridge & Irvine, 1984; Downes, 1986).

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Several factors prompted the investigation into an action of Cbz on this pathway. Firstly, Cbz shares its therapeutic action against depression with lithium which is established as having several effects on inositol lipid metabolism (Hallcher & Sherman, 1980; Batty & Nahorski, 1987; Erneux *et al.*, 1987; Ackermann *et al.*, 1987; Kendall & Nahorski, 1987; Newman & Lerer, 1988; Ragan *et al.*, 1988). Activation of protein kinase C appears to be involved in the generation of hippocampal long term potentiation (Malenka *et al.*, 1986), a phenomenon which has been compared to kindling (Baudry, 1986). Finally, increases in the products and metabolites of inositol lipid turnover have been shown to be associated with various seizure models (Bazan *et al.*, 1986; Iadorola *et al.*, 1986) and with processes linked to epilepsy, such as long term potentiation (Lynch *et al.*, 1988) and electroconvulsive shock (Vadnal & Bazan, 1988).

These studies examined the effects of Cbz on the inositol lipid signalling system in rat hippocampal slices *in vitro*. Muscarinic agonists have been demonstrated to stimulate hydrolysis of PtdInsP_2 in this tissue (Stephens & Logan, 1989a) leading to rapid formation of several inositol phosphates including Ins1,4,5P_3 and Ins1,3,4,5P_4 (Stephens & Logan, 1989b). The results indicate apparent inhibition of both basal and cholinergically-stimulated [^{3}H]-inositol phosphate accumulation when the drug is present during the labelling procedure, possibly by affecting [^{3}H]-inositol distribution in the inositol lipids. In addition, the presence of Cbz after labelling caused selective reduction of particular agonist-stimulated [^{3}H]-inositol phosphate accumulations. Such effects may be of importance in the mechanisms of action of the drug as an anticonvulsant or antidepressant. Part of this work has been published previously in abstract form (McDermott & Logan, 1988).

Methods

Male Sprague-Dawley (300–400 g) rats were killed by cervical dislocation and brains removed rapidly. Hippocampal tissue was dissected on ice and the formation chopped transversely at 350 μm intervals in three directions (60° rotation between each) by means of a McIlwain tissue chopper. The miniprisms formed were then washed several times in gassed (95%O_2/5%CO_2) Krebs-Ringer bicarbonate buffer (Stephens & Logan, 1986).

After the wash procedure, 50 μl aliquots of tissue were added to vials containing 3–7 μCi myo-2 [^{3}H]-inositol (specific activity 10–20 Ci mmol^{-1}) in gassed buffer. This produced tissue densities of 1–1.5 mg per tube. Slices were incubated for 1 h with the label before 10 μl LiCl was added to give a final concentration of 10 mM. After 10 min the agonists were added in 10 μl to give a final volume of 300 μl. Incubations were re-gassed and stoppered every 20–30 min.

Alternatively, for bulk labelling of tissue, the slices were suspended in 5 ml of buffer and preincubated for 10 min at 37°C. They were then labelled by incubating with 0.4–0.8 μM [^{3}H]-inositol for 1 h at 37°C in a shaking water bath under an atmosphere of 95%O_2/5%CO_2. Slices were washed with fresh buffer to remove excess label before incubation of 50 μl aliquots in conditions as for continuous labelling.

Incubations were terminated by addition of 250 μl of ice-cold perchloric acid (10%w/v) and put on ice for 10 min.

[^{3}H]-inositol mono- and polyphosphates were extracted from the slices by a method based on that of Sharps & McCarl (1982). After centrifugation at 2000 *g* for 5 min, the supernatant was removed and neutralised with 0.5 ml of a 1:1 (v/v) mixture of tri-n-octylamine and Freon. In later experiments maleic acid was added (final concentration 75 mM) to minimise loss of inositol trisphosphate (InsP_3) (see Wreggett *et al.*, 1987). However, no significant differences were observed in recovery of total inositol phosphates with this amendment. This may be due to metabolism of InsP_3 during long incubations. After being vortexed for 30 s the phases were separated by centrifugation at 2000 *g* for 5 min (4°C). An aliquot of the upper aqueous phase containing the [^{3}H]-inositol phosphates was then removed for separation by anion-exchange chromatography.

In experiments in which total [^{3}H]-inositol lipids were measured, lipids were separated from water soluble products using chloroform: methanol (1:2) according to the method of Donaldson & Hill (1986).

Separation of [^{3}H]-*inositol phosphates*

[^{3}H]-inositol-labelled acid-soluble metabolites were separated by use of 0.6 × 2 cm columns of BioRad AG1 anion-exchange resin (1 × 8, 200–400 mesh, formate form) made up in glass Pasteur pipettes. An aliquot of water-soluble metabolites was applied to the columns in 10 ml of 5 mM di-sodium tetraborate/0.5 mM ethylenediaminetetraacetic acid (EDTA) after the resin had been equilibrated with 20 ml of distilled water and 10 ml tetraborate/EDTA.

Before collection of the inositol phosphates, free [^{3}H]-inositol was removed with 20 ml of water; glycerophosphoinositides (GroPIns) were then eluted with 15 ml of 50 mM ammonium formate (AF). [^{3}H]-

inositol phosphates (Ins*P*s) were eluted with 8 ml of 1.025 M AF/0.1 M formic acid and 2 ml taken for liquid scintillation counting after the addition of scintillant (Optiphase-X).

Protein values were estimated by the method of Lowry *et al.* (1951) with bovine serum albumin (BSA) standards.

Materials

Carbamazepine was provided by Ciba-Geigy. Myo-2-[^{3}H]-inositol (10–20 Ci ml^{-1}) was purchased from Amersham U.K. Tri-n-octylamine and Freon were purchased from Aldrich Chemical Co; carbachol, 5-hydroxytrypamine, histamine dihydrochloride, noradrenaline, veratrin and tetrodotoxin from the Sigma Chemical Co. Inorganic salts and organic solvents were purchased from Fisons or Fluka.

Results

Incubation of hippocampal slices with Cbz during labelling with [^{3}H]-inositol resulted in decreased basal and carbachol-stimulated total [^{3}H]-inositol phosphate levels, the maximum reduction with 100 μM Cbz being 25% and 28%, respectively (Table 1a). This inhibition by the anticonvulsant appears to be dose-related and significant reductions of 17–18% were attained at approximately 10 μM Cbz. Use of higher concentrations of the drug was limited by the need for high levels of vehicle (dimethylsulphoxide) to maintain its solubility. Such concentrations also lessen the relevance of the effects to the actions seen *in vivo* at therapeutic levels. Further investigation of the action of the drug on inositol lipid hydrolysis was therefore carried out with 100 μM Cbz in 0.4% dimethylsulphoxide. Although this concentration is above the average therapeutic levels measured in plasma (10–50 μM), Cbz is absorbed rapidly and is concentrated in brain and other tissues (Morselli *et al.*, 1971) and may lead to levels of 100 μM.

The presence of LiCl (10 mM) during incubation had no effect on the inhibition caused by Cbz. This suggests that despite a common therapeutic action in manic depression, Cbz does not interfere with the activity of inositol monophosphatase as does lithium.

The lipophilic nature of Cbz suggests that its inhibitory effect on [^{3}H]-inositol phosphate levels may be related to membrane stabilisation, perhaps altering the relative amounts of phosphatidylinositol (PtdIns), phosphatidylinositol phosphate (PtdInsP) and phosphatidylinositol bisphosphate (PtdInsP_2). Moreover, since no reduction in basal [^{3}H]-inositol phosphates is seen when Cbz is present after the labelling process this suggests that there may be some effect on the distribution of the label within the lipids (Table 1b). It is unlikely that the general level of incorporation of [^{3}H]-inositol into membrane lipids is significantly decreased since measurements of ^{3}H-lipids (following a chloroform-methanol extraction) are not significantly different in control tissues (43,582 ± 5,032 d.p.m.) compared with tissues labelled in the presence of 100 μM Cbz (47,334 ± 5,656 d.p.m.). However, when Cbz was present after the labelling process the inhibition of the carbachol response remained, and was of a similar magnitude (34%) to that observed when Cbz was present during the labelling process. This suggests that there is more than simply a general membrane stabilisation involved in the reduction of the carbachol response.

Further investigations used a variety of agents to stimulate phosphoinositide turnover and showed that the inhibitory action of Cbz appears to be selective (Figure 1). Stimulation by carbachol (1 mM) was reduced by 25% from 4.5 fold to 3.4 fold, histamine (100 μM) stimulation by 60% from 4.3 fold to 1.7 fold and the veratrin (100 μM) response by 41% from 2.7 fold to 1.6 fold. However, Cbz had no effect on responses to noradrenaline (1 mM), 5-hydroxytryptamine (1 mM) or substance P (10 μM).

Table 1 Effect of carbamazepine (Cbz) on basal and carbachol-induced inositol phosphate accumulations in rat hippocampus

	Basal	*Carbachol* (1 mM)
(a) Cbz present during labelling		
Control (+Li)	100 ± 3	100 ± 5
0.1 μM Cbz	88 ± 15	97 ± 4
1 μM Cbz	100 ± 8	89 ± 7
10 μM Cbz	82 ± 4*	83 ± 1*
100 μM Cbz	75 ± 7**	72 ± 5**
100 μM Cbz (−Li)	77 ± 8**	ND
Vehicle 0.4% DMSO	106 ± 16	ND
(b) Cbz present after labelling		
Control (+Li)	100 ± 5	100 ± 9
100 μM Cbz (+Li)	99 ± 14	66 ± 10*

Results are expressed as percentage of control (i.e. incubations without Cbz). Basal d.p.m. is approximately 1000 d.p.m./50 μl slices. (a) Slices were incubated with Cbz (0.1–100 μM) for the total experiment including the 1 h labelling period with [^{3}H]-inositol. (b) Slices were first prelabelled with [^{3}H]-inositol then incubated with 100 μM Cbz for 1 h before addition of 1 mM carbachol. Values represent means ± s.e. mean (n = 4–12) with statistical analysis by Student's t test. *P < 0.05, **P < 0.01. ND—not determined.

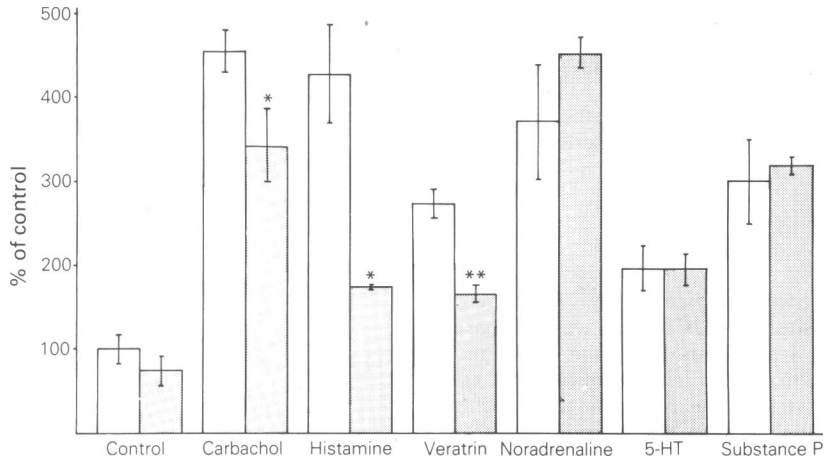


Figure 1 Effect of carbamazepine on stimulation of total [^{3}H]-inositol phosphate accumulation by various agents in rat hippocampus. Slices pre-labelled with [^{3}H]-inositol were incubated with 100 μM carbamazepine (Cbz) for 1 h before stimulation with carbachol (1 mM), histamine (100 μM), veratrin (100 μM), noradrenaline (1 mM), 5-hydroxytryptamine (5-HT, 1 mM) or substance P (10 μM). Values are expressed as percentage of unstimulated. Columns represent the mean ($n = 4$–12) and vertical bars show s.e.mean. Open columns, control responses in absence of Cbz; stippled columns, responses in presence of Cbz. Statistical analysis was by Student's t test, $^*P < 0.05$, $^{**}P < 0.005$.

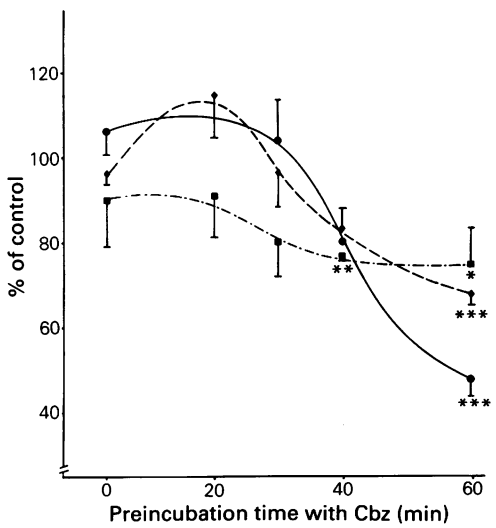


Figure 2 Time-dependence of inhibition of stimulated inositol phosphate accumulation by carbamazepine (Cbz). After pre-labelling with [^{3}H]-inositol, hippocampal slices were incubated with Cbz (100 μM) for various lengths of time before incubation with (■) carbachol (1 mM), (●) histamine (2 mM) or (◆) veratrin (100 μM) for 45 min. Values represent mean ($n = 4$–6); $^*P < 0.05$, $^{**}P < 0.02$, $^{***}P < 0.01$ by Student's t test.

Time course experiments which involved increased pre-incubation time with 100 μM Cbz before stimulation (for a constant time of 45 min) with carbachol, veratrin and histamine showed the inhibition by the anticonvulsant to be dependent on the length of time the tissue was pre-incubated with Cbz (Figure 2). A pre-incubation of approximately 40 min was needed for a statistically significant reduction of carbachol stimulation, whilst approximately 60 min was needed for reductions of both veratrin and histamine stimulations.

Comparison of the effects of Cbz (100 μM) on carbachol, histamine and veratrin dose-response curves showed differences in the apparent type of inhibition (Figure 3). The presence of Cbz caused a parallel shift to the right of the carbachol curve which was not observed with the dose-response curves of histamine and veratrin, although in these cases there was a reduction in the maximum response.

The effect of Cbz on the response to veratrin may be explained by its established action at sodium channels, as the presence of the sodium channel blocking agent tetrodotoxin (5 μM) in incubations blocked the veratrin response (Table 2). However, the toxin had no effect on stimulation of Ins$P$ accumulation by carbachol or histamine. Hence the reduction of these responses by Cbz does not appear to be due to inhibition of a polysynaptic effect and release of endogenous agonists of phosphoinositide turnover.

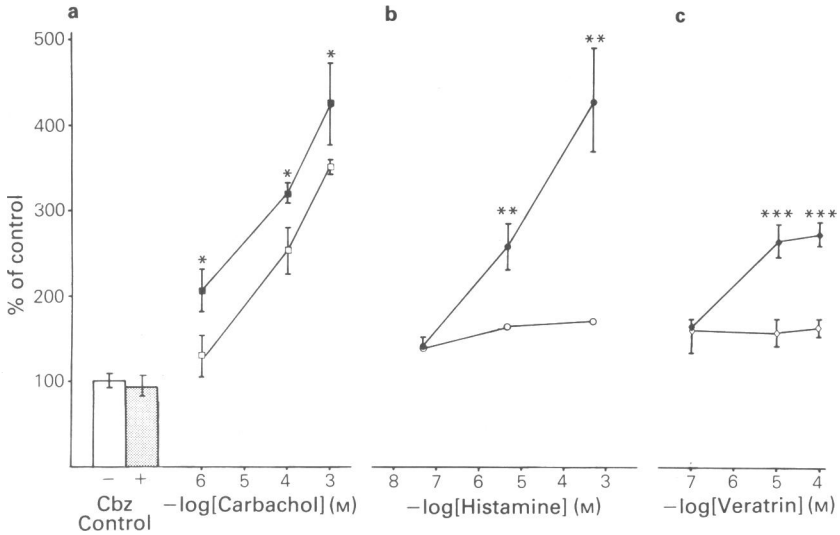


Figure 3 Effect of carbamazepine (Cbz) on (a) carbachol, (b) histamine and (c) veratrin-stimulated total [^{3}H]-inositol phosphate accumulation in rat hippocampal slices. Hippocampal slices were incubated with 100 μM Cbz for 1 h after prelabelling with [^{3}H]-inositol and before addition of agonists. Responses in absence of Cbz (solid symbols), responses after preincubation with Cbz (open symbols). Values represent mean (n = 4–9); vertical lines show s.e.mean. *$P < 0.05$, **$P < 0.025$, ***$P < 0.005$ by Student's t test.

For comparison we investigated the effect of the anticonvulsant phenytoin on [^{3}H]-inositol phosphate accumulation in response to carbachol (1 mM), histamine (100 μM) and veratrin (100 μM). Incubation of slices for one hour with 100 μM phenytoin resulted in a 38% decrease in carbachol stimulation and a 33% decrease in the veratrin response but had no effect on that of histamine (Table 2).

Table 2 The effects of tetrodotoxin (TTX) and phenytoin on inositol phosphate accumulation stimulated by carbachol, histamine and veratrin.

	Basal	*Carbachol*	*Histamine*	*Veratrin*
TTX (5 μM)	112 ± 8	95 ± 8	108 ± 6	56 ± 4
Phenytoin (100 μM)	106 ± 13	62 ± 3	100 ± 23	67 ± 9

Hippocampal slices were prelabelled with [^{3}H]-inositol for one hour before incubation with TTX (5 μM) for 10 min or phenytoin (100 μM in 0.4% DMSO) for one hour before stimulation with carbachol, histamine and veratrin (40 min). Results are expressed as percentage of basal or stimulated [^{3}H]-inositol phosphate accumulation measured in the absence of TTX or phenytoin. Values represent means ± s.e.mean of a single experiment conducted in quadruplicate, essentially identical results were obtained on two to five further occasions.

Discussion

This study demonstrates an inhibitory action of the anticonvulsant drug Cbz on inositol lipid metabolism in rat hippocampal slices. This is in common with the *in vivo* effect of Cbz on electroconvulsive-shock-induced inositol phosphate accumulation in rat cortex and hippocampus (Vadnal & Bazan, 1988). When present during the labelling procedure with [^{3}H]-inositol both basal and carbachol-stimulated inositol phosphate accumulations were reduced. This possibly reflects a general membrane stabilisation, due to the lipophilic nature of Cbz. Such an interaction could inhibit lipid turnover reducing incorporation of [^{3}H]-inositol into phosphoinositides or changing the distribution within PtdIns, PtdInsP and PtdInsP$_2$. The latter explanation may be more likely as no change in the levels of total labelled lipids was observed.

A second effect, produced by incubation with Cbz for one hour after prelabelling and before stimulation, involved a selective reduction of carbachol, veratrin and histamine-evoked [^{3}H]-Ins$P$ accumulation. The lack of effect of Cbz on phosphoinositide responses to noradrenaline, 5-hydroxytryptamine and substance P indicates that the drug does not have a general action at the phosphoinositidase. This selectivity may indicate an interaction of Cbz with particular receptors or receptor-transduction mechanisms.

Time course experiments showed inhibition of [^{3}H]-Ins*P* accumulation by Cbz (100 μM) to increase with time of incubation with the anticonvulsant (Figure 2). This time lag may suggest that some form of equilibrium is reached between Cbz and a binding site within the lipid bilayer. Alternatively it may reflect the need for a modulator substance to accumulate and reach a threshold.

Although Cbz-induced inhibition of carbachol, veratrin and histamine responses had a similar time dependence, the apparent nature of the effect on carbachol differs from that on veratrin and histamine responses. This can be seen by comparison of their respective dose-response curves in the presence or absence of Cbz (Figure 3). The parallel shift to the right of the carbachol dose-response curve is suggestive of competitive inhibition by Cbz of carbachol stimulation. However, the effect of Cbz (100 μM) on histamine and veratrin stimulation does not appear to be competitive but a consequence of a decreased maximal response.

Explanation of the action of Cbz on carbachol-evoked [^{3}H]-Ins*P* accumulation is difficult as receptor binding studies show no interaction with the muscarinic receptor at similar concentrations to those used in inositol lipid studies (Skerritt *et al.*, 1983). This discrepancy may arise from differences in time of incubation with Cbz, or perhaps reflects competition with carbachol at a step beyond the receptor-agonist interaction site. Such a selective action of Cbz on the muscarinic response is interesting in view of induction of seizures by agonists such as pilocarpine (Turski *et al.*, 1983).

The reduction in the veratrin response can be explained by the action of Cbz at the voltage-dependent sodium channel (Willow, 1986). Electrophysiological and neurochemical studies show Cbz to inhibit binding of sodium channel agents such as batrachotoxin-B and veratrin to the neurotoxin receptor 2 (Schauf *et al.*, 1974; Worley & Baraban, 1987; Willow *et al.*, 1985; Willow & Catterall, 1985). The model of this interaction proposed by Willow (1986) explains the voltage and use-dependency of the interaction of Cbz with the channel. It proposes that Cbz binds with high affinity to the inactive state of the channel, stabilising it, preventing its closure and subsequent activation. The need for the channel to pass through the activated state before binding of Cbz may also explain the time lag seen in inositol phosphate studies (Figure 2) the resulting decrease in inositol lipid turnover being due to a reduction in the depolarisation-induced release of endogenous agonists (Olpe *et al.*, 1985; Crowder & Bradford, 1987; E.E. McDermott, J.D. Turner and S.D. Logan unpublished observations). It appears, however, that the effect of Cbz on the histamine and carbachol response is not due to a reduction in Na^+ influx or of a polysynaptic response as inclusion of tetrodotoxin had no effect on these responses (Table 2). However, studies on guinea-pig synaptoneurosomes (Gusovsky & Daly, 1988) suggest that sodium influx due to such agents as veratrin may increase intracellular calcium levels via the Na^+/Ca^{2+} exchanger, resulting in a direct enhancement of phospholipase activity. If, by its action at sodium channels, Cbz produces a significant change in intracellular calcium levels then this may provide a mechanism for its effects on responses to other agonists. Selectivity could then arise from the variable calcium sensitivity in phosphoinositide turnover in response to particular agonists. For instance, the response to histamine in brain slices has been shown to require higher levels of Ca^{2+} than those of carbachol and 5-hydroxytryptamine (Kendall & Nahorski, 1984).

However, another possible explanation for inhibition of the histamine-induced response arises from the apparent interaction of histamine and adenosine receptors. In addition to the potentiation of stimulated cyclic AMP levels by H_1 agonists (Hollingsworth & Daly, 1985, Hollingsworth *et al.*, 1986), there is now evidence of an alteration in histamine-induced phosphoinositide turnover by adenosine agonists (Hill & Kendall, 1986; 1987; Kendall & Hill, 1988). An interaction of Cbz with adenosine receptors (Marangos *et al.*, 1983; 1985; 1987; Skerritt *et al.*, 1982; 1983; Fujiwara *et al.*, 1986) may affect the H_1-receptor in a manner similar to that of an A_1-agonist. Interestingly, although in guinea-pig and mouse cortical slices adenosine (A_1)-receptor activation can modulate the histamine response, no effect could be observed in rat cortical slices (Kendall & Hill, 1988). However, no such experiment appears to have been performed in rat hippocampal slices, hence an effect on Cbz via adenosine receptor interaction cannot be discounted.

In addition to the above putative mechanisms of the effect of Cbz on histamine stimulation, there is also the possibility of a direct interaction with the H_1-receptor. This is suggested by the binding studies that show tricyclic antidepressants, that are structurally similar to Cbz, act as H_1-antagonists in neuroblastoma and guinea-pig ileum (Taylor & Richelson, 1979).

Preliminary studies have examined the effects of phenytoin, an anticonvulsant with a similar therapeutic spectrum to Cbz, on phosphoinositide responses to carbachol, histamine and veratrin. As would be expected phenytoin, which has also been established to act at sodium channels (Willow, 1986), reduced veratrin-stimulated inositol phosphate accumulation to 68% of the normal response (Table 2). The response to carbachol (1 mM) was reduced to 75%, but basal and histamine-stimulated levels were unaffected. The two inhibitory actions of phenytoin

observed in these studies were of similar magnitude to those of 100 μM Cbz perhaps reflecting the common action of the drugs as anticonvulsants. The lack of effect on histamine stimulation by phenytoin could reflect the closer structural similarity between Cbz and the tricyclic antidepressants and the action of Cbz as an antidepressant.

These results support the finding of Vadnal & Bazan (1988) that Cbz and Li^+ do not share an inhibitory action on the inositol-1-phosphatase. These authors found that Cbz inhibited the increase in [^{3}H]-InsP_3 level, following electroconvulsive shock treatment in rat cortex and hippocampus, suggesting interference with signal transduction at the membrane. However, it is conceivable that as yet unclarified actions of lithium on phosphoinositide metabolism (e.g. reduction of carbachol-induced inositol tetrakisphosphate levels in brain, Batty & Nahorski, 1987) may be common to both drugs. A possible shared site of action is in the receptor/G protein/phospholipase transduction system where lithium appears to reduce agonist-stimulated GTP binding (Avissar *et al.*, 1988). Whereas the action of lithium may be due to competition with the magnesium needed for GTP binding, Cbz may take effect by altering interactions of the receptor, G protein and phospholipase C within the lipid bilayer.

Thus, we postulate that Cbz can affect the inositol lipid signalling pathway in several complex and perhaps interactive ways—(i) by altering the basal turnover of the lipids, possibly by affecting the distribution of membrane inositol lipids, (ii) by affecting the receptor-G-protein-enzyme complex and (iii) by acting directly at the receptors for some ligands that utilise this signalling pathway.

Such interactions of Cbz with this signalling system may be important in its therapeutic action against manic depression. In addition these studies suggest that the inositol lipid signalling pathway may be a site of action of anticonvulsant drugs and thus this mechanism may be important in epileptogenesis.

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Inhibitory effect of a toxin okadaic acid, isolated from the black sponge on smooth muscle and platelets

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1 Effects of okadaic acid, a toxin isolated from marine sponges, on smooth muscle contraction and platelet activation were examined.

2 Contractions in rabbit aorta induced by high concentrations of K^+ and noradrenaline were inhibited by 0.1–1 μM okadaic acid in a concentration-dependent manner. Spontaneous rhythmic contractions as well as high K^+-induced contraction in guinea-pig taenia caeci were also inhibited by 1 μM okadaic acid.

3 High K^+-induced contraction in rabbit aorta was accompanied by increased Ca^{2+} influx measured with $^{45}Ca^{2+}$ and increased cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) measured with fura-2-Ca^{2+} fluorescence. Okadaic acid inhibited the contraction without inhibiting Ca^{2+} influx and produced only a small decrease in $[Ca^{2+}]_{cyt}$.

4 In a saponin-skinned taenia, Ca^{2+}-induced contraction was not inhibited but rather potentiated by okadaic acid.

5 Okadaic acid, 1 μM, inhibited aggregation, ATP release and increase in $[Ca^{2+}]_{cyt}$ induced by thrombin in washed rabbit platelets. Okadaic acid itself did not change the platelet activities.

6 Okadaic acid did not change the cyclic AMP content of rabbit aorta although the inhibitory effects of okadaic acid were similar to those of cyclic AMP.

7 Although the mechanism of the inhibitory effect of okadaic acid was not clarified in the present experiments, it is suggested that okadaic acid acts by inhibiting protein phosphatases resulting in an indirect activation of cyclic AMP-dependent protein phosphorylation.

Introduction

Okadaic acid is a polyether monocarboxylic acid toxin (*Mr* 802) isolated from marine sponges of the genera *Halichondria* (Tachibana *et al.*, 1981) and *Pandaros* (Schmitz *et al.*, 1981). Okadaic acid induces a contraction even in the absence of external Ca^{2+} in intact (Shibata *et al.*, 1982) and also in saponin-skinned smooth muscles (Ozaki *et al.*, 1987a). Ozaki *et al.* (1987b) have shown that okadaic acid induces phosphorylation of myosin light chain in the absence of Ca^{2+} and in the presence of calmodulin inhibitors, suggesting that the contractile effect of okadaic acid is mediated by the activation of Ca^{2+}-independent protein kinase. Okadaic acid also inhibits protein phosphatase activity (Bialojan & Takai, 1988; Ishihara *et al.*, 1989). In the present experiments, we show that okadaic acid, at concentrations lower than that needed to induce smooth muscle contraction, has an inhibitory effect on smooth muscle contraction and platelet activation.

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Methods

Smooth muscle

Thoracic aorta was isolated from male New Zealand White rabbits weighing about 3 kg and muscle strips, 2–3 mm wide and 5–6 mm long, were prepared. The adventitial layer was separated from the medial layer by gently pulling apart these two layers as described by Karaki & Urakawa (1979). Endothelium was removed by gently rubbing the intimal surface with a

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moistened finger. Taenia strips, approximately 10 mm long, were isolated from the caecum of male, white guinea-pigs weighing 300–400 g. Each strip was attached to a holder under a resting tension of 1 g for aorta and 0.2 g for taenia and equilibrated for 60–90 min in a 10 ml muscle bath. Normal physiological salt solution (pH 7.4, 37°C) contained (mM); NaCl 136.9, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 23.8 and glucose 5.5. High K^+ solution was made by increasing the concentration of KCl or by substituting NaCl with equimolar KCl. Ca^{2+}-deficient solution was made by omitting $CaCl_2$ and adding 0.5 mM ethyleneglycol *bis* (β-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA). These solutions were aerated with 95% O_2 and 5% CO_2 mixture. Muscle tension was recorded isometrically with a force displacement transducer.

$[Ca^{2+}]_{cyt}$ was measured simultaneously with muscle contraction in fura-2-loaded rabbit aorta as reported by Ozaki *et al.* (1987c) and Sato *et al.* (1988). Muscle strips were loaded with 5 μM acetoxymethyl ester of fura-2 (fura-2/AM) (Grynkiewicz *et al.*, 1985) for 3 h in the presence of 0.02% cremophor EL at room temperature (23–25°C) and then placed in a tissue bath at 37°C. The muscle strip was illuminated alternately (48 Hz) with 340 nm and 380 nm light, and emission at 500 nm was detected with a spectrophotometer (CAF-100, Japan Spectroscopic Co., Tokyo). At the end of the experiment, the maximum and minimum fluorescence levels were determined using 5 μM ionomycin and 4 mM EGTA, respectively, as reported previously (Ozaki *et al.*, 1987c; Sato *et al.*, 1988). However, the absolute value for $[Ca^{2+}]_{cyt}$ was not calculated because the dissociation constant for fura-2 and Ca^{2+} in cytoplasm may be different from that obtained *in vitro* (Konishi *et al.*, 1988). Instead, the ratio of these two fluorescence ($R_{340/380}$) was used as an indicator of relative $[Ca^{2+}]_{cyt}$ (Ozaki *et al.*, 1987c; Sato *et al.*, 1988).

To prepare the saponin-skinned taenia preparation, a thin bundle of taenia (0.2 mm in width and 1.5 mm in length) was treated with 100 μg ml^{-1} saponin for 30–40 min in a solution containing (mM): KCl 136.9, $MgCl_2$ 5.0, EGTA 2.0, Tris maleate 20.0 (pH 6.8) and Na_2ATP 5, at 24°C. The Ca^{2+} concentration of this solution was changed by use of Ca^{2+}-EGTA buffers.

$^{45}Ca^{2+}$ uptake was measured as described by Karaki & Weiss (1979). Rabbit aortic strips were incubated in a solution containing $^{45}Ca^{2+}$ (1 μCi ml^{-1}) for 5 min. Okadaic acid was added 60 min before the addition of $^{45}Ca^{2+}$, and 65.4 mM K^+ was added simultaneously with $^{45}Ca^{2+}$. After the $^{45}Ca^{2+}$ incubation, muscle strips were washed for 30 min with an ice-cold lanthanum solution containing 73.8 mM $LaCl_3$, 5.5 mM glucose and 24 mM Tris adjusted to pH 6.9 at 0°C. $^{45}Ca^{2+}$ in the strips was extracted overnight with 1 ml of 20 mM EGTA solution. Scintillation mixture (ACS II, Amersham Japan, Tokyo, 1 ml) was added to the extract and radioactivity was counted with a liquid scintillation spectrometer (Packard Tri-Carb 3380, U.S.A.).

The adenosine 3' : 5'-cyclic monophosphate (cyclic AMP) content of rabbit aorta was measured as described previously (Abe & Karaki, 1988). After a 60 min incubation with 1 μM okadaic acid, muscle strips were removed from the bath, rapidly frozen in liquid N_2 and homogenized for 30 s in 6% trichloroacetic acid. Cyclic AMP in the extract was measured by radioimmunoassay using a commercial assay kit (Yamasa Shoyu, Tokyo).

Platelets

Fresh blood was obtained from male rabbits and mixed immediately with 1/10 volume of trisodium citrate solution (3.8 w/v%). The citrated blood was centrifuged at 90 *g* for 15 min to obtain platelet-rich plasma (PRP). PRP was then washed by centrifugation at 800–900 *g* for 10 min and the supernatant was removed. The pellet was resuspended in normal solution. This washing procedure was repeated three times. Normal solution contained (mM): NaCl 145, KCl 5, $MgSO_4$ 1.0, NaH_2PO_4 0.5, glucose 5.0, EDTA 0.001 and HEPES 10.0 adjusted to pH 7.4 with NaOH. The platelet suspension was restored to a final density of approximately 3×10^5 cells ml^{-1}. Aggregation was assessed by a standard turbidimetric technique using a spectrophotometer equipped with a stirrer (UVDEC-460, Japan Spectroscopic Co., Tokyo). Three min after the addition of 1.0 mM $CaCl_2$ at 37°C, thrombin was applied for 5 min and aggregation was measured. Okadaic acid was added simultaneously with $CaCl_2$. At the end of the incubation, the amount of ATP released in the external medium was measured with a luciferin–luciferase method (Strehler & McElroy, 1957) using a photomultiplier (TD-4000, Lab-Science, Tokyo).

Ca^{2+} signals in platelets were measured with fura-2. The PRP was centrifuged and the pellet was resuspended in a normal solution containing 1 μM fura-2/AM and incubated for 10 min at 37°C. Platelets were then washed twice with a normal solution to remove extracellular fura-2/AM. Changes in fura-2-Ca^{2+} fluorescence were measured with a spectrofluorometer (FP-2060, Japan Spectroscopic). Platelets were excited at a wavelength of 340 nm and emission was measured at 500 nm. At the end of the experiments, the maximum and minimum fluorescence levels were determined using 0.2% Triton X-100 and 10 mM EGTA at pH 8.5, respectively, and the $[Ca^{2+}]_{cyt}$ was calculated as reported previously (Pollock *et al.*, 1986). However, we used only relative $[Ca^{2+}]_{cyt}$ in the present results because of the uncer-

tainty of the intracellular dissociation constant of fura-2 for Ca^{2+} (Konishi *et al.*, 1988).

Statistics

Experiments were repeated on at least 4 preparations obtained from 4 different animals. Results of the experiments are expressed as mean ± s.e.mean. Student's *t* test was used for statistical analysis of the results.

Drugs and chemicals

Okadaic acid was isolated from *Halichondria okadai* as described by Tachibana *et al.* (1981) and dissolved in 100% ethanol. (±)-Noradrenaline bitartrate (Wako Pure Chemicals, Tokyo, Japan), thrombin (Sigma, St. Louis, MO, U.S.A.), ionomycin and forskolin (Calbiochem, San Diego, CA, U.S.A.), Tris (Sigma), EGTA (Wako), saponin (ICN, Cleveland, OH, U.S.A.), cremophor EL (Nakarai Chemicals, Kyoto, Japan), fura-2/AM (Dojindo Laboratories, Kumamoto, Japan) and $^{45}CaCl_2$ (Amersham Japan, Tokyo) were used.

Results

Smooth muscle contraction

Addition of 0.1–1 μM okadaic acid did not change the resting tone whereas 100 μM okadaic acid induced a sustained contraction in rabbit aorta as has been reported (Shibata *et al.*, 1982). As shown in Figure 1, addition of 0.1 to 1 μM okadaic acid during the high K^+- or noradrenaline-induced sustained contraction slowly decreased muscle tension. The cumulative concentration-response curves for KCl and noradrenaline in rabbit aorta were shifted to the right and downwards in the presence of 0.1–1 μM okadaic acid (Figure 2a and b). In a Ca^{2+}-free, high K^+ solution, cumulative addition of $CaCl_2$ induced a concentration-dependent contraction. This contraction was also inhibited by okadaic acid (Figure 2c).

In a Ca^{2+}-free solution, noradrenaline induced a transient contraction which is due to release of intracellular Ca^{2+} (Karaki, 1987). The peak tension level of the noradrenaline-induced transient contraction was 65.2 ± 5.4% ($n = 4$) of that in the presence of 1.5 mM Ca^{2+}. After a 120 min resting period in normal solution, the second application of noradrenaline in a Ca^{2+}-free solution induced a transient contraction with similar magnitude as that of the first transient contraction (68.8 ± 4.8%, $n = 4$). In the aorta pretreated with 1 μM okadaic acid for

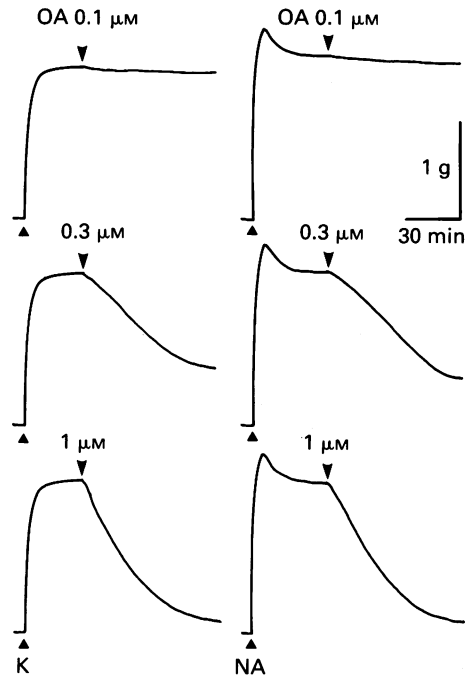


Figure 1 Inhibitory effect of 0.1–1 μM okadaic acid (OA) on the sustained contractions induced by 65.4 mM KCl (K, left) or 1 μM noradrenaline (NA, right) in rabbit aorta.

60 min, the peak tension level decreased to 9.0 ± 3.3% ($n = 6$, $P < 0.01$), as shown in Figure 3.

In guinea-pig taenia, addition of 1 μM okadaic acid inhibited the spontaneous rhythmic contractions. The high K^+ (45.4 mM)-induced contraction was also inhibited by 1 μM okadaic acid to 5.2 ± 3.1% of the contraction before the addition of okadaic acid ($n = 6$, $P < 0.01$) (Figure 4).

In saponin-skinned taenia, addition of 0.1–10 μM okadaic acid during the sustained contraction induced by 3 μM Ca^{2+} did not change the muscle tension but rather potentiated it, as reported previously (Ozaki *et al.*, 1987a).

The effects of okadaic acid on contraction and $[Ca^{2+}]_{cyt}$ in rabbit aorta are shown in Figure 5. High K^+ (27.8 mM) induced a sustained increase in both muscle tension and $[Ca^{2+}]_{cyt}$. Okadaic acid (1 μM) gradually and strongly inhibited the high K^+-induced contraction. The peak tension level of the high K^+-induced contraction measured 18 min after the addition of 1 μM okadaic acid was 18.2 ± 5.3% ($n = 4$) of that before the addition of okadaic acid. However, okadaic acid inhibited the high K^+-stimulated $[Ca^{2+}]_{cyt}$ only to 77.1 ± 5.3% ($n = 4$).

In rabbit aorta, high K^+ increased $^{45}Ca^{2+}$ uptake

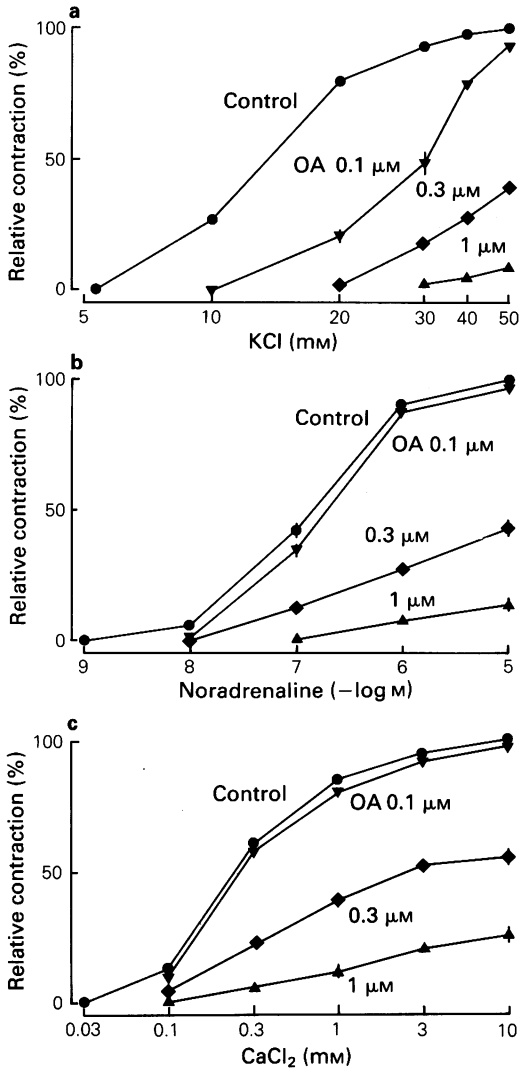


Figure 2 Cumulative concentration-response curves for KCl (a), noradrenaline (b) or $CaCl_2$ (c) in the absence or presence of okadaic acid in rabbit aorta. In (c), muscle strips were pretreated for 15 min with a Ca^{2+}-free, 55.4 mM KCl solution. Okadaic acid (OA; 0.1, 0.3 or 1 μM) was added 60 min before the cumulative addition of the stimulants. Each curve represents mean of 6 experiments and s.e.mean is shown by vertical lines.

(during 5 min) from a resting level of 78.7 ± 2.4 nmol g^{-1} ($n = 6$) to 144.7 ± 6.6 nmol g^{-1} ($n = 6$, $P < 0.01$). Okadaic acid (1 μM) changed neither the resting $^{45}Ca^{2+}$ uptake (82.7 ± 7.0 nmol g^{-1}, $n = 6$) nor the high K^+-stimulated $^{45}Ca^{2+}$ uptake (154.3 ± 4.3 nmol g^{-1}, $n = 6$).

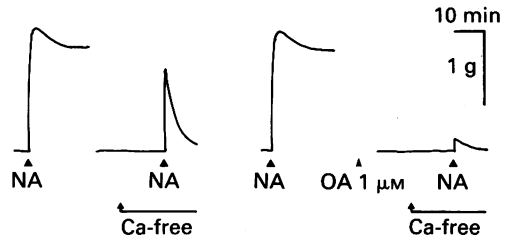


Figure 3 Inhibitory effect of 1 μM okadaic acid (OA) on 1 μM noradrenaline (NA)-induced transient contraction in the absence of external Ca^{2+} in rabbit aorta. Okadaic acid was added 60 min before the addition of noradrenaline and Ca^{2+} was removed 15 min before the addition of noradrenaline.

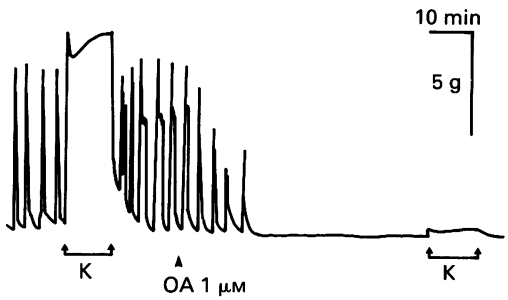


Figure 4 Inhibitory effect of 1 μM okadaic acid (OA) on guinea-pig taenia. K: 45.4 mM KCl.

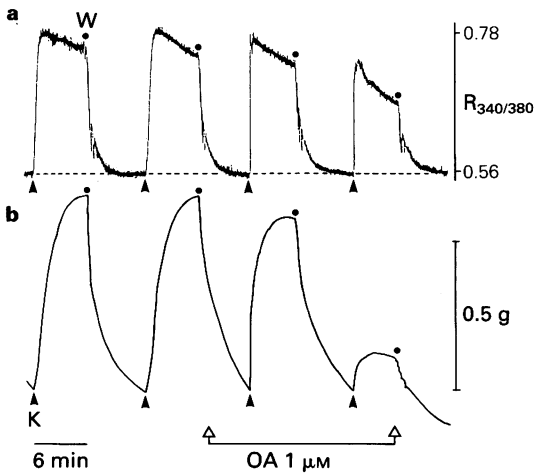


Figure 5 Inhibitory effect of 1 μM okadaic acid (OA) on relative $[Ca^{2+}]_{cyt}$ (a; shown by $R_{340/380}$, see Methods) and contraction (b) induced by 27.8 mM KCl (K) in rabbit aorta. High K^+ was applied at ▲ and removed at ●.

The cyclic AMP content of rabbit aorta was 121.9 ± 16.0 pmol g^{-1} wet weight ($n = 6$). After a 60 min incubation with 1 μM okadaic acid, this value did not change significantly (105.3 ± 7.2, $n = 6$, $P > 0.05$) although 3 μM forskolin, which almost completely inhibited the 1 μM noradrenaline-induced contraction, increased the cyclic AMP content to 459.1 ± 69.1 pmol g^{-1} wet weight ($n = 6$, $P < 0.01$).

Platelets

Figure 6 shows the effects of okadaic acid on platelet aggregation and $[Ca^{2+}]_{cyt}$. Thrombin, 1 u ml^{-1}, induced aggregation and increased $[Ca^{2+}]_{cyt}$. Okadaic acid (1 μM) itself did not change the platelet activities but strongly inhibited both the thrombin-induced aggregation (to $8.1 \pm 1.4\%$, $n = 6$) and $[Ca^{2+}]_{cyt}$ (to $17.2 \pm 2.2\%$, $n = 6$). Okadaic acid (1 μM) also strongly inhibited the thrombin-induced ATP-release (to $20.7 \pm 7.4\%$, $n = 7$). A lower concentration (0.1 μM) of okadaic acid was almost ineffective.

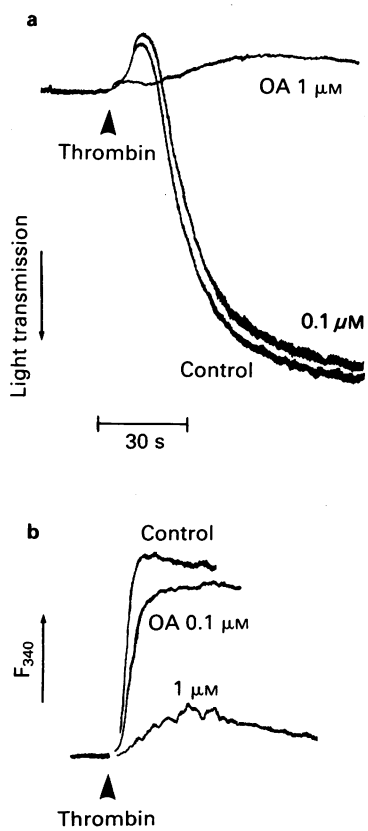


Figure 6 Inhibitory effect of okadaic acid (OA, 0.1 or 1 μM) on aggregation (a, shown by light transmission) and $[Ca^{2+}]_{cyt}$ (b, shown by F_{340}, see Methods) in rabbit platelets stimulated by thrombin (1 u ml^{-1}).

Discussion

Present results showed that 0.1–1 μM okadaic acid inhibited contractions in intact vascular and intestinal smooth muscles. However, the same concentration of okadaic acid did not inhibit the Ca^{2+}-induced contraction in skinned smooth muscle (Ozaki *et al.*, 1987a). Previously, it was shown that higher concentrations of okadaic acid (10 μM or higher) induced contraction in smooth muscle of rabbit aorta and guinea-pig taenia (Shibata *et al.*, 1982) which was attributable to the Ca^{2+}-independent phosphorylation of myosin light chain (Ozaki *et al.*, 1987b). These results indicate that okadaic acid has a dual effect on smooth muscle contraction that is dependent on the concentration of okadaic acid; lower concentrations inhibit contractions and this is not attributable to a direct effect on the contracile elements, whereas higher concentrations directly activate the contracile elements.

Contraction of smooth muscle is mediated by an increase in $[Ca^{2+}]_{cyt}$ followed by the activation of the Ca^{2+}/calmodulin-myosin light chain kinase system. This enzyme phosphorylates myosin light chain to induce contraction. When $[Ca^{2+}]_{cyt}$ is decreased, kinase activity is decreased and the phosphorylated myosin light chain is dephosphorylated by a phosphatase resulting in relaxation of smooth muscle (Hartshorne & Mrwa, 1982). Ca^{2+} is supplied by influx of extracellular Ca^{2+} by the opening of Ca^{2+} channels or by the release of Ca^{2+} from storage sites (Karaki & Weiss, 1988). The inhibitory effect of okadaic acid was accompanied by a small decrease in $[Ca^{2+}]_{cyt}$ but not by a decrease in $^{45}Ca^{2+}$ uptake, indicating that the decrease in $[Ca^{2+}]_{cyt}$ is only partially responsible for the inhibition.

A possible mechanism for the okadaic acid-induced inhibition is the decrease in Ca^{2+}-sensitivity of the contracile elements. It has been shown that cyclic AMP-dependent protein kinase phosphorylates myosin light chain kinase, inhibits the activity of this enzyme and inhibits smooth muscle contractions (Pfitzer *et al.*, 1985). In vascular smooth muscle, forskolin, an activator of adenylate cyclase, inhibits contraction with a small decrease in $[Ca^{2+}]_{cyt}$ (Abe & Karaki, 1989) and no change in $^{45}Ca^{2+}$ uptake (Hwang & Van Breemen, 1987). These effects are quite similar to those of 1 μM okadaic acid. However, okadaic acid does not seem to activate directly adenylate cyclase nor inhibit phosphodiesterase because the concentration of cyclic AMP was not altered by the okadaic acid

treatment. Recently, it was found that okadaic acid inhibits various phosphatases at different concentrations (Bialojan & Takai, 1988; Ishihara *et al.*, 1989). It is possible that okadaic acid inhibits the phosphatase which dephosphorylates the cyclic AMP-dependent phosphorylation and thus augments the cyclic AMP-dependent phosphorylation. Since cyclic GMP has similar inhibitory effects to cyclic AMP on smooth muscle contraction and $[Ca^{2+}]_{cyt}$ (Karaki *et al.*, 1988) and myosin light chain phosphorylation (Pfitzer *et al.*, 1986), okadaic acid may also augment cyclic GMP-dependent phosphorylation by inhibiting the phosphatase activity.

Activation of platelets also involves Ca^{2+}-dependent pathways: the Ca^{2+} and calmodulin-dependent myosin light chain and protein kinase C (Feinstein *et al.*, 1985). Okadaic acid inhibited the thrombin-induced aggregation, ATP release and the increase in cellular Ca^{2+} induced by thrombin. These effects are again similar to those of cyclic AMP (Feinstein *et al.*, 1985) suggesting the participation of similar mechanisms as those in smooth muscle. Further experiments are necessary to know the precise mechanism of the inhibitory action of okadaic acid.

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Do both adrenaline and noradrenaline stimulate cardiac α-adrenoceptors to induce positive inotropy of rat atria?

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1 The positive inotropic responses of rat paced left atria to adrenaline and noradrenaline were recorded. Desmethylimipramine (DMI, 1 μM) and metanephrine (10 μM) were initially present throughout.

2 The positive chronotropic responses of spontaneously beating right atria to adrenaline were used as a reference. In these, pindolol, in increasing concentrations, caused progressive shift of the concentration-response curves to the right, which yielded a pA_2 value (8.15) compatible with antagonism of β-adrenoceptors.

3 The left atrial tension responses to adrenaline showed an initial progressive displacement by pindolol (up to 3 μM) which gave an unexpectedly low pA_2 value (6.48). However, with further increases in pindolol concentration there was no additional shift of the curve. In the presence of pindolol (3 μM), prazosin (0.1 μM) displaced the curve to the right but the pA_2 value derived from this shift (7.75) was less than expected for α_1-adrenoceptor antagonism.

4 When the experiments in the presence of pindolol (3 μM) were repeated in the absence of DMI, prazosin displaced the concentration-response curves for adrenaline-induced left atrial tension to a greater extent and the pA_2 value (8.76) was now compatible with adrenaline stimulating typical α_1-adrenoceptors.

5 The concentration-response curves for noradrenaline-induced left atrial tension were also progressively displaced to the right by pindolol (0.1, 0.3 and 1.0 μM). These concentrations yielded a Schild plot of unity slope and a pA_2 value of 7.94 ± 0.04. This was not significantly different from the pA_2 value of 8.02 ± 0.07 determined for pindolol against isoprenaline in the left atria, which indicates a normal interaction of noradrenaline with β-adrenoceptors in the absence and presence of low concentrations of pindolol.

6 A further increase in the concentration of pindolol to 3 μM failed to induce an additional shift of the noradrenaline curves, whether a 'before and after' antagonist or a 'naïve tissue' design was adopted. Similarly, the rightwards shift of the concentration-response curves by timolol reached a limit as the concentration was increased. In all cases the limit of shift occurred at a noradrenaline EC_{50} value of 5–10 μM.

7 At the limit of β-adrenoceptor antagonism, prazosin and dibenamine did not displace the noradrenaline curves further. The residual inotropic response to noradrenaline therefore appeared to be mediated via neither α- nor β-adrenoceptors.

8 DMI, in the absence of β-blockade, produced the potentiation of adrenaline and noradrenaline expected of a neuronal uptake inhibitor. However, in the presence of pindolol, there was no potentiation of the right atrial rate response to adrenaline while its left atrial tension responses were antagonized. This suggested that DMI was acting as an α-adrenoceptor antagonist. It also explained the less-than-expected shift by prazosin of the adrenaline responses in the presence of both pindolol and DMI, the latter drug already exerting some α-blocking activity. In contrast, the left atrial tension responses to noradrenaline in the presence of pindolol (1 μM) were neither potentiated nor antagonized by DMI.

9 When the effects of prazosin upon left atrial tension responses to noradrenaline in the presence of pindolol (10 μM) were examined in the presence of a lower concentration of DMI (0.1 μM) or cocaine (10 μM), again there was no further shift of the curve. However, when the effect of prazosin

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was examined in the absence of DMI, but in the presence of pindolol (1 and 10 μM) or timolol (3 μM), there was a small shift of the curves by prazosin (0.1 μM). This yielded pA_2 values of 7.19, 7.34 $\pm$ 0.1 and 7.66 $\pm$ 0.09, which were at least one order of magnitude less than literature values and that obtained with adrenaline (8.76 $\pm$ 0.18), and are not consistent with noradrenaline stimulating an α_1-adrenoceptor.

10 Thus in the presence of β-adrenoceptor blockade, the increase in left atrial tension by noradrenaline does not appear to be mediated by β- or typical α-adrenoceptors. This is in contrast to adrenaline which in these conditions stimulates typical α_1-adrenoceptors.

Introduction

Myocardial α-adrenoceptors have been shown to exist by radioligand binding techniques (Williams & Lefkowitz, 1978; Karliner *et al.*, 1979; Schümann & Brodde, 1979; Steinberg & Bilezikian, 1982) and selective α-adrenoceptor agonists have been shown to exert positive inotropic responses. For example, phenylephrine (Wagner & Brodde, 1978; Shibata *et al.*, 1980; Stene-Larsen, 1980; Williamson & Broadley, 1987) and methoxamine (Shibata *et al.*, 1980; Ask & Stene-Larsen, 1984; Williamson & Broadley, 1987) increase the force of contraction of isolated left atria of rats and this response is susceptible to α-adrenoceptor blockade by phentolamine (Shibata *et al.*, 1980; Stene-Larsen, 1980) and prazosin (Williamson & Broadley, 1987).

Whether the endogenous catecholamines adrenaline and noradrenaline also stimulate cardiac α-adrenoceptors to produce positive inotropic responses is less certain. Their cardiac effects are mediated predominantly via β_1-adrenoceptors, but evidence is accumulating that stimulation of α-adrenoceptors may also contribute to the inotropic responses of both adrenaline and noradrenaline in rat atria (Ask & Stene-Larsen, 1984) and papillary muscles (Skomedal & Osnes, 1983). Earlier studies from Wagner and Schümann's group had shown that adrenaline, but not noradrenaline, stimulated α-adrenoceptors of rabbit papillary muscles (Schümann *et al.*, 1974) and human atria (Wagner *et al.*, 1980). However, this has subsequently been disputed, since noradrenaline has also been shown to exert an inotropic action via α-adrenoceptors in the presence of β-adrenoceptor blockade in rabbit papillary muscles (Aass *et al.*, 1983) and human atria (Skomedal *et al.*, 1985) and papillary muscles (Aass *et al.*, 1986). The α-adrenoceptor-mediated changes became apparent when the qualitative characteristics of individual contraction-relaxation cycles were analysed. A similar approach was used for the guinea-pig atria, in which analysis of the time-course of the response to single doses revealed an α-adrenoceptor-mediated component of the inotropic response to noradrenaline (Hull, 1986). Only in the study published by Ask & Stene-Larsen (1984) does an α-adrenoceptor-mediated inotropic response to noradrenaline appear to have been demonstrated in rat atria, by measurement of the maximum developed tension from cumulative concentration-response curves.

Thus, it is generally agreed that adrenaline stimulates cardiac α-adrenoceptors in most species, but only in the presence of β-adrenoceptor blockade, the degree of which varies considerably between studies. The situation with noradrenaline, however, remains controversial. In the present study, the maximum developed tension in response to adrenaline and noradrenaline was recorded from rat paced left atria. This tissue has been shown in these laboratories to be rich in α-adrenoceptors with substantial inotropic responses to selective α-adrenoceptor agonists (Chess-Williams & Broadley, 1987). A wide range of β-adrenoceptor antagonist concentrations was employed in an attempt to identify a 'window' where any α-adrenoceptor-mediated positive inotropic responses appear. A preliminary account of some of these findings has been presented to the British Pharmacological Society (Broadley, *et al.*, 1985).

Methods

Isolated atrial preparations

Male rats (Wistar, Animal House bred) of weight range 250–400 g were killed by a blow to the head. Separated left and right atria were rapidly removed and suspended in organ baths containing Krebs-bicarbonate solution (composition in mmol l^{-1}: NaCl 118.4, $NaHCO_3$ 25.0, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $CaCl_2 \cdot 2H_2O$ 1.9 and glucose 11.7) gassed with 95% O_2 plus 5% CO_2, and maintained at 30°C. Left atria were secured to bipolar platinum electrodes and a cotton thread through the atrial appendage was connected to a Devices strain gauge transducer (UFI, 57 g sensitivity range). Left atria were paced at 2 Hz with square-wave pulses of threshold (+50%) voltage and 5 ms pulse width with an SRI stimulator (Type 6053).

Right atria were attached to a tissue holder by means of cotton thread through the inferior vena

cava and atrioventricular junction. Cotton through the superior vena cava connected the atria to the transducer. Resting diastolic tensions of 0.5 g (right atria) and 0.8 g (left atria) were applied, and isometric tensions were recorded on a Devices M19 polygraph (Lectromed, Welwyn Garden City). The spontaneous rate of contraction of right atria was recorded from the tension signal by means of a ratemeter (Devices, Type 2751).

Experimental protocols

Tissues were allowed to equilibrate for 45–60 min, with changes of the bathing medium every 10–15 min before drug challenge. Metanephrine (10 μM) and unless otherwise indicated, desmethylimipramine (DMI, 1 μM), were present throughout the experiments to inhibit extraneuronal and neuronal uptake respectively.

Cumulative concentration-response curves were constructed by adding adrenaline or noradrenaline in half logarithmic increments of concentration, until no further effect was observed. Two experimental protocols were adopted. In the first, a single curve was obtained either in the absence or in the presence of pindolol, timolol or prazosin or a combination of prazosin with pindolol or timolol ('naïve tissues'). The second approach involved establishing a concentration-response curve for the agonist and, after washout to restore the resting levels, the antagonist under test was added to the bath. A second curve was then constructed in the presence of the inhibitor ('before and after method'). Control experiments were performed in which the inhibitor was omitted from the bath between the two curves.

All inhibitors were left in contact with the tissue for 30 min before construction of an agonist concentration-response curve.

Analysis of data

In experiments in which the first procedure was used ('naïve tissues'), increases in tension above the resting level were measured at each concentration of adrenaline or noradrenaline. These were expressed and plotted as a percentage of the maximum response.

In experiments where two concentration-response curves were obtained in each tissue ('before and after' method), the responses of the first curve were corrected for time-dependent changes in sensitivity occurring during the experiment, by application of correction factors derived from the appropriate control experiments. The mean ($n \geqslant 4$) total tension developed at each concentration on the second curve of control experiments was expressed as a fraction of the total tension at the same concentration on the first curve. These factors were then applied to the total tensions at the respective concentrations on individual pre-antagonist curves of test experiments. Increases in tension above the resting level (corrected in the case of the first curve) were then plotted as a percentage of their respective maxima.

Molar EC_{50} values from individual curves were determined by linear interpolation between points on either side of the 50% response. The geometric mean EC_{50} value was calculated together with the 95% confidence limits. In 'before and after' experiments, individual dose-ratios (DR) were calculated at the EC_{50} values. These were employed to calculate pA_2 values from the equation $pA_2 = \log (DR - 1) - \log M$, where M is the molar concentration of antagonist (Mackay, 1978; O'Donnell & Wanstall, 1979). A mean pA_2 ($\pm$s.e.mean) was then determined. Where more than one concentration of antagonist was employed, pA_2 values were also calculated by plotting the mean $\log (DR - 1)$ against $\log M$, the pA_2 being derived from the intercept on the concentration axis (Arunlakshana & Schild, 1959).

Statistical comparisons of geometric mean EC_{50} values (with 95% confidence limits) from naïve tissues and of arithmetic mean dose-ratios ($\pm$s.e.mean) and pA_2 values ($\pm$s.e.mean) from 'before and after' experiments were made by Student's unpaired t test.

Drugs used

The following drugs were used: (−)-adrenaline bitartrate (Hopkin & Williams), cocaine hydrochloride (Macarthys), desmethylimipramine hydrochloride (Sigma) or hydrobromide (Ciba Geigy), dibenamine hydrochloride (K & K Labs, California), haloperidol base (Searle), (±)-metanephrine hydrochloride (Sigma), (−)-noradrenaline bitartrate (Sigma), (±)-pindolol (Sandoz), prazosin hydrochloride (Pfizer) and (±)-timolol maleate (Merck, Sharp & Dohme). All solutions were freshly prepared in distilled water with ascorbic acid (1 $\mu g\,ml^{-1}$) being added to the catecholamines to prevent oxidation and to the pindolol to aid solution. Prazosin required sonication to dissolve.

Results

β-Adrenoceptor antagonism of adrenaline with pindolol ('naïve tissues')

Adrenaline caused concentration-dependent increases in left atrial tension and right atrial rate. Increasing concentrations of pindolol caused progressive rightward shifts of the concentration-response curves for the rate responses to adrenaline

Table 1 Geometric mean EC_{50} values (μM) for the increase in rate and tension of rat right and left atria respectively in response to adrenaline

Antagonist	*Right atrial rate*	n	*Left atrial tension*	n
None	0.07 (0.04–0.12)	5	0.77 (0.30–1.99)	5
Pindolol 0.3 μM	1.86 (0.13–27.5)*	4	1.17 (0.07–20.4)	4
Pindolol 1.0 μM	11.7 (2.39–57.5)*	5	3.39 (0.77–14.8)*	5
Pindolol 3.0 μM	24.0 (13.1–44.1)*	8	12.3 (6.87–22.0)*	8
Pindolol 10.0 μM	148.0 (85.7–255)*	4	10.5 (0.98–112)*	4
Prazosin 0.01 μM	0.07 (0.03–0.16)	6	0.27 (0.12–0.61)	6
Prazosin 0.1 μM	0.13 (0.06–0.33)	6	0.39 (0.13–1.1)	6
Pindolol 3.0 μM + prazosin 0.1 μM	46.8 (14.6–150)*	6	81.3 (37.5–176)*†	6

A single concentration-response curve was obtained either in the absence or in the presence of pindolol and/or prazosin.
Geometric mean EC_{50} values and their 95% confidence limits are shown. A significant difference ($P < 0.05$), as determined by Student's unpaired t test, between values in the absence and presence of antagonist is indicated by * and between 3 μM pindolol alone and in combination with 0.1 μM prazosin by †.

(Table 1). An Arunlakshana & Schild (1959) plot was constructed from the dose-ratios determined for these shifts (Figure 1). The slope of the regression line (1.19 ± 0.16) did not differ significantly ($P > 0.05$) from unity and a pA_2 value was therefore calculated for each concentration of pindolol from the equation $pA_2 = \log (DR - 1) - \log M$, where DR is the dose-ratio and M is the molar concentration of pindolol (Mackay, 1978). The mean pA_2 value for the antagonism of the right atrial rate responses to adrenaline by pindolol was 8.15 ± 0.09 ($n = 4$ points).

The same concentrations of pindolol were examined against the left atrial tension responses to adrenaline. There was an initial progressive rightward displacement of the concentration-response curves for adrenaline up to a pindolol concentration of 3 μM. These shifts were, however, substantially less than for the right atrial rate responses (Table 1). The Schild regression for these first three concentrations of pindolol had a correlation coefficient of 1.0 and a slope of 1.47 (Figure 1). The pA_2 value derived from these three concentrations of pindolol was 6.48 ± 0.14, which was significantly less ($P < 0.05$) than for the rate responses.

An increase in the concentration of pindolol to 10 μM produced no further shift of the concentration-response curves; the EC_{50} values for adrenaline in the presence of 3 and 10 μM pindolol did not differ significantly ($P < 0.05$) (Table 1). This concentration was therefore omitted from the Schild plot (Figure 1).

β-Adrenoceptor antagonism of noradrenaline with pindolol

'Naïve tissues' Noradrenaline increased the left atrial tension and in the absence of antagonist, the concentration-response curves were monophasic up to a concentration of 3 μM, at which the maximum response was obtained (Figure 2). Pindolol displaced the concentration-response curves for noradrenaline to the right. However, 3 μM pindolol did not displace the curve any further than 1 μM pindolol (Figure 2). An increase in pindolol concentration to 10 μM did achieve a further shift, however, the degree of antagonism was no greater when the concentration was increased again to 30 μM (Figure 2). Thus there appeared to be at least two 'stops' in the shifts of the noradrenaline concentration-response curves by pindolol.

'Before and after' method For this experimental design, lower concentrations of pindolol were used than had been used for the naïve tissue experiments. As the concentration of pindolol was increased from 0.1 to 1.0 μM, a progressive shift of the concentration-

Table 2 Mean dose-ratios for the shifts of concentration-response curves for the increase in left atrial tension in response to noradrenaline by pindolol alone or in combination with prazosin

Antagonist	*Dose-ratio*	n
Pindolol 0.1 μM	9.75 ± 2.52	4
Pindolol 0.3 μM	32.38 ± 6.93	4
Pindolol 1.0 μM	94.86 ± 16.36	4
Pindolol 3.0 μM	117.86 ± 13.12^{NS}	4
Pindolol 1.0 μM + prazosin 0.1 μM	111.6 ± 30.8^{NS}	4

Dose-ratios were measured between pre-antagonist (corrected from controls) and post-antagonist curves at the EC_{50}. NS indicates no significant difference ($P > 0.05$) from the dose-ratio with pindolol (1.0 μM) by Student's unpaired t test.

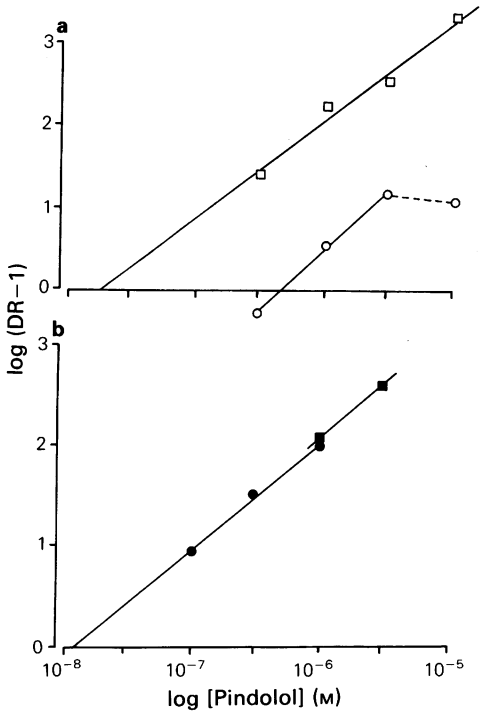


Figure 1 Schild plots for the antagonism by pindolol of (a) left atrial tension (○) and right atrial rate (□) responses to adrenaline and (b) left atrial tension responses to noradrenaline (●) and isoprenaline (■). Mean values for log (DR − 1) are plotted against log molar concentration of pindolol, where DR is the dose-ratio for the shift of the concentration-response curve measured at the EC_{50}. Only the values obtained with 0.3, 1 and 3 μM pindolol have been included in the linear regression determination for the tension responses to adrenaline. Each point represents the mean value of at least 4 determinations.

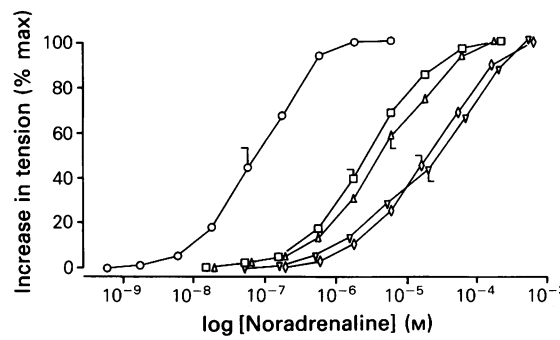


Figure 2 Mean concentration-response curves for the increase in tension of rat left atria in response to noradrenaline. Each tissue was exposed to one curve for noradrenaline either in the absence (○) or presence of pindolol 1 μM (△, $n = 12$), 3 μM (□, $n = 16$), 10 μM (▽, $n = 7$) or 30 μM (◇, $n = 4$). Increases in tension are plotted as a percentage of the maximum response and vertical lines show s.e.mean.

response curves for the left atrial tension response was achieved, as shown by the increasing dose-ratios (Table 2). However, when the concentration of pindolol was increased to 3.0 μM, there was no further shift of the concentration-response curves for noradrenaline. This failure of 3.0 μM pindolol to displace further the curves agrees with the observations made in the naïve tissue design.

The dose-ratios (DR) for the progressive shifts of the concentration-response curves by pindolol up to 1.0 μM were used to construct the Arunlakshana & Schild (1959) plot, the slope of which (1.04 ± 0.15) did not differ significantly ($P > 0.05$) from unity (Figure 1). The mean pA_2 value, calculated from these 3 concentrations of pindolol by the use of the equation $pA_2 = \log (DR - 1) - \log M$, was 7.94 ± 0.04 ($n = 12$).

As a check that the antagonism by pindolol was typical of competitive blockade of the atrial β-adrenoceptors, the two concentrations (1 and 3 μM that caused the same degree of antagonism of noradrenaline were used to antagonize isoprenaline. The 'before and after' antagonist design was used. The dose-ratios were used to construct a Schild plot (Figure 1) which formed an extrapolation of that obtained for noradrenaline and had a slope (1.05 ± 0.32) which did not differ significantly from unity ($P > 0.05$). The mean pA_2 value calculated from the equation was 8.02 ± 0.07 ($n = 8$), which was not significantly different ($P > 0.05$) from the value obtained with noradrenaline or with adrenaline on right atrial rate.

β-Adrenoceptor antagonism of noradrenaline with timolol ('naïve tissues')

The concentration-response curve for the left atrial tension response to noradrenaline was displaced to the right by timolol (0.1 μM) (Figure 3). An increase in concentration to 0.3 μM caused a further small non-significant ($P > 0.05$) shift to the right, but thereafter, no additional shift of the concentration-response curve occurred (Figure 3). Thus, as with pindolol, there was a limit to the β-adrenoceptor antagonism, although in the case of timolol there was only one. This limit occurred at an EC_{50} value for noradrenaline of between 5 and 10 μM, which compared with the first limit of shift by pindolol (1 and 3 μM) at a noradrenaline EC_{50} value of approximately 5 μM. In fact, the EC_{50} values in the presence of 1 μM pindolol (4.47(2.15–9.29) μM, $n = 12$) and

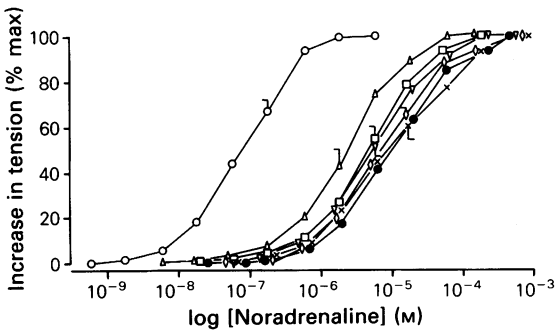


Figure 3 Mean concentration-response curves for the increase in tension of rat left atria in response to noradrenaline. Each tissue was exposed to one curve for noradrenaline either in the absence (○, $n = 6$) or presence of timolol 0.1 μM (△, $n = 4$), 0.3 μM (□, $n = 4$), 1 μM (▽, $n = 8$), 3 μM (◇, $n = 8$), 10 μM (×, $n = 6$) or 30 μM (●, $n = 6$). Increases in tension are plotted as a percentage of the maximum response and vertical lines show s.e.mean.

0.3 μM timolol (5.01(2.74–9.18) μM, $n = 4$) were not significantly different ($P > 0.05$).

α-Adrenoceptor antagonism of adrenaline with prazosin ('naïve tissues')

In the absence of β-adrenoceptor blockade by pindolol, prazosin 0.01 and 0.1 μM failed to displace the concentration-response curves for either the positive inotropic or positive chronotropic responses to adrenaline. The EC_{50} values obtained in the presence of prazosin were not significantly different ($P > 0.05$) from the values in its absence (Table 1).

To determine whether α-adrenoceptors were involved in the positive inotropic response to adrenaline at the point where pindolol produced no further shift of the concentration-response curves, the combined effects of pindolol (3 μM) and prazosin (0.1 μM) were examined. When compared with the adrenaline concentration-response curve obtained in the presence of pindolol (3 μM) alone, the additional presence of prazosin had no effect on the right atrial rate response to adrenaline (Figure 4a). The EC_{50} values in the presence of pindolol (3 μM) alone and pindolol (3 μM) plus prazosin (0.1 μM) were not significantly different ($P > 0.05$) (Table 1).

By contrast, the left atrial positive inotropic responses to adrenaline in the presence of pindolol (3 μM) were further antagonized by the additional presence of prazosin (0.1 μM) (Figure 4b). Although increasing the pindolol concentration to 10 μM failed to shift the curve further, prazosin significantly increased the EC_{50} value ($P < 0.05$) from 12.3 to

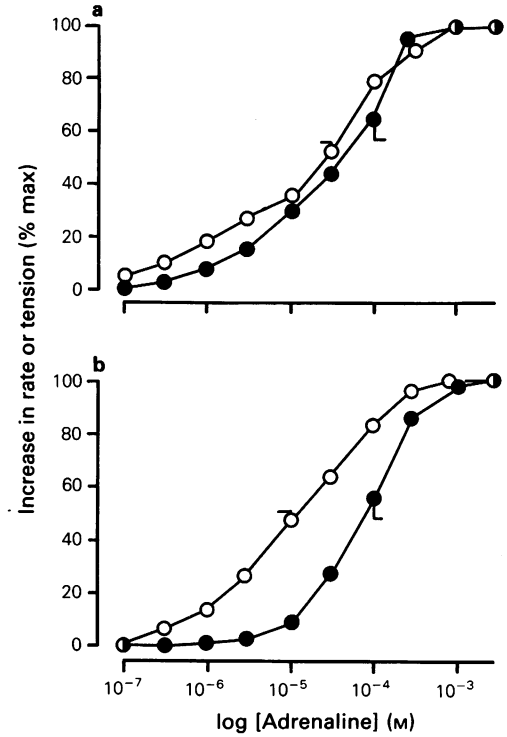


Figure 4 Mean concentration-response curves for (a) the increase in right atrial rate and (b) the increase in left atrial tension in response to adrenaline. One curve was obtained in each preparation either in the presence of pindolol (3 μM) alone (○, $n = 8$) or both pindolol (3 μM) and prazosin (0.1 μM) (●, $n = 6$). Increases in rate or tension are plotted as a percentage of the maximum response and vertical lines show s.e.mean.

81.3 μM (Table 1), a dose-ratio of 6.6. This yielded a pA_2 value of 7.75 by use of the equation of Mackay (1978).

α-Adrenoceptor antagonism of noradrenaline with prazosin or dibenamine

Naïve tissues In the absence of β-adrenoceptor blockade, prazosin (0.1 μM) failed to alter the concentration-response curve for noradrenaline on left atria (Figure 5a), the EC_{50} values for noradrenaline alone (0.083(0.031–0.222) μM, $n = 6$) and in the presence of prazosin (0.045(0.027–0.075) μM, $n = 6$) were not significantly different ($P > 0.05$). Similarly, at the first 'halt' in the antagonism by pindolol (1 μM), prazosin caused no further shift of the curves (Figure 5a), the EC_{50} values of 4.47(2.15–9.29) μM ($n = 12$) and 5.37(2.51–11.5) μM ($n = 9$) did not differ

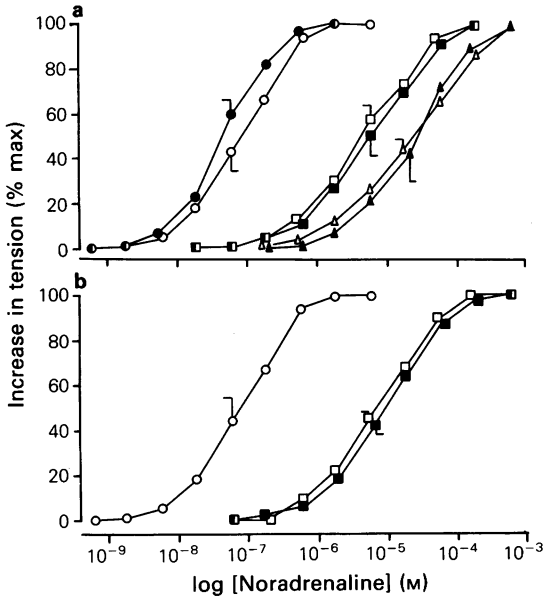


Figure 5 Effect of prazosin (0.1 μM) on the concentration-response curves for the increase in tension of rat left atria in response to noradrenaline. Each tissue was exposed to one curve for noradrenaline either in the absence of antagonist (○, $n = 6$) or in the presence of prazosin (●, $n = 6$). Curves were also obtained in the presence of pindolol (a) or timolol (b) alone or in combination with prazosin. (a) Pindolol (1 μM, □, $n = 12$), pindolol (1 μM) + prazosin (■, $n = 9$), pindolol (10 μM, △, $n = 7$), pindolol (10 μM) + prazosin (▲, $n = 8$). (b) Timolol (3 μM, □, $n = 8$), timolol (3 μM) + prazosin (■, $n = 6$). Increases in tension are plotted as a percentage of the maximum response and vertical lines show s.e.mean.

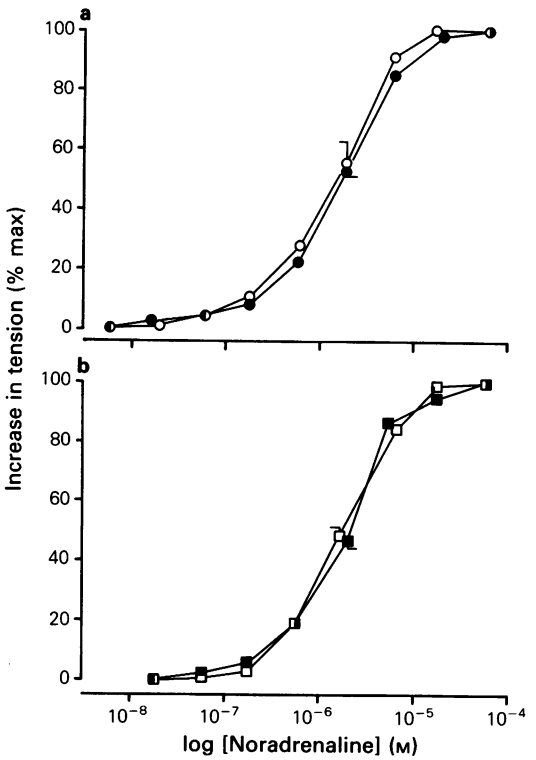


Figure 6 Effects of (a) dibenamine (1 μM) and (b) haloperidol (1 μM) on the concentration-response curves for the increases in tension of rat left atria in response to noradrenaline. In each tissue an initial curve was obtained in the presence of pindolol (1 μM) (○, □). A second curve was then repeated in the presence of pindolol (1 μM) but (a) after exposure to dibenamine for 30 min followed by a 1 h washout (●) or (b) in the additional presence of haloperidol (■). Increases in tension are plotted as a percentage of the maximum response, the first curves being corrected from time-matched controls as described in the text. Each point represents the mean ($n = 4$) with the s.e.mean on a mid-curve point.

significantly ($P > 0.05$). Also, at the second limit of antagonism in the presence of pindolol (10 μM), prazosin failed to displace the noradrenaline curves (Figure 5a). The EC_{50} values in the presence of pindolol alone (26.3(10.6–65.2) μM, $n = 7$) and with prazosin and pindolol combined (25.7(9.12–72.3) μM, $n = 8$) did not differ significantly ($P > 0.05$).

Concentration-response curves for noradrenaline on left atria were also constructed in the combined presence of prazosin and timolol. The concentration of timolol was 3 μM, above which the concentration-response curves for noradrenaline were displaced no further. Prazosin was also without effect (Figure 5b), the EC_{50} values obtained in the presence of timolol alone (8.71(5.15–14.7) μM, $n = 8$) and timolol and prazosin combined (7.08(4.52–11.1) μM, $n = 6$) were not significantly different ($P > 0.05$).

'Before and after' method The effect of prazosin was examined by constructing a concentration-response curve for noradrenaline on left atria in the presence of pindolol alone (1 μM) and then repeating it in the combined presence of prazosin (0.1 μM) and pindolol (1 μM). The mean dose-ratio for the shift of the noradrenaline curve by this concentration of pindolol alone was 94.8 ± 16.4 and when combined with prazosin, the value was 111.6 ± 30.8 (Table 2). This was not significantly different ($P > 0.05$) and therefore no additional antagonism by prazosin occurred.

To examine the effect of dibenamine, a concentration-response curve for noradrenaline was constructed in the presence of pindolol (1 μM) and after washout, the tissue was incubated with dibenamine (1 μM) for 30 min. The tissue was then washed

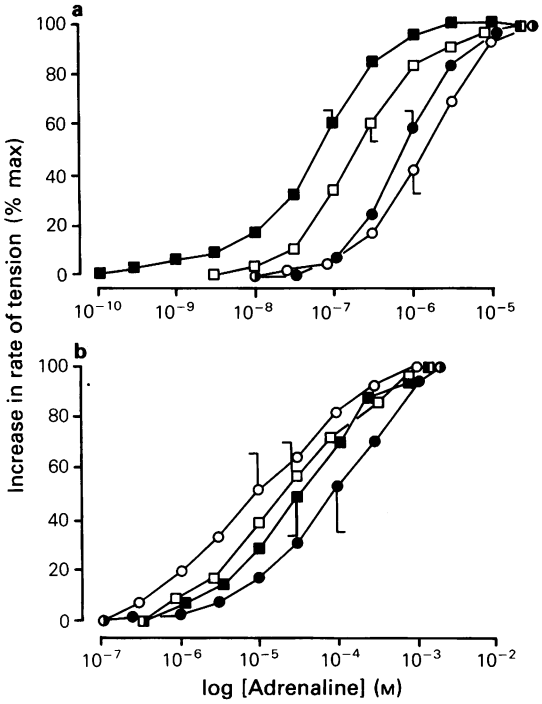


Figure 7 Effect of desmethylimipramine (DMI, 1 μM) on the left atrial tension responses (○, ●) and right atrial rate responses (□, ■) to adrenaline. Concentration-response curves were obtained before (open symbols) and repeated in the presence of DMI (solid symbols). Experiments were performed either in the absence of antagonists (a) or in the presence throughout of pindolol (3 μM, b). Increases in tension or rate are plotted as a percentage of the maximum response, pre-DMI curves being corrected from time-matched controls. Each point represents the mean (n = 4) with the s.e.mean on a mid-curve point.

for 1 h by changing the bathing medium every 10 min. Pindolol (1 μM) was returned to the bath at the final wash and then a second noradrenaline curve constructed. Dibenamine did not displace the noradrenaline concentration-response curve (Figure 6a). Thus the shift by pindolol alone was not increased by the additional incubation with dibenamine.

Dopamine receptor antagonism of noradrenaline with haloperidol

With pindolol (1 μM) and prazosin (0.1 μM) present throughout, concentration-response curves for noradrenaline in left atria were obtained in the absence and presence of haloperidol (1 μM). The curves were superimposable (Figure 6b), indicating that haloperidol had not antagonized noradrenaline.

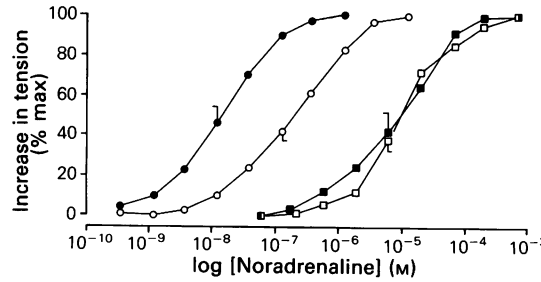


Figure 8 Effect of desmethylimipramine (DMI, 1 μM) on the increase in tension of rat left atria in response to noradrenaline. Concentration-response curves were obtained before (open symbols) and repeated in the presence of DMI (solid symbols). Experiments were performed in the absence of antagonists (n = 5, ○) or in the presence throughout of pindolol (1 μM, n = 6, □). Increases in tension are plotted as a percentage of the maximum response, pre-DMI curves being corrected from time-matched controls as described in the text. Each point represents the mean with the s.e.mean at a mid-curve point.

Inhibition of neuronal uptake by desmethylimipramine

Since DMI was present throughout all the experiments so far, experiments were next performed to determine its possible influence on the effects of adrenaline and noradrenaline in the presence of β-adrenoceptor blockade and on the lack of antagonism by prazosin. Concentration-response curves were constructed in the absence and presence of DMI (1 μM). The concentration-response curves for adrenaline on both left and right atria were displaced to the left by DMI indicating potentiation of the responses. The degree of potentiation was greater for the right atrial rate responses than the left atrial tension responses (Figure 7a). The effect of DMI upon the response to adrenaline was next examined in the presence throughout of pindolol, at the concentration (3 μM) required to demonstrate α-adrenoceptor-mediated inotropic responses to adrenaline. DMI failed to potentiate the right atrial rate response; the curves were virtually superimposed. However, the left atrial tension responses were now antagonized by DMI, the curves being displaced to the right (Figure 7b). The EC_{50} value in the presence of pindolol alone (11.5(3.8–34.7) μM was significantly less ($P < 0.01$) than the value in the presence of pindolol and DMI (95.5(23.4–389.1) μM).

In the absence of antagonists, the concentration-response curve for noradrenaline on left atria was

shifted to the left by DMI (1 μM) (Figure 8). However, in the presence of pindolol (1 μM) throughout, DMI neither potentiated nor antagonized the responses to noradrenaline; the concentration-response curves were virtually superimposed (Figure 8).

α-Adrenoceptor antagonism by prazosin in absence of DMI

To examine whether the presence of DMI (1 μM) interferes with the antagonism by prazosin, concentration-response curves for adrenaline and noradrenaline on left atria were constructed in the absence and presence of prazosin (0.1 μM). Pindolol or timolol were present throughout, but DMI was absent. The curve for adrenaline in the presence of pindolol (3 μM) was displaced to the right by prazosin (Figure 9), the mean EC_{50} value being significantly shifted ($P < 0.05$) from 1.9(0.53–6.8) μM to 112.2(77–162) μM. The mean dose-ratio was 93.9 ± 45.8 and the mean pA_2 value was 8.76 ± 0.18, which was substantially greater than the value obtained for prazosin (7.75) in the presence of pindolol (3 μM) and DMI.

The noradrenaline concentration-response curves, in contrast, were only slightly displaced to the right by prazosin (Figure 10). In the presence of pindolol 1 and 10 μM, the dose-ratios for the shift were 2.55 ± 0.90 and 3.59 ± 0.77, respectively, and these yielded mean pA_2 values of 7.19 and 7.34 ± 0.10, respectively. In the presence of timolol (3 μM), the dose-ratio was 6.22 ± 1.49 and the pA_2 value was 7.66 ± 0.09.

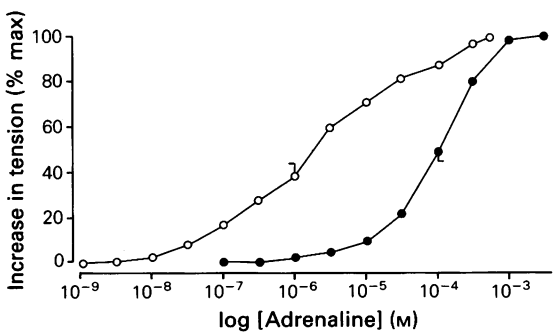


Figure 9 Effect of prazosin (0.1 μM) on the left atrial tension responses to adrenaline. Concentration-response curves were obtained before (○) and repeated in the presence of prazosin (●). Pindolol (3 μM) and metanephrine (10 μM) were present throughout, but not desmethylimipramine. Increases in tension are plotted as a percentage of the maximum response, pre-prazosin curves being corrected from time-matched controls. Each point represents the mean ($n = 6$) with the s.e.mean on a mid-curve point.

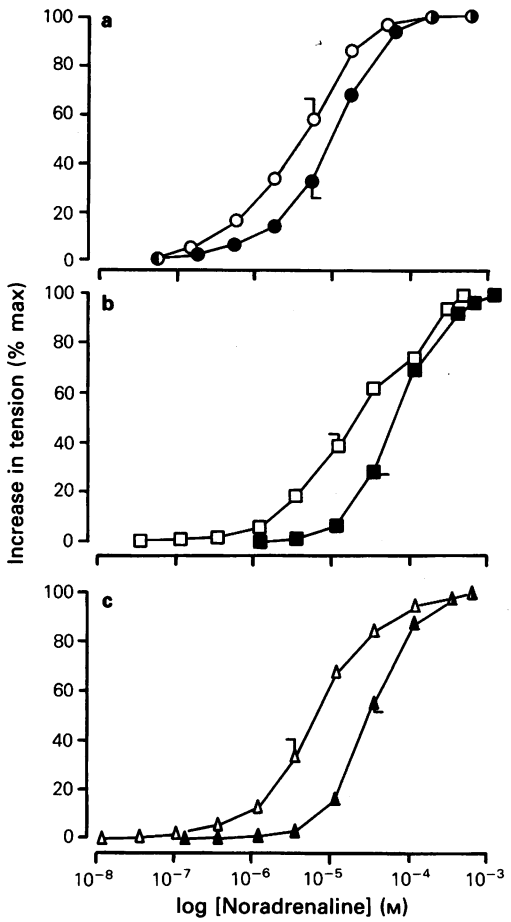


Figure 10 Effects of prazosin (0.1 μM) on the increase in tension of rat left atria in response to noradrenaline, measured in the absence of DMI. Concentration-response curves were obtained before (open symbols) and repeated in the presence of prazosin (solid symbols). Experiments were performed in the presence throughout of (a) pindolol (1 μM, $n = 4$, ○), (b) pindolol (10 μM, $n = 6$, □) or (c) timolol (3 μM, $n = 6$, △). Increases in tension are plotted as a percentage of the maximum response, the pre-prazosin curves being corrected from the corresponding time-matched control as described in the text. Each point represents the mean with the s.e.mean at a mid-curve point. The shifts of the curve at the EC_{50} were significant ($P < 0.05$) in (b) and (c) but not in (a).

α-Adrenoceptor antagonism of noradrenaline by prazosin in the presence of DMI (0.1 μM) or cocaine (10 μM)

Concentration-response curves for noradrenaline in left atria were constructed in the absence and presence of prazosin (0.1 μM). Pindolol (10 μM) was

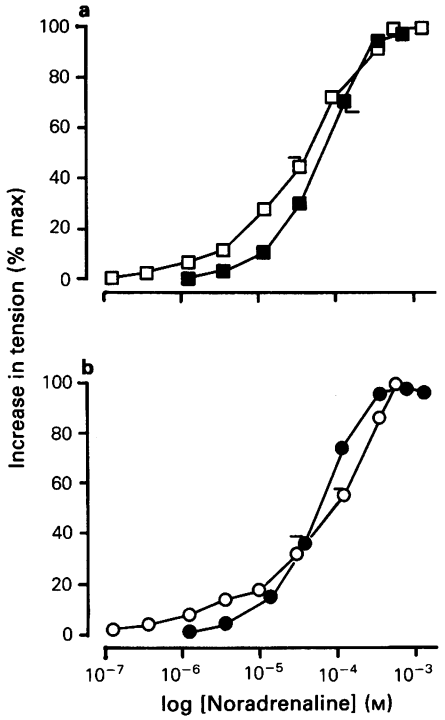


Figure 11 Effects of prazosin (0.1 μM) on the left atrial tension responses to noradrenaline, measured in the presence throughout of pindolol (10 μM), metanephrine (10 μM) and either (a) desmethylimipramine (0.1 μM) or (b) cocaine (10 μM). Concentration-response curves were obtained before (open symbols) and repeated in the presence of prazosin (solid symbols). Increases in tension are plotted as a percentage of the maximum response, pre-prazosin curves being corrected from the corresponding time-matched control. Each point represents the mean (n = 6) with the s.e.mean at a mid-curve point.

present throughout and neuronal uptake was blocked throughout by a lower concentration of DMI (0.1 μM) or by cocaine (10 μM). In both cases there was no significant shift of the curves by prazosin (Figure 11). The mean EC_{50} values before prazosin were 57.7(40–75.9) and 89.1(78.6–100.8) μM and in the presence of prazosin they were 64.9(61.4–68.6) μM and 56.4(53.3–59.8) μM respectively for DMI (0.1 μM) and cocaine (10 μM) as inhibitors of uptake.

Discussion

Pindolol and timolol were selected as the β-adrenoceptor antagonists for this study because of their non-selectivity towards β_1- and β_2-adrenoceptors and minimal membrane stabilizing properties (van Zwieten & Timmermans, 1983). This was an advantage over propranolol, for example, since relatively high concentrations were expected to be used in order to displace the curves to a 'window' where any α-adrenoceptor-mediated responses might be revealed. The concentrations of pindolol (up to 30 μM) were in excess of the 0.01 μM used by Schümann (1983) to demonstrate an α-adrenoceptor-mediated positive inotropic response to adrenaline. A predictable β-adrenoceptor antagonistic activity of pindolol was demonstrated against the right atrial rate response to adrenaline and (up to a concentration of 1 μM pindolol) the left atrial tension responses to noradrenaline. The Schild plots had slopes of unity indicating competitive antagonism and the pA_2 values were 8.15 and 7.94. These compare with values of 9.04 $\pm$ 0.11 (Horii *et al.*, 1974), 8.67 $\pm$ 0.07 (Harms, 1976) and 8.9 $\pm$ 0.1 (Kaumann *et al.*, 1980) obtained in guinea-pig left atria and 8.76 $\pm$ 0.08 for guinea-pig right atrial rate (O'Donnell & Wanstall, 1981) with racaemic pindolol; it is about a half-log unit lower than these values. However, this is unlikely to be because adrenaline and noradrenaline were the agonists, since the value obtained with isoprenaline as the agonist (8.04 $\pm$ 0.07) was no different. Admittedly only two concentrations of pindolol were used to antagonize isoprenaline, but the Schild plot formed an extension of that obtained with noradrenaline. It does appear, therefore, that rat cardiac tissue may yield lower pA_2 values for pindolol as an antagonist of β-adrenoceptors than the guinea-pig.

Notwithstanding this observation, the results do indicate that the positive chronotropic response to adrenaline is mediated via β-adrenoceptors. Furthermore, the failure of prazosin to antagonize these right atrial rate responses, either in the unblocked state or in the presence of a high concentration of pindolol, was consistent with the response being mediated solely via β-adrenoceptors, with no α-adrenoceptor involvement. Weak positive chronotropic responses mediated via α-adrenoceptors have been demonstrated with selective α-adrenoceptor agonists, but only in rat isolated atria (Wagner & Brodde, 1978; Chess-Williams & Broadley, 1987; Tung *et al.*, 1987; Williamson & Broadley, 1987) and the pithed rat (Flavahan & McGrath, 1981; Tung *et al.*, 1982). Closer inspection of the adrenaline concentration-response curves in the presence of pindolol indicates that prazosin may have displaced the lower portion of the curve, albeit slightly, which suggests that adrenaline may exert a minor positive chronotropic response through α-adrenoceptors.

The present results also indicate that the positive inotropic response to noradrenaline is mediated via β-adrenoceptors, both in the absence of antagonist and in the presence of pindolol up to a concentration of 1 μM. However, a further increase in concentration of pindolol to 3 μM did not produce additional

antagonism whether a 'before and after' or 'naïve tissue' experimental design was employed. That pindolol could have produced further β-blockade by increasing its concentration to 3 μM was indicated by the expected progressive antagonism of isoprenaline by 1 and 3 μM of pindolol. This halt in the antagonism of noradrenaline at an EC_{50} value of between 5 and 10 μM also occurred when timolol was the antagonist. The noradrenaline concentration-response curves were not displaced from this position (EC_{50} between 5 and 10 μM) by increasing the concentration of timolol over two orders of magnitude (i.e. from 0.3 to 30 μM). By contrast, when the concentration of pindolol was raised to 10 μM, there was a further shift of the noradrenaline curve, but again there was a second halt in the displacement which occurred at an EC_{50} of approximately 25 μM. If noradrenaline did not stimulate β-adrenoceptors at the limit of displacement by pindolol (first 'halt') and by timolol, then the secondary shift by larger concentrations of pindolol must be due to antagonistic properties of pindolol at a receptor other than the β-adrenoceptor. Pindolol is known to have additional blocking properties, for example, it binds to cerebral 5-hydroxytryptamine$_1$-receptors (Nahorski & Willcocks, 1983).

The positive inotropic responses to adrenaline were also antagonized in a progressive manner by pindolol and, in common with noradrenaline, there was a halt in the shift of the curves which occurred at 3 μM pindolol. The Schild plot for the concentrations of pindolol up to 3 μM had a slope similar to that for the rate responses to adrenaline, but yielded a pA_2 value of only 6.48. This was over one order of magnitude less than the values obtained for the right atrial rate responses and the left atrial tension responses to noradrenaline and those quoted in the literature (see above). This suggests that the inotropic response to adrenaline was not solely mediated by β-adrenoceptors, and this hypothesis was confirmed by the further antagonism of the response by prazosin at the limit of β-adrenoceptor blockade by pindolol.

In the presence of substantial β-adrenoceptor blockade by pindolol, adrenaline therefore exerts positive inotropic responses via α-adrenoceptors. Although in the absence of β-blockade there was no effect of prazosin. This agrees with earlier observations in rat atria (Ask & Stene-Larsen, 1984) and papillary muscle (Skomedal & Osnes, 1983) and rabbit papillary muscle (Schümann *et al.*, 1974). The shift of the concentration-response curve by prazosin was equivalent to a pA_2 value of 7.74. This is less than literature values for α_1-adrenoceptor-mediated responses in other tissues, for example the rat aorta (10.16 ± 0.1, Hamed *et al.*, 1983; 9.42 ± 0.07, Digges & Summers, 1983; 8.83, Williamson & Broadley, 1987) and rat anococcygeus muscle (8.2, Doxey *et al.* 1977; 9.1–9.4, Doggrell & Paton, 1978). It is unlikely that this smaller-than-expected shift of the adrenaline curve by prazosin is due to the receptor being an atypical α_1-adrenoceptor, since a value of 9.05 was obtained in rat left atria for the antagonism of the selective α-adrenoceptor agonist methoxamine (Williamson & Broadley, 1987).

A more likely explanation came from examination of the effects of DMI. This inhibitor of neuronal uptake (Iversen, 1967) caused a leftward displacement of the unblocked concentration-response curves for the right atrial rate and left atrial tension responses to adrenaline. The shift was greater for the right atrium, indicating that neuronal uptake was a more effective means of removal of adrenaline from the synaptic cleft in the right atrium. This is consistent with the innervation of the sinoatrial node being known to be denser than that of the left atrial myocardium (Angelakos *et al.*, 1969). The effects of DMI on the response to adrenaline obtained in the presence of pindolol (3 μM) were, however, unexpected. In contrast to the unblocked state, the right atrial responses were not potentiated by DMI. This may be due to the fact that pindolol displaces the concentration-response curve for adrenaline into a region of higher adrenaline concentration, where neuronal uptake may be saturated and therefore uptake plays little part in reducing the effective concentration of agonist at the receptor (Langer & Trendelenburg, 1969). Thus inhibition of neuronal uptake has no effect upon the response size. In the left atrium presumably the same would apply, although uptake would probably be saturated at lower concentrations of adrenaline, so that no potentiation would be expected. However, rather than the concentration-response curves being superimposed, there was a shift to the right by DMI, indicating antagonism of the left atrial tension responses. The responses to adrenaline at this concentration of pindolol are no longer β-adrenoceptor-mediated but are blocked by prazosin and therefore α-adrenoceptor-mediated. Thus DMI appears to be an antagonist of the cardiac α-adrenoceptor. Antagonism of α_1-adrenoceptors leading to orthostatic hypotension has been obtained as a side effect of tricyclic antidepressants, including DMI, and they have been shown to displace from brain α_1-adrenoceptor binding sites (U'Pritchard *et al.*, 1978). Furthermore, antagonism of noradrenaline-induced contractions of rabbit aorta by DMI has been demonstrated (Türker & Khairallah, 1967).

If α-adrenoceptors are already partially antagonized by the presence of DMI, then the effect of prazosin added after blockade with pindolol would be reduced. This would explain the less-than-expected shift of the adrenaline concentration-response curve

by prazosin in these circumstances. Indeed, when DMI was omitted, the adrenaline concentration-response curves obtained in the presence of pindolol were displaced to a greater extent by prazosin, to yield a pA_2 value (8.76) that was consistent with this being an α_1-adrenoceptor-mediated response.

The question therefore arises whether noradrenaline also exerts its inotropic effect by stimulating α-adrenoceptors at the point where pindolol and timolol produce no further shift of the curves. Unlike with adrenaline, the additional presence of prazosin or dibenamine did not displace the curves any further than in the presence of pindolol or timolol alone, which suggests that noradrenaline is not stimulating α-adrenoceptors. However, the continuous presence of the neuronal uptake inhibitor DMI may have exerted α-adrenoceptor blocking activity which could have masked the effect of prazosin. In the absence of β-adrenoceptor antagonists, DMI produced the potentiation of noradrenaline responses of the left atrium expected of a neuronal uptake inhibitor (Hawthorn & Broadley, 1982). However, in the presence of pindolol, there was no potentiation of noradrenaline by DMI, presumably due to uptake already being saturated by the higher concentration of noradrenaline required. In contrast to adrenaline, there was no antagonism of noradrenaline by DMI. This could be taken as indirect evidence that the responses were not mediated via α-adrenoceptors.

In the absence of DMI, but presence of pindolol (1 and 10 μM) or timolol (3 μM), there was a small shift of the concentration-response curve by prazosin. The pA_2 values calculated for this shift (7.19, 7.34 and 7.66) were, however, over one log unit less than those cited above for prazosin at the α_1-adrenoceptors of the rat left atria (adrenaline, 8.76) and other tissues (rat aorta and anococcygeus).

It could be argued that the pre-prazosin concentration-response curve may be displaced to the right of that occurring when uptake is inhibited, thus yielding an underestimate of the displacement by prazosin. This possibility was examined in the presence of pindolol (10 μM) by using a lower concentration of DMI (0.1 μM) which would still effectively inhibit uptake, but have reduced α-adrenoceptor blocking activity. An alternative approach was the use of cocaine as the uptake inhibitor. In both situations, there was in fact no antagonism by prazosin, which confirms the observations made in the presence of DMI (1 μM). The small antagonism by prazosin observed in the absence of uptake inhibitors but in the presence of β-adrenoceptor blockade is difficult to explain. It would appear to be a non-specific antagonism of the receptor mediating this residual response, whatever its type may be. Indeed, prazosin does have some non-classical blocking activity (Purdy *et al.*, 1980). The evidence indicates that the receptor is not of the classical α- or β-subtype. It is also not a dopamine receptor, since the residual response was unaffected by the dopamine receptor antagonist, haloperidol (Seeman, 1981). Finally, it is unlikely to be an α_2-adrenoceptor since selective α_2-adrenoceptor agonists such as UK 14,304 fail to induce positive inotropic responses in the rat left atria (Williamson & Broadley, 1987). The secondary shift of the noradrenaline concentration-response curve by pindolol may also be due to blockade of this receptor at higher concentrations, although timolol does not appear to antagonize this receptor.

What evidence is there elsewhere of atypical adrenoceptors stimulated by noradrenaline? In cardiac muscle, a positive chronotropic response to noradrenaline mediated via neither α- nor β-adrenoceptors has been identified in chick heart (Tayo, 1984). An atypical adrenoceptor stimulated by noradrenaline has also been identified for the inhibition of contractile responses of guinea-pig ileum (Bond *et al.*, 1986a,b). However, in contrast to the present study this receptor is also stimulated by isoprenaline (Bond & Clarke, 1987a) and BRL 37344 (Bond *et al.*, 1988). Sensitivity to the latter agonist suggests that it may be an atypical β-adrenoceptor related to that in adipose tissue (Arch *et al.*, 1984) or guinea-pig stomach (Coleman *et al.*, 1987). The atypical ileal response was susceptible to blockade by nadolol at high concentrations (Bond & Clarke, 1987b) and there was a halt in the antagonism by nadolol similar to that observed here with pindolol against noradrenaline. A γ-receptor for noradrenaline has been proposed in vascular smooth muscle, which is stimulated by noradrenaline released exogenously or applied iontophoretically to the synaptic cleft but is not antagonized by phentolamine (Hirst & Nield, 1980; see also Angus, 1982). Unlike the present effect of noradrenaline, the γ-receptor also appears to be stimulated by adrenaline. Finally, it is of relevance that noradrenaline but not isoprenaline has been claimed to exert a positive inotropic effect in guinea-pig papillary muscles that is mediated via an intracellular adrenoceptor not of the β-subtype and possibly not of the α-subtype (Ebner, 1982). This effect could be prevented by inhibition of extraneuronal uptake with hydrocortisone; it is therefore unlikely to explain the present results since the experiments were performed in the presence of metanephrine throughout.

The present results indicate that whereas adrenaline stimulates a typical α_1-adrenoceptor to produce a positive inotropic response of rat atria, noradrenaline does not. This conclusion supports the results of early studies by Wagner & Schümann (Schümann, 1983) who found that in the presence of β-adrenoceptor blockade, phentolamine displaced the

concentration-response curves for adrenaline but not for noradrenaline in rabbit and human cardiac muscle. The result is, however, at variance with those of Aass and co-workers who found α-adrenoceptor-mediated responses to noradrenaline in human atria (Skomedal *et al.*, 1985), human ventricle (Aass *et al.*, 1986) and rabbit papillary muscle (Aass *et al.*, 1983). Their studies are not directly comparable with the present one, since they analysed various components of each contraction cycle. The only related measure of contractility that they used was the T_{max}, the maximum developed tension of each cycle, and in only one of their studies were complete concentration-response curves for this response obtained and pD_2 values quoted (Aass *et al.*, 1983). Values for a separate α- and β-adrenoceptor-mediated component are given for noradrenaline in the presence of propranolol, the former being abolished by prazosin. No explanation for the different conclusions from the present study can be offered.

The present results also apparently conflict with the conclusion of Ask & Stene-Larsen (1984) who also used the rat left atria. They showed that in the presence of propranolol, the pD_2 value for noradrenaline was significantly reduced by prazosin. However, the level of reduction was by less than a log unit (a 4 to 5 fold shift) and the concentration of prazosin was 10^{-6} M. In other words, at this concentration of prazosin one would expect approximately a 300 fold shift for a prazosin pA_2 value of 8.5. The result is therefore in fact analogous to that obtained in the present study, which concludes that noradrenaline does not stimulate the α_1-adrenoceptor of rat isolated left atria to produce a positive inotropic response. The response in the presence of β-adrenoceptor blockade by pindolol and timolol would appear to be due to stimulation of neither β- nor α_1-adrenoceptors. This contrasts with the effect of adrenaline, which in the presence of β-adrenoceptor blockade does induce positive inotropy via a classical α_1-adrenoceptor.

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Probucol attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits

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1 Probucol was administered to rabbits fed a cholesterol-enriched (2% wt/wt) diet to determine potential anti-atherogenic effects in a preparation in which the disease process is due to elevated plasma concentrations of cholesterol ester-rich very low density lipoproteins (CER-VLDL).

2 Probucol was supplemented to the diet at 1% wt/wt which resulted in plasma concentrations rising steadily to $53 \pm 8\ \mu g\ ml^{-1}$ after 14 days, with no significant changes during continued administration. Dietary consumption and body weight gains were comparable in the drug-treated and control groups during the observation period.

3 Probucol treatment did not significantly affect plasma concentrations of total cholesterol, unesterified cholesterol, triglycerides or phospholipids.

4 The concentration of CER-VLDL in plasma and its physicochemical characteristics were not significantly changed during administration of probucol. CER-VLDL from both control and probucol-treated animals was a potent stimulant of the augmentation of the intracellular incorporation of [^{3}H]-oleate into cholesteryl-[^{3}H]-oleate in cultured macrophages.

5 Despite the lack of effect of probucol on concentrations of plasma lipids and the cell interaction characteristics of CER-VLDL, administration of the drug markedly decreased the extent of intimal aortic surface area covered by grossly discernible atherosclerotic lesions from $55.6 \pm 11.8\%$ to $11.6 \pm 1.9\%$ in thoracic sections, and from $49.1 \pm 10.2\%$ to $7.2 \pm 0.4\%$ in abdominal sections. Furthermore, probucol treatment significantly reduced the deposition of total cholesterol in vascular tissue.

6 Probucol reduced the extent of aortic atherosclerosis produced by diet-induced hypercholesterolemia in rabbits. This reduction occurred in the absence of any significant change in the characteristics of plasma lipoproteins that were determined. These results indicate that either there is a role of oxidation in the disease process of this animal model of atherosclerosis or that probucol is acting via a presently undefined mechanism.

Introduction

Probucol (4,4′-(isopropylenedithio)bis(2,6,-di,-butylphenol) is a moderately effective drug for reducing plasma concentrations of low density lipoproteins (LDL) in human subjects through a mechanism that has not been elucidated (Steinberg, 1986). Although probucol reduces plasma concentrations of LDL, the anti-atherogenic properties of this drug have been doubted due to the concomitant reduction in high density lipoprotein (HDL). Despite this caveat, anti-atherogenic properties of probucol have been demonstrated recently following administration of the drug to Watanabe heritable hyperlipidemic (WHHL) rabbits (Kita *et al.*, 1987). WHHL rabbits have elevated plasma concentrations of LDL and are thought to simulate the disease process of familial hypercholesterolemia. It has been hypothesized that the anti-atherogenic properties of probucol in WHHL rabbits may be independent of the mild reduction of plasma concentrations of cholesterol and, instead, related to the anti-oxidant properties of the drug (Carew *et al.*, 1987).

To further the understanding of the mechanism of the anti-atherogenic properties of probucol, in the present study the effects of the drug were characterized in rabbits fed cholesterol-enriched diets. In contrast to WHHL rabbits, rabbits fed a cholesterol-enriched diet transport excessive concentrations of cholesterol in cholesterol ester-rich very

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low density lipoproteins (CER-VLDL; Daugherty *et al.*, 1985). CER-VLDL is thought to be a highly atherogenic lipoprotein, that acts through promotion of uncontrolled cholesterol esterification in monocyte-derived macrophages that have been recruited to the arterial wall (Mahley, 1982). The results of this study demonstrate that administration of probucol produced a profound reduction in the extent of diet-induced atherosclerosis in the absence of any significant effects on plasma concentrations of lipids or the observed characteristics of CER-VLDL. A preliminary communication of these results has been presented (Daugherty *et al.*, 1988a).

Methods

Animals

New Zealand rabbits (2 to 3 kg body weight) were obtained from the Boswell Rabbit Farm (Pacific, MO). Animals were initially fed a standard laboratory diet (Ralston Purina, St. Louis, MO) for at least 7 days after delivery to Washington University School of Medicine. Diet and water were available *ad libitum*. All protocols concerning animals were approved by the Washington University Committee for the Humane Care of Laboratory Animals.

Diet and drug regimens

Diet enriched in cholesterol (2% wt/wt) was obtained from Ralston Purina Test Diets (Richmond, IN). Probucol (a gift from Merrell Dow Research Institute, Cincinnati, OH) was dissolved in chloroform and sprayed evenly over the diet in the proportion of 1% wt/wt. Diet for the control group was sprayed with chloroform alone. Treated diets were kept under a fume hood until the odour of the solvent had dissipated. Food intake was monitored daily for both control and drug-treated groups.

Isolation of CER-VLDL

CER-VLDL was isolated from the plasma of cholesterol-fed rabbits by ultracentrifugation as described previously (Daugherty *et al.*, 1987).

Characterization of plasma and lipoproteins

Concentrations of triglycerides, cholesterol esters, unesterified cholesterol and phospholipids in plasma and in isolated lipoprotein fractions were determined with commercially available enzyme kits (Wako Chemical Company, Dallas, TX). Concentrations of phospholipids and triglycerides were calculated based on mean molecular weights of 722 and 866 respectively. Mass of protein in lipoprotein fractions was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Pierce Chemical Company, Rockford, IL) as standard. Electrophoretic mobility of lipoproteins was assessed with agarose gels (0.5% wt/vol) stained with Fat Red 0.

Plasma concentrations of probucol were determined by reverse phase high performance liquid chromatography by the method of Satonin & Coutant (1986). Concentrations of probucol in plasma were determined relative to an internal standard supplied by the Merrell Dow Research Institute (MDL 27,272).

Determination of cholesteryl-[^3H]-oleate deposition in cultured macrophages

Pentobarbitone-anesthetized New Zealand rabbits of either sex were exsanguinated via the abdominal aortae. Saline, containing heparin ($5\,u\,ml^{-1}$), was introduced into the alveoli through tracheal cannulae. Lungs were lavaged five times with 50 ml fluid washes. Cells were plated in Dulbecco's minimum essential medium containing newborn calf serum (20% vol/vol) at a density of $3–5 \times 10^6$ cells per 35 mm well. Cells were incubated overnight at 37°C in a 5% CO_2 incubator. Cellular deposition of cholesteryl-[^{3}H]-oleate was determined as described previously (Daugherty *et al.*, 1987).

Determination of percentage of intimal area covered by atherosclerotic lesions

After 60 days of study, animals were killed by an overdose of sodium pentobarbitone ($120\,mg\,kg^{-1}$ administered intravenously). Aortae were rapidly dissected free from the ascending arch to the ileal bifurcation. Extraneous tissue was removed and full length incisions exposed intimal areas. Intimal areas were photographed with a Polaroid camera. The areas of grossly discernible normal and atherosclerotic intima were digitized from these photographs using a Numonics model 2210 tablet (Numonics Corporation, Lansdale, PA) and SigmaScan (Jandel Scientific, Corte Madera, CA) run on a XT computer.

Determination of total cholesterol and unesterified cholesterol content of aortae

Cholesterol esters and unesterified cholesterol content of vascular tissue were determined by gas chromatography as described by Ishikawa *et al.* (1974) with 5-α-cholestane as a standard.

Statistical analyses

Comparisons were made with Student's t test (two-tailed) performed with Stats-2 (Statsoft, Tulsa, OK). A P value of less than 0.05 was considered significant. Values are presented as means with s.e.means, where applicable.

Results

Nine rabbits in each group were initiated on the experimental protocol. One death occurred in both the control and drug-treated groups. Both deaths were attributable to intestinal obstruction and not as a result of either diet or drug effects. The acceptance of the diet supplemented with probucol was rapid and no significant differences were observed between the groups in the daily consumption of the diet (Figure 1a). No significant difference in body weights was observed during the experimental period (Figure 1b)

Plasma concentrations of probucol were monitored continuously throughout the study. Plasma concentrations increased progressively during the initial 20 days of administration of the drug. Thereafter, plasma concentrations of probucol were not significantly altered from a value of approximately 55 $\mu g\,ml^{-1}$, although a statistically insignificant trend toward increased concentrations of the drug was observed (Figure 2). All plasma from the probucol-treated rabbits had a distinctive green colour. This colouration was apparently due to the occurrence of an oxidized degradation product of probucol (Barnhart *et al.*, 1989). While no quantification of this colouration was performed, it appeared that a large percentage was present in the lipoproteins of density less than 1.006 $g\,ml^{-1}$. As expected, no probucol was detectable in the plasma of control rabbits.

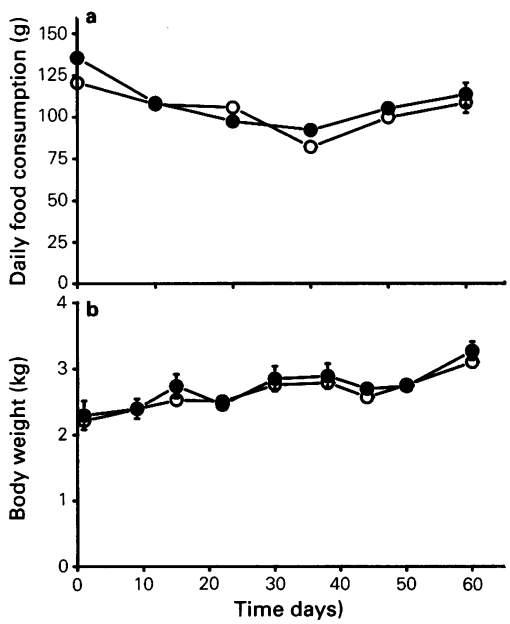


Figure 1 (a) Daily consumption of cholesterol-rich diet in control (○) and probucol-treated rabbits (●). Values are expressed as the mean daily consumption for each 10 day interval. (b) Body weight determined weekly in the same control (○) and probucol-treated (●) animals (n = 8). Vertical lines show s.e.mean.

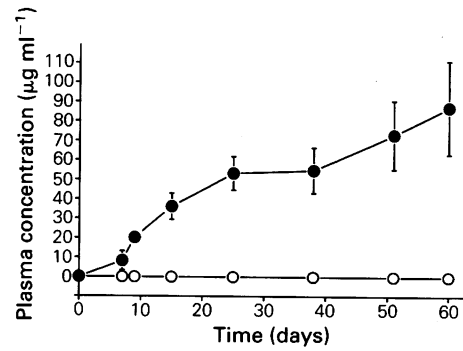


Figure 2 Plasma concentrations of probucol in control (○) and probucol-treated (●) rabbits during the study (n = 8). Vertical lines show s.e.mean.

Plasma concentrations of total cholesterol, unesterified cholesterol and phospholipids in control rabbits increased progressively for the initial 30 days, followed by no further significant increases as described previously (Daugherty *et al.*, 1988b). The highly variable plasma concentrations of triglycerides was probably due to the non-fasted conditions under which plasma was collected. The cholesterol-enriched diet did not consistently affect the plasma concentrations of trigylcerides. Probucol administration did not significantly affect any of the plasma lipid concentrations. At the end of the study, the respective plasma concentrations of controls compared to probucol-treated animals were 56.6 ± 8.6 vs $47.6 \pm 40\,mmol\,l^{-1}$ for total cholesterol, 14.1 ± 1.2 vs $13.4 \pm 1.6\,mmol\,l^{-1}$ for unesterified cholesterol, 8.8 ± 1.3 vs $8.0 \pm 0.6\,mmol\,l^{-1}$ for phospholipids, and 1.6 ± 0.4 vs 3.2 ± 1.3 $mmol\,l^{-1}$ for triglycerides (Figure 3). Although there was a trend for plasma concentrations of total and unesterified cholesterol to be reduced during the administration of probucol, none of these values was statistically significant.

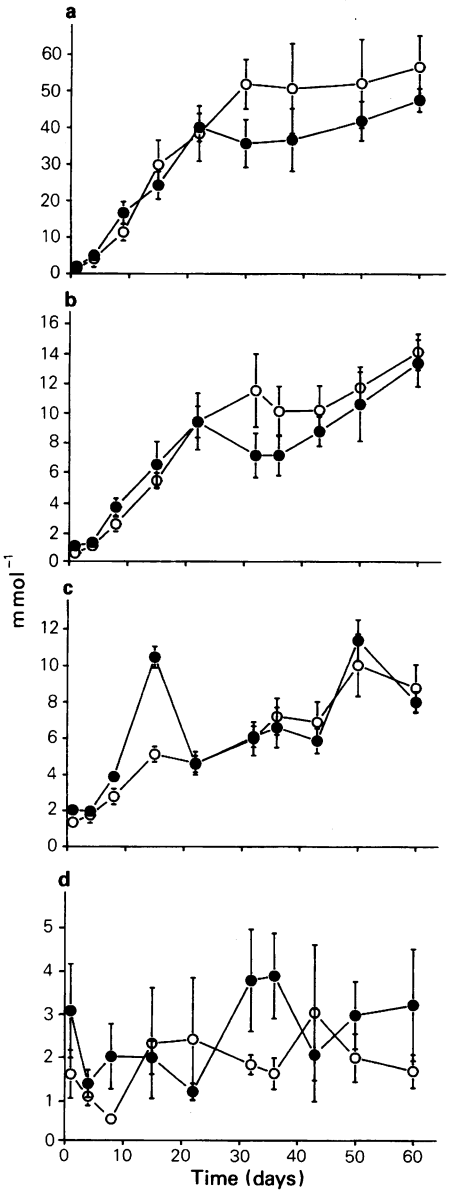


Figure 3 Plasma concentrations of total cholesterol (a), unesterified cholesterol (b), phospholipids (c), and triglycerides (d) in rabbits fed the cholesterol-enriched diet (2% wt/wt). Controls are represented by (○) and probucol-treated by (●) (n = 5–8). Vertical lines show s.e.mean.

Concentrations of CER-VLDL in plasma were unaltered by probucol treatment. In addition, the chemical composition of CER-VLDL was also unchanged (Figure 4). Furthermore, there was no

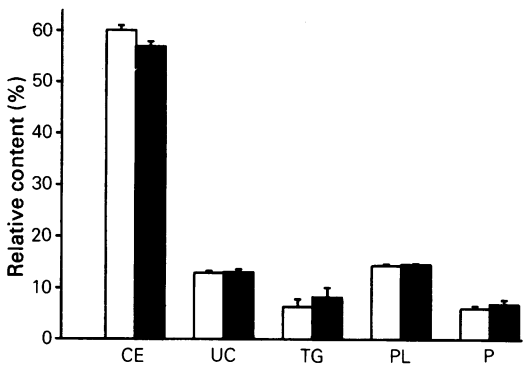


Figure 4 Chemical composition of cholesterol ester-rich very low density lipoproteins (CER-VLDL) from control (open columns) and probucol-treated (solid columns) animals. CER-VLDL was harvested at the time of excision of the aortae. The relative content of cholesterol esters (CE), unesterified cholesterol (UC), triglycerides (TG), phospholipids (PL), and protein (P) were determined as described in the Methods section (n = 8). Vertical bars show s.e.mean.

change in the electrophoretic mobility of the intact lipoprotein or in the apolipoprotein content of CER-VLDL (data not shown).

It has been observed previously that fractions of CER-VLDL that have similar physicochemical properties can differ dramatically in their ability to augment the incorporation of [^{3}H]-oleate into cholesteryl-[^{3}H]-oleate in cultured macrophages (Daugherty *et al.*, 1988b). This assay is commonly considered as one criterion to quantify the atherogenic potential of specific lipoprotein fractions.

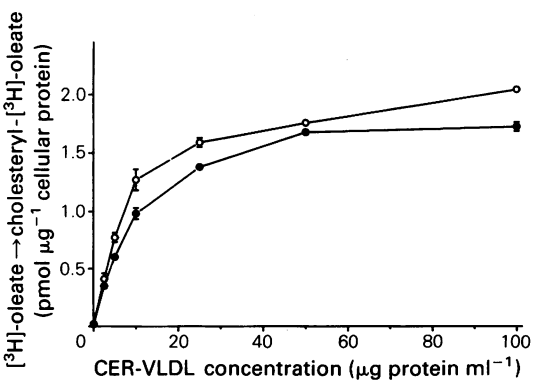


Figure 5 Ability of cholesterol ester-rich very low density lipoproteins (CER-VLDL) from control aminals (○) and probucol-treated animals (●) to augment the incorporation of [^{3}H]-oleate into cholesteryl-[^{3}H]-oleate of cultured rabbit alveolar macrophages. Points represent the mean of six values from 2 separate assays. Vertical lines show s.e.mean.

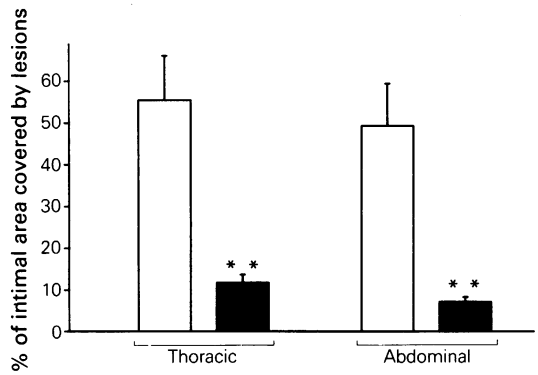


Figure 6 Percentage of intimal surface area covered by grossly discernable atherosclerotic lesions, as determined by computer-assisted planimetry, in thoracic and abdominal aortae from control (open columns) and probucol-treated (solid columns) rabbits ($n = 8$). Vertical bars show s.e.mean. ** Difference significant at $P < 0.01$.

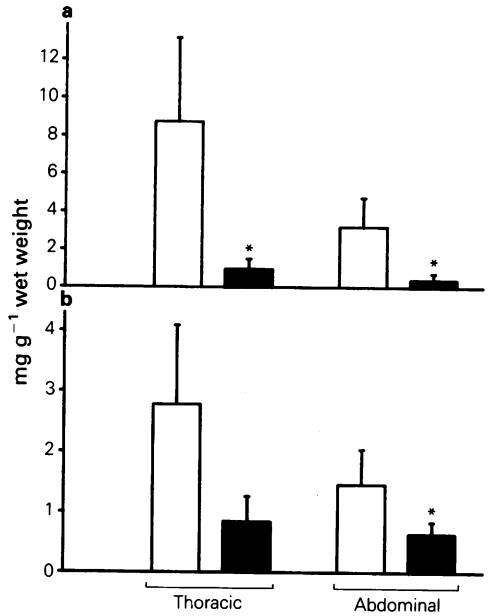


Figure 7 Total cholesterol (a) and unesterified cholesterol (b) content of thoracic and abdominal aortae of control (open columns) and probucol-treated (solid columns) animals. $n = 8$; * difference significant at $P < 0.05$.

CER-VLDL from both control and probucol-treated animals markedly stimulated the incorporation of [^{3}H]-oleate into cholesteryl-[^{3}H]-oleate in macrophages in culture, with no significant differences between the fractions (Figure 5).

Despite the lack of effect of probucol on concentrations of plasma lipids, administration of the drug profoundly reduced the severity of aortic atherosclerosis. The extent of aortic atherosclerosis was decreased significantly from $55.6 \pm 11.8\%$ to $11.6 \pm 1.9\%$ ($P < 0.01$) of surface area in the thoracic section, and from $49.1 \pm 10.2\%$ to $7.2 \pm 0.4\%$ ($P < 0.01$) in the abdominal section (Figure 6). Total cholesterol in thoracic and abdominal segments were reduced from $8.7 \pm 4.4\,\mu g\,mg^{-1}$ wet weight to 1.0 ± 0.4 ($P < 0.05$) and from 3.2 ± 1.6 to 0.4 ± 0.3 ($P < 0.05$) respectively. Probucol treatment reduced the deposition of unesterified cholesterol in thoracic and abdominal segments from $2.8 \pm 1.3\,\mu g\,mg^{-1}$ wet weight to 0.8 ± 0.4 (NS) and from 1.5 ± 0.6 to 0.6 ± 0.2 ($P < 0.05$) respectively (Figure 7).

Sections of aorta from both groups were subjected to histological analysis. Although the extent of atherosclerosis was markedly reduced by the administration of probucol, the morphology of the lesions that were present appeared similar. In both groups, the lesions consisted of lipid-engorged foam cells which are characteristic in this preparation of dietary-induced atherosclerosis (data not shown).

Discussion

The purpose of the present study was to define the effects of probucol on the severity of diet-induced aortic atherosclerosis in rabbits. The atherosclerosis produced during the feeding of cholesterol-enriched diets to rabbits is thought to be due to the presence of elevated plasma concentrations of CER-VLDL.

Probucol was administered to rabbits in doses that produced clinically relevant plasma concentrations. However, probucol treatment of rabbits fed a cholesterol-enriched diet did not significantly reduce plasma concentrations of any of the lipids that were determined, in contrast with the hypolipidemic effect of probucol in man. The mechanism of the hypolipidemic effect in man has been attributed to a variety of mechanisms. These include decreased *de novo* cholesterol biosynthesis, increased activity of hepatic LDL receptors, and increased section of bile acids (Steinberg, 1986).

The mechanism of the hyperlipidemia produced during the feeding of cholesterol-enriched diets to rabbits has been attributed to both a reduction in the catabolism (Kovanen *et al.*, 1981) and an increase in synthesis (Daugherty *et al.*, 1986) of CER-VLDL. The elevated concentrations of CER-VLDL in plasma are primarily of hepatic origin (Daugherty *et al.*, 1988b). However, plasma concentrations of CER-VLDL were not influenced by the administration of probucol in rabbits fed cholesterol-enriched diets. In WHHL rabbits, an earlier study

demonstrated the effects of probucol in reducing plasma concentrations of cholesterol. The mechanism of this effect was ascribed to the production of a modified form of LDL that was more rapidly cleared from plasma (Naruszewicz *et al.*, 1984). However, the potency of the hypolipidemic effects of probucol in WHHL rabbits was not confirmed in a more recent study (Carew *et al.*, 1987).

Although probucol reduces plasma concentrations of total and LDL-cholesterol in human subjects, its effect on the atherogenic process has been questioned since the drug also produces a concomitant reduction in plasma concentrations of HDL-cholesterol. Despite this reservation two recent studies have demonstrated that the drug may prevent the progression of atherosclerosis in WHHL rabbits. Probucol was administered to WHHL rabbits within the first two months of parturition and resulted in marked reductions in the severity of grossly discernible atherosclerotic lesions on intimal surfaces of aortae (Kita *et al.*, 1987; Carew *et al.*, 1987). The atherosclerosis that develops in WHHL rabbits is thought to be attributable primarily to the elevated plasma concentrations of LDL.

The anti-atherogenic effects of probucol in WHHL rabbits have been attributed to the anti-oxidant actions of probucol. Indeed, probucol has potent anti-oxidant properties, being a derivative of butylated hydroxytoluene. In addition, probucol is highly soluble in lipid phases and a high concentration of drug will be transported in lipoproteins. The rationale for the involvement of the anti-oxidant activity is also based on the equivalent reductions in plasma concentrations of cholesterol that were produced by both the HMG-CoA reductase inhibitor, lovastatin, and probucol, but only the latter exerted anti-atherogenic actions (Carew *et al.*, 1987). Further support for the role of oxidation of LDL in the atherogenic process in WHHL rabbits comes from recent identification (Haberland *et al.*, 1988) and isolation (Daugherty *et al.*, 1988c) of oxidized lipoprotein products in atherosclerotic aortic tissue. However, in contrast to studies with WHHL rabbits, the present study has demonstrated the anti-atherogenic effects of probucol when administered to rabbits fed cholesterol-enriched diet. At present a role for oxidation has not been established in the initiation and propagation of aortic atherosclerosis in the rabbits fed cholesterol-enriched diets. Instead, under these conditions the development of atherosclerosis is thought to be due to elevated plasma concentrations of CER-VLDL.

The interaction of lipoproteins with macrophages has been implicated as a primary influence on the initiation and progression of atherosclerosis. In the case of WHHL rabbits, it is assumed that the involvement of LDL and its oxidized products are responsible for the transformation of macrophages to foam cells in the subintimal space. In corroboration with *in vivo* findings (Kita *et al.*, 1987; Carew *et al.*, 1987), probucol both reduces the oxidized modification of LDL (Parthasarathy *et al.*, 1986) and prevents the formation of foam cells from macrophages *in vitro* (Yamamoto *et al.*, 1986). This effect of probucol may be due to a reduction in the modification of surface properties of LDL produced by oxidation (McLean & Hagaman, 1989). In contrast, during feeding of cholesterol-enriched diets to rabbits, the evolution of atherosclerosis is generally thought to occur due to the interaction of CER-VLDL with macrophages that have been recruited to the arterial tissue (Fowler *et al.*, 1979; Schaffner *et al.*, 1980). Numerous studies have demonstrated the ability of CER-VLDL to interact with macrophages leading to unregulated intracellular cholesterol esterification and storage with the subsequent transformation into foam cells (Mahley *et al.*, 1980). This property is particularly prominent in the intestinally-derived fraction of CER-VLDL (Daugherty *et al.*, 1988b). However, in the present study probucol did not influence the interaction of CER-VLDL with macrophages in the incorporation of [^{3}H]-oleate into cholesteryl-[^{3}H]-oleate. Probucol was present in high concentrations in CER-VLDL in the plasma and consequently a high concentration of the drug would have been carried in the CER-VLDL that was transported into macrophages.

The marked anti-atherogenic effect of probucol that was demonstrated in the present study is in contradiction to the lack of effect observed by Hallermayer & Schierok (1987). The reason for this discrepancy is not apparent, although plasma concentrations of probucol were not stated in this study and it is possible that sufficiently high concentrations of drug were not attained.

In summary, the present study has demonstrated that the administration of probucol to rabbits fed a cholesterol-enriched diet markedly decreases the extent of aortic atherosclerosis through a mechanism that remains to be elucidated. These anti-atherogenic effects of probucol in cholesterol-fed rabbits may be related to a currently uncharacterized effect on lipid metabolism within arterial tissue. An additional possible anti-atherogenic action of probucol in this model could be to hinder the recruitment of macrophages to arterial tissue, although there are at present no data to support such an effect.

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Multiple mechanisms in the motor responses of the guinea-pig isolated urinary bladder to bradykinin

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1 Bradykinin (1 nM–1 μM) produced a contraction of bladder strips excised from the dome of the guinea-pig urinary bladder, an effect which was greatly enhanced by removal of the mucosal layer or by thiorphan (10 μM). All subsequent experiments were performed in mucosa-free strips and in the presence of thiorphan.

2 In carbachol (5 μM)-contracted strips, bradykinin produced a concentration (1 nM–1 μM)-dependent transient relaxation.

3 Kallidin was slightly more potent than bradykinin in producing a contraction and a relaxation of the carbachol-induced tone. By contrast, [des-Arg^9]-bradykinin, a selective B_1 receptor agonist was barely effective up to 1 μM.

4 The contractile response to bradykinin was: (a) unaffected by either tetrodotoxin (1 μM), *in vitro* capsaicin desensitization (10 μM for 30 min) or apamin (0.1 μM); (b) antagonized by indomethacin (5 μM), the prostaglandin receptor antagonist SC-19220 (100 μM) or the B_2 receptor antagonist [D-Arg^0, Hyp^3, Thi^{5,8}, Phe^7]-bradykinin (10 μM) and (c) almost abolished by nifedipine (1 μM).

5 The antagonism of the contractile response to bradykinin produced by indomethacin and SC-19220 was non-additive while that produced by indomethacin and the B_2 receptor antagonist was additive.

6 The relaxant response to bradykinin was unaffected by tetrodotoxin, *in vitro* capsaicin desensitization or indomethacin but antagonized in a competitive manner by the B_2 receptor antagonist. Further, this response was abolished by apamin (0.1 μM) but unaffected by glibenclamide (1 μM).

7 Bradykinin (10 μM) produced a consistent release of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) but not substance P-LI from the guinea-pig bladder muscle. CGRP-LI release by bradykinin was greatly reduced in bladders exposed to indomethacin. [des-Arg^9]-bradykinin (10 μM) was ineffective.

8 We conclude that: (a) bradykinin-induced contraction involves activation of both B_2 receptors and prostanoid synthesis, via distinct mechanisms which act by inducing calcium influx via nifedipine-sensitive channels; (b) bradykinin-induced relaxation involves activation of B_2 receptors and opening of apamin-sensitive potassium channels; (c) bradykinin stimulates sensory nerves in this tissue largely via prostanoid production.

Introduction

Bradykinin and related kinins exert a variety of biological effects by stimulating or inhibiting smooth muscle tone in various organs (Regoli & Barabé, 1980; Regoli, 1987). Recently much attention has been directed to the study of pharmacological responses to bradykinin in various tissues and evidence has been provided indicating that multiple mechanisms mediate the smooth muscle responses to this autacoid. Regoli & Barabé (1980) recognized the existence of two distinct bradykinin receptors which were termed B_1 and B_2, respectively. Recently some evidence has been presented indicating that B_2 receptors might be heterogeneous (Rifo *et al.*, 1987; Field *et al.*, 1988; Braas *et al.*, 1988; see Plevin &

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Owen, 1988 for review). Further, bradykinin and related peptides might affect muscle motility through mechanisms not directly related to activation of B_1 or B_2 receptors. As an example, bradykinin stimulates the generation of prostanoids possibly by activating directly one of the enzymes of the arachidonic acid cascade (Rhaleb *et al.*, 1988).

An additional mechanism through which bradykinin could affect smooth muscle motility is related to activation of the 'efferent' function of capsaicin-sensitive sensory nerves (see Szolcsányi 1984, and Maggi & Meli 1988, for reviews). Bradykinin is, amongst the mediators of inflammation, one of the most potent stimulants of sensory nerves (Armstrong, 1970; Ferreira *et al.*, 1973). This action, responsible for the pain-producing action of bradykinin, also involves the release, from peripheral terminals of capsaicin-sensitive sensory nerves of several neuropeptides (Geppetti *et al.*, 1988; Manzini *et al.*, 1989) which may directly affect visceral motility.

Despite the obvious interest for its potential pathophysiological role in cystitis, relatively little information is available about the effects of bradykinin on the urinary bladder muscle. Bradykinin produces a contraction of the urinary bladder in several species, although with marked differences in sensitivity (Falconieri-Erspamer *et al.*, 1973). In the rabbit detrusor muscle the response to bradykinin was markedly reduced by cyclo-oxygenase inhibitors and biochemical evidence for prostaglandin E_2 (PGE_2) production was presented (Downie & Rouffignac, 1981; Nakahata *et al.*, 1987; Nakahata & Nakanishi, 1988). In the dog isolated urinary bladder the contractile response to bradykinin was mediated by B_2 receptors (Regoli *et al.*, 1986). Here we have addressed the question of the mechanisms responsible for the motor responses to bradykinin in the guinea-pig isolated urinary bladder. As recent evidence indicates that certain tissue components such as the bladder mucosa (Maggi *et al.*, 1987a) or metabolism via a thiorphan-sensitive mechanism (Devillier *et al.*, 1988; Dusser *et al.*, 1988) may exert a strong influence on the biological activity of exogenously administered peptides, we also investigated the possible influence of these variables on the response to bradykinin.

Methods

In vitro *experiments*

Male albino guinea-pigs (Dunkin-Hartley strain) weighing 240–300 g were used throughout the study. The animals were killed by cervical dislocation and exsanguinated. The dome of the urinary bladder was rapidly removed, placed in Krebs solution, opened along its longitudinal axis and pinned flat on a Petri dish. Bladders strips about 1 cm long and 3 mm wide were excised with fine scissors. In most strips the mucosa was separated from the muscle (see Results). The strips were placed in a 5 ml organ bath containing a standard Krebs solution gassed with 96% O_2 and 4% CO_2 of the following composition (mM): NaCl 119, KCl 4.7, $MgSO_4$ 1.5, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.

A resting load of 5 mN was applied and the preparations were allowed to equilibrate for 60 min before commencing the experiments. Tension was recorded by means of an isotonic strain gauge connected to a Basile 7050 Unirecord. The contractile response to KCl (80 mM, added to the bath) was used as internal standard. The strips were electrically field stimulated (0.03 Hz, 60 V, 0.5 ms) by means of two wire platinum electrodes placed at the top and the bottom of the organ bath (GRASS S11 stimulator). Concentration-response curves to bradykinin were constructed in a cumulative manner, the next concentration being added when the effects of the preceding one had reached a steady state. Concentrations were increased on a log 10 basis scale starting from 1 nM. Preliminary experiments (see Results) had shown that no significant desensitization occurs with this protocol.

In vitro capsaicin desensitization was achieved by prolonged (30 min) exposure of the strips to 10 μM capsaicin, followed by washing and re-equilibration, as described previously (Maggi *et al.*, 1987b; 1989).

Determination of substance P- and calcitonin gene-related peptide-like immunoreactivity by radioimmunoassay

Mucosa-free strips of bladder muscle were placed in 2 ml organ baths maintained at 37°C and superfused with standard Krebs solution, oxygenated with a mixture of O_2 and CO_2, containing 0.1% bovine serum albumin and 10 μM thiorphan (Sigma) at a rate of 2 ml min^{-1} by means of a peristaltic pump. Fractions were collected each min, in tubes containing acetic acid to a 2 N final concentration. At the end of the experiments the tissues were blotted 2–3 times on filter paper and weighed. Superfusates were freeze-dried, reconstituted with assay buffer (0.1 M, pH 7.4 phosphate buffer containing 0.9% NaCl, 0.01% NaN_3 and 0.1% bovine serum albumin). Substance P-like (SP-LI) and calcitonin gene-related peptide like activity (CGRP-LI) were measured by radioimmunoassay (RIA) as described previously (Maggi *et al.*, 1987c; 1988; Geppetti *et al.*, 1988).

For SP-LI determination (Maggi *et al.*, 1988; Geppetti *et al.*, 1988; Manzini *et al.*, 1989) the ^{125}I-Bolton and Hunter conjugated SP (Amersham, U.K.)

and No. 144 anti-SP serum (kind gift of Dr P. Pradelles, SPI-LERI, CEN/Saclay, 91191, Gif Sur Yvette, Cedex, France) were used. The sensitivity of RIA was 1.1 fmol/tube. The antiserum crossreacts to 1% with neurokinin A, 0.5% with neurokinin B and less than 0.1% with physalaemin and eledoisin.

CGRP-LI determination was made in the same samples as described previously (Geppetti *et al.*, 1988; Manzini *et al.*, 1989), by using ^{125}I-Bolton and Hunter conjugated human CGRP (Amersham) and anti human CGRP rabbit serum (Peninsula). The sensitivity of the RIA was 2.5 fmol/tube.

Statistical analysis

All data in the text are mean ± standard error of the mean (s.e.mean) of 5–7 experiments. Statistical analysis of the data was performed by means of the Student's *t* test for paired or unpaired data or by means of analysis of variance, when applicable. A P value < 0.05 was considered statistically significant. Regression analysis was made by means of the least squares method. EC_{50} and 95% confidence limits (c.l.) were calculated as the concentrations producing 50% of maximal contraction. pA_2 values were calculated as described by Van Rossum (1963).

Drugs

Drugs used were: bradykinin (Peninsula), indomethacin (Sigma), capsaicin (Sigma), nifedipine (Sigma), tetrodotoxin (Sankyo), carbachol HCl (Merck), apamin (Peninsula). SC-19220 (1-acetyl-2-(8-chloro-10,11-dihydrobenz[b,E][1,4]oxazepine-10-carbonyl)hydrazine) was a kind gift of Dr D.L. Hammond, Searle Company, Skokie, Illinois, U.S.A. [D-Arg^0, Hyp^3, Thi^{5,8}, Phe^7]-bradykinin and kallidin were a kind gift of Prof D. Regoli, Dept. of Physiology and Pharmacology, Sherbrooke University, Canada. Glibencalamide was a kind gift of Dr I. Cavero (Rhone-Poulenc, Paris, France).

Results

General

Strips excised from the dome of the guinea-pig urinary bladder responded to added KCl (80 mM) with a rapid phasic contraction the amplitude of which became reproducible after a 90–120 min equilibration period. Both in the presence or absence of the mucosa, electrical field stimulation (0.03 Hz, 0.5 ms, 60 V) induced twitches that were abolished by tetrodotoxin (1 μM). There was a striking difference in degree of spontaneous activity which averaged 40–80 and 10–30% of the response to electrical field stimulation in strips with and without mucosa, respectively.

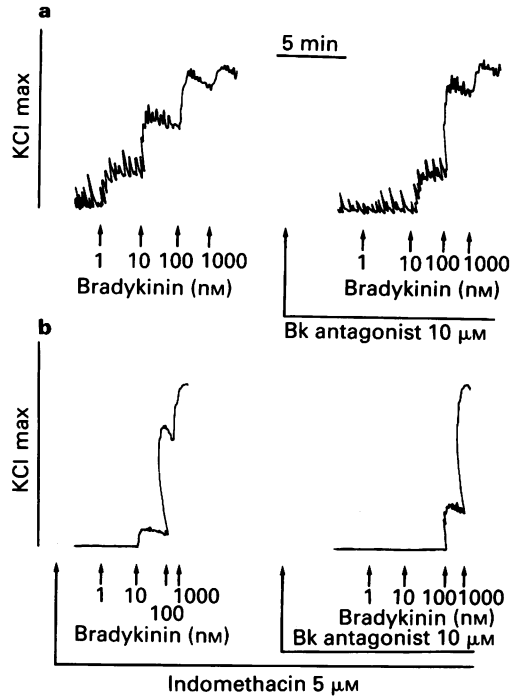


Figure 1 Typical tracings showing the contractile response of smooth muscle strips from the guinea-pig urinary bladder to cumulative addition of bradykinin (Bk) and its modification by the B_2 receptor antagonist [D-Arg^0, Hyp^3, Thi^{5,8}, Phe^7]-bradykinin (10 μM), in the absence (a) or presence (b) of indomethacin. Tension scale indicates the maximal contractile response to KCl.

Bradykinin produced a concentration-dependent contraction (Figure 1). In preliminary experiments we found no appreciable desensitization to cumulative addition of bradykinin (concentration increased on a log 10 scale, interval between doses 2–3 min), either in the presence or absence of mucosa. Thus, in strips without mucosa the response to 10 μM bradykinin amounted to 63 ± 6% of the response to KCl (80 mM) when the peptide was administered as a bolus and to 68 ± 6% when the same concentration of bradykinin was attained at the end of the cumulative concentration-response curve. Likewise, in strips with mucosa, the responses to 10 μM bradykinin were 46 ± 6 and 43 ± 8% of the response to KCl when the peptide was added to the bath as a bolus or at the end of cumulative concentration-response curves.

Effect of removal of the mucosa and thiorphan on the response to bradykinin

Removal of the mucosa increased significantly the contractile response to bradykinin which was about

Table 1 Effect of thiorphan and removal of the mucosa on the contractile response to bradykinin of the guinea-pig isolated bladder

	Without thiorphan (A)		*With thiorphan* (B)		A/B
With mucosa	6364	(835–12670)	82	(42–236)	78
Without mucosa	451	(173–2334)	26	(17–45)	17

Values are EC_{50} (nM) and 95% confidence limits (in parentheses).

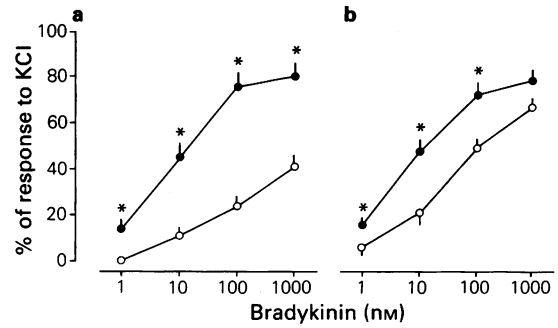


Figure 2 Effect of removal of the mucosa (b) and thiorphan (10 μM) on the contractile response of smooth muscle strips from the guinea-pig urinary bladder to bradykinin; (○) control; (●) plus thiorphan. Each value is mean of at least 6 experiments, with s.e. shown by vertical bars. Significantly different from control, $*P < 0.05$.

14 times more potent in the absence than in the presence of the mucosa (Table 1). Thiorphan (10 μM), a well known inhibitor of endopeptidase 24.11, produced a small (< 10% of KCl maximum) contraction *per se* which faded to baseline within 15 min in about 50% of preparations while it had no effect on the remainder. In the presence of thiorphan, the response to bradykinin was markedly enhanced (Figure 2). This potentiation was larger in the presence (78 times) than in the absence (17 times) of the mucosa (Table 1; Figure 2).

Mucosa-free bladder strips treated with thiorphan (10 μM, 15 min before) were then used in all the remaining experiments, unless otherwise specified.

Relaxant responses to bradykinin

To assess whether bradykinin may produce relaxation, bladder strips (mucosa-free, thiorphan 10 μM in the bath) were exposed to carbachol (5 μM). This produced a large phasic contraction followed by a steady tonic response which approached 80–90% of the response to KCl. In these conditions, bradykinin produced a concentration-dependent transient relaxation (Figure 3).

The maximal relaxant response to 10 μM bradykinin added as a bolus (48 ± 4%, $n = 4$) did not significantly differ from that observed when the same concentration was attained at the end of a cumulative concentration-response with a tenfold increase in concentration for each dose (42 ± 6%, $n = 6$). On the other hand when the relaxant response produced by a given concentration of bradykinin had faded, a second addition of the same dose produced a relaxant response much smaller (70–80% reduction) than that produced by the first dose. Bradykinin (0.1 μM) had no relaxant effect on the tonic contraction produced by KCl (80 mM, $n = 3$).

Effect of kallidin and [des-Arg^9]-bradykinin

Kallidin (1 nM–1 μM) produced motor responses similar to those produced by bradykinin, that is a concentration-dependent contraction of the unstimulated bladder and a concentration-dependent relaxation of the carbachol-induced tone (Figure 4). Kallidin was as effective as bradykinin in producing a contraction and slightly more effective in producing the relaxation (Figure 4).

[des-Arg^9]-bradykinin had little or no contractile activity nor did it induce relaxation of carbachol-

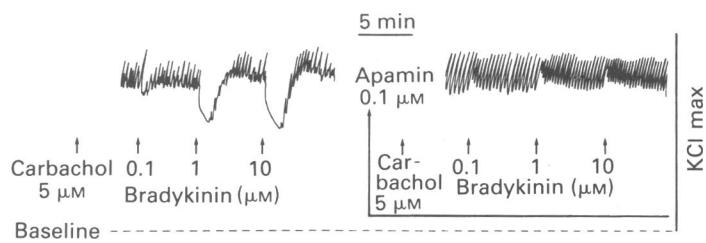


Figure 3 Typical tracings showing the relaxation produced by cumulative addition of bradykinin of smooth muscle strips from the guinea-pig urinary bladder (tone elevated by 5 μM carbachol) and blockade of this response by apamin. Tension scale indicates the maximal contractile response to KCl.

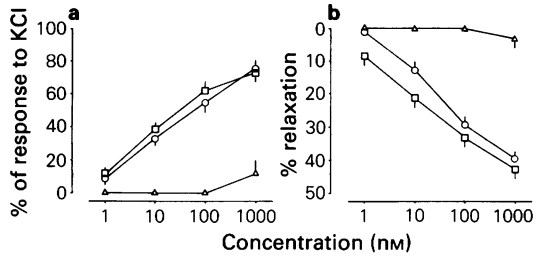


Figure 4 Contractile (a) and relaxant (b) responses to bradykinin (○), kallidin (□) and [des-Arg^9]-bradykinin (△) of smooth muscle strips from the guinea-pig urinary bladder. To study relaxations, tension was elevated by addition of 5 μM carbachol to the bath. Each value is mean of at least 6 experiments; s.e.mean shown by vertical lines.

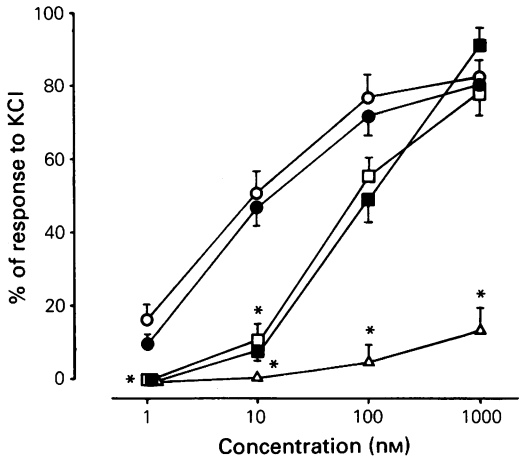


Figure 5 Effect of tetrodotoxin (1 μM, ●), indomethacin (5 μM, □), SC-19220 (100 μM, ■) or nifedipine (△) on the contractile response of smooth muscle strips from the guinea-pig urinary bladder to bradykinin; (○) control. Each value is mean of at least 6 experiments, s.e. shown by vertical bars. Significantly different from control, $*P < 0.05$.

induced tone up to 1 μM (Figure 4). In some experiments, to assess the possible involvement of a *de novo* synthesis of B_1 receptors during prolonged *in vitro* incubation (see Regoli & Barabé, 1980), the effect of the B_1 receptor agonist was investigated 4–5 h from the beginning of the experiment. Even in this case ($n = 3$) there was little or no response to [des-Arg^9]-bradykinin.

Effect of in vitro *capsaicin desensitization on the contractile response to bradykinin*

Previous exposure to capsaicin (10 μM for 30 min) did not significantly affect the contractile response to bradykinin, the EC_{50} and 95% confidence limits of this latter being 23 (7–45) and 22 (8–36) nM in controls and in preparations exposed to capsaicin, respectively. The same occurred in the absence of thiorphan, the EC_{50} of bradykinin being 251 (73–2334) and 143 (33–1815) nM in controls and in preparations exposed to capsaicin, respectively.

Effect of tetrodotoxin, indomethacin, nifedipine or SC-19220 on the contractile response to bradykinin

Tetrodotoxin (1 μM for 15 min) had a slight and not statistically significant inhibitory effect on the response to bradykinin (Figure 5, Table 2). Either indomethacin (5 μM for 90 min, Figure 1) or the prostaglandin antagonist SC-19220 (100 μM for 15 min) significantly antagonized the response to low concentrations of bradykinin (1–10 nM) while having no effect on the response to the higher concentrations (0.1–1 μM) (Figure 5). The EC_{50} of bradykinin was increased by about 10 times in the presence of either indomethacin or SC-19220 (Table 2). In other experiments the effect of both indomethacin (5 μM for 90 min) and SC-19220 (100 μM for 15 min) on the response to bradykinin was investigated. As shown in Table 2, the combined treatment with these two agents was not more effective than that produced by either of them when administered alone, as the EC_{50} of bradykinin was increased by about 13 times as compared to controls.

At the concentration tested, SC-19220 produced a slight enhancement of electrical field stimulation-induced contractions while having no effect against the response to KCl ($n = 4$). The bradykinin-induced

Table 2 Effects of various pretreatments on the contractile response to bradykinin of the guinea-pig isolated urinary bladder (mucosa-free, thiorphan 10 μM in the bath)

Treatment	EC_{50} *and 95% confidence limits* (nM)	
Controls	26	17–45
Tetrodotoxin 1 μM	36	13–90
Indomethacin 5 μM	259	64–810
SC-19220 100 μM	258	150–315
Indomethacin 1 μM plus SC-19220 100 μM	330	218–456
B_2 antagonist 10 μM	179	158–207
B_2 antagonist 10 μM plus indomethacin 5 μM	1760	1189–2347
Nifedipine 1 μM	$\geqslant$ 10000	–
Apamin 0.1 μM	26	16–53

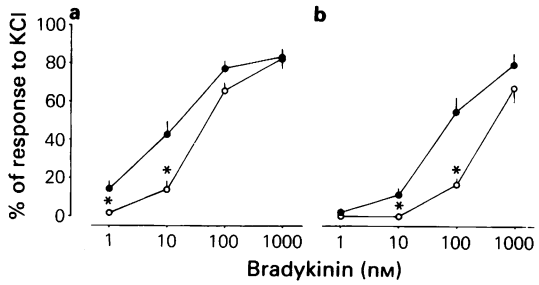


Figure 6 Effect of the bradykinin antagonist, [D-Arg^0, Hyp^3, Thi^{5,8}, Phe^7]-bradykinin (10 μM), on the contractile response of smooth muscle strips from the guinea-pig urinary bladder to bradykinin in the absence (a) or presence (b) of indomethacin: (●) control; (○) B_2-antagonist. Each value is mean of at least 6 experiments, s.e. shown by vertical lines. * Significantly different from control, *$P < 0.05$.

contraction was almost abolished by nifedipine (1 μM, 30 min before, $n = 4$, Figure 5).

Effect of [D-*Arg*^{0}, *Hyp*^{3}, *Thi*^{5,8}, *Phe*^{7}]-*bradykinin on the contractile response to bradykinin*

The B_2 receptor antagonist, [D-Arg^0, Hyp^3, Thi^{5,8}, Phe^7]-bradykinin (10 μM) itself produced either a small potentiation (5–10%) or no effect on twitches produced by electrical field stimulation and did not modify the contractile response to KCl, either in absence or presence of indomethacin ($n = 8$). This antagonist (10 μM, 15 min before) produced a rightward shift of the concentration-response curve to bradykinin which, however, attained a similar maximal response, both in the absence or presence of indomethacin (Figure 1 and 6). The B_2 antagonist increased the EC_{50} of bradykinin by 6.9 and 6.8 times in the absence and presence of indomethacin, respectively. The corresponding apparent pA_2 values, calculated as described by Van Rossum (1963), were 5.33 and 5.69 in the absence and presence of indomethacin, respectively.

Effect of tetrodotoxin, capsaicin desensitization, indomethacin, or [D-*Arg*^{0}, *Hyp*^{3}, *Thi*^{5,8}, *Phe*^{7}]-*bradykinin on bradykinin-induced relaxation*

Neither tetrodotoxin (1 μM) nor indomethacin (5 μM) had any effect on bradykinin-(1 μM) induced relaxation which was slightly but not significantly enhanced after *in vitro* capsaicin desensitization (Figure 7).

By contrast, the bradykinin-induced relaxation was antagonized, in a competitive manner by [D-Arg^0, Hyp^3, Thi^{5,8}, Phe^7]-bradykinin (10 μM) (Figure

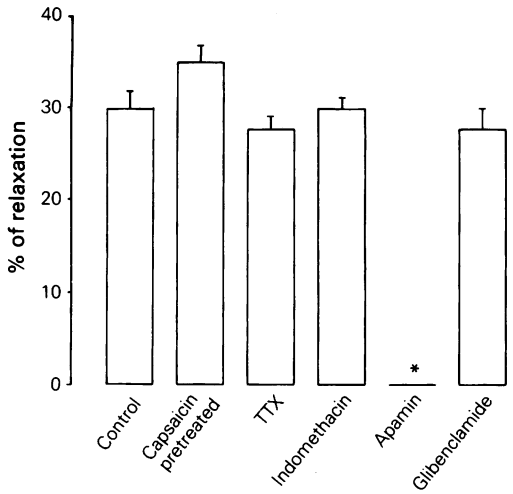


Figure 7 Effect of *in vitro* capsaicin desensitization, tetrodotoxin (1 μM), indomethacin (5 μM), apamin (0.1 μM) or glibenclamide (1 μM) on the relaxant response produced by 1 μM bradykinin on carbachol-induced tone of smooth muscle strips from the guinea-pig urinary bladder. Each column is mean of 4–6 experiments; vertical bars show s.e.mean. Significantly different from control *$P < 0.05$.

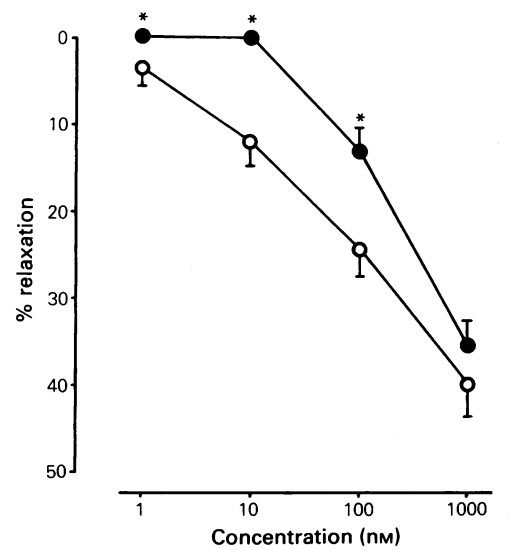


Figure 8 Effect of the bradykinin antagonist, [D-Arg^0, Hyp^3, Thi^{5,8}, Phe^7]-bradykinin (10 μM) on the bradykinin-induced relaxation of carbachol-induced tone of smooth muscle strips from the guinea-pig urinary bladder: (○) control; (●) B_2 receptor antagonist. Each value is mean of 6 experiments with s.e. shown by vertical bars. * Significantly different from control, *$P < 0.05$.

8). The corresponding pA_2 value, calculated as described by Van Rossum (1963) was 5.56.

Effect of apamin

Apamin (0.1 μM 15 min before), the peptide extracted from bee venom which blocks certain Ca-activated K channels (see Cook, 1988 for review), completely prevented the bradykinin-induced relaxations of carbachol-contracted strips (Figure 3). By contrast, glibenclamide (1 μM for 15 min) a potent blocker of ATP-sensitive K channels (Schmit-Antomarchi *et al.*, 1987) had no effect on bradykinin-induced relaxations ($n = 4$). The effect of apamin on the contractile response to bradykinin was examined. Estimation of the effect of low concentrations of bradykinin in the presence of apamin was difficult because apamin itself produced rhythmic contractile activity, the amplitude of which was 10–20% of the response to KCl. In spite of this, the estimated ED_{50} of bradykinin in the presence of apamin (0.1 μM, 15 min before) was similar to that observed in controls (Table 2).

Activation of capsaicin-sensitive nerves by bradykinin

As shown in Figure 9, bradykinin (10 μM) produced a consistent increase in CGRP-LI outflow from mucosa-free muscle strips from the guinea-pig urinary bladder. By contrast, no significant SP-LI outflow was induced (Figure 9). We have shown previously that CGRP-LI is stored and released from capsaicin-sensitive sensory nerves in this organ (Maggi *et al.*, 1988). In subsequent experiments CGRP-LI outflow was measured as an index of activation of capsaicin-sensitive fibres in this tissue. [des-Arg^9]-bradykinin (10 μM) failed to elicit any increase in CGRP-LI outflow while subsequent administration of bradykinin (10 μM) on the same samples had a consistent releasing effect ($n = 4$, data not shown). The bradykinin (10 μM)-induced CGRP-LI release was abolished by indomethacin (5 μM for 30 min, Figure 10).

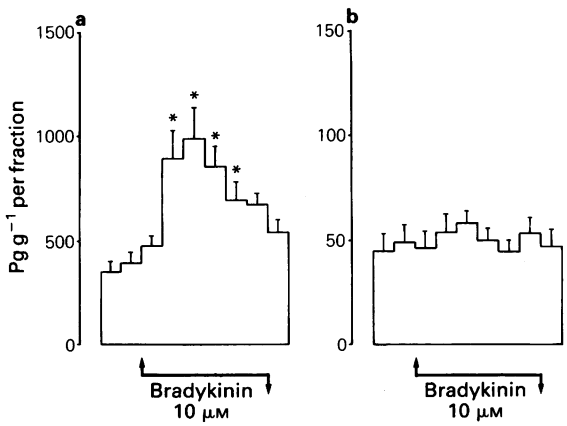


Figure 9 Effect of 10 μM bradykinin on calcitonin gene-related peptide-like immunoreactivity (a) and substance P-like immunoreactivity (b) outflow from superfused muscle of the guinea-pig urinary bladder. Each value is mean of 4 experiments; vertical bars show s.e.mean. Significantly different from baseline, $*P < 0.05$.

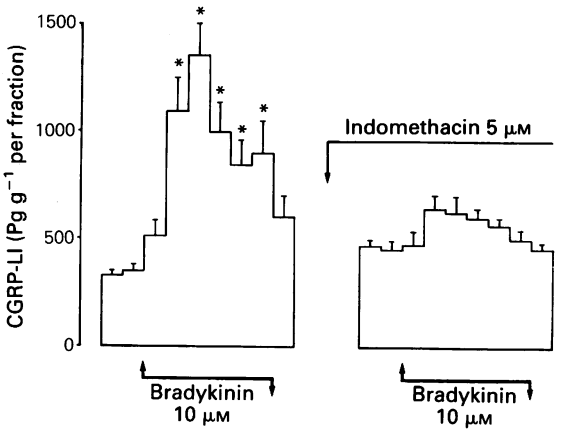


Figure 10 Effect of indomethacin (5 μM) on bradykinin (10 μM)-induced calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) released from superfused muscle strips of the guinea-pig isolated urinary bladder. These experiments were performed in parallel by comparing the effect of bradykinin in absence or presence of indomethacin on muscle strips from the same bladders. Each value is mean of 4 experiments; s.e. shown by vertical bars. Significantly different from baseline, $*P < 0.05$.

Discussion

Effect of removal of the mucosa and thiorphan

The present findings provide further evidence indicating that estimates of the biological activity of bradykinin may be strongly influenced by peptide degradation (Erdös, 1979; Regoli, 1987; Dusser *et al.*, 1988). Several proteases are able to hydrolyze kinins (Erdös, 1979; Regoli, 1987). Recently Dusser *et al.* (1988) reported that phosphoramidon and leucine-thiorphan, two inhibitors of endopeptidase 24.11, markedly enhance the contractile response to

bradykinin in the ferret isolated trachea. Here we show that thiorphan produced a marked potentiation of the response to bradykinin. Biochemical investigations have shown that bradykinin can be cleaved by endopeptidase 24.11 (Turner, 1987). We have not directly assessed whether this enzymatic activity is present in the guinea-pig urinary bladder and, for this reason, we cannot exclude that thiorphan produced its effect by blocking other enzymes capable of degrading bradykinin.

The present data indicate that the thiorphan-sensitive activity which limits the expression of the full biological activity of bradykinin resides in both the mucosal and the muscle layer of the guinea-pig urinary bladder. Indeed removal of the mucosal layer itself increased the potency of bradykinin, a finding which recalls similar observations with SP in this same organ (Maggi *et al.*, 1987a). We cannot exclude the possibility that the mucosal layer acted as a diffusion barrier toward exogenous peptides, but the observation that enhancement of bradykinin activity by thiorphan was greater in the presence (78 times) than in the absence (17 times) of the mucosa suggests that the latter plays a role in the inactivation of bradykinin, e.g. the thiorphan-sensitive activity is more concentrated in the mucosa than in the muscle.

Further studies are needed to establish the mechanism(s) whereby removal of the mucosa and addition of thiorphan enhanced the response to bradykinin. Nevertheless, these experiments allowed us to select proper experimental conditions (mucosa-free muscle strips exposed to 10 μM thiorphan) for studying the mechanisms underlying the motor responses to bradykinin.

Motor responses to bradykinin

The present findings indicate that bradykinin exerts two opposite motor effects in the guinea-pig urinary bladder muscle. These two effects can be better assessed by a further selection of the experimental conditions, that is a contraction at resting tone and a relaxation when the tone was raised by carbachol. Both effects are largely if not totally tetrodotoxin-resistant suggesting that intramural nerves do not play a key role in their genesis. The same applies to the intramural capsaicin-sensitive nerves. Although bradykinin is capable of stimulating the 'efferent' function of these nerve endings (see below), both the contraction and the relaxation produced by this peptide were unaffected by a previous exposure of the tissues to a high concentration of capsaicin, a procedure shown to produce a long-lasting blockade of sensory nerves (Maggi *et al.*, 1987b; 1989; Barthó *et al.*, 1987) (sensory neurone blocking action of capsaicin, as defined by Szolcsányi, 1984; 1989). Either tachykinins or CGRP can affect motility of the guinea-pig urinary bladder (Maggi *et al.*, 1988) but, conceivably, the motor effects of bradykinin are strong enough to overcome those produced by released tachykinins or CGRP.

The present findings suggest that B_1 receptors play no role in the action of bradykinins, as [des-Arg^9]-bradykinin, a selective B_1 receptor agonist (Regoli & Barabé, 1980) was virtually ineffective. The contractile response to bradykinin apparently involves both B_2 receptor activation and prostanoid synthesis along the cyclo-oxygenase pathway. The two mechanisms are apparently independent, because the inhibitory actions of indomethacin and the B_2 receptor antagonist were additive. By contrast, the actions of indomethacin and SC-19220 a PGE_2 antagonist (Drower *et al.*, 1987) were not additive. This conclusion agrees with the findings of Rhaleb *et al.* (1988) who proposed that activation of prostanoid synthesis by bradykinin may occur independently from B_1 or B_2 receptor activation. In any case, influx of extracellular calcium via nifedipine-sensitive calcium channels seems a likely final event for the genesis of the contractile response. B_2 receptors are apparently responsible for the bradykinin-induced relaxation which was observed when the tone was raised with carbachol but not when tone was raised by KCl. Presumably this implies that bradykinin relaxes smooth muscle cells by hyperpolarization due to opening of K channels. This could explain the sensitivity of the bradykinin-induced relaxation to apamin, a well known blocker of certain calcium-activated K channels, while glibenclamide, a blocker of certain ATP-sensitive K channels was ineffective (Schmit-Antomarchi *et al.*, 1987; Cook, 1988). These findings are reminiscent of the apamin-sensitive relaxation produced by bradykinin in the guinea-pig taenia caeci preparation (Carter *et al.*, 1987; Den Hertog *et al.*, 1988) which have been shown to involve activation of B_2 receptors. Likewise, bradykinin was shown to produce a transient rise in intracellular free neuronal calcium associated with transient hyperpolarization due to activation of an apamin-sensitive outward K current (Miller, 1987).

It is interesting to note that the B_2 receptor antagonist used in this study had a similar pA_2 value (about 5.5) when tested against bradykinin-induced contraction and relaxation. Thus the same receptor could mediate both responses in the guinea-pig bladder. [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7] bradykinin has been shown to be quite a potent ($pA_2 > 6.5$) bradykinin antagonist in some systems (rabbit jugular vein, rat vas deferens) (Rifo *et al.*, 1987; Whalley *et al.*, 1987) while in other tissue (guinea-pig taenia caeci, Field *et al.*, 1988; guinea-pig urinary bladder, this study) it showed a consistently lower

affinity for B_2 receptors (pA_2 about 5.5). Further studies are needed to assess which subtype of the B_2 receptor (see Plevin & Owen, 1988 for review) mediates the motor responses to this peptide in the guinea-pig urinary bladder.

Activation of sensory nerves by bradykinin

There is ample evidence indicating that bradykinin is a potent stimulant of sensory nerves and this action produces not only pain and activation of reflex responses (Lembeck *et al.*, 1976; Kumazawa *et al.*, 1980; Hori *et al.*, 1986; Dray *et al.*, 1988a,b; Rioux *et al.*, 1987; Staszewska-Woolley *et al.*, 1988) but also neuropeptide release from peripheral endings of capsaicin-sensitive nerves which exert a variety of motor and inflammatory actions. As discussed above, the motor effects produced by neuropeptides released by bradykinin in the guinea-pig urinary bladder are probably obscured by the motor effects produced by bradykinin itself (B_2 receptor activation) and prostanoid generation. It is however possible that peptides released from sensory nerves (Saria *et al.*, 1984; Markowitz *et al.*, 1987) play a role in the inflammatory response produced by bradykinin in the urinary tract. In a previous study (Maggi *et al.*, 1988) we demonstrated a simultaneous release of both SP-LI and CGRP-LI by capsaicin from mucosa-free muscle strips of the guinea-pig urinary bladder. Tissue content of both peptides is depleted (>90% reduction) by systemic capsaicin desensitization. Therefore we can reasonably assume that CGRP-LI released by bradykinin in the present experiments originated from peripheral endings of these primary sensory neurones. The reason why no SP-LI release was detected in response to bradykinin is at present unclear. However, the CGRP-LI release induced by bradykinin is only a fraction (<20%) of the response to capsaicin (unpublished data). When looking at the response to capsaicin in the guinea-pig urinary bladder, the released CGRP-LI/SP-LI ratio lies between 12–16/1 (Maggi *et al.*, 1988). It is therefore conceivable that a putative SP-LI release by bradykinin might have been too low to be detected with RIA.

Using different experimental models, evidence has been presented that bradykinin might excite sensory fibres both directly and through the release of prostanoids (Lembeck *et al.*, 1976; Hori *et al.*, 1986). In some instances evidence has been presented indicating the involvement of B_2 receptors (Dray *et al.*, 1988a; Staszewska-Woolley *et al.*, 1988).

The present findings indicate that SP-LI and CGRP-LI release by bradykinin in the guinea-pig urinary bladder is largely indirect, via prostanoid production. A similar conclusion was drawn about the SP-LI and CGRP-LI release from sensory nerves in the guinea-pig heart (Manzini *et al.*, 1989). When looking at the contractile response produced by bradykinin in the guinea-pig bladder (cf. Rhaleb *et al.*, 1988) one might speculate that B_2 receptors play a minor role in activating sensory fibres in this preparation, but further studies are needed to elucidate this point.

Conclusions

The present findings indicate that bradykinin produces multiple responses in the bladder which involve activation of both B_2 receptors and prostanoid synthesis, apparently via independent mechanisms. The consequences of mucosal removal and addition of thiorphan suggest that potent endogenous mechanism(s) operate to limit the activity of this peptide. When produced in biologically relevant quantities during an inflammatory process such as cystitis (cf. Marceau *et al.*, 1980) the effects of bradykinin would probably be such as to affect reflex regulation of bladder motility, by stimulation of capsaicin-sensitive nerves which regulate the afferent branch of reflex micturition in this species (Maggi *et al.*, 1987c). This may occur through stimulation of sensory nerves (either directly or via prostanoid production) and the motor response to the peptide which could itself activate bladder mechanoreceptors. Thus, bradykinin might contribute significantly to the genesis of symptoms such as urinary frequency in addition to sustaining neurogenic inflammation.

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Simultaneous measurement of endothelium-derived relaxing factor by bioassay and guanylate cyclase stimulation

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1 Endothelium-derived relaxing factor (EDRF) released by cultured endothelial cells (EC) from bovine aortae was measured by bioassay using pre-contracted strips of rabbit aorta and by radioimmunoassay of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) produced by stimulation of bovine lung soluble guanylate cyclase.

2 Bradykinin (Bk, 3 and 30 pmol) injected through a column of EC caused release of EDRF as detected by bioassay and increased cyclic GMP concentrations. Superoxide dismutase (SOD, 15 u ml^{-1}) increased the amount of EDRF detected by the activation of soluble guanylate cyclase.

3 In the absence of endothelial cells, nitric oxide (NO, 1–2 μM), arachidonic acid (AA, 3–30 μM) or sodium nitroprusside (SNP, 1–100 μM) stimulated guanylate cyclase. Superoxide dismutase strongly increased the stimulation of guanylate cyclase induced by NO, but had little effect on the stimulation induced by SNP and no effect on the stimulation induced by AA.

4 Oxyhaemoglobin (10–300 μM) abolished the stimulation of guanylate cyclase by EDRF, NO or SNP but was much less effective as an inhibitor of AA-induced stimulation of guanylate cyclase.

5 These results demonstrate that measurement of guanylate cyclase stimulation by radioimmunoassay is a viable method for detecting EDRF release, especially useful when the drugs used interfere with bioassay tissues.

Introduction

The mechanism of release of endothelium-derived relaxing factor (EDRF) has been studied mainly by bioassay using either isolated vascular strips (Furchgott & Zawadzki, 1980) or columns of cultured endothelial cells (EC; Cocks *et al.*, 1985; Gryglewski *et al.*, 1986a; de Nucci *et al.*, 1988a). This latter method made it possible to separate the EDRF-generating cells from the detecting tissues.

The use of bioassay to detect EDRF is limited because some drugs or procedures interfere with the responses of the bioassay tissues. It is also difficult to study the kinetics of EDRF release by bioassay for the tissue relaxations have a slow time course, taking minutes to wear off after the removal of the stimulus.

We have, therefore, developed another method to measure EDRF release from cultured bovine aortic EC. EDRF activates guanylate cyclase (Rapoport & Murad, 1983) and Mulsch *et al.* (1987) showed that EDRF activates purified soluble guanylate cyclase from bovine lung but did not report on the effects of other endogenous activators of guanylate cyclase such as arachidonic acid (AA), lipid peroxides or hydrogen peroxide.

We have compared activation of guanylate cyclase from bovine lungs with measurement of EDRF by bioassay tissues. The guanylate cyclase method has been used to elucidate the dynamics of EDRF release.

Methods

Bovine aortic endothelial cells

Endothelial cells were isolated by treatment of bovine aorta with 0.2% (w/v) collagenase. Cells were

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grown to confluence in plastic vessels, then removed by treatment with 0.05% (w/v) trypsin, and seeded onto Cytodex 3 microcarrier beads (Pharmacia). The beads were stirred for 3–7 days, until the cells became confluent, and then packed into columns of 1.5–2 ml containing 15–20 × 10^6 EC (de Nucci *et al.*, 1988a).

Preparation of guanylate cyclase from bovine lungs

The following procedures were carried out in an ice bath or refrigerator. Pieces (100 g) of bovine lungs were chopped and homogenized in a Polytron homogenizer with a buffer at pH 7.4 [Tris/HCl 10 mM, 300 ml, containing sucrose (0.25 M), ethylenediaminetetra-acetic acid (EDTA, 1 mM) and dithiothreitol (DTT, 2 mM)]. The homogenates were centrifuged at 500 *g* for 10 min, the supernatant collected and further centrifuged at 105,000 *g* for 1 h. The high speed supernatant (cytosol fraction) was applied to a column (4 × 15 cm) of DEAE cellulose (DE-52, Whatman) which was pre-washed (2 l) with buffer (Tris/HCl, 10 mM) containing DTT (2 mM) at pH 7.4 (buffer A). The column was further washed with the same buffer (1 l) followed by washing (1 l) with buffer A containing NaCl (0.1 M). For elution, the column was finally washed with the same buffer containing NaCl (0.5 M) and 10 ml fractions were collected. Fractions containing enzymatic activity were collected and dialysed overnight against buffer A. The dialysate was applied to a column (1.5 × 19 cm) of Blue-Sepharose CL-6B (Pharmacia) previously equilibrated with buffer A and the column washed with 1 l of buffer A containing NaCl (0.1 M). The enzyme was eluted with buffer A containing NaCl (0.5 M) and 3 ml fractions were collected. The main active fractions (absorbance at 280 nm above 0.1) were pooled and dialysed overnight against buffer A. This procedure removed most of the haemoglobin from the eluate of the column of DE-52. The dialysate was frozen in aliquots at −70°C, thawed before use and not refrozen. In SDS-polyacrylamide gel electrophoresis about 50% of the protein migrated at 72 kD, the molecular weight reported for the soluble guanylate cyclase subunit of bovine lung (Gerzer *et al.*, 1981). This method is a modified version of that described by Gerzer *et al.* (1981).

Enzyme assay

The activity of the enzyme was monitored by incubating the enzyme for 5 min at 37°C with buffer B at pH 7.4 containing Tris/HCl (50 mM), $MgCl_2$ (5 mM), ethyleneglycol-*bis*-(α-aminoethyl ether)N,N,N,N, tetraacetic acid (EGTA; 10 mM) and guanosine-5′-triphosphate (GTP, 0.2 mM). The reaction was terminated by adding two volumes of EDTA (70 mM; pH 7.4) which chelates Mg^{2+}, an essential cofactor of guanylate cyclase and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) formation measured by a radioimmunoassay kit using [^{3}H]-cyclic GMP (Amersham).

Simultaneous detection of EDRF by bioassay and guanylate cyclase activation

EDRF was measured by bioassay using a cascade of pre-contracted rabbit aortic strips (RbAs) which were denuded of endothelium and superfused with the effluent from a column of EC (Gryglewski *et al.*, 1986a). For the simultaneous detection of EDRF by bioassay and guanylate cyclase activation the effluent of the column was split into two. One half superfused the assay tissues and the other was collected in 20 s fractions in test tubes containing the same volume of buffer B (twice concentrated). The guanylate cyclase was added to the test tube just before the collection of the effluent. After collection of the effluent the reaction was continued for another 10 min at room temperature and terminated as described above.

Detection of EDRF release from a mini-column with guanylate cyclase activation

A mini-column packed with 0.5 ml of beads carrying 2.5–5 × 10^6 cells was perfused at 1 ml min^{-1} with oxygenated (95% O_2/5% CO_2) and warmed (37°C) Krebs solution. The column was immersed in a water bath at 37°C and left to stabilize for 20 min before collecting the first fraction into test tubes as described above. Bradykinin (1–10 pmol) was injected through the mini-column and the effluent collected every 15 s into tubes containing the enzyme preparation. The reaction was continued for 5 min at 37°C and terminated as described above.

Preparation of oxyhaemoglobin

Oxyhaemoglobin was prepared by reduction of bovine haemoglobin with sodium hydrosulphite followed by gel filtration with a prepacked disposable column PD-10 (Pharmacia) previously equilibrated with Tris/HCl (50 mM) at pH 7.4. The concentration of oxyhaemoglobin was determined by a spectrophotometric method (Shimadzu UV-160; E_{576nm} = 15.99 mM^{-1} cm^{-1}).

Preparation of nitric oxide

Nitric oxide solution was prepared by injecting 1 ml of NO gas into 40 ml of helium-deoxygenated water

(de Nucci et al., 1988b). The concentration of NO was calculated assuming that all the NO was dissolved (solubility of NO is 4.6 ml per 100 ml at 20°C).

Materials

Superoxide dismutase (SOD, from bovine erythrocytes) was dissolved in saline before use and kept at room temperature. Sodium arachidonate (AA, from porcine liver) was dissolved in Tris/HCl (50 mM) pH 7.4 and protected from light until used. Bradykinin, SOD, AA, atriopeptin (II) (rat sequence), ADP, sodium nitroprusside, Trizma, sodium hydrosulphite, DL-dithiothreitol, haemoglobin (from bovine blood) and EGTA were obtained from Sigma (Poole, UK). EDTA and NO cylinders were purchased from BDH (Dagenham, UK). GTP (disodium salt) was obtained from Boehringer Mannheim (West Germany) and 3-isobutyl-1-methylxanthine (IBMX) from Aldrich (Poole, UK). U46619 was a gift from Dr J. Pike (Upjohn, Kalamazoo).

Statistics

Results are shown as mean values ± s.e.mean for n experiments. Student's unpaired t test was used to determine the significance of differences between means and a P value of <0.05 was taken as significant.

Results

Simultaneous detection of EDRF by bioassay and cyclic GMP-radioimmunoassay

Bradykinin (3 and 30 pmol) injected through the column (TC) of EC caused release of EDRF, as detected by the relaxation of the RbAs (Figure 1a) and radioimmunoassay for cyclic GMP (Figure 1b). Note that the amounts of cyclic GMP reached a maximum before the relaxation of the bioassay tissues was complete. Glyceryl trinitrate (GTN, 1–10 μM) did not affect the enzyme preparation. Significant correlation ($P < 0.01$) was observed between relaxation of RbA and activation of soluble guanylate cyclase (Figure 2).

Experiments with mini-columns of endothelial cells

Bradykinin (1–10 pmol) injected through mini-columns of EC induced EDRF release in a dose-dependent manner, as detected by stimulation of soluble guanylate cyclase (Figure 3). The amounts of basal and stimulated EDRF release were significantly ($P < 0.05$) increased by infusion of SOD (15 u ml^{-1}) through the column (Figure 3a). Subsequent addition of catalase (100 u ml^{-1}, $n = 3$) did not affect bradykinin-induced release of EDRF (not shown). Oxyhaemoglobin (10 nM) inhibited the stimulation of soluble guanylate cyclase induced by EDRF (Figure 3b).

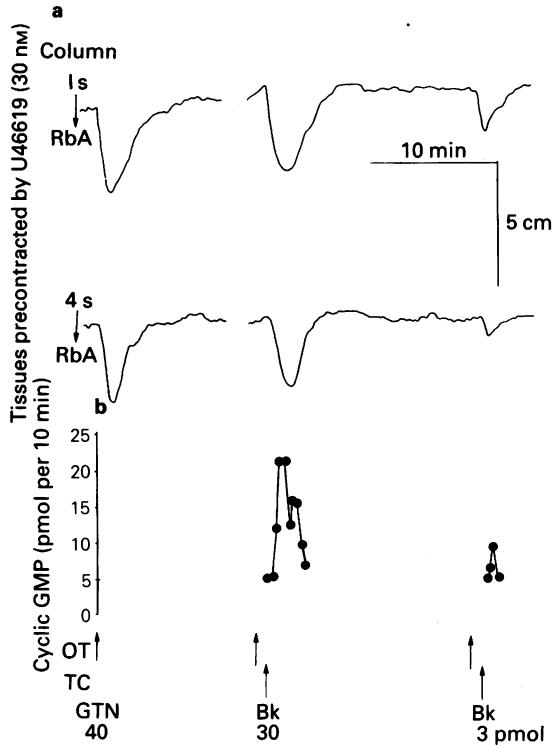


Figure 1 Endothelium-derived relaxing factor (EDRF) release measured by bioassay and guanylate cyclase assay. (a) Relaxation of rabbit aortae (RbAs) after injection of bradykinin (Bk; 3 and 30 pmol) through the column of endothelial cells (EC). EDRF released by Bk (30 pmol) caused relaxation of RbAs equivalent to that of glyceryl trinitrate (GTN, 40 pmol) U46619 = 11,9 epoxymethano-PGH_2 (b): Guanylate cyclase activation. Column effluent (0.88 ml) was collected for 20 s into each test tube containing the same volume of buffer B (twice concentrated) before and after injection of Bk. Guanylate cyclase (17 μg) was added just before collection of the effluent. Superoxide dismutase (10 u ml^{-1}) was infused through the column of EC throughout the experiment. Results of a typical experiment (one of three) are shown. OT = over the assay tissues; TC = through the column of EC.

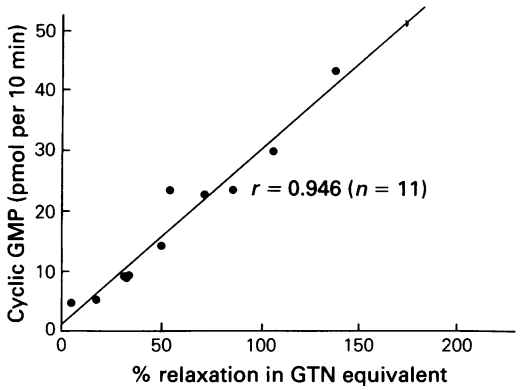


Figure 2 Correlation between bioassay and radioimmunoassay for cyclic GMP as detector methods for endothelium-derived relaxing factor (EDRF) release. The release of EDRF from a column of endothelial cells (EC) was induced by ADP (2 nmol) or bradykinin (3–100 pmol). The experiments were carried out under the same conditions as in Figure 1. The same preparation of soluble guanylate cyclase was used for all determinations. Each point represents assay of EDRF after injections of ADP or bradykinin in 4 experiments. EDRF release was measured on the first rabbit aorta and expressed as equivalents of glyceryl trinitrate (GTN).

Experiments with soluble guanylate cyclase

Nitric oxide (1–2 μM), sodium nitroprusside (SNP, 0.3–100 μM) or arachidonic acid (AA, 3–30 μM) activated soluble guanylate cyclase in the absence of endothelial cells (Figure 4). Superoxide dismutase (15 u ml^{-1}) strongly potentiated the stimulation of the enzyme induced by NO but it was less effective when guanylate cyclase was activated by SNP (Figures 4a and b). However, SOD had little or no effect on AA-induced guanylate cyclase activation (Figure 4c). When all the nine basal values (with no addition of NO, SNP or AA) were compared, there was a significant increase in guanylate cyclase activity in the presence of SOD (15 u ml^{-1}) from 4.4 $\pm$ 0.4 to 5.9 $\pm$ 0.4 pmol per 5 min ($n = 9$, $P < 0.05$). The basal activity of soluble guanylate cyclase was not affected by pre-incubation with IBMX (0.01–1 mM, $n = 3$, results not shown) or rat atrial natriuretic factor (ANF, 10–100 nM, $n = 3$, results not shown).

Oxyhaemoglobin (10–300 nM) prevented stimulation of the enzyme by SNP or NO but not that by AA (Figure 5).

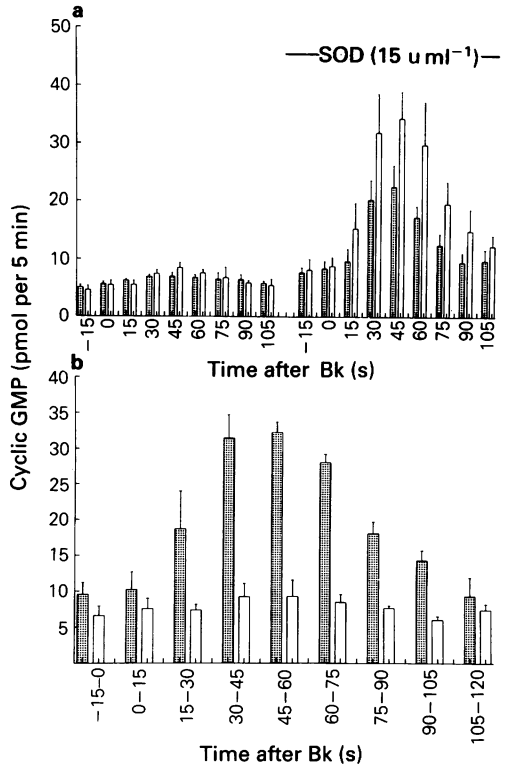


Figure 3 (a) Superoxide dismutase (SOD, 15 u ml^{-1}) increased the activation of guanylate cyclase by the effluent from a mini-column of endothelial cells (EC) stimulated by bradykinin (Bk, 1 or 10 pmol). Ten minutes after the start of infusion of SOD, Bk (1 or 10 pmol) was injected through the column of EC. Fractions (250 μl for 15 s) were collected into each test tube containing 250 μl of buffer B (twice concentrated). Guanylate cyclase (9 μg) was added to each test tube just before collection of the effluent. Each column represents mean with s.e.mean shown by vertical bars ($n = 3$). Stippled column, Bk 1 pmol; open column, Bk 10 pmol. (b) Suppression by oxyhaemoglobin of guanylate cyclase activation by the effluent from a mini-column of EC. Oxyhaemoglobin (10 nM final concentration) was added to each test tube containing 250 μl of buffer B (twice concentrated). Guanylate cyclase (9 μg) was added to the test tube just before collection of the effluent. Samples (250 μl) were collected for 15 s. Superoxide dismutase (15 u ml^{-1}) was infused through the column of EC throughout the experiment. The release of EDRF was induced by Bk (10 pmol). The guanylate cyclase activation after Bk injection was abolished by oxyhaemoglobin. Each column represents mean with s.e.mean shown by vertical bars ($n = 3$). Control, stippled column; with oxyhaemoglobin, open column.

Discussion

We describe here an alternative method using soluble guanylate cyclase for detection of EDRF release from bovine cultured EC. Simultaneous measurement of EDRF release by bioassay and activation of guanylate cyclase showed a good correlation

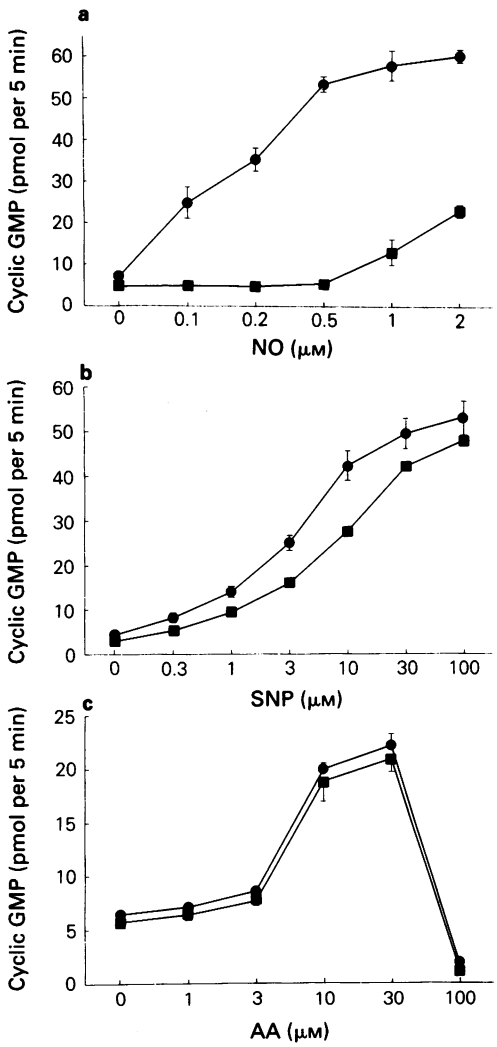


Figure 4 (a) Superoxide dismutase (SOD, 15 u ml^{-1}) strongly potentiated the activation of guanylate cyclase in test tubes by nitric oxide (NO). To imitate the conditions in experiments with columns, 250 μl of oxygenated Krebs solution was added to 235 μl of buffer B (twice concentrated) containing SOD (15 u ml^{-1}) or saline (control). Reaction mixtures were incubated at 37°C for 2 min before addition of 15 μl of guanylate cyclase (9 μg). The reaction was allowed to proceed for 5 min. Nitric oxide was added 10 s after addition of guanylate cyclase. (b) SOD (15 u ml^{-1}) also increased guanylate cyclase activation by sodium nitroprusside (SNP). The reaction was carried out under the same conditions as described in (a). (c) SOD (15 u ml^{-1}) had little or no effect on the activation of guanylate cyclase by arachidonic acid (AA). The reaction was carried out under the same conditions as described in (a). Each point represents mean of 3 determinations with s.e.mean shown by vertical bars. Control (■); SOD 15 u ml^{-1} (●).

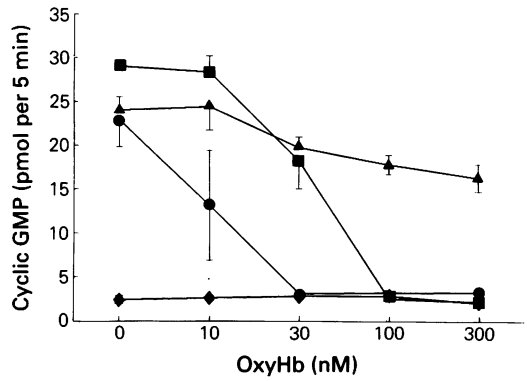


Figure 5 Oxyhaemoglobin (OxyHb) suppressed activation of guanylate cyclase by nitric oxide (1 μM, ●) and sodium nitroprusside (10 μM, ■) but not by arachidonic acid (30 μM, ▲). The reactions were started by the addition of guanylate cyclase (9 μg) to the reaction mixtures as in Figure 4. Oxyhaemoglobin was added 2 min before addition of enzyme. The basal activity of guanylate cyclase is represented by (◆). Each point is the mean of three determinations with s.e.mean shown by vertical bars.

between these two methods. The guanylate cyclase method has several advantages as compared to bioassay using columns of EC. It is sensitive enough to allow the use of smaller numbers of EC (packed in mini-columns). It allows the study of the kinetics of EDRF release, since the biological effect of EDRF on the assay tissues usually outlasts its presence. Some substances (e.g. ANF or GTN) interfere with the baseline and responses of the bioassay tissues and the guanylate cyclase method can be used to study the effect on EDRF release by these substances. Furthermore, the advantage of using several minicolumns at the same time will facilitate the study of biochemical changes occurring in the EC during EDRF release.

Although our enzyme preparation is not homogeneous and only about 50% pure, the lack of effect of IBMX or ANF indicates that there is no contamination by phosphodiesterases or by particulate guanylate cyclase.

Substances other than EDRF or NO which stimulate soluble guanylate cyclase may compromise the specificity of the assay. For instance, AA is released from EC (Moncada *et al.*, 1976) and stimulates guanylate cyclase from platelets (Glass *et al.*, 1977; Gerzer *et al.*, 1983) and spleen (Graff *et al.*, 1978). Thus, it is important to distinguish the guanylate cyclase activation by EDRF from that induced by AA or its metabolites. In our experiments, it is clear that the activation of guanylate cyclase observed following stimulation of EC by bradykinin or ADP is

due to release of EDRF and not of AA, since this activation was strongly potentiated by SOD, which had little or no effect on AA-induced activation of guanylate cyclase. Thus, AA may activate guanylate cyclase by a different mechanism from that exerted by EDRF. At higher concentrations of AA (100 μM) the guanylate cyclase appeared to be denatured as both the stimulated and basal activity were strongly inhibited. Whether the mechanism by which AA stimulates guanylate cyclase is due to metabolites of AA or AA itself is currently under investigation.

Oxyhaemoglobin inhibits NO- and SNP-induced guanylate cyclase activation by oxidising NO to NO_3^- (Doyle & Hoekstra, 1981). The finding that oxyhaemoglobin did not prevent AA-induced activation of guanylate cyclase reinforces the conclusion that a different mechanism of activation of guanylate cyclase is involved.

Superoxide dismutase increased the basal activity of guanylate cyclase, an effect also observed with hepatic guanylate cyclase (Mittal & Murad, 1977). Although this could be a direct effect of SOD, it is more likely to be due to the removal of superoxide anions (O_2^-) present in the Krebs solution (de Nucci *et al.*, 1988b). This is further supported by the finding that although SOD directly stimulated guanylate cyclase, a much stronger potentiation was observed when SOD was infused through the EC. By dismutation of O_2^-, hydrogen peroxide (H_2O_2) should be generated and H_2O_2 activates splenic guanylate cyclase (Graff *et al.*, 1978). However, it is unlikely that H_2O_2 is responsible for guanylate cyclase activation in our experiments, since catalase had no effect when infused through the EC. These data suggest that in our experiments guanylate cyclase activation by the effluent of EC only reflects EDRF release. It is interesting to note that the enzyme has basal activity in the absence of any known stimulator such as NO or AA. The finding that SOD potentiated stimulation of guanylate cyclase by NO far more than that by SNP is probably because NO free in solution is rapidly destroyed by O_2^- (Gryglewski *et al.*, 1986b; Warner *et al.*, 1989) whereas SNP provides a stable carrier for NO.

Comparison of tissue relaxation with guanylate cyclase activation showed that guanylate cyclase activation preceded tissue relaxation. Thus, the method with guanylate cyclase will make it possible to detect biochemical changes in EC during EDRF release more precisely than the bioassay method.

In conclusion, the method with guanylate cyclase will facilitate studies on the effects of vasoactive compounds on EDRF release and allow correlation of the biochemical changes in EC with EDRF release.

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Enhancement of leukotriene C_4 release from primate airway macrophages by cellular interactions

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1 Cells were obtained from the lungs of Macaque monkeys by bronchoalveolar lavage in order to study the role of cellular interactions in the release of leukotriene C_4 (LTC_4).

2 In normal monkeys, macrophages were the most abundant cell type recovered, whereas in monkeys sensitized with *Ascaris suum* there was an increase in the numbers of eosinophils and mast cells recovered.

3 Challenge of cells from both groups of animals with an optimal concentration of opsonized zymosan (OPZ) resulted in the time-dependent release of LTC_4 from macrophages. However, release was significantly greater in cells obtained from sensitized donors compared to normal donors.

4 Density-gradient centrifugation of cells lavaged from sensitized donors was used to prepare fractions containing both eosinophils and mast cells. Addition of these cells to macrophage populations obtained from non-sensitized donors caused a significant enhancement of OPZ-induced LTC_4 release. In the absence of macrophages no significant release of LTC_4 was detected from eosinophil/mast cell-containing fractions stimulated with OPZ, despite the fact that the zymosan had been phagocytosed by the eosinophils.

5 There was a significant correlation between the percentage enhancement of LTC_4 release and the number of eosinophils added. However, there was not a significant correlation with the number of mast cells added.

6 These results suggest that a cellular interaction between macrophages and eosinophils may be important in the regulation of mediator synthesis and release. The precise mechanism of this effect remains to be elucidated.

Introduction

It is now clearly recognized that cell–cell interactions play an important role in the regulation of inflammatory processes. Such interactive events may involve the physical contact of different cell types (Greenberg & Burnstock, 1983; Varsano *et al.*, 1988), or their intercellular communication by a complex network of autacoids, cytokines or as yet uncharacterized factors (Murphy & Henson, 1985; Albrightson *et al.*, 1985; Lenzi *et al.*, 1985; Silberstein *et al.*, 1986; Wei *et al.*, 1986; Robinson, 1988). The existence of this mediator network has important pharmacological implications arising from the interdependency of different cells and their chemical mediators.

The IgE-dependent activation of mast cells to release chemotactic factors and mediators which recruit secondary effector cells is one example of a mediator network (Robinson, 1988). However, other cells, such as those of the monocyte-macrophage series, have long been implicated in intercellular communication in the lung and other tissues (Bonta & Parnham, 1982). Early studies on the release of slow reacting substance of anaphylaxis (SRS-A) suggested that macrophages act in an accessory role and contribute to SRS-A release during the immunological activation of mast cells from human lung parenchyma (Paterson *et al.*, 1976; Lewis *et al.*, 1981). Even after the identification of SRS-A as a family of sulphidopeptide leukotrienes this view remains controversial (Macglashan *et al.*, 1982; Peters *et al.*, 1984).

There is no doubt that, under appropriate conditions, lung macrophages have the potential to regulate the activation state of other inflammatory cells.

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Many macrophage-derived agents may facilitate this, including leukotriene B_4 (LTB_4), platelet activating factor, interleukin-1 and tumour necrosis factor (Okusawa *et al.*, 1988). In addition, there are uncharacterized protein factors which have actions on circulating leukocytes, either causing chemotaxis or enhancing their oxidative activity (Hunninghake *et al.*, 1980; 1981; Merrill *et al.*, 1980; Pennington *et al.*, 1983; 1985). Oxygen centred radicals generated by macrophages and mast cells have also been identified as possible intercellular modulators of mediator release (Wei *et al.*, 1986). In view of the high capacity of neutrophil and eosinophil leukocytes to generate such species it is highly probable that these cells too can regulate mediator release and inactivation.

As part of a programme to investigate the cellular and molecular basis of inflammatory lung diseases, we have been investigating the role of cell–cell interactions in the regulation of lipid mediator production. In order to do this we have used *Macaca arctoides* monkeys which may be subjected to carefully controlled bronchoalveolar lavage (BAL) procedures. In normal monkeys the most abundant cell type recovered by BAL is the macrophage. However, in monkeys experimentally infected with the nematode *Ascaris suum*, there is a significant increase in the number of eosinophils and mast cells recovered by BAL. We have exploited these differences to study cell–cell interactions *in vitro* during the response to a phagocytic stimulus. We report that eosinophils obtained by BAL of Macaque monkeys sensitized to *A. suum* interact with macrophages to enhance LTC_4 release induced by opsonized zymosan (OPZ). However, in the absence of macrophages, eosinophils fail to release LTC_4 themselves in response to OPZ. A preliminary account of this work was presented to the British Pharmacological Society (Campbell *et al.*, 1988).

Methods

Bronchoalveolar lavage

Laboratory-bred stump-tailed monkeys (*Macaca arctoides*) of either sex and 10–40 kg weight were actively sensitized to *Ascaris suum* as previously described (Wells *et al.*, 1986). At 2–10 weeks after infection the monkeys were subjected to BAL (Wells *et al.*, 1986). Briefly summarized, the monkeys were anaesthetized with ketamine 10 mg kg^{-1} and xylazine 1 mg kg^{-1} i.m. prior to insertion of an AO flexible veterinary fibrescope into the second division bronchi. Lavage was performed by the instillation of three 20 ml aliquots of Hank's balanced salt solution into the airways via the bronchoscope. The recovered fluid was aspirated into cooled polypropylene tubes containing heparin (5 u ml^{-1} final). A minimum period of 4 weeks was allowed before subsequent BAL on the same lung. The monkeys were also reinfected with *A. suum* when <30% of total mast cell histamine was released in response to 2 μg ml^{-1} *Ascaris* antigen (Wells *et al.*, 1986).

Preparation of cells

In order to remove mucus and macroscopic material the fluid recovered by BAL was filtered through a 160 μm nylon mesh. The filtrate was centrifuged at 450 *g* and 4°C for 5 min and then resuspended in 5 ml pH 7.4 physiological salt solution (composition, mM: NaCl 137, NaH_2PO_4 0.4, KCl 2.7, $MgCl_2$ 1.0, $CaCl_2$ 1.8, glucose 5.6, N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid 10, gelatin 0.1% and 5 u ml^{-1} heparin). The cells were washed twice and then resuspended in 1 ml of this physiological salt solution. A differential cell count was performed on a 20 μl aliquot of the suspension after staining with Kimura's stain (Kimura *et al.*, 1973). The cells were then processed as described below.

The cell suspension was adjusted to a concentration of 0.5×10^6 macrophages per millilitre and prewarmed at 37°C for 5 min. Duplicate 50 μl aliquots of cells were challenged with either 50 μl opsonized zymosan (OPZ) solution or physiological salt solution. In time course studies a fixed concentration of OPZ 1.5 mg ml^{-1} was used and mediator release investigated over a 120 min period. In concentration-response and cell–cell interaction studies the cells were activated for 45 min with OPZ 0.03–15 mg ml^{-1}. Reactions were stopped by centrifugation in an Eppendorf microfuge and the supernatants analyzed for mediator release.

Density gradient separation of cells

Discontinuous gradients of Percoll were formed by overlaying 2 ml aliquots of Percoll solutions of four specified densities. The densities chosen (1.06, 1.076, 1.085 and 1.09 g ml^{-1}) were selected from a preliminary experiment with a continuous gradient as being able to produce discrete fractions enriched in epithelial cells, macrophages, eosinophils and mast cells respectively. Separation was achieved by centrifugation at 450 *g* and 20°C for 20 min and the cells then harvested from each interface and the cell pellet. After washing in the physiological buffer, a differential cell count was performed and the remainder challenged with either *Ascaris* antigen or OPZ as described below.

Cells from each fraction were prewarmed at 37°C for 5 min and 75 μl aliquots then challenged with 75 μl of the following at the specified final con-

centrations: *Ascaris* antigen ($1\,\mu g\,ml^{-1}$), OPZ ($1.5\,mg\,ml^{-1}$), physiological salt solution (for measurement of spontaneous release) and distilled water (for cell lysis). The release reactions were quenched on ice by adding $20\,\mu l$ divalent cation-free physiological salt solution containing 17 mM EDTA. After centrifugation at $450\,g$ and 4°C for 5 min, $50\,\mu l$ and $20\,\mu l$ aliquots were removed for the measurement of LTC_4 and histamine respectively.

Opsonization of zymosan

Zymosan A was boiled in distilled water for 5 min and then washed with buffer. It was then resuspended in macaque serum at a concentration of $20\,mg\,ml^{-1}$ and incubated for 30 min at 37°C. The serum was removed by centrifugation, the zymosan washed twice and then resuspended in physiological salt solution.

Quantitative analysis of mediators

Histamine was measured by a double isotope radioenzymatic transfer assay modified from the method of Bevan *et al.* (1972). Assay limit of detection was 2.2 ng. In routine experiments immunoreactive LTC_4 (i-LTC_4) was measured using a commercially-obtained antiserum with quoted cross-reactivities of 100%, 1% and 0.06% against LTC_4, LTD_4 and LTE_4 respectively. Assay limit of detection was 25 pg with intra- and inter-assay coefficients of variation of 8.8% and 9.3%.

In order to confirm the presence of LTC_4, selected samples were extracted on C_{18} Bond Elut AASP cartridges (Jones Chromatography, Hengoed, Mid Glamorgan) which had been preconditioned with 2 ml methanol and 2 ml 5 mM EDTA/0.5% ammonium acetate in water. The extract was then analyzed for lipoxygenase products by reversed phase high performance liquid chromatography (h.p.l.c.) with ultra-violet detection at 280 nm. The stationary phase consisted of a Novapak C_{18} 4 μm Radial PAK cartridge (Waters-Millipore, Harrow, Middlesex). The mobile phase had a flow rate of $1\,ml\,min^{-1}$ and initially comprised 60% 0.05 M sodium dihydrogen phosphate and 40% methanol:acetonitrile (1:1), with a linear gradient being run over 22 min to give a final composition of 95% organic phase.

Data analysis and presentation

Results are presented as the mean ± s.e.mean of n experiments. Significance of differences was evaluated using Student's t-test for paired or unpaired data as appropriate. Correlation coefficients were calculated by unweighted least squares linear regression.

Materials

The following were purchased as indicated: ionophore A23187, zymosan A, gelatin type 1 from swine skin, cytochalasin B, heparin, nordihydroguaiaretic acid (NDGA) and N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (Sigma, Poole, Dorset); Percoll and density marker beads (Pharmacia, Milton Keynes, Bucks.); ketamine (Parke-Davies, Eastleigh, Hants.); xylazine (Bayer, Newbury, Berkshire); Hank's balanced salt solution (Gibco, Paisley, Scotland). Radioimmunoassay kits for LTC_4 and radiotracers for histamine assay were purchased from Amersham International plc (Aylesbury, Bucks.). Reagents for electron microscopy were purchased from EM scope, Ashford, Kent and Taab, Reading, Berks. 5,8,11,14-Eicosatetraynoic acid (ETYA) was obtained from Roche Products (Welwyn Garden, Hertfordshire). *Ascaris* antigen was prepared in our laboratories by gel filtration of adult *A. suum* pseudocoelomic fluid on Sephadex G-200 (Ambler *et al.*, 1972). All other reagents were obtained from Fisons FSA (Loughborough, Leicestershire).

Results

Comparison of the differential cell counts performed on samples obtained by BAL showed that there were significant differences between sensitized and non-sensitized monkeys, despite the fact that there were similar recoveries (ca. 80%) of Hank's solution in both groups. The total number of cells recovered by BAL in non-sensitized animals was $5.3 \pm 0.5 \times 10^6$ nucleated cells and these consisted mainly of macrophages and epithelial cells (Figure 1). The total cell yield from sensitized donors was significantly larger, being $12.0 \pm 2.6 \times 10^6$ ($P < 0.001$) and consisted mainly of eosinophils and mast cells. The total numbers of macrophages were similar in both groups (Figure 1).

Studies with unfractionated cells

Initial experiments, using OPZ concentrations of $0.03–15\,mg\,ml^{-1}$, established that unfractionated BAL cells from either sensitized (5 donors) or non-sensitized (4 donors) monkeys could be maximally activated for the release of LTC_4 using an OPZ con-

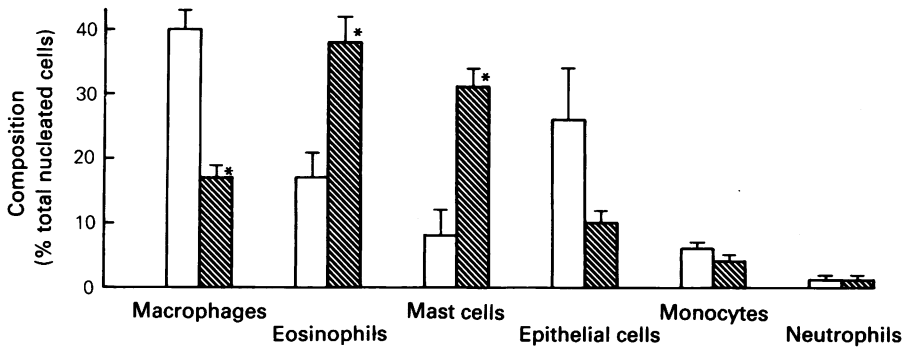


Figure 1 The cellular composition of BAL fluid obtained from Macaque monkeys. Open columns denote *Ascaris*-insensitive monkeys, the hatched columns show the corresponding values for sensitized donors. Data are presented as percentage composition of the total nucleated cell population in 24–26 lavages. Absolute cell counts ($\times 10^6$) for non-sensitized:sensitized donors were as follows: macrophages 2.0 ± 0.4:2.1 ± 0.2; eosinophils 0.9 ± 0.2:4.6 ± 0.5*; mast cells 0.4 ± 0.2:3.7 ± 0.4*; epithelial cells 1.4 ± 0.4:1.2 ± 0.3; monocytes 0.3 ± 0.1:0.5 ± 0.1; neutrophils 0.05 ± 0.03:0.12 ± 0.06*. Asterisks indicate significant differences ($P < 0.001$–0.05).

centration of $1.5\,mg\,ml^{-1}$. In both groups the maximum release of i-LTC_4 occurred 40–60 min after challenge, there being no significant increase in release at further time points up to 120 min (Figure 2). However, there was a significantly greater release of i-LTC_4 from BAL cells from sensitized donors (Figure 2). The initial rate of i-LTC_4 release in cells from sensitized monkeys was $0.37 \pm 0.08\,ng\,min^{-1}$, compared with $0.09 \pm 0.09\,ng\,min^{-1}$ in cells from non-sensitized donors. Analysis of the supernatant from challenged cells by reversed phase h.p.l.c. and ultraviolet absorbance indicated that the major sulphidopeptide leukotriene eluted with the retention time of authentic LTC_4. Pharmacological evidence also supports the view that the immunoreactive material was a lipoxygenase product. In experiments performed in duplicate with cells obtained from two non-sensitized monkeys, the formation of i-LTC_4 was inhibited by the lipoxygenase inhibitors NDGA and ETYA when the cells were preincubated with the drugs at concentrations of 0.01–10 mM for 20 min before challenge with OPZ. The IC_{50} values for the two compounds were 24 μM and 45 μM respectively.

Density gradient fractionation of cells

On discontinuous gradients, epithelial cells were found in the least dense fraction (1.000–$1.060\,g\,ml^{-1}$), macrophages at the 1.060–$1.076\,g\,ml^{-1}$ interface and mast cells at the 1.085–$1.090\,g\,ml^{-1}$ interface and in the $>1.090\,g\,ml^{-1}$ density fraction. Significant numbers of eosinophils, which co-purified with mast cells, were found only in the BAL fluid recovered from sensitized donors.

As expected, when these individual fractions were challenged with *Ascaris* antigen ($1\,\mu g\,ml^{-1}$) i-LTC_4 and histamine were only released from fractions containing mast cells obtained from sensitized donors.

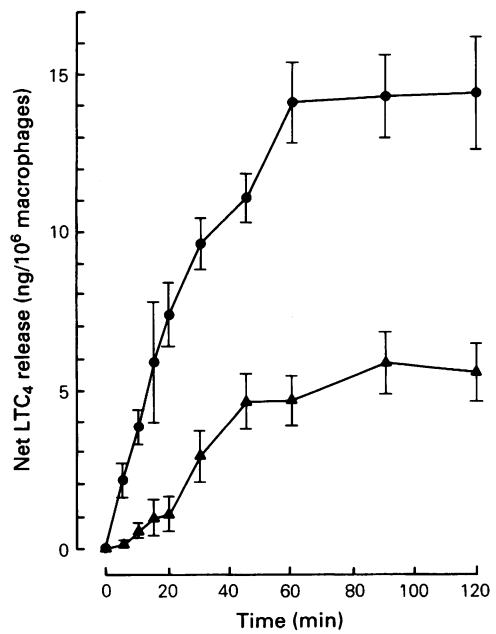


Figure 2 The time course of leukotriene C_4 (LTC_4) release induced by $1.5\,mg\,ml^{-1}$ OPZ: (●) data from cells obtained from sensitized monkeys ($n = 5$), and (▲) results obtained with cells from non-sensitized monkeys ($n = 5$).

There was a significant correlation between the release of histamine and the number of mast cells ($r = 0.85$, $P < 0.001$, $n = 18$), and between the net release of i-LTC_4 and the number of mast cells present ($r = 0.75$, $n = 34$, $P < 0.001$). In contrast, OPZ-induced release of i-LTC_4 occurred in cells from both sensitized and non-sensitized monkeys. For both groups, there was a significant correlation between the net release of i-LTC_4 and the number of macrophages in each gradient fraction ($r = 0.84$, $n = 58$, $P < 0.001$). When OPZ was the stimulus there was no release of LTC_4 from gradient fractions containing mainly mast cells and eosinophils. Representative gradient data from experiments performed with cells obtained from non-sensitized and sensitized donors are shown in Figure 3.

Cell–cell interaction studies

The finding that OPZ elicited the release of LTC_4 from macrophages prompted us to investigate why BAL cells from *Ascaris*-sensitive monkeys had an enhanced ability to release this leukotriene compared with cells from non-sensitized monkeys. As the major difference in the cells recovered by BAL from the two groups of monkeys was in the number of mast cells and eosinophils recovered (Figure 1), experiments were performed to investigate whether this could account for the difference in LTC_4 release. Fractions enriched with mast cells and eosinophils (composition: $63 \pm 8.1\%$ mast cells, $33 \pm 8.2\%$ eosinophils, $1.6 \pm 0.4\%$ macrophages, $1.4 \pm 0.5\%$ monocytes, $0.6 \pm 0.3\%$ neutrophils and $0.4 \pm 0.2\%$ epithelial cells, $n = 14$) were purified by gradient centrifugation and harvested from the $>1.090\,g\,ml^{-1}$ density fraction. When the mast cell/eosinophil fraction was added to unfractionated cells (comprising mainly macrophages) from non-sensitized donors there was a significant enhancement of OPZ-induced i-LTC_4 release (Table 1). Challenge with OPZ of the mast cell/eosinophil fraction alone did not elicit the release of LTC_4.

Table 1 Enhancement of OPZ-induced leukotriene C_4 (LTC_4) release following the addition of density-gradient fractions of mast cells/eosinophils from sensitized donors to BAL cells from non-sensitized donors

Conditions	*Net LTC_4 release* (ng per 10^6 macrophages)
Unfractionated BAL cells	4.7 ± 0.5
Unfractionated BAL cells + mast cell/eosinophil fraction	11.1 ± 1.3*

* $P < 0.001$ in 14 experiments.

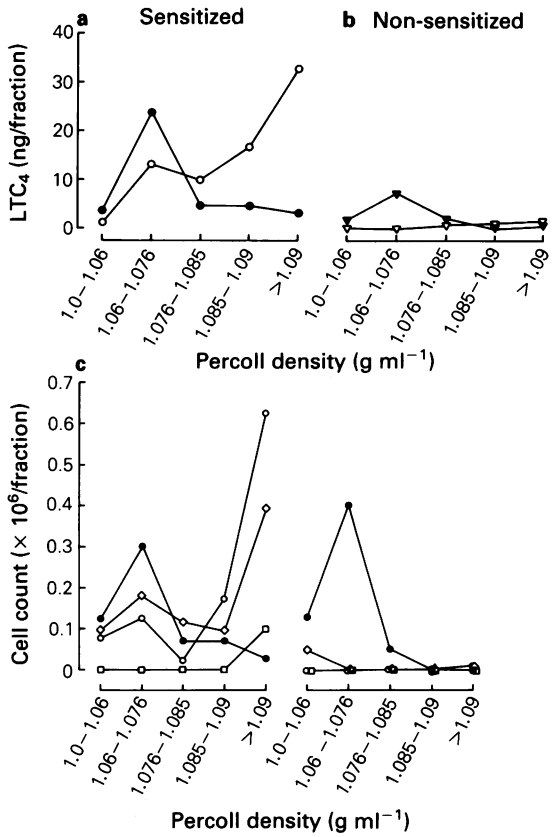


Figure 3 Representative separation of BAL cells by density gradient centrifugation, and the release of leukotriene C_4 (LTC_4) in response to challenge with OPZ (●, ▼) $1.5\,mg\,ml^{-1}$ and *Ascaris* antigen (○, ▽) $1\,\mu g\,ml^{-1}$. Symbols in (c): (●) macrophages, (○) mast cells, (□) neutrophils, (◇) eosinophils.

The enhancement of LTC_4 release on admixture of the cells was not simply a function of the number of macrophages present. When cells were prepared to yield suspensions containing 0.5×10^6 macrophages per ml the net release of i-LTC_4 was 2.9 ± 0.7 ng per 10^6 macrophages, compared to 2.4 ± 0.7 ng per 10^6 macrophages when the suspension contained 1×10^6 macrophages per ml ($n = 3$). Furthermore, immediate centrifugation at $100\,g$ for 15 s of OPZ-challenged BAL cells from non-sensitized monkeys did not alter the net release of i-LTC_4 (data not shown). This suggests that the difference was not due to the larger number of cells from sensitized donors enjoying greater contract with the OPZ. Another possibility, namely that released LTC_4 becomes bound to OPZ and thus might be removed from the incubation medium at differential rates under the two experimental conditions, was excluded by the

addition of $[^3H_2]$-LTC_4 to OPZ (0.075, 0.15, 1.5, 7.5, 15 mg ml^{-1}). In all 3 tests >95% of the added LTC_4 was recoverable at each concentration of OPZ after centrifugation at 450 *g* for 5 min.

In the cell admixture experiments shown in Table 1 there was a significant correlation ($y = 34x + 31$, $r = 0.39$, $n = 49$, $P < 0.01$) between the percentage enhancement of i-LTC_4 release and the total number of mast cells and eosinophils added. When the number of eosinophils added was considered alone there was an improved positive correlation with the percentage enhancement of LTC_4 release ($y = 105x + 23$, $r = 0.61$, $n = 49$, $P < 0.001$). However, the correlation between enhancement and the number of mast cells added was not significant ($r = 0.096$, $n = 49$).

As a more direct test of whether eosinophils were important in the enhancement phenomenon, by undertaking the synthesis and release of LTC_4, eosinophils from *Ascaris*-sensitive Macaque monkeys were purified free from mast cells by challenging the unfractionated cells with *Ascaris* antigen. This procedure resulted in mast cell degranulation, altering their density such that when the washed cells were applied to the Percoll gradient the eosinophils could be harvested at a purity of 94.0 ± 1.0% and viability of >90% ($n = 5$). The mast cell contamination of these fractions was only 6.0 ± 2.0%. Addition of an eosinophil-rich fraction (purity 88%) to unfractionated BAL cells from a non-sensitized donor resulted in an increase in the release of i-LTC_4 from 7 to 16 ng per 10^6 macrophages. However, in 5 experiments, challenge of the separated eosinophil-rich fraction with 1.5 mg ml^{-1} OPZ failed to elicit the release of i-LTC_4, even after incubation for 10 min with agents such as LTB_4 (10^{-6} M), FMLP (10^{-6} M) or FMLP plus 5 μg ml^{-1} cytochalasin b which are known to enhance ligand/receptor-dependent transmembrane events. This lack of release was not due to the absence of the appropriate enzymes because in eosinophils challenged with A23187 (3×10^{-7} M) and arachidonic acid (3×10^{-2} M) there was a net release of LTC_4 at levels of 6.4 ± 3.8 and 22.3 ± 6.5 ng per million eosinophils respectively. Furthermore, examination of the cells by electron microscopy showed that the eosinophils were capable of phagocytosing the OPZ despite the fact that this was not associated with the net synthesis and release of i-LTC_4.

In 3 experiments we investigated whether the addition of supernatants from OPZ-challenged macrophages from *Ascaris*-insensitive donors to separated, OPZ-challenged granulocytes from *Ascaris*-sensitive donors (or *vice versa*) was capable of causing the enhancement of LTC_4 release. In no case was there evidence of potentiation of release (Table 2).

Table 2 Experiments to investigate whether a transferable factor causes the enhancement of leukotriene C_4 (LTC_4) release seen after the addition of eosinophils to macrophages

Conditions	*LTC_4 content* (ng ml^{-1}) Experiment 1	2	3
(1) Challenged eosinophils	<0.25	0.37	<0.25
(2) Expected amount on admixture	<0.49	0.61	<0.44
(3) Actual amount on admixture	0.43	0.49	0.41
(4) Challenged macrophages	<1.40	<0.44	
(5) Expected amount on admixture	<1.53	<0.56	
(6) Actual amount on admixture	1.35	0.51	

In the first series of experiments (rows 1–3), incubation supernatants from OPZ-challenged macrophages from non-sensitized donors were added to OPZ challenged eosinophils from sensitized donors. As the supernatant derived from the challenged macrophages contained LTC_4 it was necessary to calculate, by addition and correction for dilution factors, the expected amount present after admixture of the macrophage supernatant to the granulocytes (row 2). The actual amount assayed after admixture is shown in row 3. In the second series (rows 4–6) supernatants from challenged eosinophils were added to macrophages in the presence of OPZ and the expected and actual amounts calculated as above. Evidence for potentiation of release was sought by comparing the actual release after admixture with the values expected from a purely additive effect.

Discussion

A key finding of these experiments was the significantly larger OPZ-induced release of LTC_4 from cells obtained from sensitized donors. As the BAL fluid recovered from the 2 groups of monkeys differed not only in the total number of cells recovered, but also in the proportion of other cells present, we investigated whether these cellular differences could account for the changes in the release of LTC_4. Addition of a cell fraction containing 63% mast cells and 33% eosinophils, prepared from donor monkeys sensitized to *A. suum*, enhanced the OPZ-induced release of LTC_4 in unfractionated cells from non-sensitized donors. This enhancement of release was significantly correlated with the number of eosinophils added to the unfractionated cells. Due to the difficulties in obtaining eosinophil preparations

which were free of mast cell contamination we cannot exclude formally the possibility that mast cells may play an intermediary role in macrophage and eosinophil activation. However, the enhancing effect was still evident to a similar degree when a fraction containing 88% pure eosinophils was employed. Our results therefore raise two important questions, namely what is the cellular origin of the additional LTC_4, and how is its expression initiated?

There are several possible explanations of how the enhanced release of LTC_4 occurs. The first of these is that macrophages themselves release larger amounts of LTC_4 in the presence of eosinophils. A second possibility is that rapid metabolism of LTC_4 is prevented when the two cell types are mixed together, perhaps by the scavenging of species involved in the oxidative degradation of LTC_4 (Lee, 1987; Owen *et al.*, 1987). Thirdly, eosinophils may respond directly to OPZ by synthesizing LTC_4. Although eosinophils purified from human peripheral blood are known to respond to OPZ in this way (Bruynzeel *et al.*, 1985) this is not a simple explanation of our data. In preparations of Macaque monkey BAL eosinophils which were relatively free from macrophage contamination, OPZ consistently failed to release significant quantities of LTC_4 despite the OPZ being phagocytosed by the cells. This view is in agreement with an observation in human peripheral blood eosinophils where the processes of phagocytosis and LTC_4 synthesis are also dissociated (Mahauthaman *et al.*, 1988). However, our experiments do establish that Macaque monkey BAL eosinophils are capable of synthesizing and releasing LTC_4 when challenged with ionophore A23187 or arachidonic acid. Thus if eosinophils are the source of the additional LTC_4, the cellular interaction with macrophages must be an important event in facilitating the release, in addition to the synthesis, of this leukotriene. This implies that in the absence of an appropriate signal for its liberation, LTC_4 must be compartmentalized within a cellular organelle or with the ingested particle. Interestingly, there is evidence in human peripheral blood eosinophils that LTC_4 release is preceded by its intracellular accumulation (Owen *et al.*, 1987).

The lipoxygenase inhibitors NDGA and ETYA were both active against OPZ-induced LTC_4 synthesis when experiments were performed in unfractionated cells, and provide pharmacological evidence that the measured immunoreactivity was due to a lipoxygenase product. By using macrophages that had been incubated with ETYA we also hoped to establish whether LTC_4 was released from eosinophils when challenged with OPZ in the presence of macrophages. However, the obligatory washing of the macrophages before their addition to the granulocyte fraction resulted in a rapid loss of enzyme inhibition and thus prevented us from performing this experiment.

Whatever the origin of the additional LTC_4 release we do know that if an intercellular factor is involved in its causation it is not easily transferable. No stimulatory activity was detected in the incubation supernatants from granulocytes or unfractionated cells stimulated with OPZ. These experiments indicate that if a soluble factor is involved in these events it is either labile, being broken down in the 2–3 min taken to transfer supernatants, or else it is membrane bound, in which case it would be removed during centrifugation. These differences suggest collectively that the macrophage-eosinophil interaction we have observed is not mediated by the relatively stable, soluble factors which have been reported by others (Dessein *et al.*, 1983; 1984; 1986; Veith & Butterworth, 1983; Vadas *et al.*, 1984), or by the simple unidirectional transfer of arachidonic acid. However, we cannot exclude the possibility that physical cell contact is important in this phenomenon.

Although the explanation for our results still remains elusive, we have provided further evidence that intercellular co-operation may be an important influence on the release of chemical mediators from inflammatory cells. These and other interactions may be particularly important in sustained phases of inflammation and tissue injury where infiltration of tissues with different cell types is known to occur (reviewed by Henson & Johnston, 1987).

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Modulation of the vasodepressor actions of acetylcholine, bradykinin, substance P and endothelin in the rat by a specific inhibitor of nitric oxide formation

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1 The effects of the specific inhibitor of nitric oxide (NO) formation, N^G-monomethyl-L-arginine (L-NMMA), on resting systemic arterial blood pressure (BP) and on the actions of both endothelium-dependent and endothelium-independent vasodilators were investigated in the anaesthetized, normotensive rat.

2 Intravenous administration of L-NMMA (12.5–50 mg kg^{-1}; 47–188 μmol kg^{-1}) but not its enantiomer, D-NMMA, induced a dose-related increase in BP, which was reversed by the intravenous administration of L-arginine (150–600 μmol kg^{-1}), but not D-arginine.

3 The vasodepressor responses to intravenous administration of the endothelium-dependent vasodilators, acetylcholine, bradykinin and substance P were significantly inhibited by L-NMMA (94 and 188 μmol kg^{-1} i.v.), but not by D-NMMA.

4 The inhibition by L-NMMA of these vasodepressor responses was reversed by administration of L-arginine, but not D-arginine.

5 Endothelin (ET-1) induced dose-related vasodepressor responses following bolus intravenous administration, which were significantly inhibited by L-NMMA but not by D-NMMA. This inhibition was reversed by administration of L-arginine.

6 The vasodepressor effects of the endothelium-independent vasodilators, glyceryl trinitrate or prostacyclin, were not significantly inhibited by L-NMMA.

7 These findings with L-NMMA suggest that resting blood pressure in the rat is modulated by endogenous NO biosynthesis and that endothelium-dependent vasodilators act through the formation of endogenous NO to exert their actions *in vivo*.

Introduction

Acetylcholine, substance P and bradykinin induce relaxation of isolated vascular tissue by the release of an endothelium-derived relaxing factor, EDRF (Furchgott & Zawadzki, 1980; Furchgott, 1983). The requirement of this factor for such vasodilatation *in vivo* has been suggested from studies in the dog femoral artery and mouse cerebral microcirculation, where an intact endothelium was necessary for these agents to act (Angus *et al.*, 1983; Rosenblum *et al.*, 1986), and from cardiovascular studies in the rabbit with gossypol, a non-specific EDRF inhibitor (Dudel & Forstermann, 1988).

Nitric oxide (NO), formed by vascular endothelial cells from the amino acid, L-arginine, accounts for the biological actions of EDRF (Palmer *et al.*, 1987; 1988a). The formation of NO and the relaxation of the rabbit aorta induced by acetylcholine, substance P or bradykinin is specifically inhibited by the L-arginine analogue, N^G-monomethyl-L-arginine (L-NMMA) but not by its enantiomer, D-NMMA (Palmer *et al.*, 1988b; Rees *et al.*, 1989a). Furthermore, intravenous administration of L-NMMA, in doses that inhibit vascular NO production *ex vivo*, increases systemic blood pressure and attenuates the vasodepressor actions of acetylcholine in the rabbit (Rees *et al.*, 1989b).

In the present study in the anaesthetized rat, the effects of intravenous administration of L-NMMA on the vasodepressor actions of acetylcholine, substance P or bradykinin have been determined. In addition, the effects of L-NMMA on the hypotensive effects of bolus administration of endothelin (ET-1,

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Yanagisawa *et al.*, 1988a,b) a 21-residue vascular peptide that elicits endothelium-dependent vasodilatation in the rat isolated mesenteric bed (De Nucci *et al.*, 1988), has been investigated. The specificity and characteristics of these actions of L-NMMA *in vivo* have been assessed by studying its actions on the endothelium-independent vasodilators, glyceryl trinitrate and prostacyclin, by reversal of its actions by L-arginine and by the use of the enantiomers, D-NMMA and D-arginine.

Methods

Male Wistar rats (230–260 g body weight) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and the trachea was cannulated to facilitate respiration. Rectal temperature was maintained at 37°C by thermistor-controlled radiant heat. Systemic arterial blood pressure was recorded from a cannula inserted into a carotid artery, and heart rate was derived from the arterial pulse. A femoral vein was cannulated, or a 25 gauge butterfly needle was inserted into tail vein, for the administration of drugs. For bolus intravenous injection, compounds were administered in randomised doses, in volumes of 1 ml kg^{-1} and flushed in with 0.25 ml of isotonic saline (0.9% w/v NaCl solution).

Drugs

Acetylcholine bromide, L-arginine, D-arginine (Sigma Chemical Co.) prepared as the acetate salts and glyceryl trinitrate (Wellcome Foundation Ltd) were freshly dissolved in isotonic saline before use. Bradykinin (Sigma), substance P (Cambridge Research Biochemicals) and endothelin (ET-1; human-porcine; Pennisula Laboratories) were dissolved in distilled water in aliquots, stored at −20°C and diluted immediately before use in isotonic saline. Prostacyclin as the sodium salt (Wellcome Foundation Ltd), was freshly dissolved in 1M Tris buffer (pH 9.6 at 4°C) immediately before use and diluted in ice-cold 1.25% w/v $NaHCO_3$ solution. Indomethacin (Sigma) was freshly dissolved in 5% w/v $NaHCO_3$ solution and diluted to 1.25% w/v with distilled water. N^G-monomethyl-L-arginine acetate and its D-enantiomer, synthesized as described previously (Patthy *et al.*, 1977) in the Department of Medicinal Chemistry, Wellcome Research Laboratories by Dr H. Hodson, were freshly dissolved in isotonic saline before use.

Statistical analysis

Results, calculated as the change in diastolic arterial blood pressure (BP) or as % inhibition of the control response to the vasodilator agent, are expressed as mean ± s.e.mean, where (n) is the number of values. The difference between groups was evaluated by Student's t test for paired or unpaired data as appropriate, with $P < 0.05$ being taken as significant.

Table 1 Vasopressor effects of intravenous administration of L-NMMA (47, 94 and 188 μmol kg^{-1}) on resting diastolic blood pressure (BP) in the anaesthetised rat, and reversal by the administration of L-arginine in a 3 fold excess (150, 300 and 600 μmol kg^{-1} i.v. respectively)

L-NMMA dose (mg kg^{-1})	*ΔBP* (mmHg) *above basal*	
	L-NMMA	*L-NMMA + L-Arginine*
12.5	18 ± 1***	8 ± 2†
25	27 ± 1***	8 ± 2†
50	28 ± 5***	8 ± 3†

Results, shown as the change in BP from its basal value (ΔBP) are the mean ± s.e.mean of 5–8 experiments in each group, where the significant increase from basal BP by L-NMMA is shown as ***$P < 0.001$, and where † indicates significant difference ($P < 0.05$) between the L-NMMA and L-arginine group and the corresponding group with L-NMMA alone.

Results

Effect of L-NMMA on resting blood pressure

Mean diastolic BP was 100 ± 2 mmHg ($n = 102$). Bolus intravenous administration of L-NMMA (12.5–50 mg kg^{-1}; 47–188 μmol kg^{-1}) induced a dose-related increase in resting BP, as shown in Table 1. The increase in BP reached its maximal level within 5 min and its duration was dose-dependent, being maintained for 45 min with L-NMMA (188 μmol kg^{-1}). The increase in BP was accompanied by a minor fall in heart rate, with L-NMMA (188 μmol kg^{-1}) inducing a fall of 34 ± 4 beats min^{-1} ($n = 16$, $P < 0.01$) from the resting value of 409 ± 16 beats min^{-1}.

Intravenous administration of L-arginine (150–600 μmol kg^{-1}) in a 3 fold molar excess, significantly reversed the L-NMMA-induced increase in BP, as shown in Table 1, whereas D-arginine (1.2 mmol kg^{-1}, i.v.) had no significant effect ($n = 4$). The enantiomer, D-NMMA (188 or 376 μmol kg^{-1}, i.v.) did not significantly affect either resting BP or heart rate ($n = 4$ for each dose).

Effect of L-NMMA on vasodepressor actions of acetylcholine

Bolus intravenous administration of acetylcholine (0.3–1.2 nmol kg^{-1}) induced a dose-related fall in

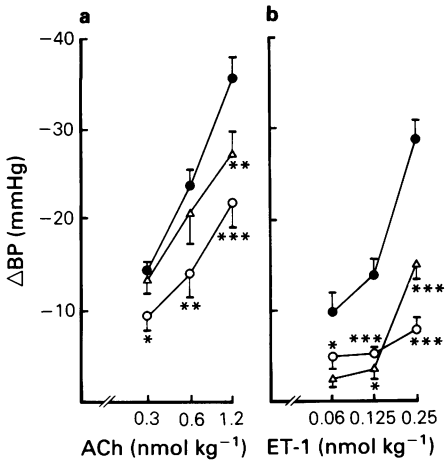


Figure 1 Inhibition of the vasodepressor responses to bolus intravenous administration of acetylcholine (●; ACh, 0.3–1.2 nmol kg^{-1}; a) or endothelin (●; ET-1, 0.06–0.25 nmol kg^{-1}; b) by L-NMMA (Δ 25 and ○ 50 mg kg^{-1}; 94 and 188 μmol kg^{-1}) in the anaesthetized rat. Results, shown as the fall in diastolic BP (ΔBP), are the mean ± s.e.mean of 6–8 experiments in each group, where significant difference from the control response is shown as $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

diastolic BP (Figure 1), with minimal changes in heart rate at these doses. Whereas pretreatment (10 min) with the low dose of L-NMMA (47 μmol kg^{-1}; $n = 4$) had no significant effect on these vasodepressor actions, pretreatment with L-NMMA (94 and 188 μmol kg^{-1}, i.v.), significantly inhibited the fall in diastolic BP induced by acetylcholine (Figure 1). Higher doses of L-NMMA (376 μmol kg^{-1}) did not further augment significantly the maximal degree of inhibition ($n = 4$). Pretreatment with D-NMMA (376 μmol kg^{-1}, i.v.) did not significantly alter the vasodepressor response to acetylcholine ($n = 3$).

Intravenous administration of L-arginine (600 μmol kg^{-1}) but not D-arginine (600 μmol kg^{-1}; $n = 4$), significantly reversed the inhibition by L-NMMA (188 μmol kg^{-1}) of the vasodepressor actions of acetylcholine (Table 2). This dose of L-arginine alone did not significantly affect either the resting BP or the vasodepressor response to acetylcholine ($n = 4$).

Effect on the vasodepressor actions of endothelin

In these preparations, with a resting diastolic BP of 112 ± 3 mmHg ($n = 30$), rapid bolus intravenous administration of endothelin (ET-1; 0.06–0.25 nmol kg^{-1}) induced a dose-dependent fall in BP (Figure 1). No consistent secondary increase in BP

Table 2 Inhibition of the vasodepressor responses to intravenous bolus administration of acetylcholine (1.2 nmol kg^{-1}), bradykinin (3.2 nmol kg^{-1}) substance P (30 pmol kg^{-1}) and endothelin (ET-1, 0.25 nmol kg^{-1}) by L-NMMA (188 μmol kg^{-1}, i.v.)

	% *inhibition*			
	L-NMMA	(n)	*L-NMMA + L-Arginine*	(n)
Acetylcholine	41 ± 6**	(8)	−10 ± 9†	(4)
Bradykinin	39 ± 11*	(4)	−38 ± 14†	(4)
Substance P	40 ± 8*	(4)	−15 ± 10†	(4)
Endothelin	72 ± 4**	(14)	19 ± 16†	(4)

Results, shown as % inhibition of the control responses to the vasodepressors, are mean ± s.e.mean of (*n*) animals, where significant inhibition is shown as $*P < 0.05$, $**P < 0.01$. L-arginine (600 μmol kg^{-1}) reversed the inhibition by L-NMMA, which led in some experiments to the potentiation of the vasodepressor responses (indicated by the negative inhibition), where † reflects significant difference ($P < 0.05$) from L-NMMA alone.

was observed following the return of BP to its resting values. Whereas the low dose of L-NMMA (47 μmol kg^{-1}, i.v.; $n = 4$) had no significant effect on these vasodepressor actions, pretreatment (10 min) with L-NMMA (94 and 188 μmol kg^{-1}, i.v.) significantly inhibited the fall in diastolic BP induced by ET-1 (Figure 1). No consistent secondary increase in BP was observed under these conditions. Higher doses of L-NMMA (376 μmol kg^{-1}) did not further augment the degree of inhibition of the responses to ET-1 ($n = 4$). D-NMMA (188 μmol kg^{-1}, i.v.) did not significantly alter the vasodepressor response to ET-1 ($n = 4$). Pretreatment (10 min) with indomethacin (5 mg kg^{-1}, i.v.), did not significantly affect the responses to ET-1 ($n = 4$), or the extent of L-NMMA (188 μmol kg^{-1})-induced inhibition of the ET-1 (0.25 nmol kg^{-1}) response (73 ± 6% inhibition, $n = 8$).

Administration of L-arginine (600 μmol kg^{-1}, i.v.), reversed the inhibition of the vasodepressor actions of ET-1 induced by L-NMMA (188 μmol kg^{-1}, i.v.) as shown in Table 2.

Effect on the vasodepressor actions of bradykinin and substance P

Bradykinin (0.4–3.2 nmol kg^{-1}, i.v.) or substance P (3.75–30 pmol kg^{-1}, i.v.) induced dose-dependent falls in diastolic BP (Figure 2). Pretreatment with L-NMMA (188 μmol kg^{-1}, i.v.) significantly inhibited the vasodepressor responses of all but the lowest

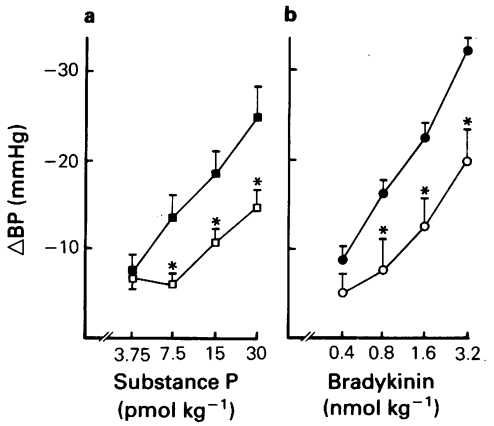


Figure 2 Inhibition of the vasodepressor responses to substance P (■, 3.75–30 pmol kg^{-1} i.v.; a) or bradykinin (○, 0.4–3.2 nmol kg^{-1} i.v.; b) by L-NMMA (□, ○, 188 μmol kg^{-1}, i.v.) in the anaesthetized rat. Results, shown as the fall in diastolic BP (ΔBP), are the mean of 4 experiments in each group, where significant inhibition is shown as $*P < 0.05$; s.e.mean shown by vertical bars.

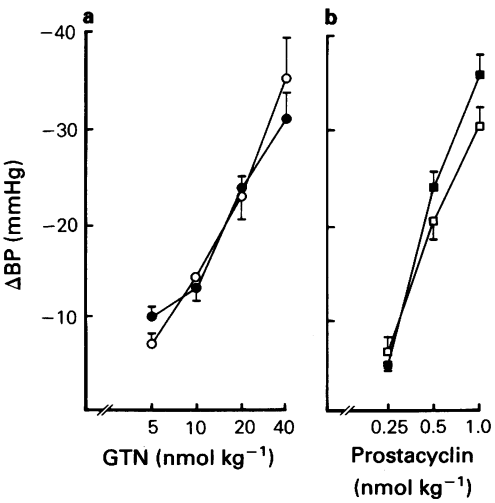


Figure 3 Lack of significant inhibition of the vasodepressor responses to glyceryl trinitrate (○, GTN, 5–40 nmol kg^{-1}, i.v.; a) or prostacyclin (■, 0.25–1.0 nmol kg^{-1}, i.v.; b) by L-NMMA (○, □, 188 μmol kg^{-1}; i.v.) in the anaesthetized rat. Results shown as the fall in diastolic BP (ΔBP), are the mean of 4 and 8 experiments respectively; s.e.mean shown by vertical bars.

dose of either agent (Figure 2). The inhibition by L-NMMA (188 μmol kg^{-1}) of these vasodepressor responses was reversed by the administration of L-arginine (600 μmol kg^{-1}) as shown in Table 2.

Effect on the vasodepressor actions of glyceryl trinitrate and prostacyclin

Glyceryl trinitrate (5–40 nmol kg^{-1}, i.v.) or prostacyclin (0.25–1 nmol kg^{-1}, i.v.) induced dose-dependent falls in diastolic BP (Figure 3). The vasodepressor actions of either glyceryl trinitrate or prostacyclin were not significantly inhibited by L-NMMA (188 μmol kg^{-1}, i.v.), as shown in Figure 3.

Discussion

In the present study in the anaesthetized rat, intravenous administration of the L-arginine analogue, L-NMMA, which specifically inhibits the formation of nitric oxide (NO) from L-arginine (Palmer *et al.*, 1988b) induced a dose-related increase in BP, with an accompanying bradycardia. The prolonged hypertensive effect of L-NMMA was rapidly reversed by the administration of a 3 fold excess of L-arginine but not D-arginine, thus confirming the recent observations with L-NMMA in the rabbit (Rees *et al.*, 1989b). The enantiomer-specific nature of the action of L-NMMA was also confirmed, since D-NMMA, which does not inhibit the synthesis of NO by endothelial cells (Palmer *et al.*, 1988b), did not affect resting BP in the rat. In a previous study in the rabbit with gossypol, a putative inhibitor of endothelium-dependent relaxation, no such effect on resting BP was observed (Dudel & Fosterman, 1988). However, that agent has a broad pharmacological profile, including inhibition of membrane enzymes involved with phospholipid metabolism and calcium transport (Reyes *et al.*, 1984; Kimura *et al.*, 1985), and its lack of specificity and possible vascular tissue disruption may therefore obscure any direct actions on the cardiovascular system.

L-NMMA inhibited the vasodepressor actions of the endothelium-dependent vasodilators, acetylcholine, bradykinin and substance P to a comparable degree, suggesting an action on a common mechanism. D-NMMA did not, however, inhibit the vasodepressor responses to these vasodilators, while L-arginine but not D-arginine reversed the inhibition by L-NMMA, as observed in the rabbit with acetylcholine-induced vasodepression (Rees *et al.*, 1989b). This rapid reversal, as well as the gradual disappearance of the inhibitory effects of L-NMMA in the absence of exogenous L-arginine, indicates that the reduction in endothelium-dependent vasodepressor responses is not just a consequence of endo-

thelial cell damage by this compound. These present findings in the rat thus support the involvement of an L-arginine-dependent mechanism in the hypotensive actions of these vasodilators.

The vasodepressor responses to the endothelium-dependent vasodilators were substantially inhibited, but not abolished, by L-NMMA in the present study. However, the mechanism by which L-NMMA interferes with either the uptake or utilisation of L-arginine by endothelial cells is not yet known, although competition with this substrate for NO synthesis is apparent. Thus, sufficiently high concentrations of L-NMMA in the microenvironment of the vascular endothelial cell may not be achieved at these doses to compete effectively with the endogenous levels of L-arginine, and hence abolish NO formation, following stimulation with exogenous agents such as acetylcholine. Knowledge of the uptake of L-NMMA into vascular tissue, its distribution and its pharmacokinetic profile, as well as its effects on endogenous L-arginine levels may, therefore, be useful in interpreting its *in vivo* profile of actions.

The failure of L-NMMA to inhibit substantially the small vasodepressor effects of the threshold doses of substance P and bradykinin is not yet understood. This may reflect a non-NO-dependent component of these agents at low doses, such as through the release of an endothelium-derived hyperpolarizing factor (Feletou & Vanhoutte, 1988; Chen *et al.*, 1988). However, since an elevation of basal BP may augment the apparent vasodepressor activity of these agents, as shown previously in the rabbit (Rees *et al.*, 1989b), such effects of L-NMMA on basal BP may lead to an underestimation of its degree of inhibition of the vasodepressor responses. Both such possibilities may also contribute to the failure of L-NMMA to abolish the responses to endothelium-dependent vasodilators. It will be of interest to compare the effects of L-NMMA on resting tone and on endothelium-dependent vasodilatation in specific vascular beds *in vivo*, to determine whether there is a differential sensitivity to this inhibitor in diverse tissues.

The selectivity of L-NMMA in the rat was confirmed by its failure to inhibit the vasodepressor responses to glyceryl trinitrate or prostacyclin, both endothelium-independent vasodilators. In studies in the rabbit, gossypol likewise did not inhibit the actions of glyceryl trinitrate or prostaglandin E, (PGE_1), but did attenuate the vasodepressor actions of acetylcholine, substance P and adenosine triphosphate (Dudel & Fostermann, 1988). Whether this reflects inhibition of NO formation or is due to other actions on the endothelial cells of gossypol is not known. In other *in vivo* studies, local infusion of methylene blue attenuated the vasodilatation induced by either acetylcholine or glyceryl trinitrate in the dog femoral artery (Sobey *et al.*, 1988). Although not fully selective, methylene blue can inhibit vascular guanylate cyclase (Martin *et al.*, 1985; 1989), and thus these latter findings could support the involvement of cyclic GMP in the mechanisms underlying endothelium-dependent and nitrate-induced vasodilatation (Rapoport & Murad 1983) and hence the actions of endogenous NO *in vivo*.

Porcine-human endothelin (ET-1) induces endothelium-independent contraction of isolated strips of vascular smooth muscle (Yanagisawa *et al.*, 1988a,b). However, in the isolated perfused mesenteric bed of the rat, low doses of ET-1 can induce vasodilatation which is inhibited by oxyhaemoglobin or removal of the endothelium, indicating the release of EDRF (De Nucci *et al.*, 1988). Studies in the rat *in vivo* with ET-1 have also demonstrated a complex cardiovascular profile, with an initial vasodepressor action preceding any vasopressor effects, these responses being dependent on the resting BP (Yanagisawa *et al.*, 1988a,b; Wright & Fozard, 1988; De Nucci *et al.*, 1988; Whittle *et al.*, 1989). In the current study, the fall in BP induced by ET-1 in the anaesthetized normotensive rat was substantially inhibited by pretreatment with L-NMMA but not D-NMMA, and this inhibition was abolished by concomitant administration of L-arginine. The inhibition of the vasodepressor actions of ET-1 by L-NMMA did not, however, lead to a consistent appearance of a vasoconstrictor phase under these conditions, while the inhibition was not further augmented by the cyclo-oxygenase inhibitor, indomethacin. However, it is pertinent that the degree of inhibition by L-NMMA of the vasodepressor responses of ET-1 was significantly greater than that of the other endothelium-dependent vasodilators. Such actions may reflect an unmasking of the inherent vasoconstrictor properties ET-1 following the inhibition of endothelium-derived NO *in vivo*, or a greater contribution of NO formation to the vasodepressor responses of this peptide.

Since EDRF has been now characterized both biologically and chemically in a variety of systems as NO (Palmer *et al.*, 1987; Hutchinson *et al.*, 1987; Radomski *et al.*, 1987; Ignarro *et al.*, 1987, Khan & Furchgott, 1987; Moncada *et al.*, 1988; Kelm *et al.*, 1988), whose synthesis can be inhibited by L-NMMA (Palmer *et al.*, 1988b; Rees *et al.*, 1989a,b) the current findings with L-NMMA in the rat further implicate endothelium-derived NO in the regulation of cardiovascular tone and blood pressure, and as a mediator of the hypotensive actions of endogenous endothelium-dependent vasodilators *in vivo*. The modulation of local substrate levels and enzymic activity involved with the biosynthesis of NO in the vascular endothelium may, therefore, make an

important contribution to the physiological regulation and pathological conditions of the cardiovascular system.

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Interaction of the Paf antagonist WEB 2086 and its hetrazepine analogues with human platelets and endothelial cells

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1 Intact platelets and confluent human umbilical vein endothelial cells bound [^{3}H]-Paf-acether (platelet activating factor, [^{3}H]-Paf) at 20°C in the presence of 0.25% (w/v) bovine serum albumin (BSA).

2 [^{3}H]-Paf binding to platelets was inhibited in a concentration-dependent manner by WEB 2086. An excess of WEB 2086 indicated the presence of specific, saturable Paf binding which reached a maximum of 28.3 ± 3.7 fmol [^{3}H]-Paf per 5×10^7 platelets. In platelets, different hetrazepines (WEB 2098, 2105, but not 2118) also inhibited [^{3}H]-Paf binding in a concentration-dependent manner.

3 WEB 2086 partially displaced platelet-bound [^{3}H]-Paf in a concentration-dependent manner reaching a plateau at 400 nM WEB 2086. No further displacement was observed when WEB 2086 and an excess of unlabelled Paf were added together.

4 The hetrazepines inhibited platelet aggregation. Platelet aggregation IC_{50} values correlated well with the IC_{50} values of the hetrazepines against [^{3}H]-Paf binding ($r^2 = 0.99$). WEB 2086 shifted the Paf dose-response curve rightwards in a parallel manner. Tested against platelet aggregation the pA_2 obtained for WEB 2086 was 7.9.

5 WEB 2086 inhibited [^{3}H]-Paf binding to endothelial cells in a concentration-dependent manner. WEB 2086 also inhibited the Paf-mediated cytosolic calcium increase in endothelial cells with an IC_{50} value of 23.1 ± 10.4 nM as compared with an IC_{50} of 21.6 ± 10.4 nM WEB 2086 for platelet aggregation.

6 These results demonstrate an inhibition of [^{3}H]-Paf binding to platelets and endothelial cells by different hetrazepines, most probably at the Paf receptor level.

Introduction

Paf-acether (Paf, 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) (Benveniste *et al.*, 1972; 1979; Demopoulos *et al.*, 1979; Blank *et al.*, 1979) exhibits potent inflammatory and hypotensive properties (Vargaftig *et al.*, 1981; Pinckard *et al.*, 1982; Chung & Barnes, 1988). Paf activates platelets independently of the cyclo-oxygenase pathway via a receptor-dependent mechanism (Cazenave *et al.*, 1979; Valone *et al.*, 1982; Korth *et al.*, 1986). Intact washed human platelets do not metabolise Paf, but when platelets are activated or lysed they release their Paf-degrading cytosolic acetylhydrolase (Korth *et al.*, 1988a). Binding sites or receptors for Paf have been reported in platelets (Valone *et al.*, 1982; Korth & Benveniste, 1987), neutrophils (Valone & Goetzl, 1983), macrophages (Lambrecht & Parnham, 1986), membrane preparations of human lung tissue (Hwang *et al.*, 1985) and recently in human cultured umbilical vein endothelial cells (Korth *et al.*, 1987).

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In the latter work, binding sites for Paf on endothelial cells were shown to display a higher affinity than those in platelets.

Several putative antagonists of Paf have recently been described, such as CV 3988 (Terashita *et al.*, 1983), Kadsurenone (Shen *et al.*, 1985), BN 52021, a chemically-defined extract from *Ginkgo biloba* (Braquet, 1984; Nunez *et al.*, 1986; Korth & Benveniste, 1987) and the Ginkgolide-mixture BN 52063 (Guinot *et al.*, 1986; Korth *et al.*, 1988b). The Ginkgolides specifically inhibit platelet aggregation, produce a shift in Paf concentration-response curves and displace labelled Paf from platelets in a concentration- and time-dependent manner suggesting competition at the binding site (Korth *et al.*, 1988b). Certain calcium channel antagonist drugs also inhibit Paf binding (Wade *et al.*, 1986).

Another class of Paf antagonists has recently been proposed. Triazolo-benzodiazepines such as alprazolam and triazolam and triazolo-thieno-1,4-benzodiazepines such as brotizolam inhibit Paf-induced platelet activation (Kornecki *et al.*, 1984; Chesney *et al.*, 1987) and these *in vitro* and *in vivo* effects are dissociated from their CNS activity (Casals-Stenzel & Weber, 1987; Casals-Stenzel, 1987a; Casals-Stenzel *et al.*, 1987b). One specific Paf-antagonist derived from triazolodiazepines, WEB 2086, inhibited Paf-induced platet aggregation (Weber *et al.*, 1986), endotoxin shock (Casals-Stenzel, 1987b), anaphylaxis (Casals-Stenzel, 1987c) and Paf binding to human platelets and endothelial cells (Korth *et al.*, 1987).

In various organs Paf triggers vascular effects such as vasoconstriction and acute oedema formation which are inhibited by Paf receptor antagonists (Benveniste *et al.*, 1983; Guinot *et al.*, 1986; Hwang *et al.*, 1986; Casals-Stenzel *et al.*, 1987a). Several reasons argue for these vascular Paf effects being directly mediated by endothelial cells. Firstly, Paf elicits vascular effects even in the absence of blood cells (Casals-Stenzel, 1987a); secondly, endothelial cells possess Paf receptors with a higher affinity than those in platelets (Korth *et al.*, 1987) and thirdly, Paf receptor antagonists specifically inhibit vascular effects *in vivo* and *in vitro* (Guinot *et al.*, 1986; Casals-Stenzel *et al.*, 1987a; Hwang *et al.*, 1986). The shape change, the Ca^{2+} flux and the release of prostacyclin from endothelial cells in response to Paf (Bussolino *et al.*, 1985; Brock & Gimbrone, 1986; D'Humières *et al.*, 1986; Grigorian & Ryan, 1987; Hirafuji *et al.*, 1988), as well as the Paf-mediated adherence of inflammatory cells (McIntyre *et al.*, 1986; Kimani *et al.*, 1988), could explain the important pathophysiological role of the mediator in allergy and inflammation (Chung & Barnes, 1988; Arnoux *et al.*, 1988). Therefore we investigated the antagonism of Paf receptors in human platelets and endothelial cells using radioligand binding assays and, in the latter, increase in cytosolic Ca^{2+}. We describe in platelets the comparative characteristics of the triazolo-thieno-benzodiazepines WEB 2086, 2098, 2105 and 2118 as antagonists of Paf and of [^{3}H]-Paf binding.

Methods

Preparation of washed human platelets

Platelets were prepared as described previously (Lalau Keraly *et al.*, 1984 modifed by Korth *et al.*, 1988b). For binding studies platelets were incubated in Tyrode buffer containing Aspegic (0.1 mM) for 30 min before the last of three washing procedures.

After the last centrifugation, platelets (1×10^9 ml^{-1}) were kept in acid:citrate:dextrose anticoagulant-free Tyrode buffer (pH 6.4) in the presence of 0.25% bovine serum albumin (BSA) (Korth *et al.*, 1986). The platelet count in the solution was adjusted to 1×10^9 ml^{-1} as determined with a Coulter Counter.

Platelets for aggregation studies were prepared either by the method of Mustard *et al.* (1972) or that described by Korth *et al.* (1988b). The former method was used when the release reaction was studied. Platelets from 150 ml of blood obtained from healthy donors were labelled in the first washing fluid with 2 μCi of [^{14}C]-5-hydroxytryptamine (5-HT). The platelets were finally resuspended at 5×10^8 ml^{-1} in modified Tyrode solution (pH 7.35) at 37°C and containing 0.35% BSA, 2 mM Ca^{2+}, 1 mM Mg^{2+}, 5 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), apyrase (0.7 μl ml^{-1} of platelet suspension), and 5 μM imipramine.

Platelet aggregation and release reaction in the presence of antagonists

Platelets were incubated in the aggregometer cuvette with WEB 2086 and 2098 (1, 5, 10, 50, 100, 1000 nM, final concentrations), 2105 or 2118 (0.1, 0.5, 1, 5 μM) or with their solvents. After stirring for 30 s at 37°C, fibrinogen was added and platelets were submitted to the 5-HT release and the aggregation procedures in the presence of Paf (0.66 ± 0.08 μM) or ADP (2.5 μM). After 3 min, platelets were pelleted at 11.000 *g* for 1 min in an Eppendorf centrifuge and the radioactivity in 100 μl of supernatant was counted under standard conditions in ACS scintillant.

The antagonism of Paf-induced aggregation was studied by incubating platelets for 30 s at 37°C in the presence of different concentrations of WEB 2086

(11, 22, 33, 44, 88, 100 and 500 nM, final concentrations) before constructing a concentration-response curve to Paf. Control experiments were performed in the presence of drug vehicle alone. Schild plots were constructed as described by Arunlakshana & Schild (1959).

[^2H]-Paf binding studies in intact human platelets

The platelet suspension was diluted (1:10, v/v) in pH 7.4 Tyrode buffer supplemented with 1.3 mM Ca^{2+} and 0.25% BSA before binding and displacement studies which were performed at 20°C. Platelets were separated by vacuum filtration through Whatman GF/C filters (Ferrière, France). The filter-associated radioactivity in the absence of platelets was subtracted from that measured in the presence of platelets to derive the total binding to cells.

Increasing concentrations of all four hetrazepines (4, 10, 20, 40, 200, 400 nM, 1, 2 μM) were incubated for 30 min together with both platelets and 0.065 nM [^{3}H]-Paf. Platelet-bound radioactivity in the presence of hetrazepines was expressed as the percentage inhibition of total platelet-bound [^{3}H]-Paf.

To establish the extent of specific, saturable Paf binding, platelets (5×10^7 in 500 μl) were incubated with increasing concentrations of [^{3}H]-Paf (0.65–6.53 nM) in the presence of either WEB 2086 (2 μM) or 0.2% (v/v) ethanol vehicle for 30 min. The incubation was performed in pH 7.4 Tyrode buffer in the presence of 0.25% BSA and 1.3 mM Ca^{2+}. Binding was expressed as fmol [^{3}H]-Paf bound per 5×10^7 platelets. Non-specific binding to the cells was defined as that not inhibited by 2 μM WEB 2086. The specific binding was defined as the difference between total and non-specific binding.

In a second type of binding experiment, displacement of platelet-bound [^{3}H]-Paf by WEB 2086 was investigated. Platelets (5×10^7 in 450 μl pH 7.4 Tyrode buffer containing 1.3 mM Ca^{2+} and 0.25% BSA) were preincubated for 15 min with 50 μl 0.65 nM [^{3}H]-Paf before addition of 1 μl WEB 2086 (4, 40, 400, 2000 nM) for different time periods. In other experiments WEB 2086 alone, unlabelled Paf (50 nM) alone, WEB 2086 (2000 nM) together with unlabelled Paf (50 nM) or WEB 2098, 2105 and 2118 alone (40 nM) or vehicle alone were added for 30 min to the prelabelled platelets. Platelet-bound radioactivity in the presence of hetrazepines and/or unlabelled Paf was calculated as a percentage of total platelet-bound radioactivity before the additions.

[^3H]-Paf degradation by platelets in the presence and absence of hetrazepines

Platelets (4×10^7 in 400 μl pH 7.4 Tyrode buffer containing 0.25% BSA and 1.3 mM Ca^{2+}) were incubated at 20°C or 37°C with 0.65 nM [^{3}H]-Paf for 30 min. The suspension was then filtered and the filters, in 400 μl water (1 v), were then extracted in 1 ml of dichloromethane/methanol (1:2, v/v). Dichloromethane and water containing 2% acetic acid (1:1, v/v) were added after 24 h extraction at 4°C. The dichloromethane phase was recovered and the water phase was washed with dichloromethane (1 v) three times. Dichloromethane extracts were submitted to high performance liquid chromatography (h.p.l.c.) (Benveniste *et al.*, 1979). All water phases were also counted to exclude the abnormal presence of radioactivity. Using appropriate authentic markers, a retention time of 3–7 min was defined as void volume, 11–13 min as alkyl-acyl-(long-chain)-*sn*-glycero-3-phosphocholine (AAGPC), 18–25 min as Paf and 28–33 min as lyso-Paf. Radioactivity of different fractions was calculated as a percentage of the radioactivity in all fractions after subtraction of background values.

[^3H]-Paf binding to endothelial cells

Endothelial cells were isolated from umbilical cord veins according to the method of Jaffe *et al.* (1973) with modifications (Hirafuji *et al.*, 1987). In order to remove culture medium (Ham's F-12 plus 10% heated inactivated foetal calf serum) confluent endothelial cells were washed twice with pH 6.4 Tyrode buffer containing 0.25% BSA and 900 μl of the same buffer adjusted to pH 7.4 and containing 1.3 mM Ca^{2+}, 100 μl 0.65 nM [^{3}H]-Paf with different concentrations of WEB 2086, or its vehicle, added. After 30 min at 20°C, free radioactivity was washed twice from confluent endothelial cells with pH 6.4 Tyrode buffer containing BSA, and then once with cold isotonic saline. Endothelial cells were detached by adding cold isotonic saline containing 5 mM EDTA, followed by incubation at 4°C for at least 30 min. The detached cells were filtered from the medium under vacuum. Incubation buffer was filtered to avoid loss of cells during the washing procedure after the binding period. Filters were washed twice with 10 ml cold Tyrode buffer and radioactivity bound to filters was counted under standard conditions in PCS scintillant. Filter-bound radioactivity in the absence of cells was subtracted from radioactivity measured in their presence. The inhibitory effects of different concentrations of WEB 2086 (1–1000 nM) were tested and calculated in fmol [^{3}H]-Paf per $5.2 \pm 0.15 \times 10^5$ endothelial cells.

Measurement of the increase of cytosolic calcium in endothelial cells and its inhibition by WEB 2086 was performed as previously described (Hirafuji *et al.*, 1988). Endothelial cells were grown to confluence onto coverglasses and continuously superfused (1 ml min^{-1}) with Hank's balanced salt solution

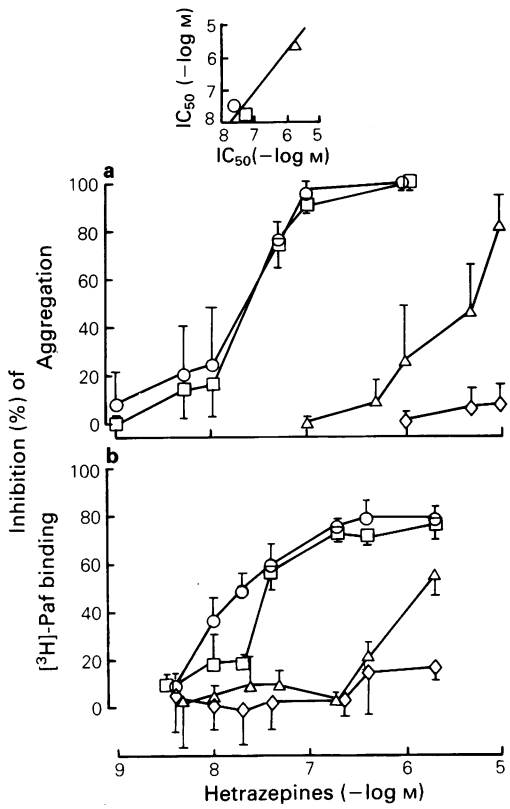


Figure 1 Inhibition by WEB 2086 (○), 2098 (□), 2105 (△) and 2118 (◇) of platelet aggregation in response to Paf (a) and, after 30 min incubation, [^{3}H]-Paf binding to human platelets (b). Each point represents the mean ± 1 s.d. (n = 3–4) inhibition of maximal aggregation or binding; s.d. shown by vertical bars. Inset: correlation between inhibitory hetrazepine effects (IC_{50} in −log M) obtained from dose-response curves (a, b) on Paf-induced platelet aggregation (ordinate scale) and binding of 0.065 nM [^{3}H]-Paf (abscissa scale). Correlation coefficient (r^2) = 0.99.

(HBSS, pH 7.4) containing 10 mM HEPES and 0.1% BSA and incubated for 30 min with 5 μM fura 2/AM. After loading, excess fura 2/AM was removed by washing. When appropriate, cells were superfused with WEB 2086 for 3 min before and during the stimulation.

Confluent endothelial cells were incubated at 20°C for 30 min with 0.65 nM [^{3}H]-Paf under binding conditions. Free radioactivity was carefully washed from confluent endothelial cells as described above. Cells were then detached by freezing and thawing at −20°C three times and the whole cell suspension was extracted as described above in dichloromethane/methanol (1:2, v/v) for h.p.l.c.

Drugs and buffers

Tyrode buffer comprised (in mM): NaCl 137, KCl 2.58, $NaHCO_3$ 11.9, $MgCl_2$ 1.0, NaH_2PO_4 0.41, dextrose 0.5 and HEPES 5.0. Acid citrate dextrose (ACD) comprised citric acid (0.8%), trisodium citrate (2.2%) and glucose (2.45%); citric acid (0.15 M) (all from Merck-Darmstadt, F.R.G). Aspirin was used as its lysine salt (Aspegic, Egic Laboratory, Amilly, France). The following were obtained and prepared as indicated: fatty acid-free BSA, imipramine, collagenase and ADP were from Sigma Chemical Co, St. Louis, MO, U.S.A. [^{3}H]-Paf, (1-O-[^{3}H]-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) sp. act. 80 Ci mmol^{-1}, [^{14}C]-5-hydroxytryptamine-3-creatinine sulphate, sp. act. 50 Ci mmol^{-1} and PCS or ACS scintillation fluids were from Amersham International plc, Aylesbury, Bucks, U.K. Culture media were from Hyclone, Logan, Utah, U.S.A and laboratory ware from Falcon, Oxnard, CA, U.S.A. Paf, 1-octacecyl-2-acetyl-*sn*-glycero-3-phosphocholine and its enantiomer (Bachem, Bubendorf, Switzerland) were dissolved in saline containing 0.25% BSA. WEB 2086, 2098, 2105 and 2118 (3-(4-(2-chlorophenyl)-9-methyl-; -9-cyclopropyl-; 3-(4-(2-chlorophenyl)- and -9-cyclohexyl-6H-thieno-(3,2-f)(1,2,4)triazolo-(4,3-a)(1,4)diazepin-2yl)-1-(4-morpholinyl)-1-propanone (Boehringer-Ingelheim, Ingelheim, F.R.G.) were dissolved freshly each day either in water by adding 0.1 N HCl with ultrasonication, or in ethanol. Apyrase from potatoes was used in the platelet-suspending media at a concentration that converted 0.25 μM ATP to AMP within 120 s at 37°C. Human fibrinogen (grade L, AB Kabi, Stockholm, Sweden) was pretreated with diisopropylfluorophosphate and partially purified. Trypsin-EDTA and Hank's balanced salt solution (Gibco, Paisley, Scotland), and specific antiserum to human factor VIII (Nordic Immunology, Silburg, the Netherlands). Acetic acid was from UCB, Leuven, Belgium. Fura 2/AM was from Dojin (Tohoku, Japan).

Data analysis

All results are given as mean ± 1 s.d. of 3–4 experiments, except when indicated.

Results

Platelet aggregation and 5-HT release

Preliminary studies showed that platelet aggregation IC_{50} values for the antagonists yielded similar values whatever the method used for platelet preparation. WEB 2086, 2098 and 2105 all inhibited, in a concentration-dependent manner, platelet aggre-

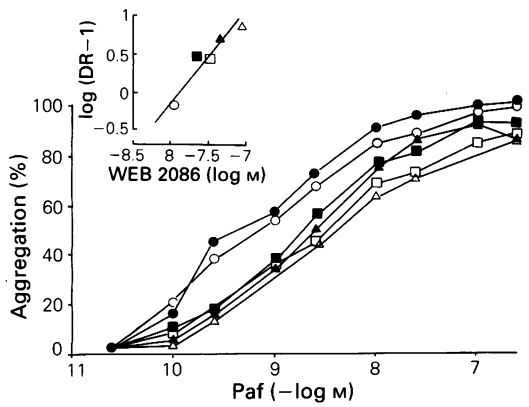


Figure 2 Concentration-dependent aggregation of platelets by Paf in the absence (●) and in the presence of WEB 2086. Platelets were incubated 30 s at 37°C with 11 nM (○), 22 nM (■), 33 nM (□), 44 nM (▲) or 88 nM (△) WEB 2086 prior to aggregation. Each point represents the mean of six experiments. Inset: results from Figure 2 calculated by Schild plot analysis. Each point represents the mean of 6 experiments.

gation induced with 0.66 ± 0.08 μM Paf (Figure 1a). WEB 2105 showed only weak, and WEB 2118 no inhibitory potency. The inhibitory profile for all hetrazepines was the same after preparation in either of the vehicles (results not shown). The 50% inhibitory concentrations (IC_{50}) for platelet aggregation of WEB 2086, 2098 and 2105 were 21.6 ± 10.4 nM, 25.6 ± 4.5 nM and 1.19 ± 0.27 μM respectively (Figure 1a). Similar results were obtained in the presence of 0.25% BSA using the method of Lalau Keraly *et al.* (1984). Platelet aggregation induced by 25 μM ADP was not inhibited by 1 μM WEB 2086 since 72%

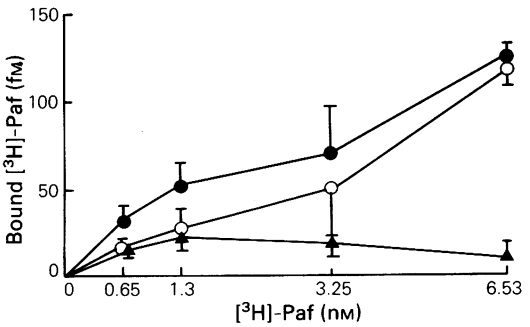


Figure 3 [^{3}H]-Paf binding to intact human platelets in the absence or presence of 2 μM WEB 2086. Saturable specific Paf binding (▲) was calculated in fmol from the difference of total (●) and non-specific binding (○). In this and following figures, values without platelets are subtracted from values in the presence of platelets and are means of 3 experiments with s.d. shown by vertical bars.

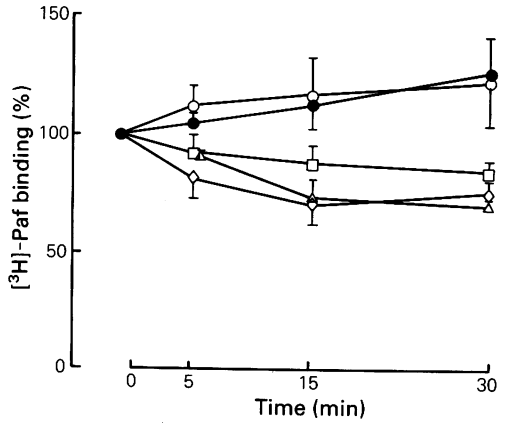


Figure 4 Displacement of platelet-bound [^{3}H]-Paf by increasing concentrations of WEB 2086 (4 ○, 40 □, 400 △, 2000 nM ◇, final concentrations) or vehicle (●, 0.2% ethanol, v/v). WEB 2086 was added to labelled platelets at time zero and platelet-bound radioactivity at various times thereafter was calculated as the percentage of platelet-bound radioactivity at time zero without WEB 2086.

aggregation was obtained with the antagonist versus 66% in the presence of vehicle (one experiment representative of three). WEB 2086 shifted the Paf dose-response curve rightwards in a parallel manner (Figure 2). The pA_2 was 7.9 with a slope of 0.81 and a correlation coefficient of 0.95 (Figure 2 inset).

WEB 2086, 2098 and 2105 exhibited similar inhibitory potencies on Paf-induced [^{14}C]-5-HT release as on platelet aggregation. The IC_{50} values for WEB 2086, 2098 and 2105 were 26.9 ± 25.0 nM, 30.3 ± 1.5 nM and 3.6 ± 4.0 μM, but could not be calculated for WEB 2118 (means ± 1 s.d. of three experiments).

[^{3}H]-*Paf binding to platelets in the presence of hetrazepines*

WEB 2086, 2098 and 2105 inhibited binding of 0.065 nM [^{3}H]-Paf in a concentration-dependent manner, with IC_{50} values of 26 ± 6 nM, 40.3 ± 3.5 nM and 1.76 ± 0.55 μM respectively (Figure 1b). At 0.065 nM nearly all [^{3}H]-Paf was specifically bound to platelets and the non-specific binding in the presence of an excess of WEB 2086 and 2098 was low. At concentrations up to 2 μM WEB 2118 failed to inhibit [^{3}H]-Paf-binding to platelets. There was a significant correlation between the IC_{50} values of the hetrazepines for inhibition of aggregation and inhibition of [^{3}H]-Paf binding when data were analysed by linear regression (Figure 1 inset).

Both total and non-specific binding of [^{3}H]-Paf in the absence and presence of WEB 2086 increased

with Paf concentration but did not reach a plateau (Figure 3). WEB 2086 indicated a saturable reversible component to Paf binding accounting for a maximum of 28.3 ± 3.7 fmol [^{3}H]-Paf per 5×10^7 platelets.

After 15 min preincubation of platelets with 0.65 nM [^{3}H]-Paf, platelet-bound label could be partially displaced by WEB 2086 in a concentration- and time-dependent manner (Figure 4). The displacement induced 30 min after the addition of 40–2000 nM WEB 2086 is shown in Figure 5. No additional displacement was obtained when 2 μM WEB 2086 was added to platelets either with or without 50 nM unlabelled Paf (Figure 5). At 40 nM WEB 2098 induced a moderate displacement, but similar concentrations of WEB 2105 and WEB 2118 failed to displace platelet-bound [^{3}H]-Paf (Figure 5).

Lack of metabolism of [^{3}H]-Paf in the presence of platelets

Platelets were incubated at 20°C or 37°C for 30 min with 0.65 nM [^{3}H]-Paf in the absence or presence of 40 nM WEB 2086. In all cases only intact Paf was identified by h.p.l.c. after extraction of filter-bound platelets (Figure 6).

Binding and metabolism of Paf to endothelial cells

WEB 2086 inhibited total [^{3}H]-Paf binding to endothelial cells in a concentration-dependent manner (Figure 7). In three experiments WEB 2086 inhibited the increase of cytosolic calcium in human endothelial cells triggered by a submaximal concentration of Paf (100 nM). After 3 min preincubation of cells with the antagonist, the WEB 2086 IC_{50} value was 23.1 ± 2.9 nM. After incubation of confluent endothelial cells with 0.65 nM [^{3}H]-Paf for 30 min only

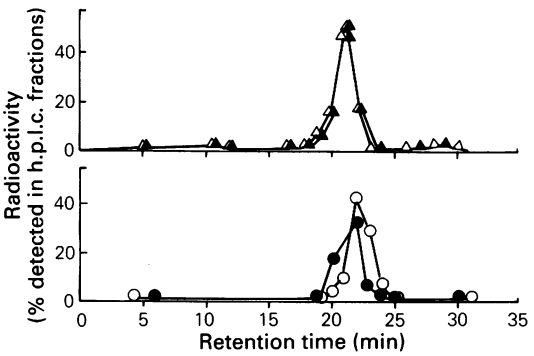


Figure 6 Lack of [^{3}H]-Paf metabolism in the presence of platelets and hetrazepines. Experiments were conducted in the absence (● ▲) or presence (○ △) of 40 nM WEB 2086 for 30 min at 37°C (▲ △) or 20°C (● ○). The figure shows 1 experiment representative of 3.

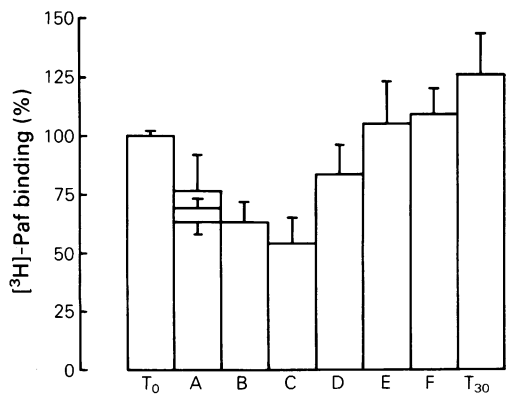


Figure 5 Displacement of platelet-bound [^{3}H]-Paf (0.65 nM) by WEB 2086. Platelets were preincubated for 15 min with 0.65 nM [^{3}H]-Paf alone (T_0). Hetrazepines were then added for 30 min before platelets were separated by vacuum filtration and binding measured: (A) from top to bottom 40, 1000, 2000 nM WEB 2086 in the absence of unlabelled Paf; (B) in the presence of 2000 nM WEB 2086 and 50 nM unlabelled Paf; (C) 50 nM unlabelled Paf alone; (D) WEB 2098; (E) WEB 2105; (F) WEB 2118, all at 40 nM; T_{30}, vehicle alone after 30 min incubation (T_{30}).

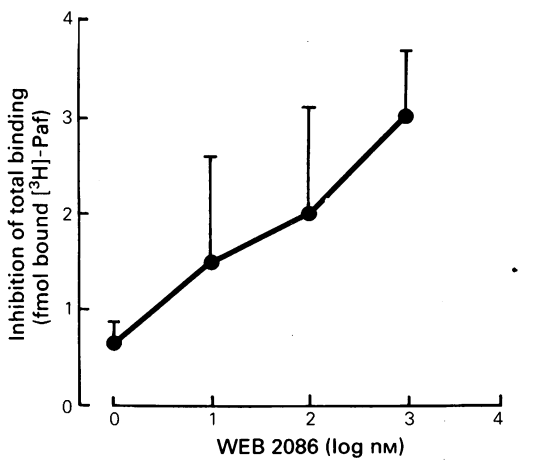


Figure 7 Concentration-dependent inhibition by WEB 2086 of total [^{3}H]-Paf binding to confluent endothelial cells. After 30 min incubation of endothelial cells with increasing concentrations of WEB 2086 (1–1000 nM) in the presence of 0.65 nM [^{3}H]-Paf, cells were detached and filtered. The inhibitory effect of WEB 2086 (●) on [^{3}H]-Paf binding to endothelial cells was calculated in fmol per 5.2 ± 0.15 × 10^5 cells. Values without cells were subtracted from filter-bound radioactivity in the presence of endothelial cells and are means of three experiments; s.d. shown by vertical bars.

$1.4 \pm 0.9\%$ and $3.4 \pm 3.4\%$ [^{3}H]-lyso-Paf and AAGPC were detected respectively.

Discussion

This study demonstrates that WEB 2086 inhibits platelet aggregation and Paf binding to platelets and endothelial cells in a concentration-dependent manner. The functional relevance of putative Paf receptors is supported by the fact that inhibition of platelet aggregation correlated closely with inhibition of Paf binding. The lack of inhibition of ADP-induced platelet aggregation by hetrazepines supports their specificity as described before with ADP and arachidonic acid (Casals-Stenzel, 1987a and our unpublished data).

Saturation of the inhibitory effect of WEB 2086 indicates its competition for a limited number of Paf binding sites in platelets. Similar results were obtained previously with unlabelled Paf, but not with its synthetic enantiomer (Korth & Benveniste, 1987). Platelets did not degrade Paf irrespective of whether antagonists were present, consistent with our observations with Ginkgolides (Korth & Benveniste, 1987; Korth *et al.*, 1988b). Competitive binding studies were performed at low [^{3}H]-Paf concentrations where nearly all [^{3}H]-Paf is specifically bound to putative Paf receptors. Platelet aggregation IC_{50} values of the hetrazepines correlated closely with their IC_{50} values versus Paf binding, sharing the same linear regression line with the Ginkgolides (Korth *et al.*, 1988b). Thus the same direct competitive inhibition which was previously demonstrated for Ginkgolides applies to hetrazepines. Substitution in the 9-position of the hetrazepine molecule modulates the inhibitory potency since WEB 2086 and WEB 2098 inhibited platelet aggregation and [^{3}H]-Paf binding at lower concentrations than WEB 2105 and WEB 2118 analogues (this article and Weber *et al.*, 1986).

In the aggregation studies WEB 2086 caused a rightward parallel shift with a slight depression in the maximum of the Paf dose-response curve at a concentration of 88 nM WEB 2086 (see Figure 2). We were not able to exclude an interaction between Paf, hetrazepines and albumin (our unpublished data). Therefore the same experiments were performed in the absence of albumin and using rabbit platelets which aggregate at lower Paf concentrations than human platelets. Rabbit platelets reached a maximal response to 1 μM Paf even in the presence of 88 nM WEB 2086 (unpublished results). The latter results are in agreement with those in the literature (Stewart & Dusting, 1988).

Association and dissociation binding studies (displacement) were performed since association studies alone cannot exclude allosteric effects or non-competitive receptor alterations. WEB 2086 displaced saturated platelet-bound [^{3}H]-Paf in a concentration- and time-dependent manner. No additive effects on Paf displacement were observed when WEB 2086 was incubated together with unlabelled Paf. The latter experiments favour a direct competitive hetrazepine/Paf receptor interaction, although more analysis would be helpful to exclude the presence of allosteric effects.

WEB 2086 also inhibited the binding of [^{3}H]-Paf and the Paf-mediated cytosolic calcium increase in cultured human vein endothelial cells. This might be of interest for further pharmacological and clinical studies since Paf binding sites in endothelial cells are different from those described in platelets (Korth *et al.*, 1987). Casals-Stenzel described an inhibitory effect of WEB 2086 on Paf-mediated vascular and bronchial effects in the isolated perfused rat lung even in the absence of blood cells indicating that endothelial cells may mediate pathophysiological effects of Paf (Casals-Stenzel *et al.*, 1987a).

Given the present results, it seems of interest to investigate hetrazepines in clinical studies since WEB 2086 is a very potent antagonist of Paf-induced effects in endothelial cells and platelets. The close correlation between inhibition of Paf binding and of platelet aggregation further emphasizes the importance of Paf receptors in the onset of the biological effects of this mediator.

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Effect of cromakalim on bronchoconstriction evoked by cholinergic nerve stimulation in guinea-pig isolated trachea

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1 Cromakalim reduced intraluminal pressure in the guinea-pig isolated, innervated trachea.

2 Preganglionic stimulation of the cervical vagus nerve elicited a frequency-dependent increase in intraluminal pressure. Cromakalim attenuated responses to vagal stimulation in a concentration-dependent manner at all frequencies tested.

3 Field stimulation caused a frequency-dependent increase in intraluminal pressure mediated by muscarinic cholinoceptors. Cromakalim did not affect the amplitude of responses at any frequency of stimulation, even at high concentrations.

4 Acetylcholine, added to the Krebs solution bathing the adventitial surface of the trachea, evoked a concentration-dependent increase in intraluminal pressure. The concentration-effect curve for acetylcholine was unaltered in the presence of cromakalim.

5 It is concluded that cromakalim modulates cholinergic neuroeffector transmission in the trachea chiefly by a prejunctional mechanism. However, cromakalim probably does not interfere with acetylcholine release from postganglionic cholinergic neurones.

Introduction

Cromakalim is a smooth muscle relaxant which is thought to act by promoting K^+-efflux through specific K^+-channels in the plasma membrane (Hamilton *et al.*, 1986; Weir & Weston, 1986a,b). In guinea-pig trachealis, relaxation is associated with marked hyperpolarisation, close to the K^+-equilibrium potential for these cells (Allen *et al.*, 1986), which would be expected to reduce the general excitability of the tissue. However, cromakalim did not markedly attenuate the bronchoconstriction induced by a variety of agents including potassium chloride (>10 mM), histamine and acetylcholine (ACh) (Allen *et al.*, 1986). Although the effects of applied ACh were relatively unaffected by cromakalim, it seemed possible that cromakalim might be more effective against the bronchoconstriction induced by endogenous ACh released from parasympathetic nerve terminals, perhaps by modulating cholinergic neurotransmission. To test this hypothesis the guinea-pig isolated trachea was used to examine the effects of cromakalim on bronchoconstriction evoked in three different ways: (1) stimulation of the cervical vagus nerve (preganglionic), (2) field stimulation (postganglionic) and (3) applied ACh. Preliminary accounts of these and similar experiments by Hall & Maclagan have been published (Hall & Maclagan, 1988; McCaig & De Jonckheere, 1989).

Methods

Preparation

Guinea-pigs (male, approximately 400 g body weight) were killed by a blow to the head and the trachea rapidly excised with or without the right vagus nerve and its recurrent laryngeal branch, as required (Blackman & McCaig, 1983). The trachea was cannulated at each end and mounted horizontally in an organ bath, maintained at 37°C, through which Krebs solution flowed at a rate of 5 ml min^{-1}. The composition of the Krebs solution was (mM): Na^+ 127, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 121, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2, HCO_3^- 25 and glucose 11. The trachea was filled with Krebs solution, then one end was closed and the other attached to a pressure transducer (Statham). Intraluminal pressure (ILP) was recorded continuously on a pen recorder (Devices). Increases and decreases in ILP reflect bronchoconstriction and bronchodilatation, respectively.

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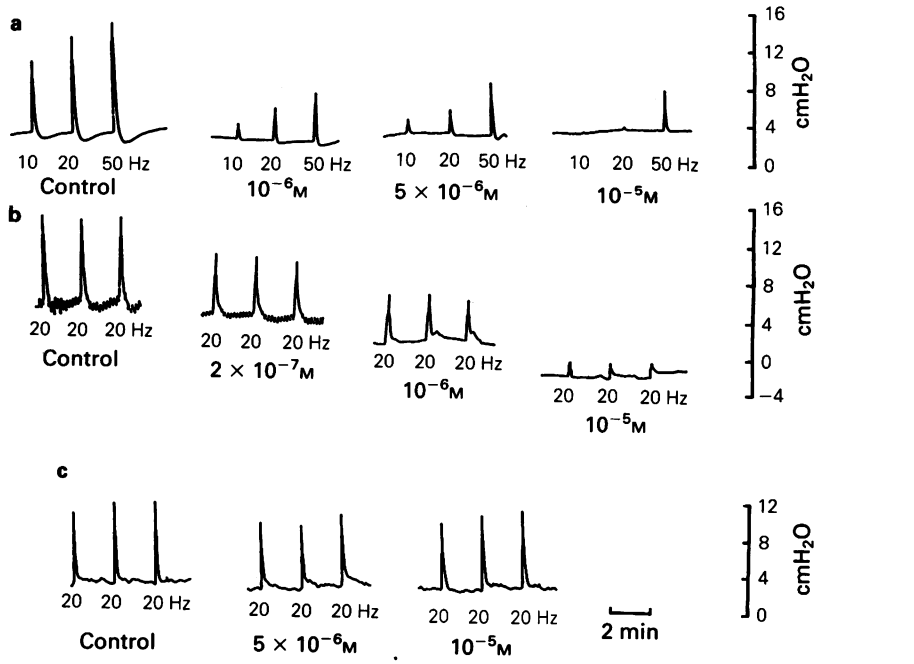


Figure 1 Changes in intraluminal pressure in guinea-pig isolated trachea evoked by (a) stimulation of the vagus nerve at frequencies indicated for 5 s at 2 min intervals, (b) stimulation of the vagus nerve at 20 Hz for 5 s at 2 min intervals and (c) field stimulation at 20 Hz for 5 s at 2 min intervals. Each trace is taken from a single preparation before and 30 min after cumulative additions of cromakalim at concentrations indicated.

Procedure

The cervical vagus nerve was stimulated through a suction or, occasionally, a bipolar electrode (pulses 20 V, 1 ms duration) for 5 s every 2 min at frequencies of 1–50 Hz. Field stimulation was achieved by means of a bipolar electrode placed close to the dorsal aspect of the trachea and the same stimulus parameters. Cumulative concentration-effect curves to applied ACh were constructed by adding ACh directly to the organ bath (with perfusion pump off) in 10 fold increments (10^{-8}–10^{-2} M).

In vagal- or field-stimulated preparations a control frequency-response curve was obtained, then the tissue was stimulated at 20 Hz every 2 min and cromakalim (10^{-7}–10^{-5} M) was added to the reservoir of Krebs solution. (It should be noted that solution from the reservoir was pre-heated by passing it through a coil system (volume approximately 20 ml) in a water bath so that a delay of about 4 min occurred before the tissues were exposed to cromakalim). After 25 min, a second frequency-response curve was obtained. The procedure was then repeated once, or at the most twice, with successively higher concentrations of cromakalim. Cumulative concentration-effect curves to ACh were obtained before and 25 min after addition of cromakalim (10^{-5} M) to the reservoir of Krebs solution. In one group of preparations repeated frequency-response or concentration-effect curves were obtained in the absence of cromakalim to serve as time-matched controls. In another group of preparations frequency-response curves to vagal or field stimulation were obtained in the absence and presence of hexamethonium (2.5×10^{-4} M added to the reservoir of Krebs solution), to determine whether responses were due to pre- or post-ganglionic activation of cholinergic nerve fibres.

Drugs

The following drugs were used: acetylcholine chloride (Sigma), atropine sulphate and hexamethonium bromide (Sigma), cromakalim (Beecham Research Laboratories) and propranolol HCl (ICI).

Statistics

Mean responses were compared by use of t test for independent or paired data as appropriate. Values of $P < 0.05$ were regarded as significant.

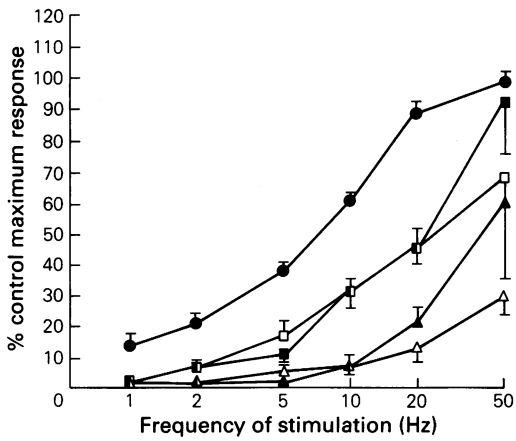


Figure 2 Guinea-pig isolated trachea: relationship between increase in intraluminal pressure, expressed as percentage control maximum response (ordinate scale) and frequency of stimulation (abscissa scale) of the vagus nerve in the absence (●) and presence of cromakalim, 2×10^{-7} M (■), 10^{-6} M (□) 5×10^{-6} M (▲) and 10^{-5} M (△). Values are the mean of 5–8 observations; vertical lines show s.e.mean. All values (except the response at 50 Hz in the presence of 2×10^{-7} M cromakalim) were significantly different from the corresponding control ($P < 0.05$, paired t test).

Results

Relaxant effect of cromakalim

Tracheal preparations had a positive resting ILP of approximately 2–4 cmH_2O, as described previously (Blackman & McCaig, 1983), indicating the presence of spontaneous resting tone in the trachealis muscle. Cromakalim, 10^{-7}–10^{-5} M, evoked a concentration-dependent reduction in ILP which developed gradually, reaching a maximum at 15 ± 1 min (mean ± s.e.mean, $n = 14$). The maximum effect was not maintained in the continued presence of the drug. The amplitude of the response varied considerably between preparations and was probably determined by the initial level of ILP (compare a and b in Figure 1).

Effect of cromakalim on responses to stimulation of the vagus nerve

Stimulation of the vagus nerve elicited frequency-dependent increases in ILP (Figures 1a and 2). When preparations were stimulated at 20 Hz for 5 s at 2 min intervals highly reproducible increases in ILP were obtained. On exposure to cromakalim (10^{-7}–10^{-5} M) the amplitude of this response gradually diminished (see Figure 1b). Maximum attenuation was found to occur after 22 ± 2 min ($n = 17$). Responses were attenuated at all frequencies of stimulation tested (1–50 Hz, Figures 1a and 2), but at 50 Hz the effects of cromakalim were more variable, especially at lower concentrations. The degree of attenuation of vagal responses was concentration-dependent (Figures 1 and 2). At the lowest concentration of cromakalim tested (10^{-7} M) attenuation was already substantial (e.g. at 20 Hz, $50 \pm 15\%$, $n = 3$) and was marked at the highest concentration (10^{-5} M) (at 20 Hz, $86 \pm 5\%$, $n = 5$).

When time-matched control tissues were examined, it was found that the amplitude of vagal responses was well maintained during the first 40 min but declined somewhat thereafter by approximately 20%. Attenuation of vagal responses was significantly greater during exposure to cromakalim at all concentrations tested, than in time-matched controls, as shown in Figure 3a. Cromakalim therefore attenuated markedly responses to stimulation of the vagus nerve.

Responses to vagal stimulation at all frequencies tested were virtually abolished in the presence of hexamethonium (2.5×10^{-4} M, Figure 4a). This confirms that activation of the cholinergic pathway is preganglionic in this preparation.

Effect of cromakalim on responses to field stimulation

In these experiments field stimulation elicited a response which was predominantly an increase in ILP. In some tissues the initial increase in ILP was followed by a reduction in ILP. These responses were blocked respectively by atropine (6×10^{-7} M) and propranolol (3.5×10^{-6} M) indicating muscarinic cholinoceptor-mediated excitation and β-adrenoceptor-mediated inhibition. The amplitude of the initial increase in ILP was unaffected by propranolol pretreatment. This was anticipated since the responses have largely separate time courses, the excitatory response being rapid and brief and the inhibitory response of slower onset and rather longer duration (Blackman & McCaig, 1983). Experiments with cromakalim, therefore, were performed in the absence of an antagonist of β-adrenoceptors.

Field stimulation evoked frequency-dependent increases in ILP. Cromakalim, 2×10^{-7}–10^{-5} M had no demonstrable effect on responses to field stimulation (Figure 1c). Frequency-response curves obtained in the absence and presence of cromakalim were superimposable (Figure 5). In time-matched control tissues there was some reduction in the amplitude of responses with time (Figure 3b). There were no significant differences between responses in time-matched control tissues and cromakalim-treated tissues at any of the concentrations tested. In

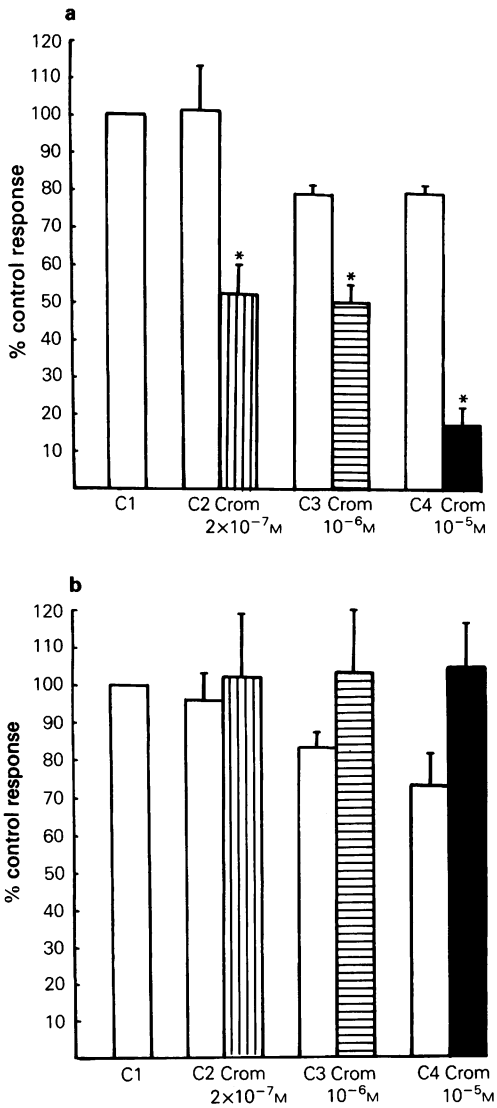


Figure 3 Guinea-pig isolated trachea: increases in intraluminal pressure induced by (a) stimulation of the vagus nerve and (b) field stimulation, each of 20 Hz for 5 s at 2 min intervals, expressed as percentage of the initial control response (C1), in time-matched controls (C2–C4 open columns) and in the presence of cromakalim (Crom) 2×10^{-7} M, 10^{-6} M and 10^{-5} M. Values are the mean of 4–8 observations; vertical bars show s.e.mean. Asterisks denote values significantly different from time-matched controls at $P < 0.01$ (t test).

contrast to the marked attenuation of vagal responses therefore, cromakalim had no effect on cholinergic excitation elicited by field stimulation.

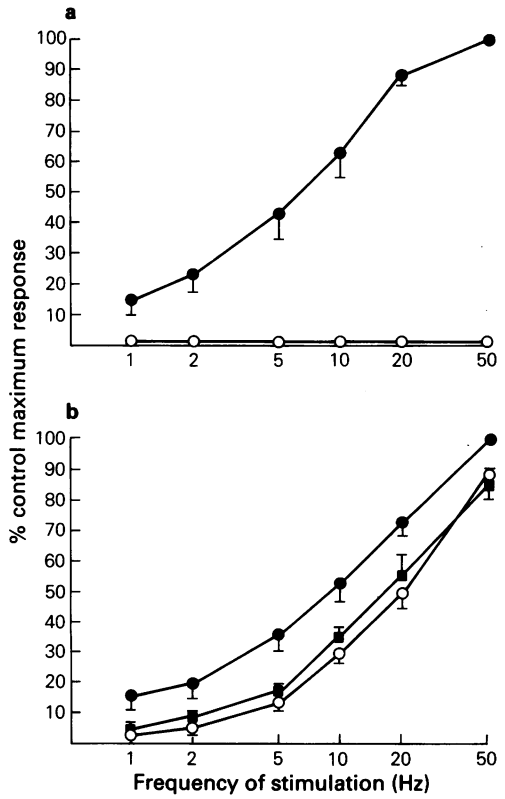


Figure 4 Guinea-pig isolated trachea: relationship between increase in intraluminal pressure, expressed as percentage of the initial control maximum (ordinate scale) and frequency of vagal (a) or field (b) stimulation (abscissa scale). (a) Responses before (●) and 30 min after (○) the addition of hexamethonium, 2.5×10^{-4} M, to the reservoir of Krebs solution. (b) Responses before (●), 30 min after (○) the addition of hexamethonium, 2.5×10^{-4} M, to the reservoir of Krebs solution and in time-matched controls (■). Values are the mean of 5–10 observations; vertical lines show s.e.mean. The response to vagal stimulation was virtually abolished by hexamethonium (a). In (b) responses to field stimulation in time-matched controls were significantly reduced ($P < 0.05$, paired t test) at frequencies of 1 and 2 Hz and in the presence of hexamethonium responses were significantly reduced at 5 and 10 Hz (each as compared with corresponding initial control responses); responses in the presence of hexamethonium were not significantly different from the corresponding time-matched control response at any frequency of stimulation.

When a second frequency-response curve to field stimulation was obtained 30 min after the first curve a small rightward shift was observed, with a significant reduction in response amplitude at low frequencies of stimulation (1 and 2 Hz, Figure 4b). The frequency-response curve obtained in the presence of hexamethonium (2.5×10^{-4} M) also exhibited a

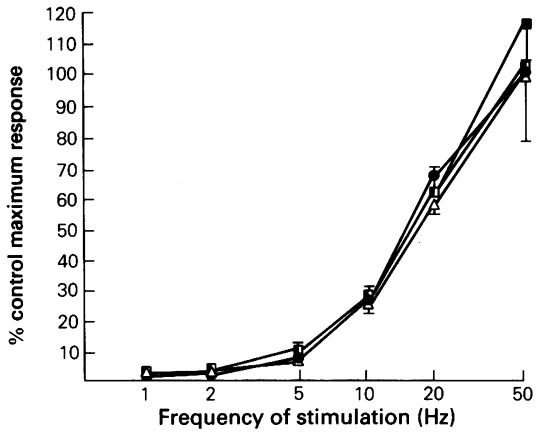


Figure 5 Guinea-pig isolated trachea: relationship between increase in intraluminal pressure, expressed as percentage control maximum (ordinate scale), and frequency of field stimulation (abscissa scale) in the absence (●) and presence of cromakalim 2×10^{-7} M (■), 10^{-6} M (□) and 10^{-5} M (△). Values are the mean of 4–6 observations and none of the values was significantly different from the corresponding control (paired t test). Vertical lines show s.e.mean.

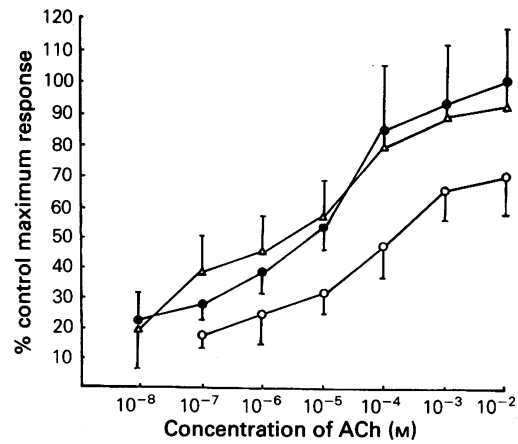


Figure 6 Guinea-pig isolated trachea: relationship between increase in intraluminal pressure, expressed as percentage of the initial control maximum, (ordinate scale) and concentration of applied acetylcholine (ACh) (abscissa scale) in the absence (●) and presence of cromakalim 10^{-5} M (△) and in time-matched controls (○). Values are the mean of 5 observations; vertical lines show s.e.mean. Responses in time-matched controls at ACh 10^{-3} M and 10^{-2} M were significantly reduced ($P < 0.05$, t test) compared with the corresponding responses in the initial curve. Responses in the presence of cromakalim were not significantly different from either the initial control or the time-matched control at any concentration of ACh.

slight rightward shift compared to the initial control curve, but was not significantly different from the time-matched control curve at any frequency of stimulation (Figure 4b). These results indicate that responses to field stimulation are not susceptible to ganglion blockade. Activation of the cholinergic pathway, therefore, is postganglionic in this situation.

Effect of cromakalim on responses to applied ACh

ACh (10^{-8}–10^{-2} M) elicited concentration-dependent increases in ILP. There was a slight rightward shift of the concentration-effect curve with time, with responses at higher concentrations of ACh being significantly smaller than those observed in the initial curve (Figure 6). In the presence of cromakalim, 10^{-5} M, the concentration-effect curve was close to the initial control curve and not significantly different from either this or the time-matched control curve at any concentration of ACh. Cromakalim, therefore, did not attenuate the bronchoconstrictor response to applied ACh.

Discussion

These results indicate that cromakalim can effectively reduce bronchoconstriction mediated by stimulation of the vagus nerve in the isolated guinea-pig trachealis. Hall & Maclagan (1988) obtained a similar finding. Such attenuation could arise pre- and/or post-junctionally and attempts were made, therefore, to localise the effect. Cromakalim had no effect on the response of the trachealis to applied ACh (present study). Allen *et al.* (1986) and Hall & Maclagan (1988) both observed that the concentration-effect curve for ACh in guinea-pig trachea was shifted only slightly to the right when constructed in the presence of cromakalim. Together these results provide strong evidence that the effect of cromakalim against vagal nerve stimulation is predominantly prejunctional. Hall & Maclagan (1988) suggested that this might involve inhibition of ACh release from the cholinergic nerve terminals. However, in the present study, it was found that cromakalim had no effect on bronchoconstriction evoked by field stimulation, suggesting that transmitter output from the postganglionic cholinergic nerve terminals was normal and that the attenuation arises at some earlier point in the cholinergic transmission process. Cromakalim might be acting, for example, to promote K^+-channel opening in tracheal ganglion cells, thus modifying ganglionic transmission, or in the membrane of the pre- or

postganglionic nerve fibres themselves, thus compromising action potential conduction. However, non-specific effects of cromakalim on neuronal function cannot be ruled out.

It is believed that ACh can bring about contraction in smooth muscle through at least three, possibly independent, mechanisms, namely by promoting Ca^{2+}-influx through both receptor-operated Ca^{2+}-channels (ROCs) and voltage-operated Ca^{2+}-channels (VOCs) and by releasing Ca^{2+} from intracellular storage sites (Bolton & Large, 1986). By enhancing K^+-efflux and thus causing hyperpolarisation, cromakalim would be expected to oppose the depolarisation evoked by bronchoconstrictor agents and thereby reduce influx of Ca^{2+} through VOCs. However, it has been suggested that Ca^{2+} influx through VOCs plays a relatively minor role in ACh-induced bronchoconstriction (Farley & Miles, 1977; 1978), which might account for the failure of cromakalim to combat this response in guinea-pig trachealis. It should be noted that it has not yet been shown directly that cromakalim reduces agonist-induced depolarisation. Hyperpolarisation does not always reduce the amplitude of depolarisation by excitatory stimuli. In guinea-pig trachealis, for example, sympathetically-mediated hyperpolarisation can be associated with a reduction, no change or even an increase in amplitude of vagally-mediated depolarisation (McCaig, 1987).

It has been observed that cromakalim is more effective against carbachol-induced bronchoconstriction in human bronchioles than in guinea-pig trachea (Taylor *et al.*, 1988). It is possible that the relative contribution of VOC opening to contraction is greater in human airways than in guinea-pig trachea. Direct determination of the degree of depolarisation induced by carbachol in the absence and presence of cromakalim and/or Ca^{2+}-channel blocking agents would help resolve this question.

Resting tone in guinea-pig trachea is associated with spontaneous fluctuations in membrane potential (slow waves), which are thought to involve cyclical influx of Ca^{2+} through VOCs, since they are abolished in Ca^{2+}-free medium (Foster *et al.*, 1983; McCaig, 1986) or in the presence of Ca^{2+}-channel blockers such as gallopamil and nifedipine (Small, 1982; Ahmed *et al.*, 1985). The hyperpolarising effect of cromakalim would be expected to reduce VOC opening, thus abolishing slow wave activity and resting tone, as demonstrated by Allen *et al.* (1986). It was noted in the present study that the reduction in tone caused by cromakalim was maximal after 15 min, but that maximal attenuation of vagal responses occurred rather later (22 min) at a time when the relaxation was starting to wear off. The different time courses of these two effects would be consistent with the view the relaxation represents a direct effect of cromakalim on the smooth muscle, whereas attenuation of vagal responses occurs independently by a largely prejunctional mechanism.

In conclusion, cromakalim effectively reduces vagally-mediated bronchoconstriction in guinea-pig isolated trachealis, seemingly through prejunctional modulation of cholinergic neurotransmission. Cromakalim, therefore, may be particularly useful in bronchoconstriction where there is a significant reflex component involving vagal efferent pathways.

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Effects of calcium entry blockers on contractions evoked by endothelin-1, [Ala^{3,11}]endothelin-1 and [Ala^{1,15}]endothelin-1 in rat isolated aorta

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1 The aim of the present study was to examine the contractile responses evoked by the recently characterized vasoactive peptide endothelin-1 (ET-1) and by two of its structural analogues, [Ala^{3,11}]ET-1 and [Ala^{1,15}]ET-1 in endothelium-denuded rat isolated aorta, and also to assess the extent of dependence of these responses on extracellular calcium entry.

2 ET-1 (0.3 to 10 nM), [Ala^{3,11}]ET-1 (2.25 to 225 nM) and [Ala^{1,15}]ET-1 (0.04 to 1.36 μM) evoked concentration-dependent contractions in normal, calcium-containing medium with the order of potency: ET-1 > [Ala^{3,11}]ET-1 > [Ala^{1,15}]ET-1.

3 Preincubation of tissues for 60 min with diltiazem (1 μM) induced a significant 3 fold rightward shift of concentration-effect curves to ET-1 without affecting maximal responses elicited by 10 nM of this peptide, whereas the same treatment failed to modify concentration-effect curves to [Ala^{3,11}]ET-1.

4 Preincubation of tissues for 60 min with nifedipine (0.1 or 1 μM) markedly inhibited contractions elicited by either ET-1 (10 nM) or [Ala^{1,15}]ET-1 (0.41 μM). Furthermore, when added cumulatively to tissues maximally contracted by ET-1 (10 nM), nifedipine (3 nM to 1 μM) induced concentration-dependent relaxations with an IC_{50} value of 21.8 $\pm$ 5.9 nM. Maximal relaxation to nifedipine, 1 μM, amounted to 56.9 $\pm$ 11.5%.

5 Submaximal concentrations of ET-1 (3 nM), [Ala^{3,11}]ET-1 (75 nM) and [Ala^{1,15}]ET-1 (0.41 μM), gave about 85% of maximal contractions elicited by noradrenaline (1 μM) in normal, calcium-containing medium. These contractile responses were all reduced by about 70% in calcium-free medium. Pretreatment of tissues with diltiazem (1 μM) or with nifedipine (0.1 or 1 μM) did not affect these residual contractions.

6 Upon readdition of calcium (10 μM to 10 mM) to tissues in calcium-free medium, preincubated with submaximal concentrations of one or the other of the peptides, concentration-dependent contractions were elicited with EC_{50} values of 1.21 $\pm$ 1.1 mM (ET-1-exposed rings), 74.6 $\pm$ 9.1 μM ([Ala^{3,11}]ET-1-exposed rings) and 102 $\pm$ 27 μM ([Ala^{1,15}]ET-1-exposed rings). Calcium-induced responses were significantly inhibited by diltiazem (1 μM) in both ET-1- and [Ala^{3,11}]ET-1-exposed tissues and by nifedipine (0.1 and 1 μM) in ET-1-exposed tissues. However, nifedipine did not significantly affect calcium induced responses in [Ala^{1,15}]ET-1-exposed rings.

7 Readdition of calcium to a calcium-free medium containing 40 mM K^+, evoked concentration-dependent responses that were unaffected by the presence of ET-1 (3 nM).

8 Overall, these results indicate that contractions to ET-1 and its analogues in rat aorta are to some extent dependent on extracellular calcium. However, the mode of antagonism by diltiazem and nifedipine of responses to the peptides in normal medium, as well as of contractions induced by readdition of calcium in peptide-exposed tissues in calcium-free medium, argue against a direct activation by endothelin or its analogues of L-type calcium channels. Finally, removal of one or the other of the disulphide bonds of ET-1 reduces the potency of the peptide.

Introduction

A potent vasoactive peptide was identified in the medium of porcine endothelial cells in culture and named endothelin (Yanagisawa *et al.*, 1988). Recently it has been reported that there is a family of related peptides and the peptide that was originally described has been designated endothelin-1 (ET-1;

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Inoue *et al.*, 1989). However, ET-1 has since been demonstrated to exert multiple actions. It is a potent secretagogue, releasing atrial natriuretic factor from rat myocytes (Fukuda *et al.*, 1988), inhibits renin release from rat glomeruli (Rakugi *et al.*, 1988), has positive inotropic effects on guinea-pig atria (Ishikawa *et al.*, 1988), contracts guinea-pig ileal smooth muscle, interferes with sympathetically-induced twitch responses in the rat vas deferens (Hiley *et al.*, 1989) and possesses bronchoconstrictor qualities (Uchida *et al.*, 1988). Early reports suggested that its vasoconstrictor effects were dependent to a large extent on extracellular calcium and were inhibited by the dihydropyridine calcium entry blockers nifedipine and nicardipine (Hirata *et al.*, 1988; Yanagisawa *et al.*, 1988). These observations correlate with the reported failure of the peptide to induce accumulation of phosphoinositides in rat vascular smooth muscle cells in culture (Hirata *et al.*, 1988). More recent data however, suggest that calcium entry through dihydropyridine-sensitive calcium channels might play only a minor role in the vasoconstrictor responses to the peptide (Auguet *et al.*, 1988; Hagiwara *et al.*, 1988; Miasiro *et al.*, 1988).

Therefore, it was of interest to study the dependence on extracellular calcium of responses to ET-1 in rat aorta *in vitro*. The role of intracellular and extracellular calcium in agonist-induced contractile responses and the effects of calcium entry blockers thereon have been extensively studied in this artery (Godfraind *et al.*, 1986). Few structure-activity studies of the bicyclic peptide ET-1 have been undertaken, but disulphide bonds in cyclic peptides such as somatostatin, oxytocin and atrial natriuretic factor for example are required for maintaining their biologically active conformations (Veber & Saperstein, 1979; Misono *et al.*, 1984, Hruby, 1985). The relative importance of the disulphide bonds for the activity of ET-1 is therefore an interesting question, and this has been assessed by comparing its activity with that of two analogues, [$Ala^{3,11}$]- and [$Ala^{1,15}$] ET-1. The results show that contractions elicited by ET-1 and some of its vasoactive analogues can be partially antagonized by the calcium entry blockers diltiazem and nifedipine. Elimination of one or other of the disulphide bonds by replacement of the appropriate cysteine residues with alanine, reduced potency and changed some of the functional characteristics of the peptide in this tissue. A preliminary account of this work has been published (Topouzis *et al.*, 1989).

Methods

Aortae were obtained from male Sprague-Dawley rats weighing 200–225 g, taking care not to damage the endothelium. Four rings were taken from each aorta and stripped of their endothelium by rubbing with a wooden match stick. Tissues were placed in a physiological solution (composition in mM: NaCl 112, KCl 5, $NaHCO_3$ 25, KH_2PO_4 1, $MgSO_4$ 1.2, $CaCl_2$ 1.25 and glucose 11.5), gassed with 95% O_2 and 5% CO_2. The tissues were allowed to equilibrate for 60 min then contracted with noradrenaline, 1 μM. This maximal noradrenaline-induced contraction served as a reference for all subsequent contractile responses. After the response had reached a plateau, acetylcholine, 1 μM, was added to the bath, to test for the absence or presence of endothelium (Furchgott & Zawadzki, 1980). Preparations were then washed and left to equilibrate for 2 h, during which period the medium was changed 4 times. During the second hour tissues were incubated with either one of the calcium-entry blockers or vehicle. The blockers were then present for the duration of the experimental protocol. At the end of the incubation period, concentrations of ET-1 or an analogue of ET-1 were added to the bath in a cumulative fashion. Only one concentration-effect curve to the peptides was constructed in each tissue. Alternatively, in some experiments nifedipine (3 nM to 1 μM) or diltiazem (1 μM) was added cumulatively to preparations precontracted with 10 nM ET-1.

It has been suggested that ET-1 may activate calcium channels (Yanagisawa *et al.*, 1988) and to examine this possibility contractions induced by readmission of calcium to tissues in the presence of the peptides or noradrenaline were examined. At the end of the 1 h incubation period in the presence or absence of a calcium entry blocker, tissues were washed 4 times with calcium-free medium over a 10 min period. For the first 2 washes the medium contained EGTA 0.2 mM. The calcium-free solutions were obtained by omitting calcium and contained a calcium-entry blocker when necessary. A submaximal concentration of ET-1, one of its analogues or noradrenaline was then added to the bath. When the contraction elicited reached a plateau, Ca^{2+} (10 μM to 10 mM) was added cumulatively to contract the tissues further. These responses to calcium are expressed as a percentage of the maximal response to noradrenaline (1 μM). In some of these latter experiments 2 min before the addition of ET-1 the calcium-free solution was substituted by one containing 40 mM K^+ with an equivalent reduction in Na^+ content.

Materials

Noradrenaline bitartrate (Boyer, France), acetylcholine HCl (Sigma, France) and diltiazem HCl (Synthelabo, France) were dissolved in deionized water. Nifedipine HCl (Sigma, France) was dissolved

in ethanol to a concentration of 1 mM. Solutions were prepared daily. Care was taken when using nifedipine to work in conditions of diminished light. Human/porcine endothelin (Peninsula Laboratories, U.K.), [Ala^{3,11}]ET-1 and [Ala^{1,15}]ET-1 (synthesized at Merrell Dow Research Institute, Strasbourg, France) were prepared as 0.1 mM stock solutions in deionized water and kept at 4°C. Dilutions were prepared daily in deionized water.

Statistics

Comparisons were made by the unpaired Student's t test or analysis of variance (StatView 512 + programme for Macintosh computers, Brain Power Inc.). $P < 0.05$ was taken as denoting significant differences.

Results

ET-1, [Ala^{3,11}]ET-1 and [Ala^{1,15}]ET-1 all contracted rat aortic preparations without endothelium in a concentration-dependent manner (Figure 1). Contractions to all three peptides developed slowly. ET-1 was about 13 and 200 fold more potent than the [Ala^{3,11}]- and [Ala^{1,15}]-ET-1 derivatives respectively. Concentrations of the peptides eliciting a contraction equal to 50% of that elicited by noradrenaline (1 μM) in the same tissue and the maximal responses obtained are shown in Table 1.

Preincubation of tissues with diltiazem (1 μM) for 60 min did not significantly affect maximal responses elicited by ET-1 (Figure 2a) but increased EC_{50} values, from 0.88 ± 0.24 nM to 2.95 ± 1.0 nM (t test, $P = 0.013$; $n = 4$). However, neither maximal responses evoked by [Ala^{3,11}]ET-1 nor EC_{50} values (39.4 ± 14.5 nM and 28.2 ± 10.3 nM respectively) were significantly altered (Figure 2b).

When rat aortic rings were preincubated with nifedipine (0.1 μM) for 60 min, contractile responses induced by ET-1 3 and 10 nM were significantly reduced (Figure 3a) as compared to responses elicited in parallel in tissues not exposed to nifedipine. A 10 fold higher concentration of nifedipine had no greater effect (Figure 3a). Similarly, nifedipine (0.1 μM) inhibited responses elicited by [Ala^{1,15}]ET-1 0.41 μM (Figure 3b).

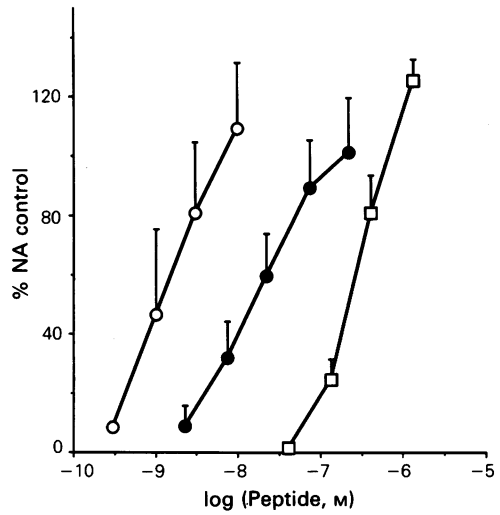


Figure 1 Contractile responses evoked by endothelin (○, $n = 8$), [Ala^{3,11}]endothelin (●, $n = 7$) and [Ala^{1,15}]endothelin (□, $n = 8$) in rat isolated aorta. Contractions are expressed as a percentage of responses induced by noradrenaline (NA, 1 μM) in the same tissues. Vertical bars represent s.e.mean.

When added to endothelium-denuded tissues precontracted maximally by ET-1, 10 nM, nifedipine (3 nM to 1 μM) elicited a concentration-dependent relaxation (Figure 4). At 1 μM, nifedipine relaxed ET-1-induced contractions maximally by 56.9 ± 11.5% and the IC_{50} value was 21.8 ± 5.9 nM ($n = 4$). Diltiazem (1 μM) relaxed precontracted tissues maximally by only 29.8 ± 8.7% ($n = 5$).

In a calcium-free medium, addition of equieffective concentrations of the three peptides (producing about 85% of the maximal response to noradrenaline in normal medium), ET-1 (3 nM), [Ala^{3,11}]ET-1

Table 1 Concentrations of endothelin and endothelin analogues that evoke a response equal to 50% of the maximal contraction evoked by noradrenaline ($EC_{50,NA}$), and the maximal effects ($E_{max,NA}$) of the peptides at the highest concentrations tested (EP_{max}) relative to those of noradrenaline in rat isolated aorta

Peptide	$EC_{50,NA}$ (nM)	EP_{max} (nM)	$E_{max,NA}$ (%)
Endothelin	1.4 ± 0.3	10	109.2 ± 22.3
[Ala^{3,11}]endothelin	18.3 ± 4.0*	225	101.1 ± 18.6
[Ala^{1,15}]endothelin	299 ± 71*†	1360	125.2 ± 7.4

Values are the means ± s.e.mean of 7 or 8 observations.
* Significantly different from endothelin value ($P \leq 0.006$).
† Significantly different from [Ala^{3,11}]endothelin value (t test, $P = 0.002$).

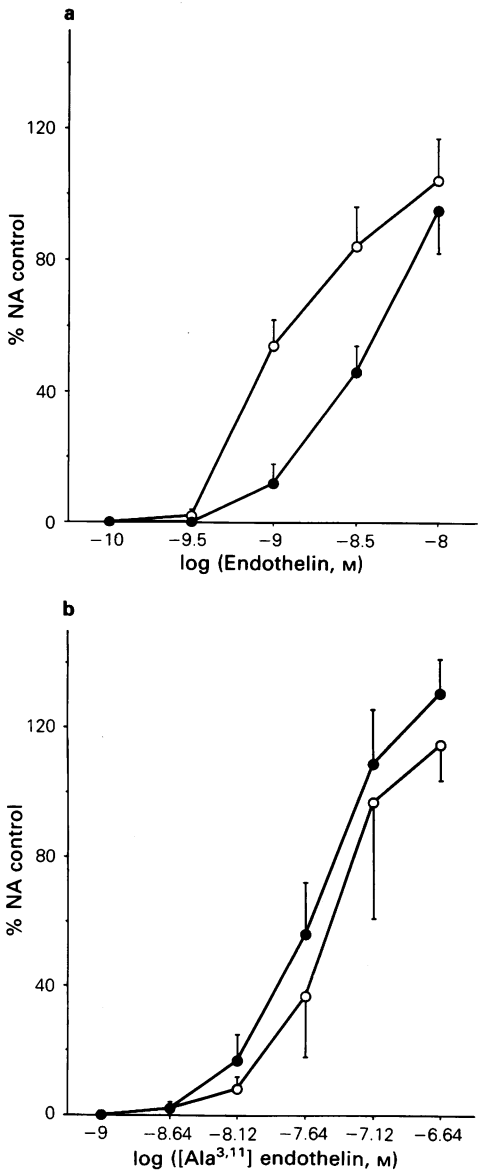


Figure 2 Contractile responses elicited by (a) endothelin and (b) [$Ala^{3,11}$]endothelin in rat isolated aorta in the absence (○, $n = 4$) and in the presence (●, $n = 4$) of diltiazem (1 μM). Contractions are expressed as a percentage of responses induced by noradrenaline (NA, 1 μM) in the same tissues. Vertical bars represent s.e.mean of 4 observations.

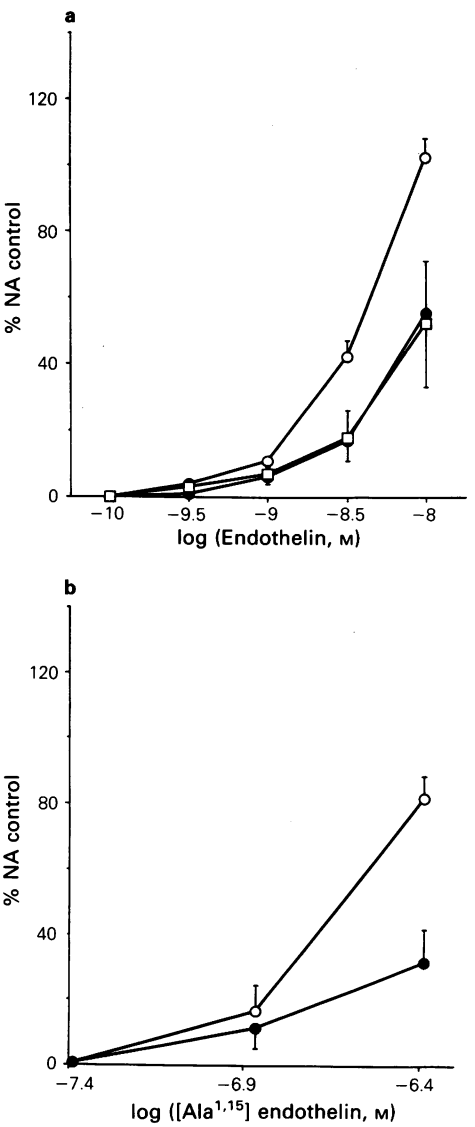


Figure 3 Contractile responses elicited by (a) endothelin and (b) [$Ala^{1,15}$] endothelin in rat isolated aorta. (a) Responses elicited in the absence (○, $n = 10$) and in the presence of 0.1 (●, $n = 6$) or 1 (□, $n = 4$) μM nifedipine. (b) Responses elicited in the absence (○, $n = 4$) and in the presence (●, $n = 4$) of 1 μM nifedipine. Contractions are expressed as a percentage of responses induced by noradrenaline (NA, 1 μM) in the same tissues. Vertical bars represent s.e.mean.

(75 nM) or [$Ala^{1,15}$]ET-1 (0.41 μM) evoked slowly developing responses that were sustained for at least 20 min and amounted to 17.35 ± 2.5% ($n = 16$), 40.9 ± 15.5% ($n = 7$) and 18.8 ± 1.9% ($n = 7$) respectively of maximal responses evoked by noradrenaline (1 μM) (Figure 5). These responses were not significantly different from each other and were approximately 35%, 45% and 24% respectively of responses

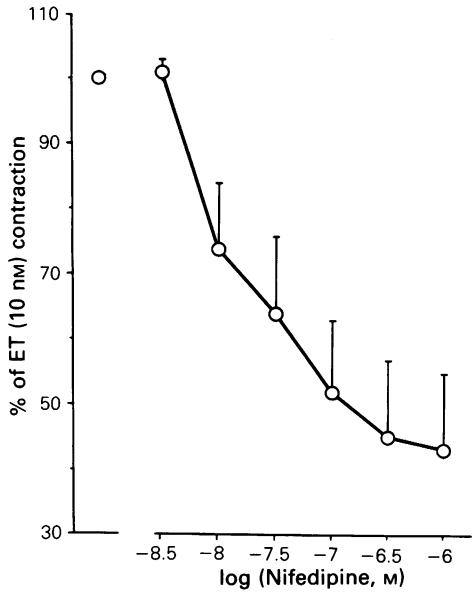


Figure 4 Relaxation evoked by addition of nifedipine (1 nM to 1 μM) to rat isolated aorta maximally precontracted with endothelin, 10 nM. Results are the means of 4 observations. Vertical bars represent s.e.mean.

elicited by the same concentrations of the peptides in normal medium. These residual contractions to ET-1 were not affected by either diltiazem (1 μM) or nifedipine (1 μM), those to [Ala^{3,11}]ET-1 were not affected by diltiazem (1 μM) and those to [Ala^{1,15}]ET-1 were not affected by nifedipine (0.1 μM) (Figure 5).

Readdition of calcium (30 μM to 10 mM) to tissues washed with Ca^{2+}-free medium elicited no significant change in tension. However, in tissues washed in Ca^{2+}-free medium and then exposed to 3 nM ET-1, readdition of Ca^{2+} evoked a concentration-dependent response with an EC_{50} value of 1.21 $\pm$ 1.1 mM. In the presence of diltiazem (1 μM), these ET-1-dependent concentration-effect curves to calcium were significantly modified (analysis of variance, $P = 0.0009$) but neither maximal responses to calcium nor the EC_{50} values (Figure 5a) were significantly different (t test). Nifedipine (0.1 μM) also antagonized responses elicited by 10 μM to 10 mM calcium in tissues pre-exposed to ET-1 3 nM (analysis of variance, $P = 0.0001$) but EC_{50} values were not altered. A ten fold higher concentration of nifedipine had no greater effect (Figure 5b).

Readdition of calcium (10 μM to 10 mM) to tissues partially precontracted with 75 nM [Ala^{3,11}]ET-1 elicited concentration-dependent responses, EC_{50} 74.6 $\pm$ 9.1 μM ($n = 7$). This EC_{50} value is significantly less (t test, $P = 0.042$) than that found in ET-1-exposed rings. Preincubation with diltiazem (1 μM) significantly ($P = 0.0073$) shifted the calcium concentration-effect curves to the right (EC_{50} 348.3 $\pm$ 169.7 μM, $n = 7$) with a concomitant significant increase of maximal contractions (Figure 5c). Analysis of variance indicates a significant difference between these concentration-effect curves ($P = 0.0007$). Concentrations of 0.1 mM and 0.3 mM calcium were equieffective in the absence and presence respectively of diltiazem and an apparent K_b value for diltiazem of about 0.5 μM was calculated.

Readdition of calcium (10 μM to 10 mM) to tissues partially precontracted with 0.41 μM [Ala^{1,15}]ET-1 in the absence of Ca^{2+}, elicited concentration-dependent responses, EC_{50} 102 $\pm$ 27 μM (Figure 5d, $n = 4$). This EC_{50} value is significantly less than that found in ET-1-exposed rings (t-test, $P < 0.0078$), but is not significantly different from the EC_{50} value for Ca^{2+} determined in the presence of [Ala^{3,11}]ET-1. Preincubation with nifedipine, 0.1 μM, did not significantly alter these maximal responses or EC_{50} values for Ca^{2+} (Figure 5d) and analysis of variance indicated no significant difference between these 2 concentration-effect curves ($P = 0.498$). Readdition of calcium to tissues exposed to 0.3 μM noradrenaline in the absence of Ca^{2+} also elicited concentration-dependent responses (Figure 6). In the presence of diltiazem, 1 μM, these concentration-effect curves were significantly shifted to the right, the EC_{50} values were increased by about 3 fold ($P < 0.012$) and the maximal responses depressed ($P = 0.046$).

In the presence of 40 mM K^+ and the absence of calcium, residual responses to 3 nM ET-1 were unchanged compared to those in a Ca^{2+}-free medium with a normal concentration of K^+. Readmission of calcium (10 μM to 10 mM) to tissues in Ca^{2+}-free medium containing 40 mM K^+, induced concentration-dependent contractions with an EC_{50} value of 0.7 $\pm$ 0.2 mM ($n = 4$). In the presence of both 3 nM ET-1 and 40 mM K^+, EC_{50} values for Ca^{2+} (0.4 $\pm$ 0.1 mM, $n = 4$) were not significantly altered and neither were maximal responses (t-test; Figure 7). Analysis of variance did not demonstrate any significant difference ($P = 0.301$).

Discussion

Endothelin (ET-1) was recently described as a vasoconstrictor peptide released from endothelial cells in culture (Yanagisawa *et al.*, 1988). This peptide and two of its analogues [Ala^{3,11}] and [Ala^{1,15}]ET-1 elicited concentration-dependent contractions of rat isolated aorta preparations (Figure 1). It is noteworthy that removal of one or the other of the disulphide bridges reduced potency but did not

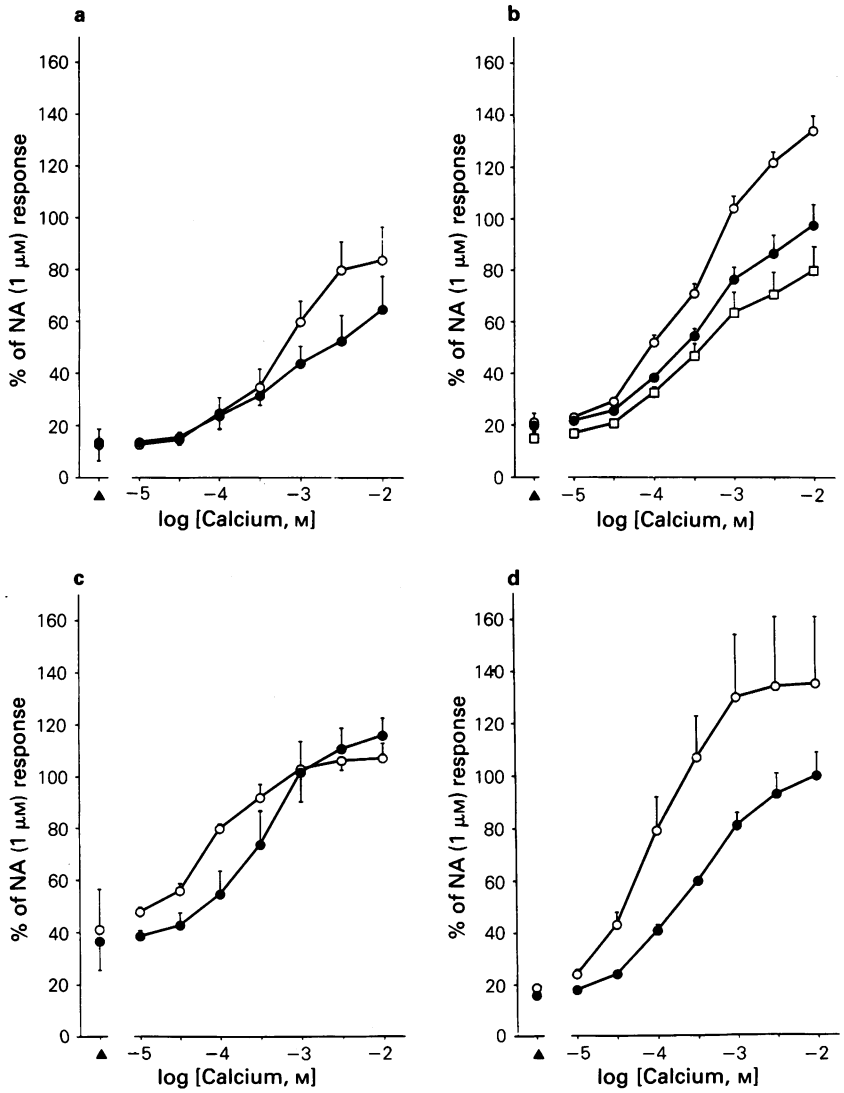


Figure 5 Contractions evoked by either 3 nM endothelin (a, b), 75 nM [$Ala^{3,11}$]endothelin (c) or 0.41 μM [$Ala^{1,15}$]endothelin (d) in Ca^{2+}-free medium (▲) and responses to the subsequent addition of Ca^{2+} (○). Inhibitory effects of 1 μM diltiazem (●; a and c) and 0.1 (●) or 1 μM (□) nifedipine (b and d) are also shown. Results are expressed as percentage of contraction evoked by 1 μM noradrenaline (NA). Vertical bars represent s.e.mean of 4 to 10 observations.

markedly affect maximal evoked responses. Alanine was chosen to replace the cysteine residues because this substitution retains a chiral amino acid, it is also a neutral residue and does not introduce any new charge at the substituted position and is pseudo isosteric with cysteine. Evidently, the presence of both disulphide bonds is not essential for activity of ET-1. However, inhibition of the formation of one or other of these bonds by the inclusion of blocking groups on the sulphur of the appropriate cysteine residues results in reduction (Kimura *et al.*, 1988) or loss of activity (Hirata *et al.*, 1989). This probably indicates that steric hindrance from the large blocking groups distorts the tertiary structure of the peptide while simple removal of one or other of the bridges has a relatively small effect.

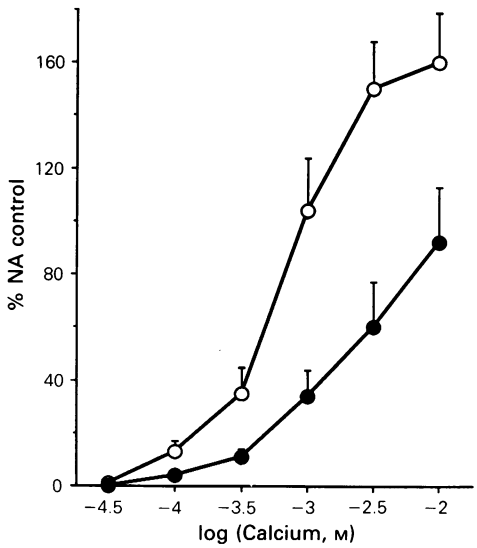


Figure 6 Contractions induced by calcium in rat isolated aortic rings exposed to 0.3 μM noradrenaline in the absence (○) and presence (●) of 1 μM diltiazem. Results are means of 4 observations. Vertical bars represent s.e.mean.

Removal of extracellular calcium, a procedure that abolished responses to 100 mM K^+, reduced, but did not abolish, responses to equieffective submaximal concentrations of ET-1 and its analogues. Results of this type, obtained with a variety of other agonists, have been interpreted as indicating that contractions are partially dependent on intracellular and partially on extracellular calcium (Godfraind *et al.*, 1986). Diltiazem antagonized contractions elicited by ET-1 in normal calcium-containing medium. However, the concentration-effect curves in the presence of diltiazem were parallel to those obtained in its absence and maximal responses were unchanged (Figure 2a). This sort of antagonism by calcium entry blockers of agonist-induced responses that are partly dependent on extracellular calcium in this tissue is not typical. It was expected that maximal concentrations would be reduced with little or no change in EC_{50} values (see for example Godfraind & Dieu, 1981; Godfraind & Miller, 1983). However, diltiazem failed to antagonize responses elicited by [$Ala^{3,11}$]ET-1. Since ET-1, [$Ala^{3,11}$]ET-1 and [$Ala^{1,15}$]ET-1 all have affinity for the same binding site, at least in the central nervous system (Jones *et al.*, 1989), the results indicate that the antagonism by diltiazem of ET-1-induced responses is unlikely to be due to an effect at ET-1 receptors. Also, diltiazem and ET-1 do not interact with each others specific binding sites (Hirata *et al.*, 1988; Van Renterghem *et al.*, 1988).

Responses elicited by the readdition of calcium in the presence of ET-1 and [$Ala^{3,11}$]ET-1 were significantly affected by diltiazem although the type of inhibition did not seem to be the same in each case. This is surprising if the peptides activate the same receptors (Jones *et al.*, 1989). These results (Figures 5a, c) are also not characteristic of antagonism of calcium-induced contractions in the presence of other agonists such as noradrenaline in rat aorta by high concentrations of calcium entry blockers (Figure 6).

On the other hand nifedipine inhibited responses elicited by both ET-1 and [$Ala^{1,15}$]ET-1 in a manner typical of its antagonism of other agonists such as noradrenaline, whose contractile responses are partly dependent on extracellular calcium (Godfraind *et al.*, 1986). This inhibition is unlikely to be due to an effect on ET-1 receptors since dihydropyridines do not interact with ET-1 binding sites (Hirata *et al.*, 1988; Van Renterghem *et al.*, 1988). Furthermore, nifedipine relaxed tissues precontracted with ET-1 with an IC_{50} value of about 22 nM. The maximal relaxation elicited by nifedipine was comparable to the reduction in maximal responses to ET-1 in the absence of extracellular calcium. The IC_{50} value for nifedipine was similar to that of 19 nM determined for inhibition of noradrenaline-induced contractions in rat aorta (Godfraind & Miller, 1983). Contractions induced by readdition of calcium to tissues in calcium-free medium in the presence of ET-1 were also antagonized by nifedipine in a

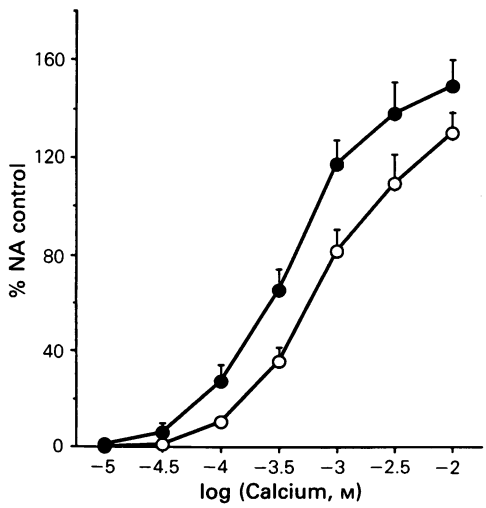


Figure 7 Contractions induced by calcium in rat isolated aorta in a solution containing 40 mM K^+ in the absence (○) and presence (●) of 3 nM endothelin. Vertical bars represent s.e.mean of 4 observations.

manner that would be expected if dihydropyridine sensitive calcium channels were being activated (Godfraind, 1983). Overall, these experiments using nifedipine are in agreement with reports of antagonism of ET-1-mediated responses by dihydropyridines and therefore with the idea that ET-1 activates dihydropyridine-sensitive calcium channels (Yanagisawa *et al.*, 1988). However, it has been reported that ET-1-induced contractions in rat aorta were not antagonized by diltiazem, D600 or nifedipine (Auguet *et al.*, 1988). Also, ET-1 did not potentiate calcium-induced contractions in depolarized tissues (Figure 7). The calcium channel activator, Bay K 8644, enhances contractions (Hwang & van Breemen, 1985) and ^{45}Ca influx (Chiu *et al.*, 1986) under these conditions and thus it seems unlikely that ET-1 directly activates dihydropyridine-sensitive calcium channels.

The sensitivity of ET-1-exposed tissues to calcium, as indicated by the EC_{50} values of the Ca^{2+}-curves, was significantly lower than in tissues exposed to equieffective concentrations of [$Ala^{3,11}$] or [$Ala^{1,15}$]ET-1 (Figure 5). The reason for this difference is unknown at present but it might indicate that ET-1 is a less effective activator of membrane channels that permit the passage of calcium than are the other analogues. Alternatively the analogues might reduce calcium extrusion from the cells to a greater extent than ET-1.

To summarize, the results obtained in this study using diltiazem and nifedipine suggest that there is a limited role for extracellular calcium entry via L-type calcium channels in the responses to ET-1 and to its analogues. This may be because ET-1 and its analogues only activate these channels secondarily to the activation of other channels that permit the entry of extracellular calcium (Van Renterghem *et al.*, 1988).

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Effects of iloprost (ZK 36374) on glutathione status during ischaemia and reperfusion of rabbit isolated hearts

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1 Reperfusion of rabbit isolated hearts after 60 min of ischaemia resulted in poor recovery of mechanical function, release of creatine phosphokinase (CPK) and of reduced (GSH) and oxidized (GSSG) glutathione, reduction of mitochondrial superoxide dismutase (Mn SOD) activity and of tissue GSH/GSSG ratio with a shift of cellular thiol redox state toward oxidation, suggesting the occurrence of oxidative stress.

2 Pretreatment of the isolated heart with the stable prostacyclin analogue (iloprost) at 27 or 270 nM, but not at 2.7 nM, improved the functional recovery of the myocardium, reduced CPK, GSH and GSSG release, maintained Mn SOD activity and attenuated the occurrence of oxidative stress.

3 This effect of iloprost cannot be explained by a decreased demand or an enhanced delivery of oxygen during ischaemia or by a direct effect on glutathione peroxidase and reductase activity.

Introduction

In the experimental animal, prostacyclin (PGI_2) and stable prostacyclin-mimetics, such as iloprost, ZK 36374, (5-{(E)-(1S,5S,6R,7R)-7-hydroxy-6-|(E)-(3S,4RS)-3-hydroxy-4 methyl-oct-1-en-6-yn-yl|-bicyclo-|3 3.0-octan-3-yliden}-pentanoic acid) exert a cardioprotective action on ischaemic and reperfused myocardium (Lefer *et al.*, 1978; Schrör *et al.*, 1981a,b,c; De Langen *et al.*, 1985; Van Der Giessen *et al.*, 1986; Darius *et al.*, 1987; Farber *et al.*, 1988). Despite considerable efforts to find explanations for these beneficial effects, a commonly accepted basic mechanism is still lacking. Recently, it has been suggested that PGI_2 and iloprost protect the myocardium by reducing the formation of ischaemic and reperfusion-induced oxygen free radicals (Fantone *et al.*, 1983; Thiemermann *et al.*, 1984; Simpson *et al.*, 1987).

We now describe the effect of iloprost on oxidative stress and/or damage which occurs in the isolated and perfused rabbit heart during post-ischaemic reperfusion (Adams *et al.*, 1983; Ferrari *et al.*, 1985; Curello *et al.*, 1987). Oxidative stress was determined by measuring the rate of release and the tissue changes in reduced and oxidized glutathione content.

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Iloprost was administered in the perfusate at three different concentrations: 2.7, 27 and 270 nM. As indices of myocardial function and of tissue damage, the pressure developed by isolated heart and the rate of creatine phosphokinase (CPK) release were also determined.

Methods

Perfusion of the hearts

Adult New Zealand white rabbits (2.5 to 3.0 kg) were stunned by a blow on the head. The hearts were rapidly excised and perfused with a modified Krebs-Henseleit buffer solution by the non-recirculating Langendorff technique, as previously described (Ferrari *et al.*, 1985). The buffer solution was delivered to the aortic cannula at 37°C and at a perfusion pressure of 60 to 80 mmHg, maintained with a Watson Marlow rotary pump (MHRE MK3). The perfusion pressure was monitored at the head of the aortic inflow cannula with a Statham P 23 transducer. This provided a constant flow of 25 ± 1.7 ml min^{-1}. The hearts were paced at 180 beats min^{-1} by suprathreshold rectangular pulses of 1.0 ms duration.

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A period of 30 min equilibration was allowed before any experimental intervention. Then an infusion of iloprost (at 2.7, 27 and 270 nM) or of iloprost vehicle was started into the aortic inflow tract and maintained until the end of the experiment. Thirty minutes later, the hearts were divided into three groups. Those in the first group were further perfused under aerobic conditions (coronary flow 25 ± 1.7 ml min^{-1}) for 90 min. The hearts of the second group were made ischaemic by reducing coronary flow from 25 to 1 ml min^{-1} for 60 min and those of the third group were made ischaemic for 60 min and then reperfused. Reperfusion was for 30 min at an aerobic, coronary flow of 25 ml min^{-1}. Left ventricular wall temperature was maintained at 36° to 37°C, irrespective of coronary flow.

Left ventricular pressure measurements

To obtain an isovolumetrically beating preparation, a fluid-filled balloon was inserted into the left ventricular cavity via the atrium. The intraventricular balloon was then connected by a fluid-filled polyethylene catheter to a Statham pressure transducer (P 23 D6) for the determination of left ventricular pressure.

Coronary effluent analysis

During each perfusion the coronary effluent was collected in chilled glass vials and assayed, on the same day, for creatine phosphokinase (CPK) activity and glutathione content, either reduced (GSH) or oxidized (GSSG). CPK activity was determined spectrophotometrically by the method of Oliver (1955). GSH and GSSG content were determined as described later in this section.

Reduced (GSH) and oxidized glutathione (GSSG) determination

The tissue (~100 mg) was deproteinized with 3 M $HClO_4$ and the supernatant obtained after centrifugation at 6000 *g* for 15 min was neutralized with 2 M K_2CO_3. A sample of the neutralized extract was analysed for total glutathione by the method of Tietze (1969) as modified by us (Curello *et al.*, 1987). The reaction mixture (1.0 ml) contained: 0.05 M potassium phosphate buffer pH 7.4, 1 mM EDTA, 0.1 mM 5-5'-dithiobis-(2 nitrobenzoic acid) (DTNB), 0.15 mM NADPH and an appropriate volume of sample. After 2 min of preincubation, the reaction was initiated by the addition of 1 u of glutathione reductase and the rate of reduction of the DTNB was continuously monitored at 412 nm; the slope was proportional to glutathione concentration over the range 0–1 μM. Oxidized glutathione was measured as described above after the preliminary reaction of GSH with 20 mM N-ethyl maleimide, followed by complete removal of unreacted sulphydryl reagent with diethylether.

Determination of protein sulphydryl groups

To determine the content of tissue thiol (SH) groups, a portion of the left ventricle (~300 mg) was homogenized, as described by Sedlack & Lindsay (1968), with 6 ml of 20 mM EDTA pH 4.7 and filtered on nylon. Samples of homogenates (0.5 ml) were mixed with 0.2 M Tris HCl pH 8.2, 0.01 M DTNB and methanol to a final volume of 10 ml. A reagent blank (without sample) and a sample blank (without DTNB) were prepared in a similar manner. The mixture was incubated for 30 min at room temperature. The absorbance was read at 412 nm and a molar extinction coefficient of 13,600 M^{-1} cm^{-1} was used.

The acid-soluble SH group content (as an expression of non-protein sulphydryl groups) was determined in the same manner, in the supernatant fluid obtained after denaturation of the homogenate with ice-cold 50% trichloroacetic acid (TCA).

Protein SH groups were determined by subtracting the acid-soluble from the total SH-group content.

Determination of glutathione peroxidase (GPD) and glutathione reductase (GRD)

Frozen tissues (~500 mg) were homogenized in 10 vol of ice-cold 0.05 M potassium phosphate buffer, pH 7.2, centrifuged at 3000 *g* for 10 min and the supernatants used for enzymatic determinations.

Glutathione peroxidase activity was assayed by a modification of the method of Paglia & Valentine (1967). The reaction mixture consisted, in a final volume of 1 ml, of 50 mM Tris-HCl buffer pH 7.3, 0.1 mM EDTA, 0.12 mM NADPH, 0.25 mM GSH, 1 unit ml^{-1} of glutathione reductase (1 unit ml^{-1} corresponds to 1 μmol of NADPH oxidized min^{-1}) and an appropriate volume of supernatant (0.05 ml). After 5 min of incubation, 0.2 mM cumene hydroperoxide was added and the rate of disappearance of NADPH was monitored at 340 nm. The activity of GPD was calculated as nmol of NADPH oxidized min^{-1} mg^{-1} protein.

Glutathione reductase activity was assayed in a medium which contained in a final volume of 1 ml: 0.1 M Tris buffer pH 8.0, 0.94 mM EDTA, 4.6 mM GSSG, 0.16 mM NADPH and an appropriate volume of supernatant (0.05 ml) (Bergmeyer, 1974). The activity of GRD was calculated as nmol of NADPH oxidized min^{-1} mg^{-1} protein.

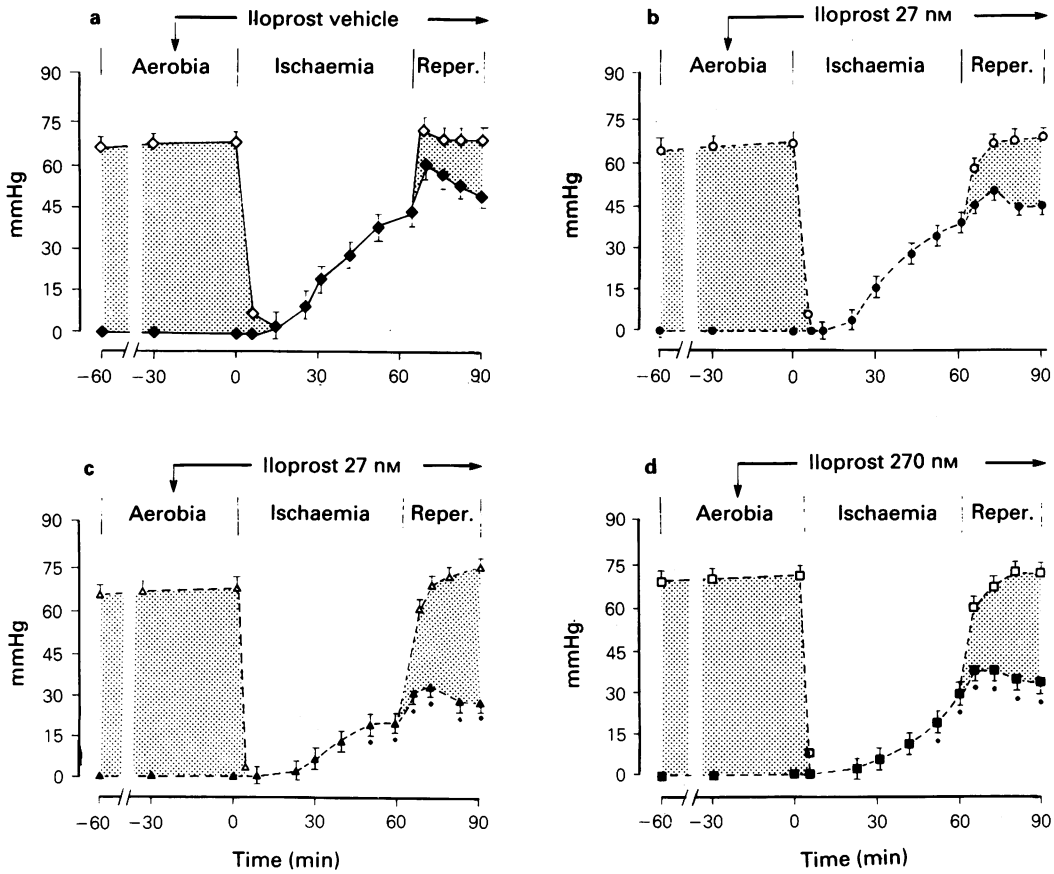


Figure 1 Effect of iloprost, 2.7 nM (b), 27 nM (c) and 270 nM (d), on the ischaemia and reperfusion induced alterations of left ventricular developed (open symbols) and diastolic (solid symbols) pressure. (a) Effect of the iloprost vehicle. Each point is the mean of at least six separate experiments; vertical lines show s.e.mean. $^{*}P < 0.01$, relates to the significance of the difference between the control (vehicle-treated) and the respective iloprost-treated group.

Superoxide dismutase determination

Left ventricular tissue was homogenized in 10 volumes of 10 mM potassium phosphate buffer pH 7.4, supplemented with 30 mM KCl. The homogenates were then sonicated (Braun Labsonic 1510; 40 w min^{-1}) and centrifuged at 20,000 *g* for 15 min. Superoxide dismutase in the supernatant was determined, in terms of its ability to inhibit the cytochrome c reduction mediated by $O_2^{\cdot-}$ generated during the oxidation of xanthine catalyzed by xanthine oxidase, by the method of Crado *et al.* (1977) as modified by us (Ferrari *et al.*, 1985). Total SOD activity was measured at 25°C in 0.05 M Na_2CO_3 pH 10, which contained in a final volume of 1 ml, 0.1 mM EDTA, 0.1 mM xanthine, 10 μM cytochrome c, 1 mM NaN_3 and sufficient xanthine oxidase to produce a rate of ferricytochrome c reduction of 0.025 absorbance units per min at 550 nm. The Mn-SOD was determined, as described above, in the presence of 1 mM KCN. The Cu-Zn SOD activity was calculated by subtracting Mn SOD from total SOD activity. Under these defined conditions, the amount of SOD required to inhibit the rate of reduction of cytochrome c by 50% is defined as 1 unit of activity.

Protein determination

Protein determination was carried out according to Bradford (1976), with bovine serum albumin as standard.

Experimental compounds and statistical evaluation

The reagents were of analytical grade. All enzymes used for the biochemical determinations were

obtained from the Sigma Company (U.S.A.). Iloprost was kindly supplied by Schering S.p.A., Milan (Italy).

The data are expressed as mean ± s.e.mean of at least 6 experiments, where each experiment is an individual perfusion. A one-way analysis of variance was first carried out to test for any differences between all groups. If a difference was established, each of the groups was analysed by the Dunnett's test, and results were considered to be significantly different if P values were below 0.01.

Results

Figure 1 summarizes the data for left ventricular developed and diastolic pressure obtained during aerobic perfusion, ischaemia and reperfusion from vehicle and iloprost-treated hearts.

Infusion of vehicle or of iloprost at the different concentrations did not cause changes in cardiac performance or in the rate of CPK release during aerobic perfusion. During ischaemia, left ventricular developed pressure decreased rapidly to zero in all groups and the period of detectable contractile activity was the same (2.5 to 3.5 min). The ischaemic-induced contracture, as indicated by the increase in diastolic pressure, was significantly less pronounced in hearts receiving iloprost at 27 or 270 nM (from 42 ± 2.4 (mean value ± s.e.) to 19 ± 1.7 and to 26 ± 3.0 mmHg, $P < 0.001$, respectively), whilst it was not affected by administration of iloprost 2.7 nM. Because of the severe reduction in coronary flow, no change in the rate of CPK release was observed during ischaemia in all groups.

The percentage of recovery of developed pressure after reperfusion was significantly improved in hearts treated with iloprost at 27 or 270 nM (from 26% to 69% and to 48%, respectively) but not in those receiving 2.7 nM iloprost. Reperfusion of vehicle-treated hearts resulted in a marked and sustained increase in the rate of CPK release (from 87 ± 9 to 603 ± 32 mu $min^{-1} g^{-1}$ wet wt.) which was not modified by administration of 2.7 nM iloprost. However, CPK release on reperfusion was completely abolished when 27 nM iloprost was administered. A further increase of iloprost concentration (270 nM) significantly reduced (from 603 ± 32 to 165 ± 29 mu $min^{-1} g^{-1}$ wet wt., $P < 0.01$) but did not abolish CPK release.

The data on release and cellular content of GSH and GSSG are shown in Figure 2 and Table 1, respectively. In vehicle-treated hearts there was a small release of GSH and almost no release of GSSG during 60 min of aerobic perfusion (Figure 2). Ischaemia did not alter the rate of GSH or GSSG release (Figure 2), whilst tissue content of GSH and of protein-SH was significantly reduced (Table 1).

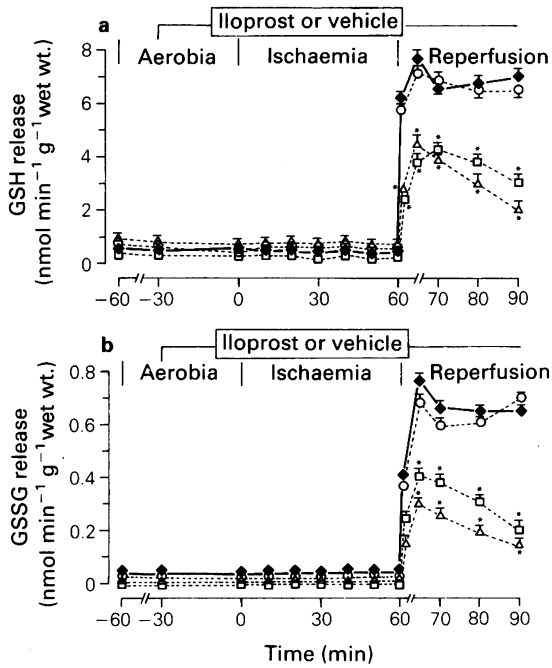


Figure 2 Effects of iloprost on the ischaemia- and reperfusion-induced alterations in the rate of (a) reduced (GSH) and (b) oxidized (GSSG) glutathione release. Effects of iloprost vehicle (◆) and iloprost 2.7 nM (○), 27 nM (△) and 270 nM (□) are shown. Each point is the mean of at least six separate experiments; vertical lines show s.e.mean. *$P < 0.01$, relates to the significance of the difference between the control (vehicle-treated) and the respective iloprost-treated group.

Tissue content of GSSG after ischaemia was not different from that obtained after aerobic perfusion (Table 1). Reperfusion of these hearts resulted in a marked and sustained increase of the rate of GSH and of GSSG release with a further reduction of cellular GSH (Figure 2, Table 1). Furthermore, in this group tissue content of GSSG was significantly increased after the return of coronary flow (from 0.26 ± 0.06 to 0.46 ± 0.07 nmol mg^{-1} protein, $P < 0.01$, Table 1), despite the reperfusion-induced release of GSSG. The concentration of protein-SH after reperfusion remained low (Table 1).

Administration of iloprost at the different concentrations did not change tissue content or rate of GSH and GSSG release under aerobic conditions (Figure 2, Table 1). Iloprost at 2.7 nM failed to modify the alteration of glutathione status induced by ischaemia and reperfusion. However, iloprost at 27 and at 270 nM had important effects on the changes in cellular redox state occurring on isch-

Table 1 Effect of iloprost on tissue content of reduced (GSH) and oxidized (GSSG) glutathione, protein SH groups and on the activity of Cu-Zn and Mn superoxide dismutase (SOD)

	Aerobia (150 min)	*Ischaemia* (60 min)	*Reperfusion* (30 min)
	GSH (nmol mg^{-1} protein)		
Iloprost vehicle	12.1 ± 0.8 (6)	6.1 + 0.2 (5)	4.9 + 0.2 (6)
Iloprost 2.7 nM	11.3 + 0.6 (5)	5.9 + 0.4 (5)	4.2 + 0.3 (6)
Iloprost 27 nM	11.9 + 0.9 (5)	8.1 + 0.3 (5)*	10.1 + 0.7 (6)*
Iloprost 270 nM	12.6 + 0.7 (5)	7.9 + 0.4 (5)*	10.9 + 0.6 (6)*
	GSSG (nmol mg^{-1} protein)		
Iloprost vehicle	0.26 + 0.06 (6)	0.18 + 0.04 (5)	0.46 + 0.07 (6)
Iloprost 2.7 nM	0.23 + 0.06 (5)	0.20 + 0.03 (5)	0.51 + 0.02 (6)
Iloprost 27 nM	0.24 + 0.03 (5)	0.21 + 0.09 (5)	0.23 + 0.04 (6)*
Iloprost 270 nM	0.26 + 0.06 (5)	0.24 + 0.07 (5)	0.29 + 0.05 (6)*
	Protein SH (nmol mg^{-1} protein)		
Iloprost vehicle	312 + 12.1 (6)	139 + 10.3 (5)	209 + 11.9 (6)
Iloprost 2.7 nM	336 + 19.1 (4)	105 + 12.4 (5)	219 + 19.3 (6)
Iloprost 27 nM	307 + 14.2 (5)	193 + 9.1 (5)*	297 + 12.3 (6)*
Iloprost 270 nM	327 + 13.1 (5)	167 + 12.1 (5)	283 + 14.5 (6)
	CuZn SOD (units mg^{-1} protein)		
Iloprost vehicle	62.3 + 2.1 (6)	60.0 + 2.0 (6)	64.3 + 1.9 (6)
Iloprost 2.7 nM	63.1 + 1.9 (6)	62.1 + 1.9 (5)	63.0 + 1.0 (6)
Iloprost 27 nM	59.7 + 2.0 (6)	60.1 + 2.1 (6)	61.3 + 1.3 (6)
Iloprost 270 nM	61.0 + 2.1 (6)	59.1 + 1.7 (6)	59.3 + 2.1 (6)
	Mn SOD (units mg^{-1} protein)		
Iloprost vehicle	40.1 + 1.6 (6)	18.0 + 1.9 (6)	20.1 + 1.0 (6)
Iloprost 2.7 nM	42.0 + 1.3 (6)	20.1 + 2.1 (5)	17.6 + 1.4 (6)
Iloprost 27 nM	41.0 + 1.2 (6)	35.7 + 1.9 (6)*	37.8 + 1.1 (6)*
Iloprost 270 nM	43.1 + 1.6 (6)	39.1 + 1.1 (6)*	40.0 + 1.2 (6)*

Results are given as mean ± s.e.mean. The number of experiments is shown in parentheses. *$P < 0.01$, relates to the significance of the difference between control (iloprost vehicle) and the iloprost-treated groups.

aemia and reperfusion: (1) the ischaemic and reperfusion-induced decline of tissue GSH was significantly reduced (Table 1); (2) protein-SH content was almost completely re-established after reperfusion (Table 1); (3) during reperfusion there was significantly less release of GSH and GSSG (Figure 2) and no increase of tissue concentration of GSSG (Table 1). These effects were independent of the dose of iloprost employed.

The GPD and GRD activities were not different from the respective control values of 21.2 ± 1.6 and 44.3 ± 2.3 nmol during ischaemia and reperfusion, and iloprost treatment had no effects on these enzymes. As in the myocardium the first line of defence against oxygen toxicity is reflected by superoxide dismutase concentrations (SOD), we have also determined the activity of this enzyme. The data are presented in Table 1, which shows that in vehicle-treated hearts 60 min of ischaemia significantly reduced the activity of Mn SOD, while that of Cu-Zn SOD remained unaffected. Reperfusion of these hearts did not restore Mn-SOD activity and did not cause significant changes of Cu-Zn SOD. Administration of iloprost at the different concentrations had no effect on the activity of either SOD in hearts perfused under aerobic conditions. Iloprost at 2.7 mM failed to modify alterations of Mn SOD induced by ischaemia and reperfusion. In contrast, when utilized at the higher concentrations, iloprost significantly reduced the decline in Mn SOD activity of the ischaemic and reperfused hearts.

Discussion

Our results indicate that in the rabbit isolated and reperfused heart after a prolonged period of ischaemia there is a marked reduction of Mn SOD activity and of the ratio of GSH/GSSG, with a shift of cellular thiol redox state towards oxidation. The decline in intracellular GSH and in protein-SH which we found after ischaemia in control hearts cannot be accounted for by release of GSH or GSSG, nor was it accompanied by release of GSH or GSSG. It is probable that in these hearts, ischaemia caused important changes in the thiol status of the myocardium, leading to the formation of mixed-disulphides. It follows that, under these conditions, the glutathione system, which represents an important mechanism of defence against oxygen toxicity, is under stress, the reduced availability of cellular GSH becoming a rate limiting factor for detoxification of

oxygen metabolites (Ferrari *et al.*, 1985; Curello *et al.*, 1987).

These alterations are known to cause disturbances in calcium transport (Hess *et al.*, 1981; Harris *et al.*, 1982), changes in membrane permeability (Tappel, 1973; Meerson *et al.*, 1982), and impairment of muscle contraction, which could account for the poor recovery of function and for the CPK release which we observed after reperfusion.

Administration of iloprost to the rabbit isolated heart at 27 and at 270 nM provided protection against ischaemia-induced changes. The increase of diastolic pressure, the loss of Mn SOD activity and the depletion of cellular GSH and protein-SH during ischaemia were attenuated. On reperfusion there was a significantly better recovery of mechanical function, no enzyme leakage, reduced rate of GSH and GSSG release and no accumulation of GSSG, which is considered to be a reliable index of oxidative stress (Adams *et al.*, 1983; Ishikawa & Sies, 1984; Curello *et al.*, 1987).

Iloprost at 27 nM seemed to be the optimal dose, as a lower concentration (2.7 nM) was ineffective and a higher concentration (270 nM) did not result in further protection.

The mechanism by which iloprost improved functional recovery and reduced the occurrence of oxidative stress during reperfusion is unclear, although a number of factors can be eliminated. A lowered myocardial oxygen demand at the time of ischaemia is an unlikely factor, as iloprost in our preparation did not modify the maximum developed pressure before ischaemia or the onset of quiescence during ischaemia. In addition, heart rate and afterload were kept constant. As coronary flow was also kept constant, the beneficial effect of iloprost is unlikely to be due to an enhanced delivery of oxygen during ischaemia.

Pretreatment with iloprost did not increase tissue GSH or protein-SH content after aerobic perfusion and had no effect on the activity of GRD and GPD. Thus, it is unlikely that the protection afforded by iloprost is secondary, like that of other compounds (Ceconi *et al.*, 1988), to the maintenance of a reduced thiol pool or to a direct effect on the enzymes regulating the cellular glutathione status.

In our opinion, the most likely explanation relies on the ability of iloprost to reduce oxygen toxicity. Several groups (Fantone & Kinnes, 1983; Simpson *et al.*, 1987; Farber *et al.*, 1988) have demonstrated that PGI_2 and iloprost inhibit, in a concentration-dependent manner, the *in vitro* production of superoxide anion by canine neutrophils. In addition, Schrör *et al.* (1981a) and Thiemermann *et al.* (1984) have shown that iloprost attenuates the loss of total superoxide dismutase (SOD) specific activity that occurs in the ischaemic myocardium.

From the present study it appears that the protective effect of iloprost on SOD activity is specific for the enzymatic form mainly present in the mitochondria. This finding is of particular relevance as in our experimental preparation SOD represents the first line of defence against oxygen-free radicals, which are likely to be generated exclusively by the mitochondria, as the rabbit myocardium lacks xanthine-oxidase and an asanguineous medium without neutrophils was used. We have previously demonstrated that ischaemia and/or hypoxia enhances mitochondrial $O_2^{\cdot-}$ production, whilst it reduces mitochondrial SOD activity (Ferrari *et al.*, 1982; 1985; 1986). This, in turn, leads to a stimulation of the second line of defence against oxygen toxicity, GPD, yielding a reduction of GSH/GSSG ratio with enhanced GSSG content. It is therefore most likely that the effects of iloprost on SOD activity are important in the cardioprotective action and reduction of oxidative stress induced by iloprost in the isolated heart. It is difficult to determine from our data whether mitochondrial SOD preservation was due to some type of scavenging effect of iloprost or to a direct action of the drug on free-radical generating systems. It is interesting to note here that, when used in the same experimental preparation, iloprost has previously been shown to have a profound protective effect on isolated mitochondrial function and prevented mitochondrial membrane deterioration (Ferrari *et al.*, 1988).

In conclusion, the prostacyclin-mimetic, iloprost, ameliorates post-ischaemic dysfunction in the isolated heart in a dose-dependent manner. The best protection was obtained by using iloprost at 27 nM and not at 270 nM. This could be explained in terms of saturation of iloprost binding sites on heart membranes. These effects do not appear to be mediated by changes in classic oxygen supply-demand mechanisms. Instead, our *in vitro* experiments, suggest, for the first time, that the mechanism by which iloprost improves recovery of myocardial function may be mediated via a reduction of oxidative stress due to oxygen-derived free radicals.

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Vascular activities of endothelin-1 and some alanyl substituted analogues in resistance beds of the rat

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1 The effects of endothelin-1 and of three analogues in which alanyl residues had been substituted in place of cysteinyl residues were studied in the rat, isolated, Krebs-Henseleit-perfused mesenteric bed and the *in situ*, blood-perfused, mesenteric and hindquarters circulations of the rat. The effects on the vascular responses to these peptides of removing the endothelium by detergent perfusion or of cyclo-oxygenase inhibition by indomethacin were also determined.

2 In all three preparations, endothelin-1 was a potent vasoconstrictor (ED_{50} values ranged from 40 to 400 pmol) although it was rather less potent in the hindquarters than in the mesentery. Also, the maximum response was very much smaller in the isolated mesentery (24.7 ± 2.1 mmHg) than in the *in situ* mesentery (81.8 ± 2.6 mmHg) or hindquarters (107 ± 10 mmHg).

3 Removal of the endothelium by perfusion with detergent significantly enhanced the potency of endothelin as a vasoconstrictor in the *in situ* messentery, but reduced the maximum response obtained, whereas removal of the endothelium *in vitro* significantly increased the maximum response without changing the ED_{50}. The presence or absence of indomethacin had no significant effects in the blood-perfused hindquarters preparation or the isolated mesentery but, after administration of 5 mg kg^{-1} indomethacin to the *in situ* mesenteric preparation, the maximal response to endothelin-1 was enhanced.

4 When the preparations were preconstricted with α_1-adrenoceptor agonists, endothelin-1 had modest vasodilator effects. These vasodilator effects were abolished when the endothelium was destroyed by detergent perfusion.

5 Both [$Ala^{3,11}$]endothelin-1 and [$Ala^{1,15}$]endothelin-1 were also vasoconstrictor agents in the mesenteric preparations but they were less potent than endothelin-1 itself; [$Ala^{3,11}$]endothelin-1 was intermediate in potency between endothelin-1 and [$Ala^{1,15}$]endothelin-1. In the *in situ* preparation these analogues gave similar maximal responses to the parent peptide but, *in vitro*, they gave maximal responses that were much greater than that of endothelin-1 and which were similar to those found with all 3 peptides in the *in situ* mesentery. Destruction of the endothelium *in vitro* had no effect on the responses to these 2 analogues and the log dose-response curve for [$Ala^{1,15}$]endothelin-1 in the isolated mesentery was biphasic.

6 A third analogue possessing no disulphide bridges ([$Ala^{1,3,11,15}$]endothelin-1) was a partial agonist in the *in situ* preparations but had no vasoconstrictor effect in the *in vitro* mesentery. It had no vasorelaxant effect in the hindquarters preparation but it enhanced the responses to endothelin-1 when the 2 peptides were administered together in all 3 preparations.

7 It is concluded that it is not essential for the endothelin family of peptides to possess 2 disulphide bridges for them to be vasoconstrictor agents. However, only endothelin-1, of the 4 peptides studied, showed either endothelium-dependent vasorelaxant activity or modulation by the endothelium of its pressor effects. This, together with some differences in the vasoconstrictor log dose-response curves to the peptides and the results of co-administration of [$Ala^{1,3,11,15}$]endothelin-1 and endothelin-1, suggests that there may be more than one receptor type mediating vascular responses to the peptides studied.

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Introduction

The endothelins are a class of recently discovered 21 amino acid peptides which are amongst the most potent vasoconstrictor agents known. The first characterized of the family, endothelin-1, was detected in culture medium conditioned by porcine aortic endothelial cells (Yanagisawa *et al.*, 1988). It produces prolonged pressor responses in the ganglion-blocked rat which are preceded by transient depressor responses. Endothelin-1 has also been shown to have actions in a very wide range of tissues, not only those associated with the cardiovascular system. Thus, it contracts isolated arterial and venous segments (D'Orleans-Juste *et al.*, 1988), releases endothelium-derived relaxing factor (Warner *et al.*, 1989), causes bronchoconstriction (Turner *et al.*, 1989), inhibits the release of renin from juxtaglomerular cells (Takagi *et al.*, 1988), inhibits twitch responses in the field-stimulated guinea-pig ileum but augments those of the rat vas deferens (Hiley *et al.*, 1989b), inhibits platelet aggregation (Thiemermann *et al.*, 1988), produces ventral root depolarization in newborn rat spinal cord preparations (Yoshizawa *et al.*, 1989), possesses mitogenic activity on cultured smooth muscle cells (Komuro *et al.*, 1988) and is ulcerogenic in the stomach (Wallace *et al.*, 1989; Whittle *et al.*, 1989; MacNaughton *et al.*, 1989).

Since the original description of endothelin-1, two other sequences have been detected in the human genome and these encode very similar 21 amino acid peptide structures to the parent compound (Inoue *et al.*, 1989); these have been designated endothelin-2 and endothelin-3. Human endothelin-3 has an identical sequence to the peptide originally found to be coded in the rat genome. The endothelins contain disulphide bridges between positions 1–15 and 3–11, forming an intramolecular loop structure which is not only conserved between the three forms of endothelin that have currently been characterized, but is also found in a family of snake toxins, the sarafotoxins S6. Not only are the positions of the two disulphide bridges conserved between the endothelins and sarafotoxins, but there is also considerable conservation in the remainder of the amino acid sequence and sarafotoxin S6b, which is the best characterized of the toxins, is a potent vasoconstrictor (Kloog *et al.*, 1988). The relatively unusual possession of two disulphide bridges in this family of vasoactive peptides made it of particular interest to examine the effect of removing them from endothelin-1 by chemical substitution of the bridge-forming cysteinyl residues with alanyl residues. This yielded a series of analogues: one with a disulphide bridge at the 3-11 position ([$Ala^{1,15}$]endothelin-1), one with a bridge between positions 1 and 15 ([$Ala^{3,11}$]endothelin-1) and an analogue without either bridge ([$Ala^{1,3,11,15}$]endothelin-1). The vascular actions of this series were assessed, and compared to those of endothelin-1, in the superior mesenteric bed of the rat both *in vivo*, perfused with blood, and *in vitro*, perfused with modified Krebs-Henseleit solution. The blood-perfused hindquarters of the rat were also studied. These beds are of particular interest as haemodynamic studies have revealed that, in them, endothelin-1 can have either constrictor or relaxant actions, or both (Thomas *et al.*, 1989; Gardiner *et al.*, 1989).

A preliminary account of some of these results was presented at the First William Harvey Workshop on Endothelin, London, December 1988 (Hiley *et al.*, 1989a).

Methods

The isolated, Krebs-Henseleit-perfused superior mesenteric arterial bed of the rat

Male Wistar rats (250–350 g; Bantin & Kingman, Hull) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1} i.p.: Sagatal; Rhône Poulenc, Dagenham, U.K.) and heparinized (1000 u kg^{-1}, i.p.). The superior mesenteric artery was cannulated and the vascular bed perfused, by a Harvard Type 1203A peristaltic perfusion pump, according to the method of McGregor (1965), at 2 ml min^{-1} with Krebs-Henseleit solution containing (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and D-glucose 5.5. In addition, the buffer contained 10 μM indomethacin. The perfusing fluid was saturated with 95% O_2/5% CO_2 and, together with the tissue, was maintained at 37°C. Perfusion pressure was recorded by a Bell & Howell Type 4-422-0001 pressure transducer connected to a T-piece placed in the perfusion circuit close to the point of insertion into the superior mesenteric artery. Recordings were made on a Grass Model 79D polygraph.

After a 30 min equilibration period, peptides were administered into the perfusion circuit in volumes not exceeding 100 μl. Administration of peptides was repeated 5 min after the tissue response to the previous dose of peptide had returned to the resting basal perfusion pressure. In co-administration studies, the peptides were mixed and immediately injected into the perfusion circuit in volumes of 200 μl or less.

Destruction of the endothelium was achieved by perfusion of the vascular bed with a 0.3% (w/v) solution of the detergent CHAPS (3-[3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate; Sigma Chemical Co., Poole, Dorset) in distilled water for

120–150 s followed by 45 min re-equilibration. This procedure has been shown by histology to lead to loss of the endothelium (Hiley *et al.*, 1987). In each case functional loss of the endothelium was confirmed by showing that acetylcholine was unable to oppose noradrenaline-induced vasoconstriction before construction of dose-response curves to the peptide.

The vasorelaxant properties of endothelin-1 were assessed by recording reductions in tone that had been established by infusion of methoxamine into the perfusate at a concentration of 10^{-4} M. It was found necessary to include bovine serum albumen (Sigma) in the perfusate at a concentration of $5\,g\,l^{-1}$ and the flow rate used was $4\,ml\,min^{-1}$. In some control experiments, indomethacin was omitted from the perfusion fluid and, where this was done, it is specifically stated in the text.

In situ *blood perfusion of the superior mesenteric arterial bed*

Male Wistar rats (250–330 g; Bantin & Kingman, Hull) were anaesthetized with a mixture of $60\,mg\,kg^{-1}$ sodium thiopentone (Trapanal; BYK Gulden, Konstanz, F.R.G.) and $30\,mg\,kg^{-1}$ sodium pentobarbitone (Sagatal) given i.p. A tracheal cannula was inserted to allow them to breath air spontaneously and the left common carotid artery cannulated for administration of drugs and infusion of 0.9% saline at $6\,ml\,h^{-1}$ (to prevent volume depletion). The right common carotid artery was cannulated and connected to a Bell & Howell Type 4-422-0001 pressure transducer coupled to a Grass Model 79D polygraph to record central arterial pressure. Heart rate was derived from the pressure wave by means of a Grass 7P44 tachograph preamplifier. Rectal temperature was maintained at 37°C by means of a homeothermic blanket system (BioScience, Sheerness, Kent).

Preparation for the *in situ* blood perfusion of the superior mesenteric arterial bed was similar to that described by Jackson & Campbell (1980) and has been described in detail elsewhere (Hiley *et al.*, 1985). Briefly a mid-line abdominal incision was made and ligatures placed around the aorta distal to the renal arteries and around the superior mesenteric artery close to the aorta. Following a 20 min period to allow haemostasis, the animal received $1000\,u\,kg^{-1}$ heparin into the jugular vein. The abdominal aorta was cannulated with PP60 tubing which led to a Harvard Type 2903 servo-controlled pump and then an air compliance chamber which was inserted into the circuit to act as a bubble trap. The blood was reheated to 37°C by means of a heat exchanger and returned to the animal through the superior mesenteric artery via a PP50 inflow cannula. The ischaemic period experienced by the bed during cannulation did not exceed 1 min. Perfusion pressure in the mesentery was measured by means of a second Bell & Howell pressure transducer placed a fixed distance from the end of the inflow cannula. Following a 20 min equilibration period drugs were administered into the extracorporeal circuit proximal to the heat exchanger in volumes not exceeding $100\,\mu l$, except in the co-administration experiments when the volume did not exceed $200\,\mu l$. In the relaxation experiments the volume of injection did not exceed $10\,\mu l$.

Destruction of the endothelium *in vivo* was achieved by perfusing the mesenteric vascular bed with a 0.3% solution (w/v) of CHAPS in 0.9% saline at $2\,ml\,min^{-1}$ for 150 s in place of blood. In order to limit the contact of the detergent with the blood the bed was initially loaded with 2–3 ml of 0.9% saline. The use of CHAPS in this way has been shown by electron microscopy to lead to destruction of the endothelium without morphological changes in the vascular smooth muscle (Randall, 1989). Following such treatment the vasorelaxation to acetylcholine is lost, while dilatation to sodium nitroprusside and constriction to noradrenaline remain intact (Randall & Hiley, 1988).

Administration of endothelin-1 into the portal vein was achieved by injecting it directly into the splenic pulp by means of a steel needle. Haemostasis was achieved by cyanoacrylate adhesive. All rats used in these experiments were pretreated with $5\,mg\,kg^{-1}$ indomethacin to eliminate the vascular actions of prostaglandins released from the spleen by endothelin (Rae *et al.*, 1989).

Blood perfusion of the hindquarters of the rat in situ

The preparation is essentially as described for the blood perfusion of the mesenteric bed except that blood from the extracorporeal circuit is returned to the animal through a cannula (PP50) located proximal to the iliac bifurcation in the abdominal aorta.

In both blood-perfused preparations, when the relaxant properties of the peptides were examined, tone was established by a close arterial infusion of a $50\,\mu g\,ml^{-1}$ solution of (−)-phenylephrine at $5\,\mu g\,min^{-1}$. Pretreatment of either blood-perfused preparation with indomethacin was by administration of $5\,mg\,kg^{-1}$ into the jugular vein before perfusion was commenced through the extracorporeal circuit. The injection volume was $2\,ml\,kg^{-1}$.

Statistical and data analysis

All values are given as the mean ± s.e.mean and the number of animals in each group is represented by *n*.

Comparison between means was carried out by Student's unpaired t test.

Dose-response curves were analysed by fitting the logistic equation:

$$R = \frac{R_{max} A^{n_H}}{ED_{50}^{n_H} + A^{n_H}}$$

where R is the increase in perfusion pressure, A the dose of peptide, R_{max} the maximum pressor response, n_H the slope function and ED_{50} the dose of peptide giving the half-maximal response. Where appropriate, the data were fitted to a double hyperbola according to the following equation:

$$R = \frac{R_{max_1} \cdot A^{n_{H_1}}}{ED_{50_1}^{n_{H_1}} + A^{n_{H_1}}} + \frac{R_{max_2} \cdot A^{n_{H_2}}}{ED_{50_2}^{n_{H_2}} + A^{n_{H_2}}}$$

in which the subscripts 1 and 2 serve to denote the values of R_{max}, n_H and ED_{50} for each of the two sites. A modified Marquardt procedure, as implemented in the Harwell routine VB01A on the Cambridge University IBM 3081 mainframe computer, was used to carry out the curve fitting (Aceves *et al.*, 1985).

Drugs and peptides

Apart from the peptides, all drug solutions were made freshly on the day of the experiment. (−)-Phenylephrine hydrochloride and (−)-noradrenaline bitartate (Sigma Chemical Co., Poole, Dorset) were both dissolved in saline (0.9% NaCl w/v), which contained 1 mg ml^{-1} of ascorbic acid (Fisons, Loughborough, U.K.), to give stock solutions of 1 mg ml^{-1} and 0.5 mg ml^{-1} respectively. Methoxamine hydrochloride (Sigma) was dissolved in saline as was acetylcholine bromide (Sigma); serial dilutions were prepared for the latter drug. Indomethacin (Sigma) was dissolved to give a 1 mg ml^{-1} solution in 5% sodium bicarbonate and then diluted to the required concentration in saline or Krebs-Henseleit solution. Solutions (0.3% w/v) of the non-denaturing, zwitterionic detergent CHAPS (Sigma) were also prepared either in distilled water (*in vitro* studies) or saline (*in situ* studies). Endothelin-1 (originally designated as human/porcine endothelin) was obtained from Novabiochem (Läufelfingen, Switzerland). The three alanyl substituted analogues of endothelin-1, [Ala^{1,15}]endothelin-1, [Ala^{3,11}]endothelin-1 and [Ala^{1,3,11,15}]endothelin-1, were synthesized by Dr J.T. Pelton of the Merrell Dow Research Institute, Strasbourg. The peptides were dissolved in distilled water to give 100 μM solutions (except for [Ala^{1,3,11,15}]endothelin-1; 10 μM) and frozen until used.

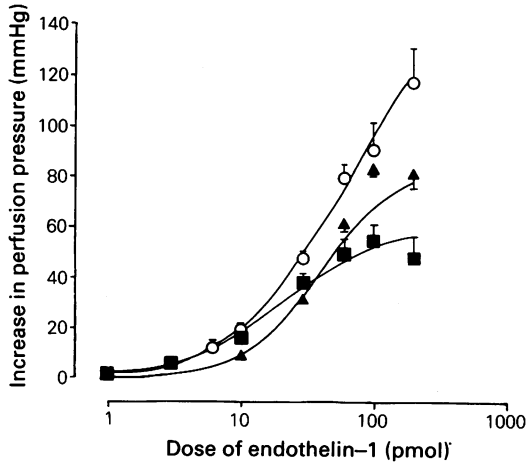


Figure 1 Pressor responses to endothelin-1 in the blood-perfused mesenteric arterial bed of the rat. (▲) shows the control responses to endothelin-1 ($n = 5$); (■) represents the responses to the peptide after destruction of the endothelium by perfusion with CHAPS ($n = 5$–6); and (○) gives the responses obtained with endothelin-1 in animals pretreated with 5 mg kg^{-1} indomethacin ($n = 8$). The points show the mean and the vertical lines show s.e.mean.

Results

Pressor activity of endothelin-1 in the in situ *blood-perfused, superior mesenteric arterial bed and the isolated, Krebs-Henseleit-perfused mesenteric preparation*

Endothelin-1 (1–200 pmol, *in situ*, results shown in Figure 1; 1–1000 pmol, *in vitro*, data shown in Figure 2) when injected into either preparation produced dose-dependent increases in perfusion pressure which reached a maximum 1–2 min after injection and returned to baseline within 20–25 min in the *in situ* preparation and within 30–45 min in the *in vitro* preparation.

The ED_{50} for vasoconstrictor activity of the peptide *in situ* was 40.3 ± 4.5 pmol, the calculated maximum increase in perfusion pressure (R_{max}) was 81.8 ± 2.6 mmHg and the slope function (n_H) was 1.58 ± 0.03 ($n = 5$). Figure 1 also shows that endothelial cell destruction *in situ* resulted in an increase in the potency of the peptide as a pressor agent; the ED_{50} was significantly ($P < 0.05$) lower at 22.2 ± 5.5 pmol. However, the R_{max} was reduced significantly ($P < 0.05$) to 60.9 ± 6.7 mmHg ($n = 5$–6).

In the isolated, Krebs-Henseleit-perfused mesenteric bed endothelin-1 had a similar ED_{50} (66.2 ± 16.3 pmol) to that *in vivo*, but the calculated

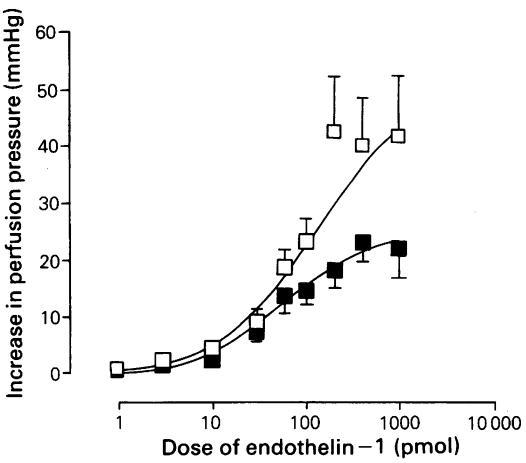


Figure 2 Pressor responses to endothelin-1 in the mesenteric arterial bed of the rat perfused *in vitro* with Krebs-Henseleit solution containing 10 μM indomethacin. (■) show the control responses ($n = 9$) and (□) the responses obtained in preparations in which the endothelium had been destroyed with CHAPS ($n = 7$). The points show the mean and the vertical lines show s.e.mean.

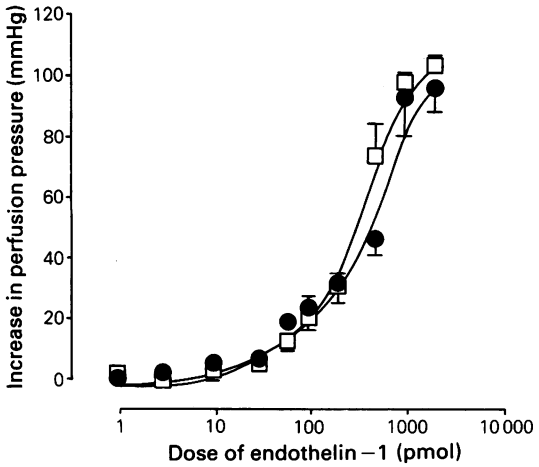


Figure 3 Pressor responses to endothelin-1 in the blood-perfused hindquarters preparation of the rat. (●) shows the control responses to endothelin-1 ($n = 4$) and (□) represents the responses to the peptide obtained in animals pretreated with 5 mg kg^{-1} indomethacin ($n = 4$). The points show the mean and the vertical lines show s.e.mean.

maximum pressor response was significantly ($P < 0.001$) lower than that in the *in situ* preparation with a value of 24.7 ± 2.1 mmHg ($n = 9$). Destruction of the vascular endothelium *in vitro* significantly ($P < 0.01$) increased the R_{max} of the peptide to 50.3 ± 7.4 mmHg without affecting the ED_{50} value (Figure 2) which was 133.2 ± 54.6 pmol ($n = 7$). The slope functions, 1.03 ± 0.10 and 0.84 ± 0.08 for the control curve and that obtained following CHAPS perfusion respectively, showed no statistical differences. Omission of indomethacin from the Krebs-Henseleit solution did not significantly alter the dose-response relationship for vasoconstriction by endothelin-1 (1–200 pmol) in the presence of endothelium; the R_{max} was 18.6 ± 4.1 mmHg, the ED_{50} was 25.6 ± 12.3 pmol and n_H was 1.34 ± 0.34 ($n = 6$).

Pressor effects of endothelin-1 in the blood-perfused hindquarters of the rat

Endothelin-1 (1 pmol–2 nmol) produced dose-related increases in the perfusion pressure in the blood-perfused hindquarters ($n = 4$), similar in time course to those in the mesenteric circulation. The ED_{50} was 419 ± 136 pmol, the calculated maximum pressor response was 107 ± 10 mmHg and the slope function was 0.85 ± 0.10 (Figure 3).

Effect of indomethacin on pressor activity of endothelin-1 in the blood-perfused superior mesenteric arterial bed and hindquarters of the rat

Pretreatment of the rats with indomethacin (5 mg kg^{-1}, i.v.) had no significant effects on either mean systemic arterial pressure, heart rate or basal perfusion pressure in either the mesenteric or hindquarters preparations. However, Figure 1 shows that the treatment significantly ($P < 0.05$) increased the maximum constrictor response of the peptide in the mesentery ($R_{max} = 133.4 \pm 16.2$ mmHg; $n = 8$) as compared to the control value (see above). The ED_{50} (47.1 ± 10.0 pmol) did not differ significantly from the control value but n_H (1.19 ± 0.08) was significantly lower than the control ($P < 0.05$). In the blood perfused hindquarters indomethacin treatment had no significant effects (Figure 3); after its administration the ED_{50} was 322 ± 64 pmol, the R_{max} was 117 ± 7 mmHg and n_H was 1.29 ± 0.13 ($n = 4$).

Effects of intrasplenically administered endothelin-1 on mesenteric perfusion pressure and mean arterial pressure

Injections of endothelin-1 (100–600 pmol) intrasplenically into rats pretreated with indomethacin had limited effects on mesenteric perfusion pressure; the maximum dose used, 600 pmol, produced an increase of 6.96 ± 0.99 mmHg ($n = 3$). The systemic effects of

this dose of the peptide given intrasplenically consisted of a transient depressor response of 22.7 ± 1.6 mmHg and subsequently mean arterial pressure was only 3.33 ± 1.67 mmHg greater than the pressure before administration of the endothelin-1 (104 ± 15 mmHg).

Effect of endothelin-1 and [Ala^{1,3,11,15}]endothelin-1 on phenylephrine-induced tone in the blood-perfused mesentery and hindquarters of the rat

Infusion of 5 μg min^{-1} (−)-phenylephrine increased perfusion pressure by 69.2 ± 9.3 mmHg (n = 7) and 93.9 ± 8.5 mmHg (n = 9) respectively in the mesenteric and hindquarters vascular beds. In the latter preparation, endothelin-1 (0.01–10 pmol) caused a modest dilatation, which was maximal, 12.2 ± 2.4% (n = 7) of the induced tone, at 0.3 pmol (Figure 4a). The vasodilatation in the hindquarters was unaffected by pretreatment of the animals with 5 mg kg^{-1} indomethacin; the maximum effect observed was a reduction in established tone of 8.4 ± 1.8% which was obtained at 0.3 pmol (n = 5).

In the mesenteric arterial preparation, endothelin-1 (0.01–10 pmol) produced small decreases in this established tone (Figure 4b); the maximum observed inhibition of tone was 14.8 ± 4.3% at 0.3 pmol (n = 7). At doses above 6 pmol vasodilatation was no longer observed and a pressor response was obtained.

The only one of the alanyl-substituted analogues of endothelin-1 that was tested for direct vasorelaxant actions was [Ala^{1,3,11,15}]endothelin-1 and it had no significant activity in the hindquarters over the dose range 7 fmol–6.5 pmol (Figure 4c; n = 5).

Relaxation of methoxamine-induced tone in the isolated, Krebs-Henseleit-perfused superior mesenteric arterial bed

In the isolated mesenteric arterial bed, endothelin-1 (0.5–500 pmol) also relaxed tone established by infusion of methoxamine (Figure 5); 25 pmol endothelin-1 gave the maximum effect which was a reduction of 32.9 ± 7.2% of the established tone (n = 6). Figure 5 also shows that the inclusion of indomethacin in the perfusion fluid changed the concentration at which the maximum relaxation occurred to 50 pmol although the maximum degree of relaxation (22.2 ± 3.4%; n = 7) was not significantly different from the reduction of induced tone observed in the absence of indomethacin. In the experiments in which indomethacin was absent from the perfusion fluid, the tone established by methoxamine infusion was 63.1 ± 7.6 mmHg, whereas in the presence of indomethacin it was 91.6 ± 5.5 mmHg. Experiments carried out in the presence of indo-

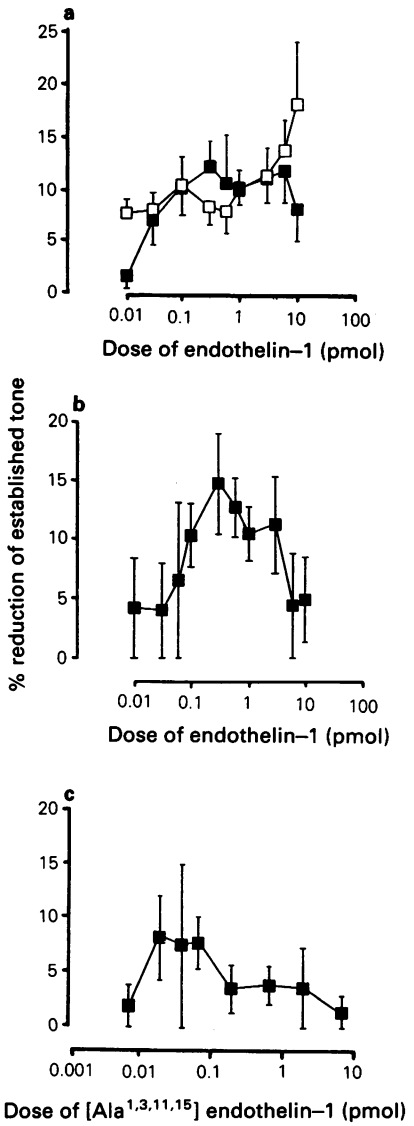


Figure 4 Relaxation by endothelin-1 or [Ala^{1,3,11,15}]endothelin-1 of vascular tone induced by infusion of 5 μM phenylephrine into either the *in situ* blood-perfused mesenteric bed (b) or the blood-perfused hindquarters preparation (a and c) of the rat. (a) Reductions in established tone by endothelin-1 in the hindquarters in control preparations (■; n = 9) and in preparations pretreated with 5 mg kg^{-1} indomethacin (□; n = 6). (b) Vasorelaxation in response to endothelin-1 in the *in situ* mesenteric arterial bed in the absence of indomethacin (n = 7). (c) Reductions in established tone by [Ala^{1,3,11,15}]endothelin-1 in the blood perfused hindquarters preparation (n = 5). The points show the mean and vertical lines show s.e.mean.

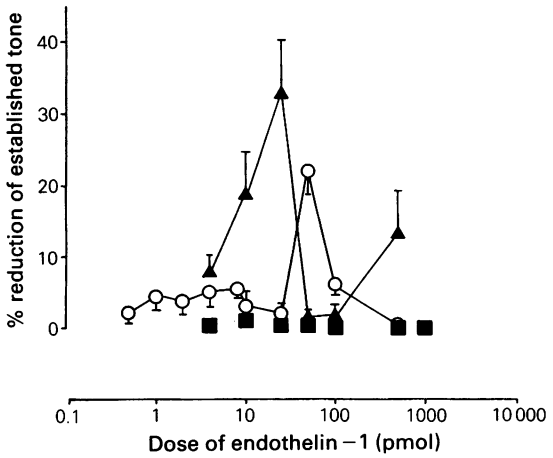


Figure 5 Relaxation by endothelin-1 of vascular tone established by infusion of 100 μM methoxamine into the *in vitro*, Krebs-Henseleit-perfused mesenteric arterial bed preparation of the rat. (■) shows the responses obtained in the presence of the endothelium and 10 μM indomethacin (n = 6–7); (○) represents the responses obtained in the presence of 10 μM indomethacin after destruction of the endothelium with CHAPS (n = 4–5); and (▲) gives the results obtained in the absence of indomethacin but in the presence of the endothelium (n = 6). The points show the mean and vertical lines show s.e.mean.

methacin showed that the relaxation in response to endothelin-1 was abolished by removal of the endothelium (n = 4–5).

Pressor actions of alanyl substituted analogues of endothelin-1 in the in situ, *blood-perfused, and in the isolated, Krebs-Henseleit-perfused, mesenteric arterial beds*

[Ala^{3,11}]endothelin-1 (0.75 pmol–2.25 nmol) increased perfusion pressure in the *in situ* mesentery (Figure 6a), but was significantly ($P < 0.01$) less potent than endothelin-1, with an ED_{50} of 270 ± 52 pmol, while the calculated R_{max}, 94.0 ± 6.2 mmHg (n = 7), was similar to that of endothelin-1 (Figure 6a). *In vitro*, [Ala^{3,11}]endothelin-1 produced a maximum increase in perfusion pressure (128 ± 10 mmHg; n = 3) comparable to that *in vivo* (Figure 7), but significantly ($P < 0.01$) greater than that for endothelin-1 *in vitro* (24.7 ± 2.1 mmHg; Figure 2); the ED_{50}, 0.94 ± 0.17 nmol (n = 3), was also significantly ($P < 0.001$) greater than that found for endothelin-1 in the same preparation. After the endothelium had been removed with CHAPS *in vitro* neither the R_{max} (110 ± 13 mmHg) nor the ED_{50} (0.62 ± 0.08 nmol) for this analogue were changed significantly from those found in the preparations with endothelium (n = 3; Figure 7).

[Ala^{1,15}]endothelin-1 (1.36 pmol–6.80 nmol) also produced dose-related vasoconstrictor responses in the blood-perfused mesentery; the dose-response curve for these responses had two components and was best fitted to a double hyperbola (Figure 6a). The component describing its action at the lower doses (1.36–1360 pmol) had an R_{max} of 19.1 ± 1.7 mmHg and an ED_{50} of 40.7 ± 19.6 pmol (n = 8). Two higher doses, 4.08 nmol and 6.80 nmol, produced pressor responses of 70.4 ± 4.9 mmHg (n = 6) and 89.3 ± 8.5 mmHg (n = 3) respectively. The actual maximum response could not be obtained owing to the acute systemic effects of the peptide (see below). Figure 7 shows that, *in vitro*, this analogue was significantly less potent than either endothelin-1 ($P < 0.001$) or [Ala^{3,11}]endothelin-1 ($P < 0.01$); the ED_{50} was found to be 3.5 ± 0.5 nmol (n = 4). The R_{max} for [Ala^{1,15}]endothelin-1 was 107 ± 14 mmHg which is comparable to that for [Ala^{3,11}]endothelin-1, but significantly ($P < 0.001$) greater than that of endothelin-1. Destruction of the vascular endothelium *in vitro* had no significant effect on either the ED_{50} or R_{max}, with values of 3.9 ± 0.2 nmol and 71.0 ± 7.5 mmHg, respectively (n = 5).

[Ala^{1,3,11,15}]endothelin-1 produced modest vasoconstrictor responses in both the blood-perfused mesenteric (dose range 0.65–390 pmol; Figure 6b) and hindquarters (dose range 6.5–650 pmol; Figure 6c) preparations. In the intact, blood-perfused mesentery this analogue had an R_{max} of 20.9 ± 3.0 mmHg (n = 7), which was significantly ($P < 0.001$) lower than that for endothelin-1 in this vascular bed over a similar dose range. Its ED_{50} was 45.3 ± 22.6 pmol, comparable to that of endothelin-1. In the isolated mesentery [Ala^{1,3,11,15}]endothelin-1 had no pressor activity in the dose range 0.65–1300 pmol (n = 3); endothelial cell destruction did not reveal any pressor activity of this analogue. In the blood-perfused hindquarters preparation [Ala^{1,3,11,15}]endothelin-1 had similar activity to the *in situ* mesentery; the R_{max} was 17.6 ± 1.7 mmHg, which is significantly ($P < 0.001$) lower than that for endothelin-1 in this bed and the ED_{50} was 38.2 ± 5.4 pmol, which is significantly ($P < 0.001$) less than the corresponding value for endothelin-1 (n = 5).

Effects on mean systemic blood pressure of close arterial administration of endothelin-1 and its alanyl-substituted analogues into the mesenteric or hindquarters circulations

Administration of endothelin-1 (1–200 pmol) into the superior mesenteric artery had no significant effects on mean arterial blood pressure. On administration into the lower abdominal aorta perfusing the hind-

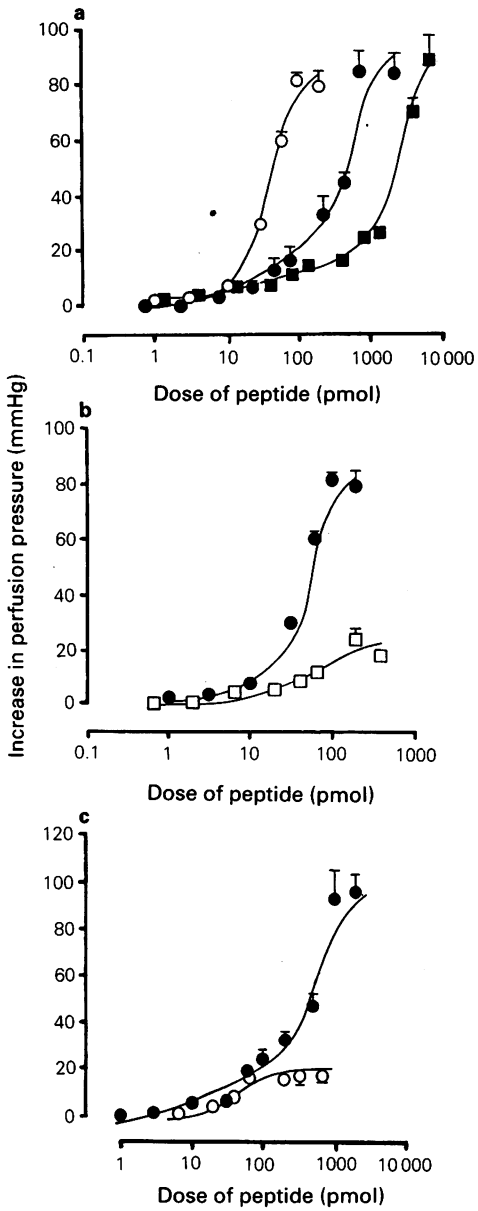


Figure 6 Pressor responses to alanyl-substituted analogues of endothelin-1 in the blood-perfused mesenteric or hindquarters preparations of the rat. (a) Responses to endothelin-1 (○; $n = 5$), [Ala^{1,15}]endothelin-1 (■; $n = 3$–8) and [Ala^{3,11}]endothelin-1 (●; $n = 7$) in the mesenteric arterial bed. (b) Responses to endothelin-1 (●; $n = 5$) and [Ala^{1,3,11,15}]endothelin-1 (□; $n = 7$) in the mesenteric bed. (c) Responses to endothelin-1 (●; $n = 4$) and [Ala^{1,3,11,15}]endothelin-1 (○; $n = 5$) in the hindquarters circulation. In (a) and (b) the curve for endothelin-1 is shown for comparison and the data are the control results for endothelin-1 in Figure 1. Similarly, the data for endothelin-1 in the hindquarters are the control data shown in Figure 3. The points are the mean and vertical lines show s.e.mean.

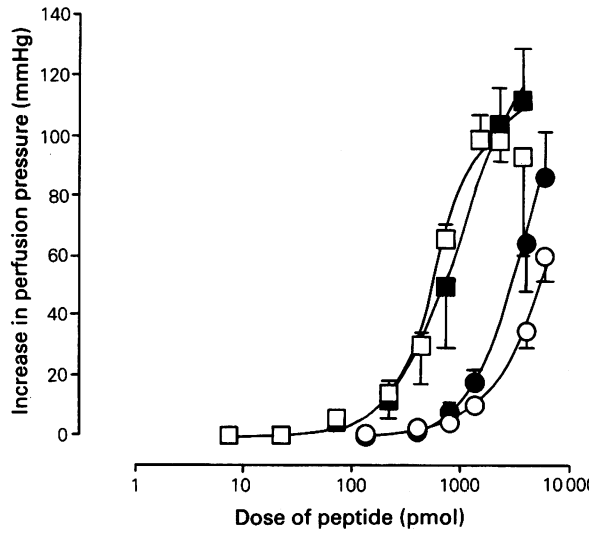


Figure 7 Pressor responses to [Ala^{1,15}]endothelin-1 (○, ●) and [Ala^{3,11}]endothelin-1 (□, ■) in the isolated mesenteric arterial bed of the rat perfused with Krebs-Henseleit solution containing 10 μM indomethacin. The closed symbols (●, ■) show results obtained in preparations with an intact endothelium and the open symbols (○, □) give results from preparations in which the endothelium had been destroyed with CHAPS. The points are the mean and the vertical lines show 1 s.e.mean. For [Ala^{3,11}]endothelin-1, $n = 3$ for both intact and endothelium-denuded preparations, but for [Ala^{1,15}]endothelin-1, $n = 4$ for the intact preparations and $n = 5$ for the preparations without endothelium.

quarters, endothelin-1 (1–2000 pmol) produced a modest elevation of mean arterial blood pressure commencing at 60 pmol, at 2000 pmol the pressor response was 28.3 ± 4.4 mmHg ($n = 3$).

On mesenteric administration, both [Ala^{3,11}]-endothelin-1 (45 pmol–2.25 nmol) and [Ala^{1,3,11,15}]-endothelin-1 (19.5–390 pmol) produced relatively long lasting (5–20 min) falls in mean systemic arterial blood pressure which were not followed by pressor responses. [Ala^{3,11}]endothelin-1 injected close arterially into the mesenteric circulation produced a systemic depressor response at doses above 45 pmol; at the maximum dose used (2250 pmol), the decrease in mean arterial blood pressure was 21.7 ± 6.7 mmHg ($n = 7$). [Ala^{1,3,11,15}]endothelin-1 also caused depressor responses at doses greater than 19.5 pmol; at the maximum dose used

(390 pmol) the decrease in mean arterial pressure was 10.5 ± 4.5 mmHg ($n = 7$).

[Ala^{1,15}]endothelin-1, on administration into the mesenteric arterial bed, produced systemic depressor responses at the lower doses (81.6–816 pmol; $n = 8$), with a maximum fall of 16.1 ± 1.8 mmHg at 816 pmol of the peptide. Higher doses (1–6.8 nmol) produced pressor responses, with 6.8 nmol increasing mean systemic arterial pressure by 25.0 ± 1.0 mmHg.

Of these alanyl-substituted analogues, only [Ala^{1,3,11,15}]endothelin was administered into the hindquarters and it had no significant systemic actions up to the maximum dose given (650 pmol).

Effect of [Ala^{1,3,11,15}]endothelin-1 on the pressor activity of endothelin-1 in the mesentery in vivo *and* in vitro

[Ala^{1,3,11,15}]endothelin-1 (130 pmol), which produced a pressor response of 11.3 ± 0.9 mmHg ($n = 4$) in the blood-perfused mesenteric arterial bed augmented the pressor activity of endothelin-1 when the two peptides were co-administered (Figure 8a). A higher dose of 260 pmol [Ala^{1,3,11,15}]endothelin-1 also caused an increase in mesenteric perfusion pressure (15.5 ± 2.2 mmHg; $n = 4$), while on co-administration with endothelin-1 it produced a leftward shift in the log dose-response curve for endothelin-1 compared to that for endothelin-1 alone (Figure 8b). Figure 8a also shows that the positions of the curves for endothelin-1 obtained in the presence of either dose of [Ala^{1,3,11,15}]-endothelin-1 were very similar. Further, the mean responses at each dose of endothelin-1 obtained in the absence of [Ala^{1,3,11,15}]endothelin-1 were significantly different ($P < 0.05$) from those obtained in the presence of either 130 or 260 pmol [Ala^{1,3,11,15}]endothelin-1 with only two exceptions, 400 pmol endothelin-1 with 260 pmol analogue and 10 pmol endothelin-1 with 130 pmol analogue.

Figure 8b shows that, in the blood-perfused hindquarters preparation, the pressor activity of endothelin-1 co-administered with 260 pmol [Ala^{1,3,11,15}]endothelin-1 was greater than that of endothelin-1 alone in the lower dose range (up to 100 pmol), while from 300 pmol to 2 nmol endothelin-1 there was no significant difference between the two groups. In the absence of the analogue the dose-response relationship for endothelin-1 was described by an ED_{50} of 436 ± 90 pmol, an R_{max} of 99.7 ± 3.5 mmHg and a slope function of 1.72 ± 0.37 but, on co-administration with the analogue, the dose-response curve for endothelin-1 became distinctly biphasic; significant enhancement of the response to the parent peptide occurred with the 4 lowest doses tested, that is, 10 pmol ($P < 0.05$), 30 pmol, 60 pmol and 100 pmol (all $P < 0.01$; Student's unpaired t test; $n = 6$ for both the control and co-administration groups).

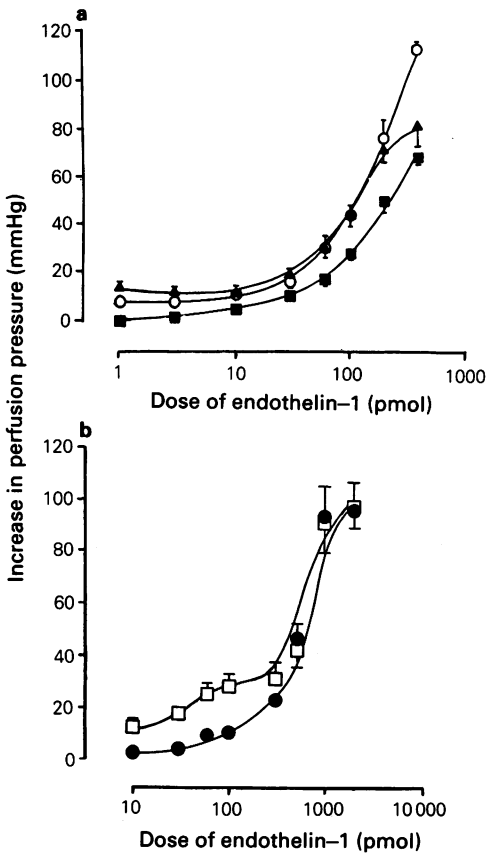


Figure 8 Pressor responses obtained either for endothelin-1 alone or for the parent peptide co-administered with [Ala^{1,3,11,15}]endothelin-1 in the blood-perfused superior mesenteric or hindlimb preparations. (a) Results obtained in the superior mesenteric arterial bed. (■) shows the responses obtained with endothelin-1 alone ($n = 4$); (○) represents the responses obtained with endothelin-1 administered together with 130 pmol [Ala^{1,3,11,15}]endothelin-1 ($n = 4$); and (▲) shows the responses obtained when endothelin-1 was co-administered with 260 pmol endothelin-1 ($n = 5$). (b) Pressor responses to endothelin-1 alone (●; $n = 3$–6) or endothelin-1 co-administered with 260 pmol [Ala^{1,3,11,15}]endothelin-1 (□; $n = 3$–6) in the hindquarters circulation. The points are the mean and vertical lines show s.e.mean.

In vitro, co-administration of 130 pmol [Ala^{1,3,11,15}]endothelin-1 with endothelin-1 (60–400 pmol) in the mesenteric bed did not alter the pressor activity of endothelin-1 significantly (Figure 9a). However, Figure 9b shows that co-administration of 260 pmol [Ala^{1,3,11,15}]endothelin-1

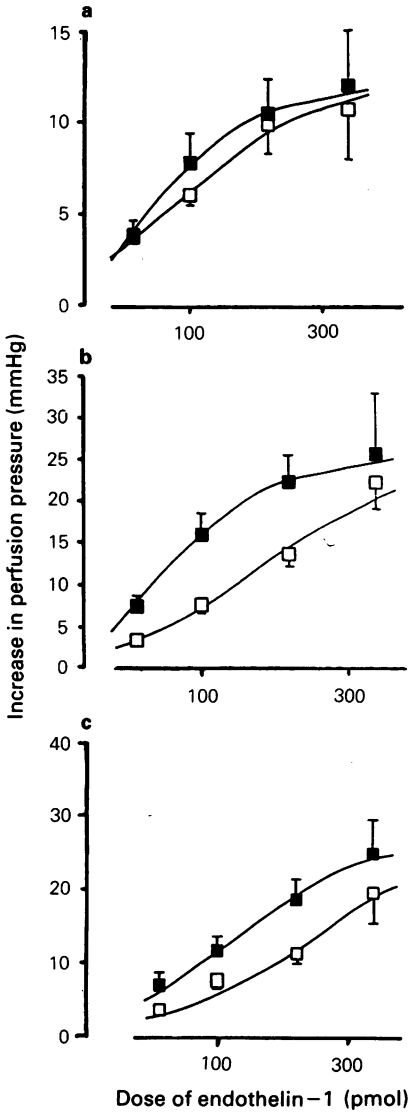


Figure 9 Increases in perfusion pressure in response to endothelin-1 either administered alone or in conjunction with [Ala^{1,3,11,15}]endothelin-1 in the rat isolated superior mesenteric arterial bed preparation. (a) Responses to endothelin-1 either alone (□; $n = 7$) or when administered with 130 pmol [Ala^{1,3,11,15}]-endothelin-1 (■; $n = 5$–7) in preparations with intact endothelium. (b) Pressor responses to endothelin-1 administered either alone (□; $n = 8$) or together with 260 pmol [Ala^{1,3,11,15}]endothelin-1 (■; $n = 8$) in preparations with intact endothelium. (c) Effects of endothelin-1 given either separately (□; $n = 6$) or together with 260 pmol [Ala^{1,3,11,15}]endothelin-1 (■; $n = 5$–7) in preparations denuded of endothelium by perfusion with CHAPS. Values are the mean with vertical lines showing s.e.mean.

with the parent peptide ($n = 8$) significantly augmented the pressor responses relative to those observed in response to endothelin-1 alone ($n = 8$) at doses of 60 pmol ($P < 0.01$), 100 pmol ($P < 0.01$) and 200 pmol ($P < 0.05$). This potentiating action of the alanyl-substituted analogue on the pressor responses produced by endothelin-1 was shown to be an endothelium-independent action. After endothelial cell destruction by perfusion of the mesenteric bed with CHAPS, co-administration of 260 pmol [Ala^{1,3,11,15}]endothelin-1 with endothelin-1 resulted in significant increases in the pressor responses to doses of 100 pmol ($P < 0.05$) and 200 pmol ($P < 0.05$) of endothelin-1 (Figure 9c).

Discussion

This study shows that endothelin-1 is a potent vasoconstrictor of both the mesenteric and the hindquarters circulations of the rat. Furthermore, analogues of endothelin-1, in which one or other of the disulphide bridges was absent, also caused vasoconstriction of the mesenteric arterial bed and an analogue in which there was no disulphide bridge was a partial agonist, relative to the parent peptide, in the two blood-perfused preparations investigated.

Endothelin-1 was 10 fold less potent in the blood perfused hindquarters preparation than in the *in situ* mesenteric arterial bed. This difference is interesting since it has been shown that profound changes in mesenteric blood flow, associated with systemic pressor responses to endothelin-1, are not accompanied by reductions in hindlimb blood flow in conscious, spontaneously hypertensive (Wright & Fozard, 1988) or normotensive (Gardiner *et al.*, 1989) rats. Also, in pithed rats we have found that a modest increase in systemic blood pressure caused by endothelin-1 is associated with a large rise in mesenteric vascular resistance, which is not accompanied by an increase in skeletal muscle vascular resistance (MacLean *et al.*, 1989).

It is of interest that the peptide has a higher maximal pressor activity in the *in situ* blood-perfused mesentery compared to the isolated, buffer-perfused preparation. The difference in activity probably reflects the anatomical differences between the two different preparations; the *in situ* bed is essentially intact and contains the smaller arterioles, capillaries, venules and hepatic portal vein which are absent in the isolated bed. The increased maximal pressor activity *in situ* may thus reflect a substantial constrictor action on the venous side of the bed. This hypothesis would accord with the finding that endothelin-1 appears to be a more potent vasoconstrictor in venous tissues than it is on arterial preparations (D'Orleans-Juste *et al.*, 1988). However, this

anatomical difference in potency of endothelin-1 may only apply to the larger blood vessels on the venous side of the bed as Brain *et al.* (1988) have found that, in the hamster cheek pouch, endothelin-1 was only an arteriolar vasoconstrictor and had no apparent actions on the venules. In the present study the administration of endothelin-1 into the hepatic portal venous system via the spleen had only very small effects on the arterial perfusion pressure in the mesentery. Since systemic effects were apparent after intrasplenic administration of endothelin-1, the peptide must have entered the portal circulation, as the spleen drains into this vein. Therefore the small rises in mesenteric perfusion pressure found in this study may be the result of portal venous constriction, recirculation of the peptide through the systemic circulation or both. However, a substantial contribution by portal venoconstriction to the pressor activity of endothelin-1 in the mesentery seems very unlikely. Alternatively, the increased constrictor activity *in vivo* may be due to the fact that the intact bed is a closed circuit and thus constrictor activity generally may be enhanced. This would seem unlikely in view of the comparable maximum pressor responses to [$Ala^{3,11}$]endothelin-1 *in vivo* and *in vitro* obtained in this study. Thus it would seem that the isolated bed has a diminished number of responsive sites for endothelin-1 compared to the intact bed and that such a difference does not exist for [$Ala^{3,11}$]endothelin-1.

Endothelin-1 releases EDRF in the isolated Krebs-Henseleit perfused mesentery (De Nucci *et al.*, 1988; Warner *et al.*, 1989) which leads to a modulation of the pressor activity of the peptide. Enhanced pressor activity for endothelin-1 was also found in this study after endothelial cell destruction in the isolated preparation and this supports a role for the endothelium in the modulation of the pressor actions of endothelin-1 by the release of EDRF. Release of EDRF by endothelin-1 probably also accounts for the relaxation of induced tone that the peptide produced in both the isolated and *in situ* mesenteric beds and in the hindquarters circulation. Alternatively, the enhancement of the pressor responses to endothelin-1 in the isolated mesenteric bed may reflect the loss of a diffusional barrier, facilitating the access of the peptide to the vascular smooth muscle (Pohl & Busse, 1989). However, selective destruction of the endothelium *in vivo* resulted in decreased vascular reactivity towards the peptide. The same method of endothelial destruction *in vivo* had no effect on the maximum pressor responses to noradrenaline (Randall *et al.*, 1988), suggesting that the change observed with endothelin-1 is not general for vasoconstrictor agents. It is possible that, during blood perfusion, some of the pressor activity of endothelin-1 may be due to release of endothelium-derived vasoconstrictors, although the reduction in maximum response may be due to an action of the detergent on the processes underlying endothelin-induced vasoconstriction while leaving the pressor activity to noradrenaline intact.

Cyclo-oxygenase inhibition by indomethacin potentiates the pressor activity of endothelin-1 in the pithed rat (De Nucci *et al.*, 1988) and in the isolated kidney and spleen (Rae *et al.*, 1989). In the current study, cyclo-oxygenase products appeared to be able to influence the activity of endothelin-1 in the *in situ* mesentery, but not the hindquarters, since indomethacin, at a dose which inhibits peripheral cyclo-oxygenases (Jackson & Campbell, 1980), increased the maximal response to the peptide in the mesentery but not the hindquarters. This suggests that eicosanoids, which are either tonically released, released by stimulation or produced by the two processes together, may modulate the activity of this peptide in the mesenteric circulation but not the hindquarters. The prostanoids are unlikely to be of endothelial origin as destruction of the vascular endothelium *in vivo* does not have the same effect as indomethacin pretreatment. Modulation of pressor responses to endothelin-1 has been shown to be due to eicosanoid activity in the intact rat, since indomethacin or piroxicam pretreatment increased responses to second and subsequent doses of endothelin-1 (De Nucci *et al.*, 1988); this potentiation is abolished by adrenal ligation (Walder *et al.*, 1989). Thus it was proposed that eicosanoids derived from the adrenal glands modulate the constrictor actions of endothelin-1. Adrenal-derived eicosanoids are unlikely to account for the modulation found in the current study, due to the lack of effect of the cyclo-oxygenase inhibitor on endothelin-1-stimulated constriction in the hindquarters. Further, there were no systemic effects observed after endothelin-1 administration into the mesentery *in situ*. Thus the modulation observed *in situ* is probably due to non-endothelial cyclo-oxygenase activity in the mesentery.

A slight effect of cyclo-oxygenase products in the isolated mesentery was also found; the vasodilator effect of the peptide could be observed at lower doses in the absence of indomethacin than in its presence. Thus, in these circumstances, modulation is apparently primarily by vasoconstrictor products of the enzyme. However, it should be noted that there were no significant changes in the pressor responses to endothelin-1 when indomethacin was omitted from the perfusing fluid and thus it would seem that there is not a very great capability for modification of the effects of endothelin-1 by cyclo-oxygenase products in this isolated preparation.

The observation that endothelin-1 caused systemic vasoconstriction when administered close arterially

into the hindquarters, but not when given into the superior mesenteric artery, probably reflects greater overspill of the peptide from the hindquarters to the systemic circulation. Reduced access from the mesenteric bed is probably due to extensive binding of the peptide in the hepatosplanchnic circulation. This may possibly be in the liver as ^{125}I-labelled endothelin-1 has been shown to bind extensively in this organ (Shiba *et al.*, 1989; Neuser *et al.*, 1989). Similar studies performed by Ånggård *et al.* (1989) have led to the conclusion that the liver, along with the lungs and kidneys, may be an important organ in the removal of circulating endothelin-1 from the blood stream.

The two disulphide bridges present in endothelin-1 represent a relatively unusual structure in vasoactive peptides. Since this 'double loop' structure is conserved not only between the various forms of endothelin encoded in the human genome, but is also found in the sarafotoxin group of snake venoms which possess vasoconstrictor activity (Kloog *et al.*, 1988), it was thought that this structure might be important for biological activity. Selective substitution of the bridge cysteinyl with alanyl residues, leading to removal of the bonds, has led to a series of vasoactive analogues, suggesting that the disulphide bonds are not an absolute requirement for activity. Kimura *et al.* (1988) have also shown that vasoconstrictor activity is retained when the disulphide bridges are removed from the parent peptide, but their analogues included blocked cysteinyl residues and they were much less potent than the alanyl substituted analogues used here. However, the 1–16 fragment of endothelin-1, containing the intramolecular loops but not the 5 C-terminal amino-acids, was without constrictor activity on the porcine coronary artery (Kimura *et al.*, 1988). From these structure-activity relationships they concluded that the activity of the peptide resides in the hydrophobic C-terminus and that this is enhanced by the intramolecular loop. They proposed that enzymic degradation of either the loop structure or C-terminus could represent *in vivo* mechanisms for inactivation of the peptide. However, the present data suggest that metabolic products with disrupted disulphide bridges may have appreciable biological activity.

[Ala^{3,11}]endothelin-1, which lacks the 3-11 bridge forming the smaller loop, was less potent than endothelin-1 as a vasoconstrictor both *in vivo* and *in vitro*. *In vivo* this analogue was less potent than endothelin-1 by a factor of about seven and *in vitro* by a factor of about twenty-four; but these potency ratios are comparable to the 12 fold lower potency obtained for [Ala^{3,11}]endothelin-1 in the rat isolated aorta (Topouzis *et al.*, 1989) and the 8 fold lower potency, found for the analogue compared to endothelin-1, at increasing the twitch response in the electrically-stimulated vas deferens of the rat (Hiley *et al.*, 1989b). The greater maximum responses to this analogue and to [Ala^{1,15}]endothelin-1 *in vitro* compared to that of endothelin-1 are particularly interesting and show that endothelin-1 is a partial agonist relative to its analogues on the arterial side of the bed. This has also been observed with respect to [Ala^{3,11}]endothelin-1 in the rat aorta (Topouzis *et al.*, 1989). However, in the intact, *in situ* mesenteric circulation there was no apparent difference in the maximal reactivities of [Ala^{3,11}]endothelin-1 and endothelin-1, thus endothelin-1 is behaving as a full agonist here. Therefore, it is likely that the reduced reactivity in the isolated bed is due to a limited receptor population for endothelin-1 relative to the analogues, but the data do not allow differentiation of whether this is due to endothelin-1 having a lower efficacy than the analogues at the same receptor or to its having a different receptor. It is also of interest that endothelial cell destruction led to enhanced reactivity for endothelin-1 but not the analogues *in vitro*, this is in accord with the contractile responses of the rat isolated aorta being enhanced to endothelin-1 but not to [Ala^{3,11}]endothelin-1 on removal of the endothelium (Topouzis *et al.*, 1989). However, the data presented here do not suggest that endothelium-dependent modulation of the pressor response occurs for either [Ala^{1,15}]-endothelin-1 or [Ala^{3,11}]endothelin-1; with neither of these peptides *in vitro* was there either an enhancement of the maximum response or an increase in potency. These observations suggest that the alanyl analogues and endothelin-1 interact with receptors in different ways, such that the putative ability to release EDRF is dependent on the possession of both disulphide bridges, and add further weight to the proposition that these alanyl-substituted peptides are not acting on exactly the same population of receptors as endothelin-1 itself. However, previous work has shown that both [Ala^{1,15}]endothelin-1 and [Ala^{3,11}]endothelin-1 inhibit the binding of [^{125}I]endothelin-1 from homogenates of rat cerebellum and hindbrain (Jones *et al.*, 1989b,c), and that [Ala^{3,11}]endothelin-1 inhibits iodinated endothelin-1 binding to rat kidney cortex (Jones *et al.*, 1989a) indicating that there are some sites at which they interact directly.

Removal of the 1–15 disulphide bridge, which forms the outer ring structure, in [Ala^{1,15}]-endothelin-1 leads to a greater loss of pressor activity in the isolated mesentery, suggesting that for actions on the arterial side of the bed the possession of the 1–15 bridge is more important for activity than the 3–11 bridge. In the intact bed the dose-response relationship for the pressor activity of this analogue was best fitted to a double hyperbola, indicating that the actions of this analogue may well be

the result of it interacting with two independent sites. The higher of the two ED_{50}s for [$Ala^{1,15}$]endothelin-1 is close to that of this analogue in the isolated bed. Thus it is likely that the sites with the lower ED_{50} (and perhaps higher affinity) are located on the smaller arterioles or on the venous side of the bed. Hence, the vascular actions of these two analogues, when compared to those of endothelin-1, make it probable that there is more than one receptor type for endothelin-like peptides.

Removal of both of the disulphide bridges resulted in an analogue ([$Ala^{1,3,11,15}$]endothelin-1) with partial agonist activity in both the blood-perfused mesentery and hindquarters, while in the isolated mesentery this tetra-alanyl analogue had no pressor activity at the doses examined. This lack of pressor activity is not due to a concomitant induction of release of endothelium-derived vasorelaxants since destruction of the vascular endothelium did not reveal any pressor activity. Thus, the lack of effect of the analogue in the isolated mesentery, whilst it possesses activity in the intact bed, may be due to differences in either receptor reserve or types of receptor. These findings would be compatible with those of Spokes *et al.* (1989) who have compared the activities of endothelin-1 and endothelin-3 on rat stomach strips, guinea-pig ileum and guinea-pig trachea. These workers have also produced data which would appear to suggest the existence of multiple receptor subtypes for endothelin. The results of Warner *et al.* (1989) also suggest different receptor types on endothelium and vascular smooth muscle, since endothelin-3 was equipotent with endothelin-1 as a vasodilator but less potent as a vasoconstrictor agent.

The systemic depressor actions of [$Ala^{3,11}$]-endothelin-1 and [$Ala^{1,3,11,15}$]endothelin-1 following mesenteric administration are interesting in view of the observations that these analogues did not have their pressor activity changed by removal of the vascular endothelium. The depressor actions at the lower doses and the pressor activity of the higher doses of [$Ala^{1,15}$]endothelin-1 mirror the two-site model for the action of this analogue on the mesentery. The systemic pressor activity occurs at doses which form the component of the dose-response curve with the higher ED_{50} in the mesentery. This supports the proposition that the peptides may act at two receptors in the periphery. The systemic vasodepressor actions of these three analogues might reflect a specific activation by the peptides of receptors which bring about vasodilatation in vascular beds not investigated here, or it might be due to changes in cardiac performance. Alternatively, there might be competitive interactions with endogenous endothelin. However, the co-administration experiments with [$Ala^{1,3,11,15}$]endothelin-1 and endothelin-1 did not show any antagonism of the pressor responses to the parent peptide by the analogue but rather an enhancement.

The partial agonist activity of [$Ala^{1,3,11,15}$]-endothelin-1 in the blood-perfused vascular beds led us to examine the effect of this analogue on the activity of endothelin-1 in all the preparations used. The enhancement of the pressor activity of endothelin-1 in the isolated mesentery is of particular interest as the tetra-alanyl analogue had no pressor activity in this bed and suggests a pharmacological action of the analogue in this preparation. Its action may be to enhance the activity of endothelin-1 at the smooth muscle level or possibly the analogue might antagonize the action of endothelin-1 on endothelial receptors, so attenuating the release of humoral modulators. However, this latter explanation appears to be unlikely as the *in vitro* studies have shown this effect to be endothelium-independent. In both of the blood-perfused beds there is a clear-cut summation of the activities of the analogue with those of endothelin-1 at the lower doses of endothelin-1, and no evidence of antagonism at higher dose levels as would be expected with a partial agonist. This lends further support to the contention that there are two independent receptor classes and that there is selectivity between endothelin-1 and its analogues.

The results presented here show that endothelin-1 is a potent vasoconstrictor of the two vascular beds studied. It may cause release of EDRF which modulates its pressor activity. The vasoconstrictor actions of the endothelin family of peptides are not dependent on the retention of the two disulphide bridges within the molecule, but there is evidence that there may be more than one subtype of receptor for these agents.

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Endothelium-dependent calcium-induced relaxation in the presence of Ca^{2+}-antagonists in canine depolarized coronary arteries

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1 We examined the mechanisms underlying Ca^{2+}-induced relaxation in the presence of clentiazem, a new Ca^{2+}-antagonist, in depolarized coronary arteries of the dog.

2 Ca^{2+} (3×10^{-5}–3×10^{-3} M) caused an unexpected relaxation in the presence of a high concentration of clentiazem (10^{-6} M) in coronary, but not in mesenteric or renal arteries.

3 The Ca^{2+}-induced relaxation was also observed in the presence of established Ca^{2+}-antagonists such as diltiazem (3×10^{-6} M), nifedipine (3×10^{-8} M) and verapamil (3×10^{-6} M).

4 The Ca^{2+}-induced relaxation was inhibited by removal of the endothelium, treatment with oxyhaemoglobin (1.5×10^{-6} M) or methylene blue (10^{-5} M), but not by treatment with indomethacin (5×10^{-6} M).

5 The Ca^{2+}-induced relaxation was observed in an endothelium-denuded coronary artery segment when closely apposed to an endothelium-containing segment of coronary or mesenteric artery.

6 These results suggest that Ca^{2+}-induced relaxation in the presence of high concentrations of Ca^{2+}-antagonists is mediated through endothelium-derived relaxing factor (EDRF). In addition, Ca^{2+}-antagonists do not affect the Ca^{2+}-influx necessary for the release and/or synthesis of EDRF.

Introduction

The endothelial cell is a source of various vasodilator and vasoconstrictor factors. Furchgott & Zawadzki (1980) first proposed the existence of endothelium-derived relaxing factors (EDRFs), and recently, one EDRF was identified as nitric oxide (Ignarro *et al.*, 1987; Palmer *et al.*, 1987).

EDRF stimulates soluble guanylate cyclase leading to an elevation of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) in vascular smooth muscle (Griffith *et al.*, 1985). The increased cyclic GMP has been shown to cause relaxation by activation of Ca^{2+}-activated ATPase (Popescu *et al.*, 1985), by an elevation of K^+ conductance (Ignarro & Kadowiz, 1985), by inhibition of the interaction of Ca^{2+} with myosin light chain (Pfitzer *et al.*, 1984) and by inhibition of IP_3 production (Rappoport & Murad, 1986).

Ca^{2+} influx into endothelial cells has also been shown to play an important role in the release and/or synthesis of EDRF (Loeb *et al.*, 1988). However, the involvement of the voltage-dependent Ca^{2+} channel in the release and/or synthesis of EDRF is still controversial. Singer & Peach (1982) demonstrated that methacholine-induced endothelium-dependent relaxation of rabbit aorta was inhibited by verapamil and nifedipine. On the other hand, Jayakody *et al.* (1987) showed that the release and/or synthesis of EDRF in the rabbit aorta was not affected by Ca^{2+}-antagonists, such as diltiazem and nicardipine.

In a previous paper, we found that Ca^{2+} produced a relaxation of K^+-depolarized coronary artery of the dog in the presence of clentiazem, a new benzothiazepine Ca^{2+}-antagonist (Kikkawa *et al.*, 1988). In this study, we investigated further the mechanism of action of this Ca^{2+}-induced relaxation. Evidence was obtained to suggest that EDRF was involved in the Ca^{2+}-induced relaxation.

Methods

Animals and general procedures

Male mongrel dogs weighing 8 to 15 kg were anaesthetized with an intravenous injection of sodium pentobarbitone (30 mg kg^{-1}), and killed by exanguination. The heart, renal and mesenteric

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arteries were removed immediately, and stored overnight in physiological salt solution (PSS) at 4°C.

The circumflex branch of the left coronary artery (o.d. 1.0–1.5 mm), the distal portion of the mesenteric artery (o.d. 1.0–1.5 mm) and the renal artery (o.d. 1.2–1.6 mm) were cleaned of surrounding connective tissue and fat.

The arteries were cut into ring segments of 5 mm in length. Ring segments were suspended in 15 ml organ baths containing PSS, which was maintained at 37.0°C and gassed with 95% O_2 and 5% CO_2. Care was taken not to touch the luminal surface. Resting tension was adjusted to 1.5 g. The composition of PSS, prepared with deionized water, was as follows (mM): NaCl 147.2, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 2.2, $NaHCO_3$ 14.5, dextrose 5.4 (pH 7.3 to 7.4). Isometric tension was measured by means of a strain gauge transducer (UL-10, Minebea, Tokyo, Japan), and recorded on a Multicorder (MC-6621, graphtec, Tokyo, Japan).

After equilibration for 90 to 120 min, preparations were exposed to Ca^{2+}-free, K^+-depolarizing PSS containing 80 mM KCl in equimolar replacement for NaCl. No Ca^{2+}-chelating agent was present. The first exposure to Ca^{2+}-free, K^+-depolarizing PSS produced a transient contraction, but this was abolished by washing several times with Ca^{2+}-free, K^+-depolarizing PSS. All experiments were carried out in Ca^{2+}-free, K^+-depolaring PSS. Resting tension did not change after replacement with Ca^{2+}-free, K^+-depolaring PSS.

Ca^{2+}-induced relaxation and the effect of various agents

Ca^{2+} (10^{-5}–3×10^{-3} M) was added cumulatively to preparations several times in order to obtain steady responses. After stabilization of responses, clentiazem (10^{-7} or 10^{-6} M), diltiazem (3×10^{-6} M), nifedipine (3×10^{-8} M) or verapamil (3×10^{-6} M) was added 10 min before the next cumulative administration of Ca^{2+}.

In some experiments, indomethacin (5×10^{-6} M) or oxyhaemoglobin (1.5×10^{-6} M) was added 15 min before the cumulative administration of Ca^{2+}, and 5 min before the administration of clentiazem (10^{-6} M). Oxyhaemoglobin was prepared according to the method of Martin *et al.* (1985a). Briefly, oxyhaemoglobin was obtained by adding to a 1 mM solution of commercial haemoglobin, in double distilled water, a 10 fold molar excess of the reducing agent, sodium dithionite ($Na_2S_2O_4$). Sodium dithionite was then removed by dialysis against 100 volumes of double distilled water for 5 h at 4°C. The solution was frozen and stored at −20°C for up to 14 days.

In experiments with methylene blue, this agent was added to preparations during the plateau of the response to Ca^{2+} in the presence of clentiazem (10^{-6} M).

Removal of endothelium on the Ca^{2+} response

The endothelium was removed from some preparations by gently rubbing the intimal surface with a cotton bar. The effectiveness of the removal procedure was checked functionally by the failure of acetylcholine (10^{-6} M) or calcium ionophore A23187 (10^{-6} M) to elicit a relaxation response. Some preparations were also examined by scanning electron microscopy.

Sandwich preparation

A sandwich preparation, similar to that described by Tayo and Bevan (1986), was used to investigate the release of endothelium derived relaxant factor(s). Briefly, the endothelium of the recipient coronary segment was removed by rubbing as described above. The donor tissue (endothelium-containing coronary or mesenteric artery) was turned inside-out so that the intimal surface was on the outside. The donor tissue was then inserted into the recipient.

Statistical analysis

Results are expressed as means ± s.e.mean, and statistical analysis was performed by Student's *t* test.

Drugs

The compounds used were as follows: clentiazem (TA-3090, (+)-(2S,3S)-3-acetoxy-8-chloro-5-(2-(dimethylamino)ethyl)-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4-(5H)-one maleate) and diltiazem hydrochloride (Tanabe Seiyaku, Co., Ltd., Osaka, Japan); nifedipine (synthesized by organic chemistry research laboratory, Tanabe Seiyaku Co., Ltd.); verapamil hydrochloride (Vasolan, Eizai Co., Ltd., Tokyo, Japan); acetylcholine (Ovisot, Dai-ichi Seiyaku, Tokyo, Japan); methylene blue (Junsei Chem. Co., Tokyo, Japan); A23187, indomethacin and haemoglobin (Type 1) (Sigma Chemical Co.). A23187 and indomethacin were dissolved in dimethyl sulphoxide and ethanol (10^{-3} M stock solution), respectively. Other agents were dissolved in double distilled water.

Results

Ca^{2+} response in the presence of Ca^{2+}-antagonists

Typical tracings showing the effects of clentiazem pretreatment on Ca^{2+}-induced contraction in K^+

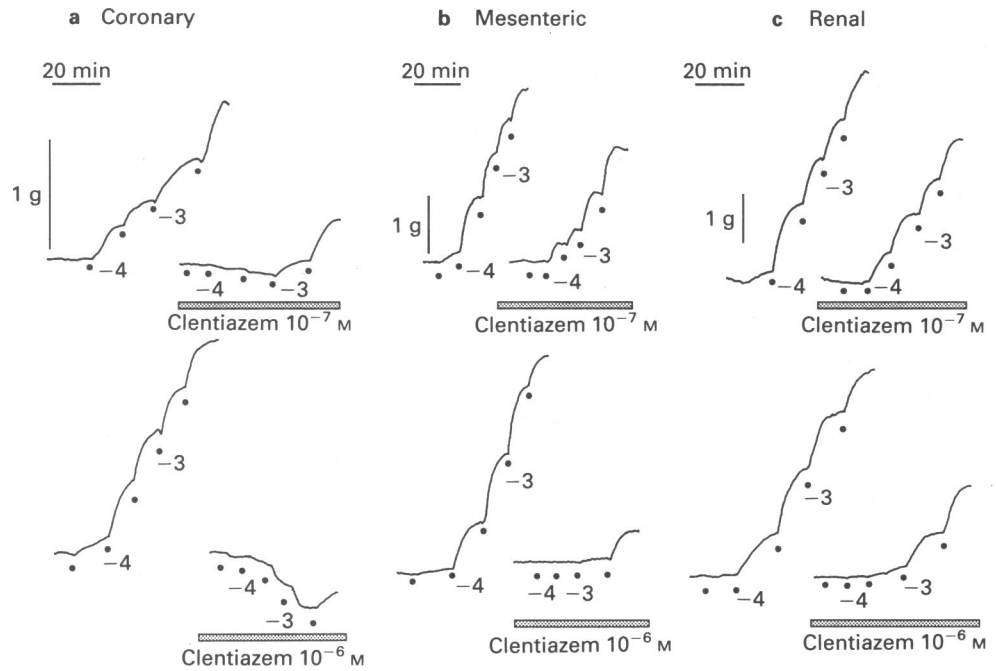


Figure 1 Typical tracings showing the effects of clentiazem on the concentration-response curve for Ca^{2+} in Ca^{2+}-free, K^+ (80 mM)-depolarized arteries of the dog. (a) Coronary artery, (b) mesenteric artery and (c) renal artery. Clentiazem (10^{-7} M or 10^{-6} M) was added to the preparations 10 min before the cumulative administration of Ca^{2+} (3×10^{-5}–10^{-3} M). Ca^{2+} was added at the points indicated, in incremental doses.

(80 mM)-depolarized canine coronary, mesenteric and renal arteries are shown in Figure 1. Ca^{2+} (3×10^{-5}–3×10^{-3} M) produced concentration-dependent contractions, which were inhibited partially by pretreatment with clentiazem (10^{-7} M) in all arterial segments tested. However, addition of Ca^{2+} produced relaxation in the presence of a high concentration of clentiazem (10^{-6} M) in coronary but not mesenteric or renal arteries. The maximum relaxation was obtained at 10^{-3} M Ca^{2+}.

Concentration-response curves to Ca^{2+} (3×10^{-5}–3×10^{-3} M) in K^+-depolarized canine coronary arteries in the presence of clentiazem (10^{-6} M), diltiazem (3×10^{-6} M), verapamil (3×10^{-6} M) and nifedipine (3×10^{-8} M) are shown in Figure 2. Maximum relaxations were observed at a concentration of 10^{-3} M Ca^{2+}, and reversed at concentrations of 3×10^{-3} Ca^{2+} or more.

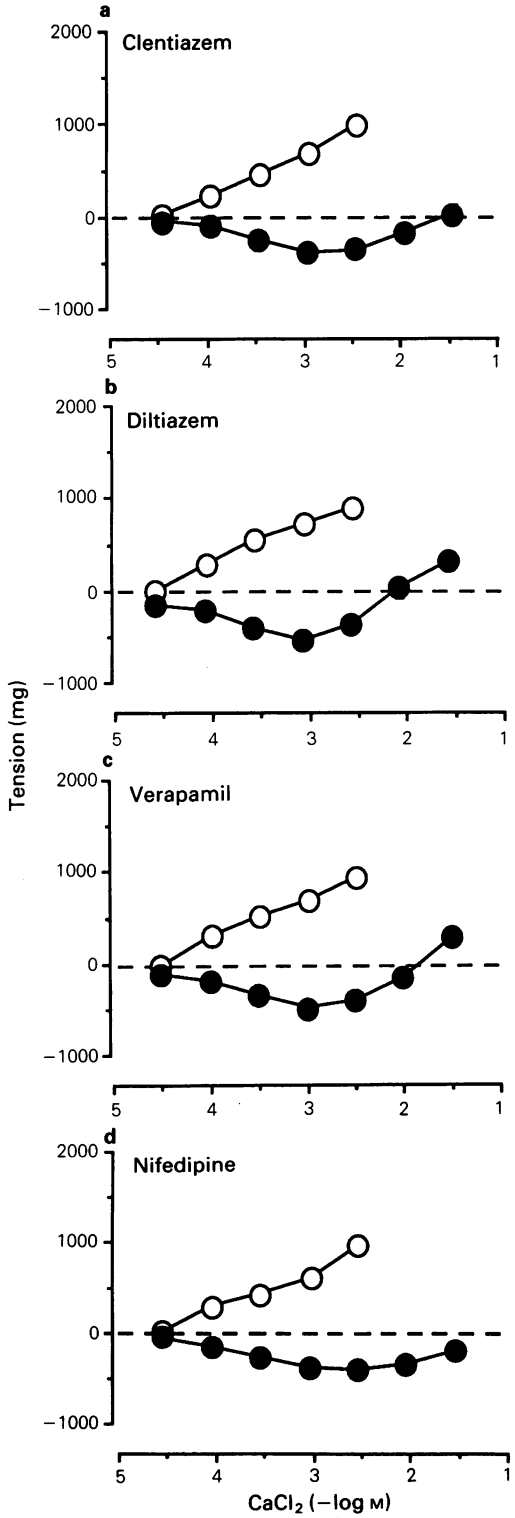
Removal of endothelium

Figure 3 shows that the maximum contraction induced by Ca^{2+} in K^+-depolarized canine coronary arteries was enhanced about 3 fold by removal of the endothelium, but the threshold concentration for Ca^{2+} did not change. Ca^{2+}-induced relaxation in the presence of clentiazem (10^{-6} M) was almost abolished by removal of the endothelium.

Actions of indomethacin, oxyhaemoglobin and methylene blue

Contractions obtained after addition of Ca^{2+} (3×10^{-5}–3×10^{-3} M) to K^+-depolarized coronary arteries were unaffected by treatment with indomethacin (5×10^{-6} M). The relaxations obtained to Ca^{2+} in the presence of clentiazem (10^{-6} M) were also unaffected by indomethacin (data not shown).

Figure 4 shows typical tracings of the effects of oxyhaemoglobin (1.5×10^{-6} M) on the Ca^{2+}-induced responses in K^+-depolarized coronary artery in the presence and absence of clentiazem (10^{-6} M). Oxyhaemoglobin had no effect on basal tone but the maximum contraction induced by Ca^{2+} was almost doubled (1.5 ± 0.12 g vs 2.74 ± 0.3 g, $n = 6$) and occurred without any change in the threshold concentration for Ca^{2+}. Ca^{2+}-induced relaxation in the presence of clentiazem (10^{-6} M) was, however, almost abolished by haemoglobin (Figure 4). Oxyhaemoglobin did not enhance Ca^{2+}-induced



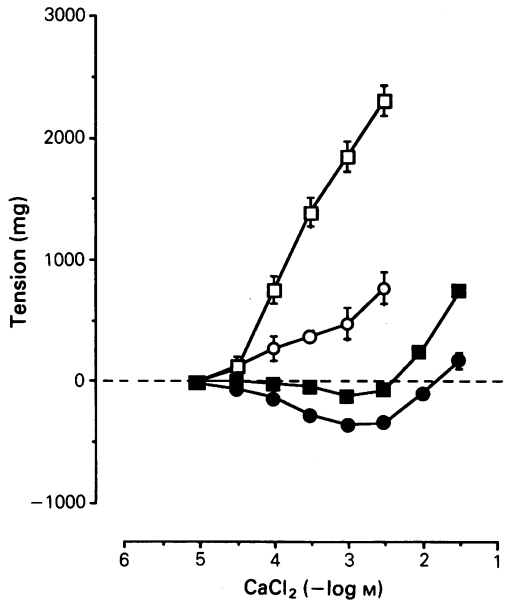


Figure 3 Effect of removal of endothelium on Ca^{2+}-induced responses in the presence (●, endothelium-containing, ■, endothelium-denuded) and absence (○, endothelium-containing, □, endothelium-denuded) of clentiazem in K^+ (80 mM)-depolarized coronary arteries of the dog. Clentiazem (10^{-6} M) was added to the preparation 10 min before the cumulative administration of Ca^{2+}. Responses are expressed as absolute values of developed tension (mg). Each point represents the mean of 6 observations and vertical bars indicate s.e.mean. Some s.e.mean bars are contained within the symbols. Broken lines indicate resting tension.

contractions in rubbed coronary artery segments (Figure 4).

Methylene blue (10^{-5} M) abolished the relaxation induced by Ca^{2+} (10^{-3} M) in K^+-depolarized, clentiazem (10^{-6} M)-treated coronary artery segments (Figure 5).

Figure 2 Effect of Ca^{2+}-antagonists (○, untreated group, ●, treated group) on the concentration-response curves for Ca^{2+} in K^+ (80 mM)-depolarized coronary arteries of the dog. (a) Clentiazem (10^{-6} M), (b) diltiazem (3×10^{-6} M), (c) verapamil (3×10^{-6} M) and (d) nifedipine (3×10^{-8} M) were added to the preparations 10 min before the cumulative administration of Ca^{2+}. Responses are expressed as absolute values of developed tension (mg). Each point represents the mean of 6 observations. All s.e.mean bars are contained within the symbols. Broken lines indicate resting tension.

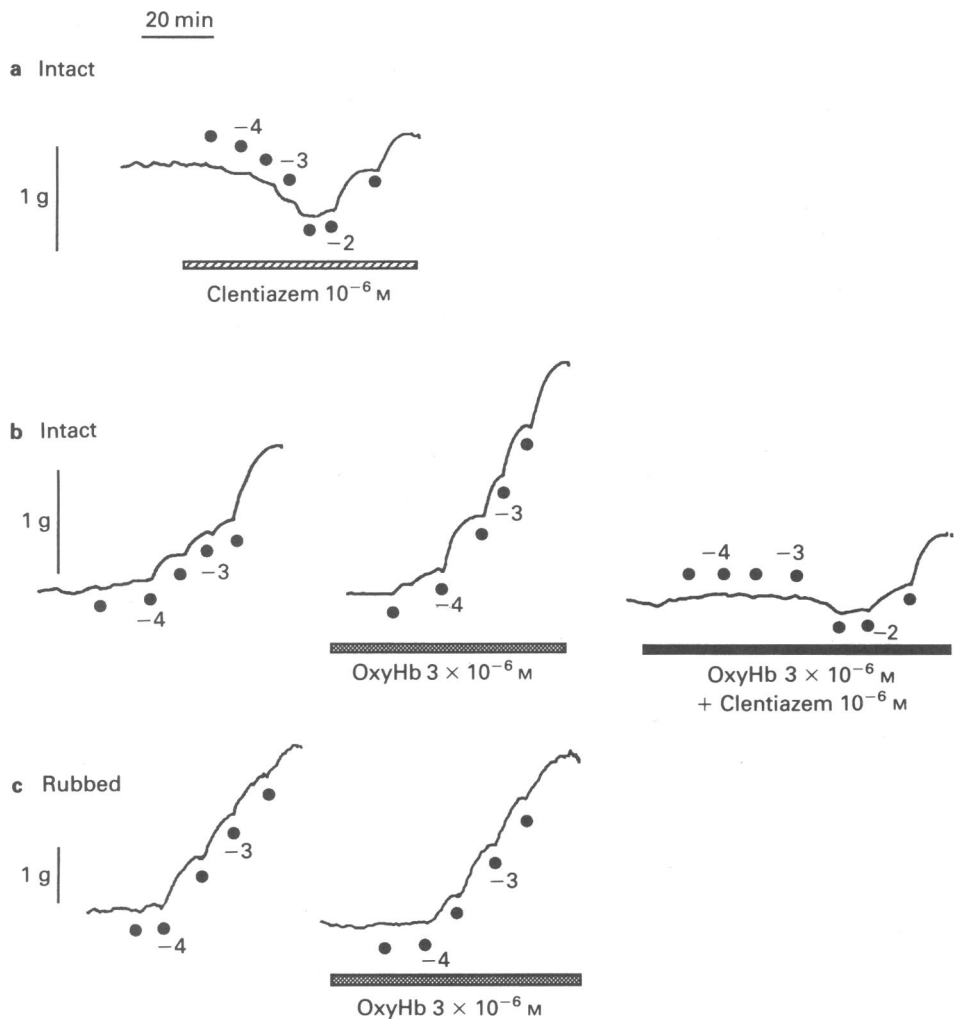


Figure 4 Typical tracings showing the effects of oxyhaemoglobin (OxyHb) on the Ca^{2+}-induced responses in the presence and absence of clentiazem in K^+ (80 mM)-depolarized coronary arteries of the dog. Clentiazem (10^{-6} M) and oxyhaemoglobin (1.5×10^{-6} M) were added 10 and 15 min before cumulative administration of Ca^{2+}, respectively. Ca^{2+} (3×10^{-5}–3×10^{-2} M) was added at the points indicated, in incremental doses.

Sandwich preparation

Following contraction with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 2×10^{-6} M), acetylcholine (10^{-6} M) produced relaxations of about 50% in endothelium-denuded recipient rings of coronary artery in sandwich preparations with an endothelium-containing donor ring ($n = 5$, data not shown). After the addition of Ca^{2+} (3×10^{-5}–3×10^{-3} M), in the presence of clentiazem (10^{-6} M) endothelium-denuded segments relaxed to about 50% of that observed in endothelium-containing coronary segment (Figure 6a). When a mesenteric artery segment was inserted as a donor into the recipient coronary segments, similar results were obtained (Figure 6b).

Discussion

In a previous study, we examined the Ca^{2+}-antagonistic effect of a new 1,5-benzothiazepine derivative, clentiazem. In K^+-depolarized coronary arteries of the dog, we found that Ca^{2+} produced an unexpected reduction of basal tone in the presence of high concentrations of clentiazem (Kikkawa *et al.*,

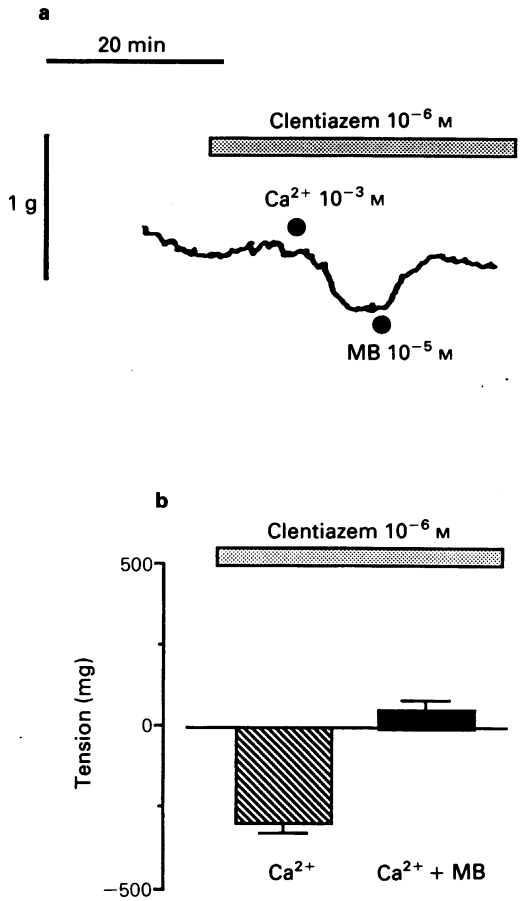


Figure 5 Effect of methylene blue on Ca^{2+}-induced relaxation in the presence of clentiazem (10^{-6} M) in K^+ (80 mM)-depolarized coronary arteries of the dog. (a) Typical tracing. Methylene blue (MB, 10^{-5} M) was added to the preparation during a Ca^{2+} (10^{-3} M)-induced relaxation. (b) Relaxant responses obtained on 5 preparations. Relaxations are expressed as absolute values of developed tension (mg). Vertical bars indicate the s.e.mean.

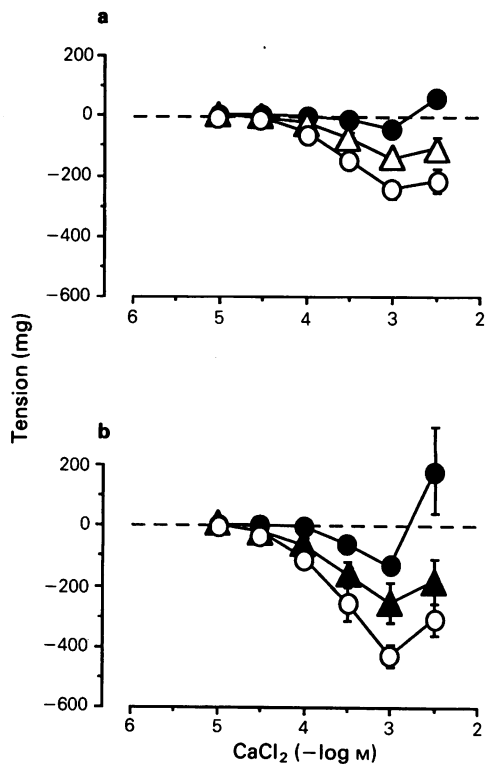


Figure 6 Reappearance of Ca^{2+}-induced relaxation in canine endothelium-denuded coronary segments when apposed to endothelium-containing coronary (a) or mesenteric arterial segments (b) in the presence of clentiazem (10^{-6} M). Ca^{2+} (10^{-5}–3×10^{-3} M) was added to endothelium-containing (○) and endothelium-denuded (●) coronary segments and to sandwich preparations (△, coronary, ▲, mesentery, as donors of EDRF). Responses are expressed as absolute values of developed tension (mg). Each point represents the mean of 6 observations and vertical bars indicate the s.e.mean. Some s.e.mean bars are within the symbols. Broken lines indicate resting tension.

1988). We therefore studied further the mechanisms underlying the action of this Ca^{2+}-induced relaxation.

As with clentiazem, Ca^{2+}-induced relaxation was observed in coronary arteries in the presence of high concentrations of diltiazem, verapamil and nifedipine. It is clear that the Ca^{2+}-induced relaxations are seen with all Ca^{2+}-antagonists. Ca^{2+}-induced relaxation was found to be abolished by removal of endothelium.

It is known that endothelial cells modulate vascular tone by releasing vasodilator factors such as prostacyclin (PGI_2) and EDRF. Therefore, we examined the effect of indomethacin, which inhibits the production of PGI_2, oxyhaemoglobin, which inactivates EDRF (Martin *et al.*, 1985b) and methylene blue, which blocks the soluble guanylate cyclase of smooth muscle, to determine which endothelium-derived substance was responsible for the relaxation seen. Ca^{2+}-induced relaxation was inhibited by treatment wiht oxyhaemoglobin or methylene blue but was not affected by indomethacin. Furthermore, Ca^{2+}-induced relaxation in the presence of clentiazem was reproduced in sandwich preparations in which an intact coronary was inserted into rubbed

coronary segments as a donor. These results suggest that the substance released by Ca^{2+} was EDRF.

EDRFs have been shown to be composed of at least two factors: nitric oxide and an unknown hyperpolarizing factor (Feletou & Vanhoutte, 1988; Komori *et al.*, 1988). The Ca^{2+}-induced relaxation observed in this study is probably due to nitric oxide because K^+-depolarized preparations were used.

We propose that under K^+-depolarized conditions, addition of Ca^{2+} triggers both contraction of smooth muscle and release of EDRF. In the presence of high concentrations of Ca^{2+} antagonists, contraction is reversed to relaxation. It is possible that relaxation results from the ability of Ca^{2+} antagonists to inhibit Ca^{2+} entry into smooth muscle but not endothelium (Jayakody *et al.*, 1987).

It is interesting that Ca^{2+}-induced relaxation was observed in canine coronary but not in mesenteric or renal arteries. This tissue difference could be due to a difference in sensitivity of smooth muscle to EDRF, or to different amounts of EDRF released. However, our study showed that Ca^{2+} caused a relaxation in sandwich preparations when intact mesenteric artery as well as intact coronary artery was used as a donor of EDRF. Therefore, the tissue difference may be due to variations in the sensitivity of smooth muscle to EDRF rather than to a difference in the amount of EDRF released and/or synthesized.

In conclusion, the present study suggests that Ca^{2+}-antagonists inhibit vascular contraction in response to K^+-depolarization without affecting the release and/or synthesis of EDRF. These data may underly the clinical utility of Ca^{2+}-antagonists in the treatment of coronary vasospasm.

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Modulation of neurotransmission in the guinea-pig vas deferens by capsaicin: involvement of calcitonin gene-related peptide and substance P

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1 The effects of capsaicin, calcitonin gene-related peptide and substance P were studied via three parameters in the guinea-pig vas deferens: the overflow of ATP and of tritiated noradrenaline, the mechanical responses to field stimulation and the mechanical responses to exogenous noradrenaline and α,β-methylene ATP.

2 At 2 Hz, capsaicin inhibited the stimulus-evoked release of ATP, whereas it was without effect on the release of noradrenaline. At 20 Hz capsaicin did not affect the release of either of the co-transmitters. Capsaicin enhanced responses to α,β-methylene ATP, but not to exogenous noradrenaline.

3 Calcitonin gene-related peptide, like capsaicin, inhibited the release of ATP, but not noradrenaline at 2 Hz and was without effect on release at 20 Hz. However, calcitonin gene-related peptide inhibited responses to α,β-methylene ATP and was without effect on responses to exogenous noradrenaline.

4 Substance P had no effect on the release of either noradrenaline or ATP at either frequency. However, like capsaicin it enhanced responses to α,β-methylene ATP and was without effect on exogenous noradrenaline.

5 These results suggest that the actions of capsaicin on the guinea-pig isolated vas deferens are mediated via the release of both calcitonin gene-related peptide and substance P. Furthermore, as capsaicin and calcitonin gene-related peptide prejunctionally modulate purinergic, but not noradrenergic transmission, this suggests that the mechanisms for the storage and release of the sympathetic co-transmitters noradrenaline and ATP may not be the same.

Introduction

Capsaicin, the pungent ingredient of red peppers, is known to degenerate a certain population of primary sensory nerves (Nagy, 1982; Szolcsányi, 1982; 1984). This degeneration is paralleled by the release of calcitonin gene-related peptide (CGRP), substance P (SP) and related tachykinins (Gamse *et al.*, 1981; Lee *et al.*, 1985; Lundberg *et al.*, 1985a,b; Saria *et al.*, 1985; Skofitsch & Jacobowitz, 1985). By examining the effects of capsaicin and comparing them to responses obtained from the exogenous addition of SP and CGRP, studies have been able to elucidate which peptide is responsible for mediating the effects of capsaicin. For example, SP has been implicated as the mediator of the capsaicin-induced contraction of the guinea-pig isolated trachea (Szolcsányi & Barthó, 1982) and ileum (Szolcsányi & Barthó, 1978; Barthó & Szolcsányi, 1979), the rat urinary bladder (Maggi *et al.*, 1984; 1985) and iris sphincter muscle (Banno *et al.*, 1985), whereas CGRP has been shown to mediate the effects of capsaicin in several tissues including the guinea-pig atrium (Franco-Cereceda & Lundberg, 1985) and the rat duodenum (Maggi *et al.*, 1986).

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More recently, studies on the effects of capsaicin on the rat vas deferens have been carried out (Maggi *et al.*, 1987; Saito *et al.*, 1987; Moritoki *et al.*, 1987). The first two studies showed an inhibition of responses to electrical field stimulation by capsaicin, whereas the latter study demonstrated an augmentation by capsaicin at concentrations employed by the first two studies, although an inhibition was seen at the highest concentration of capsaicin used. As similar procedures were used in each of the studies it is not immediately clear why the results differ.

The guinea-pig vas deferens responds to electrical field stimulation with an initial twitch response followed by a slow second component (Swedin, 1971). The initial twitch is purinergic in nature being mediated by adenosine 5'-triphosphate (ATP) (Westfall *et al.*, 1978; Fedan *et al.*, 1981; Meldrum & Burnstock, 1983; Sneddon & Burnstock, 1984; Sneddon & Westfall, 1984; Stjärne & Åstrand, 1985), whereas noradrenaline (NA) mediates the sustained second phase (Swedin, 1971; McGrath, 1978). Recently, a technique has been devised to allow the simultaneous measurement of ATP and NA release in the guinea-pig vas deferens (Kasakov *et al.*, 1988). This technique enables the pre- and postjunctional effects of neuromodulators in this tissue to be studied directly.

The purpose of this study was to determine the effects of capsaicin, CGRP and SP on noradrenergic and purinergic transmission in order to ascertain, if possible, which neuropeptide(s) was mediating the effects of capsaicin.

Methods

General

Vasa deferentia of male guinea-pigs (250–400 g) were used. Preparation of the tissues and the measurement of [^{3}H]-NA and ATP overflow was as previously described by Ellis & Burnstock (1989). During the experiment which lasted 52 min the preparations were stimulated electrically twice in the absence of uptake inhibitors, at 9 and 45 min for 240 pulses with trains of rectangular pulses of 0.1 ms duration, 50 V, and either at 2 or 20 Hz delivered by Grass SD9 stimulators. Perfusate was collected for periods corresponding to the length of the stimulus train. Therefore at 2 Hz the collection period was 120 s, whereas at 20 Hz the collection period was 12 s. Samples were collected for the first 16 min, the period 26–30 min and the period 40–52 min. For the periods when samples were not collected a linear relationship for tritium efflux was assumed. Results are expressed as the ratio of responses to the second stimulation (S_2) versus responses to the first stimulation (S_1), that is the S_2/S_1 ratio. The S_2/S_1 ratio for ATP release was calculated from the absolute value of release and for NA release from the fractional value of release. Control values for S_2/S_1 ratios were obtained when the second stimulation was carried out in the absence of any drug. Experimental data were obtained by the superfusion of the preparation with modified Krebs solution, which contained either capsaicin, CGRP or SP, 10 min before the second stimulation. This superfusion continued until the end of the experiment. Tissues from different animals were used to assess the effects of these drugs.

Analysis of results

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

Drugs

Capsaicin, NA, α,β-methylene ATP (α,β-mATP) and luciferin-luciferase were obtained from Sigma. Noradrenaline-(−)[ring,2,5,6-^{3}H] (specific activity 1624.3 GBq mmol^{-1}) was obtained from New England Nuclear. SP and CGRP were obtained from Cambridge Research Biochemicals Ltd. SP, CGRP and α,β-mATP were first prepared as stock solutions in distilled water. Capsaicin was made up in absolute ethanol at an original concentration of 0.1 M. NA was prepared in a 0.4% ascorbic acid solution to prevent oxidation. Luciferin-luciferase was prepared in sterile water.

Results

Field stimulation

'No drugs' responses The contractile response of the vas deferens to electrical field stimulation at 2 Hz (stimulation period lasting 2 min) exhibited two distinct phases: an initial twitch response which reached its maximum (0.86 ± 0.08 g, $n = 6$) in 2–3 s and then faded rapidly and a second slow phase which reached its maximum (0.70 ± 0.10 g, $n = 6$) in 10–15 s and whose amplitude slowly decreased, but

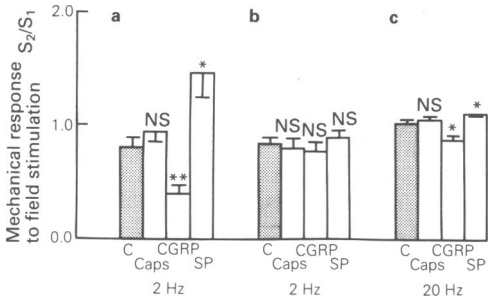


Figure 1 Effects of capsaicin (Caps), calcitonin gene-related peptide (CGRP) and substance P (SP) on the mechanical response of the guinea-pig vas deferens to electrical field stimulation. Alterations in the mechanical responses are shown by comparing the control S_2/S_1 ratio when no drug is present throughout the experiment to the S_2/S_1 ratio obtained when the drug is added to the superfusate 10 min before the second stimulation. (a) The purinergic response at 2 Hz in the presence of Caps (1 μM), CGRP (10 nM) and SP (1 μM) compared to control (C, stippled columns). (b) The noradrenergic response at 2 Hz in the presence of Caps (1 μM), CGRP (10 nM) and SP (1 μM) compared to control. (c) The total response at 20 Hz in the presence of Caps (1 μM), CGRP (10 nM) and SP (1 μM) compared to control. Each column is the mean of at least six observations and vertical lines indicate s.e.mean. Tests for the significance of drug effects were made by use of Student's t test for unpaired observations (NS, not significant; $^*P < 0.05$, $^{**}P < 0.01$).

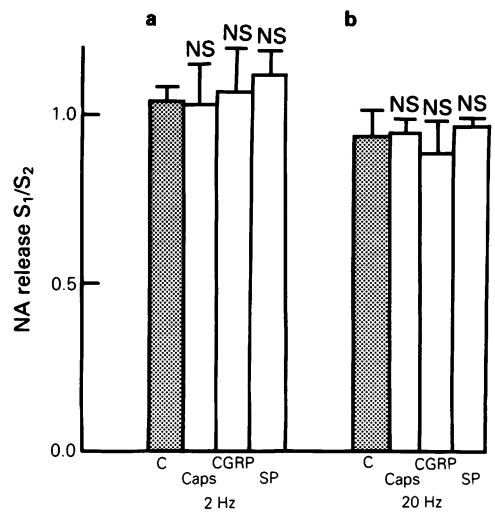


Figure 2 Effects of capsaicin (Caps), calcitonin gene-related peptide (CGRP) and substance P (SP) on the overflow of [^{3}H]-NA from the guinea-pig vas deferens induced by electrical field stimulation. Alterations in the overflow of [^{3}H]-NA are shown by comparing the control S_2/S_1 ratio when no drug is present throughout the experiment to the S_2/S_1 ratio obtained when the drug is added to the superfusate 10 min before the second stimulation. (a) Release at 2 Hz in the presence of Caps (1 μM), CGRP (10 nM) and SP (1 μM) compared to control (C, stippled columns). (b) Release at 20 Hz in the presence of Caps (1 μM), CGRP (10 nM) and SP (1 μM) compared to control. Each column is the mean of at least six observations and vertical lines indicate s.e.mean. Tests for the significance of drug effects were made by use of Student's t test for unpaired observations (NS, not significant).

which lasted for the duration of the stimulation period. The second stimulation period 30 min later, exhibited similar contractions but both phases were slightly reduced in amplitude (S_2/S_1 ratio for 1st phase was 0.81 ± 0.09, 2nd phase was 0.84 ± 0.06, $n = 6$) (Figure 1). During both stimulation periods there was a marked rise in [^{3}H]-NA and ATP release over basal levels. Levels for the first stimulation period were 1.61 ± 0.14 and 1.95 ± 0.17 ($n = 6$) times basal efflux for [^{3}H]-NA and ATP release respectively. [^{3}H]-NA release differed little between the two stimulation periods (S_2/S_1 ratio was 1.04 ± 0.04, $n = 8$) (Figure 2). However, ATP release during the second phase was reduced (S_2/S_1 ratio was 0.49 ± 0.08, $n = 6$) (Figure 3).

At 20 Hz (stimulation period lasting 12 s) the distinction between the two phases was not clear, therefore mechanical responses are given as the overall magnitude of the response (2.38 ± 0.12 g, n = 6) and not divided into two distinct phases. The magnitude of the response elicited by the second stimulation was unchanged (S_2/S_1 ratio was 1.02 ± 0.03, $n = 6$) (Figure 1). Again, during both stimulation periods there was a marked rise in [^{3}H]-NA and ATP release over basal levels. Levels for the first stimulation period were 13.22 ± 1.82 and 6.30 ± 1.11 ($n = 6$) times basal efflux for [^{3}H]-NA and ATP release respectively. Thus release of [^{3}H]-NA over basal levels was approximately eight times higher at 20 than at 2 Hz and similarly, ATP release was approximately three times higher. [^{3}H]-NA release elicited by stimulation was unchanged during the second stimulation (S_2/S_1 ratio was 0.94 ± 0.08, $n = 6$) (Figure 2). ATP release was only slightly diminished during the second stimulation (S_2/S_1 ratio was 0.79 ± 0.10, $n = 9$) (Figure 3).

Capsaicin The addition of capsaicin (1 μM) to the superfusate 10 min before the second stimulation was without effect on basal efflux of either [^{3}H]-NA or ATP, although it produced a small increase (50 mg) in the baseline tension in 3 out of 18 preparations. At 2 Hz capsaicin was without significant effect on either phase of the mechanical response. The S_2/S_1

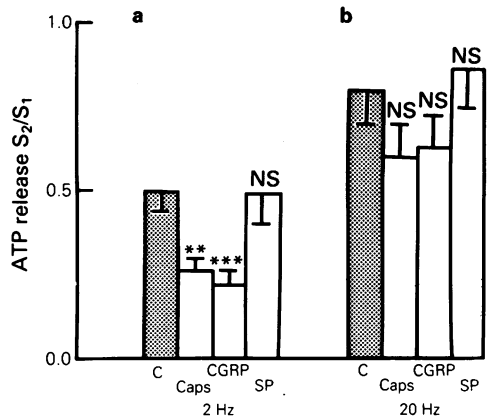


Figure 3 Effects of capsaicin (Caps), calcitonin gene-related peptide (CGRP) and substance P (SP) on the overflow of ATP from the guinea-pig vas deferens induced by electrical field stimulation. Alterations in the overflow of ATP are shown by comparing the control S_2/S_1 ratio when no drug is present throughout the experiment to the S_2/S_1 ratio obtained when the drug is added to the superfusate 10 min before the second stimulation. (a) Release at 2 Hz in the presence of Caps (1 μM), CGRP (10 nM) and SP (1 μM) compared to control (C, stippled columns). (b) Release at 20 Hz in the presence of Caps (1 μM), CGRP (10 nM) and SP (1 μM) compared to control. Each column is the mean of at least six observations and vertical lines indicate s.e.mean. Tests for the significance of drug effects were made by use of Student's t test for unpaired observations (NS, not significant; $^{**}P < 0.01$, $^{***}P < 0.001$).

ratio for the purinergic phase of the response was 0.95 ± 0.09 ($n = 10$) (Figure 1) and for the noradrenergic phase it was 0.80 ± 0.09 ($n = 8$) (Figure 1). At this frequency capsaicin inhibited the stimulus-evoked release of ATP (S_2/S_1 ratio was 0.26 ± 0.04, $n = 6$) (Figure 3), whereas it was without effect on the release of [^{3}H]-NA (S_2/S_1 ratio was 1.03 ± 0.12, $n = 6$) (Figure 2). At 20 Hz capsaicin had no effect on the overall magnitude of the response (S_2/S_1 ratio was 1.06 ± 0.02, $n = 9$) (Figure 1). At this frequency capsaicin still had no effect on the release of [^{3}H]-NA (S_2/S_1 ratio was 0.95 ± 0.04, $n = 9$) (Figure 2), nor did it significantly inhibit ATP release (S_2/S_1 ratio was 0.59 ± 0.10, $n = 10$) (Figure 3).

CGRP CGRP (10 nM) added 10 min before the second stimulation was without effect on basal efflux of either [^{3}H]-NA or ATP and had no effect on the baseline tension of the tissue. At 2 Hz, CGRP inhibited the purinergic phase of the response (S_2/S_1 ratio was 0.39 ± 0.08, $n = 6$) (Figure 1), whereas it was without effect on the noradrenergic phase (S_2/S_1 ratio was 0.78 ± 0.07, $n = 6$) (Figure 1). At this frequency CGRP inhibited ATP release (S_2/S_1 ratio was 0.22 ± 0.04, $n = 6$) (Figure 3), whereas, it had no significant effect on [^{3}H]-NA release (S_2/S_1 ratio was 1.07 ± 0.13, $n = 6$) (Figure 2). At 20 Hz, CGRP slightly inhibited the overall magnitude of the response (S_2/S_1 ratio was 0.88 ± 0.03 $n = 12$) (Figure 1). At this frequency it no longer inhibited the release of ATP (S_2/S_1 ratio was 0.62 ± 0.10, $n = 6$) (Figure 3) and was still without effect on [^{3}H]-NA release (S_2/S_1 ratio was 0.89 ± 0.10, $n = 7$) (Figure 2).

Substance P SP (1 μM) added to the superfusate 10 min before the second stimulation was without effect on basal efflux of either [^{3}H]-NA or ATP, although it produced a small rise in baseline tension of about 50 mg in 4 out of 12 preparations. At 2 Hz, SP had no effect on the noradrenergic phase of the response (S_2/S_1 ratio was 0.90 ± 0.06, $n = 6$) (Figure 1), whereas it enhanced the purinergic phase (S_2/S_1 ratio was 1.46 ± 0.22, $n = 6$) (Figure 1). However, at 2 Hz SP was without effect on either ATP release (S_2/S_1 ratio was 0.49 ± 0.09, $n = 6$) (Figure 3), or on [^{3}H]-NA release (S_2/S_1 ratio was 1.12 ± 0.07, $n = 6$) (Figure 2). SP at 20 Hz enhanced the overall magnitude of the response (S_2/S_1 ratio was 1.11 ± 0.02, $n = 6$) (Figure 1), but still had no effect on either ATP release (S_2/S_1 ratio was 0.86 ± 0.12, $n = 6$) (Figure 3), or on [^{3}H]-NA release (S_2/S_1 ratio was 0.98 ± 0.02, $n = 6$) (Figure 2).

Pharmacological responses

Responses to exogenously added NA (30 μM) (1.89 ± 0.20 g, $n = 6$) and α,β-mATP (1 μM) (1.78 ± 0.07 g, $n = 6$) did not show tachyphylaxis, provided that once the tissues had reached their maximum response they were washed immediately. Capsaicin (1 μM) added 10 min before the addition of α,β-mATP produced an increase in the response to a value $125 \pm 6\%$ ($n = 7$) (Figure 4) of the control level, whereas it did not alter responses to NA; value was $106 \pm 6\%$ compared to control ($n = 6$) (Figure 4). CGRP (10 nM) inhibited the response to α,β-mATP to $37 \pm 4\%$ of the control value ($n = 7$) (Figure 4), whereas it had no effect on responses to NA; value was $95 \pm 9\%$ compared to control ($n = 8$) (Figure 4). SP (1 μM) produced a large increase in the response to α,β-mATP, to $272 \pm 34\%$ of the control value ($n = 7$) (Figure 4), whereas it had no effect on responses to NA; value was $103 \pm 13\%$ compared to control ($n = 7$) (Figure 4).

Discussion

The biphasic nature of the response and the concomitant release of both [^{3}H]-NA and endogenous

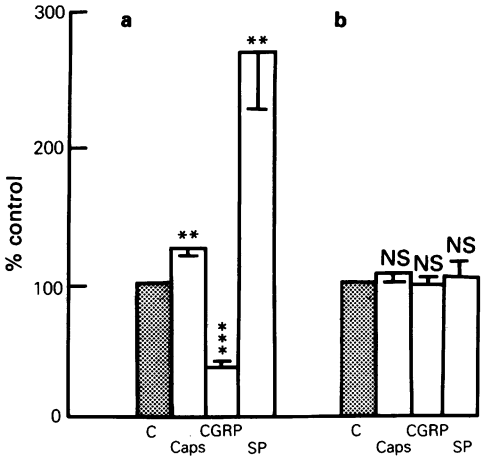


Figure 4 Effects of capsaicin (Caps), calcitonin gene-related peptide (CGRP) and substance P (SP) on the response of guinea-pig isolated vas deferens to exogenous α,β-methylene ATP (α,β-mATP) and NA. Responses to drugs are compared to the control value (C, stippled columns). (a) Responses to α,β-mATP in the presence of Caps (1 μM), CGRP (10 nM) and SP (1 μM). (b) Responses to NA in the presence of Caps (1 μM), CGRP (10 nM) and SP (1 μM). Each column is the mean of at least six observations and vertical lines indicate s.e.mean. Tests for the significance of drug effects were made by use of Student's t test for paired observations (NS, not significant; $^{**}P < 0.01$, $^{***}P < 0.001$).

ATP confirms that the motor response to field stimulation in this tissue is due to NA and ATP acting as co-transmitters (see Burnstock, 1982; 1985).

The release of both CGRP and SP have been suggested to mediate the effects of capsaicin in a variety of tissues. In the guinea-pig isolated ileal longitudinal muscle, SP has been shown to mediate the initial contraction and CGRP the inhibition of responses to field stimulation seen upon application of capsaicin (Maggi *et al.*, 1988a). Similarly, in the guinea-pig urinary bladder, SP was found to mediate contractions and CGRP the inhibition of contractions induced by field stimulation seen upon addition of capsaicin (Maggi *et al.*, 1988b). Our present findings suggest that capsaicin is also able to release CGRP and SP in the guinea-pig vas deferens and that the effects of capsaicin on sympathetic neurotransmission in this tissue are mediated via the release of these two neuropeptides. The inhibition of ATP release at 2 Hz by CGRP closely mimics the action of capsaicin. On the other hand, it is unlikely that the inhibition of ATP release produced by capsaicin is due to the release of SP, as SP is without effect on ATP release. However, the difference between the purinergic mechanical response at 2 Hz in the presence of capsaicin and that for CGRP can be explained by the concomitant release of CGRP and SP. CGRP prejunctionally inhibits ATP release and postjunctionally inhibits the action of ATP at its receptors, whereas in antagonism to this, SP, while not having an action prejunctionally, substantially enhances the action of ATP on its receptors, as seen by the enhancement of contractile responses to α,β-mATP. The lack of effect of capsaicin on the mechanical response at 20 Hz is probably due again to the opposing actions of CGRP and SP postjunctionally on ATP receptors. This is borne out by the actions of capsaicin, CGRP and SP on the pharmacological responses to α,β-mATP. Capsaicin slightly enhances responses to α,β-mATP and CGRP inhibits responses, whereas SP produces a greater than two fold increase in the response to α,β-mATP.

Evidence for capsaicin-induced release of SP also comes from the effect of these two substances in producing small contractions in some of the tissues. The very small size of the contractions, and the infrequency with which they occurred, is probably due to the concentrations of these drugs used being close to the threshold for direct activation of the smooth muscle.

That capsaicin is without action on the noradrenergic mechanical response at 2 Hz is due to the complete lack of effect of CGRP and SP both pre- and postjunctionally on noradrenergic transmission.

Similar studies in the rat vas deferens have produced opposing results (Maggi *et al.*, 1987; Moritoki *et al.*, 1987; Saito *et al.*, 1987). Maggi *et al.* (1987) found that capsaicin and CGRP produced an inhibition of twitch responses to field stimulation, whereas SP potentiated nerve-mediated contractions. From this they concluded that the inhibition of motility of the rat vas deferens by capsaicin was due to the release of CGRP, which postjunctionally inhibited neurotransmission. Similarly, Saito *et al.* (1987) showed that capsaicin and CGRP inhibited the twitch response of the rat vas deferens. As neither CGRP nor capsaicin inhibited the amplitude of the excitatory junction potentials, they concluded that the effect of capsaicin was due to the release of CGRP, which acts directly on the smooth muscle, and not prejunctionally, to inhibit transmission. However, in disagreement with these studies, Moritoki *et al.* (1987) found that capsaicin enhanced the non-adrenergic twitch response of the rat vas deferens. This study also showed that SP augmented the twitch response, whereas CGRP inhibited this response. It is not clear why these results differ, as similar stimulation parameters were used. However, what these studies do indicate is that in the rat vas deferens, similar to this study, the actions of capsaicin may be mediated by the release of CGRP and possibly SP. Our present study also shows that

CGRP may act prejunctionally to inhibit the release of ATP, while SP may act postjunctionally to enhance responses to ATP.

Of considerable interest is the action of CGRP and capsaicin in inhibiting the release of ATP at 2 Hz, but not having an effect on NA release at this frequency. That CGRP and capsaicin are able to modulate the release of ATP, while not having an effect on NA release, raises questions as to whether these two co-transmitters share the same storage and/or release mechanisms in this tissue. There is increasing evidence in a number of species that the release of ATP and NA from the vas deferens can be differentially affected by neuromodulators. For example, in the guinea-pig vas deferens it has been shown that angiotensin III inhibits the release of endogenous ATP at 2 Hz, whereas it enhances the overflow of [^{3}H]-NA at this frequency (Ellis & Burnstock, 1989). Similarly, in the rabbit vas deferens, angiotensins II and III were shown to potentiate noradrenergic transmission, whereas they inhibited purinergic transmission (Trachte, 1988). Also in the rabbit, prostaglandin E_2 almost abolishes purinergic transmission at 6 Hz, whereas it only partially inhibits noradrenergic transmission at this frequency (Trachte, 1985). The observation in the present study that neuromodulation of ATP release by CGRP and capsaicin is frequency-dependent is in agreement with a previous study in this tissue which demonstrated that the modulation of ATP release by angiotensins II and III was not evident at high frequency (Ellis & Burnstock, 1989).

If the release of these two peptides can occur in differing ratios, this would allow dual control of purinergic transmission, with transmission being able to be enhanced or inhibited depending on the needs of the tissue. This study also provides further evidence that purinergic and noradrenergic transmission may be under separate control mechanisms.

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MEETINGS CALENDAR

22–25 October 1989

Molecular and Cellular Biology of IL-1, TNF and Lipocortins in Inflammation and Differentiation. Siena, Italy. (L. Parente, Sclavo Research Centre, via Florentina 1, 53100 Siena, Italy).

8–11 November 1989

Tenth International Symposium on Drugs affecting Metabolism, Houston, USA. (Meeting Manager, 4550 Post Oak Place, Suite 248, Houston, Texas 77027, U.S.A.)

27–29 November 1989

Biological Actions of Extracellular ATP, Philadelphia, USA. (Conference Dept., New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021, U.S.A.)

4–5 December 1989

Sensory Nerves and Neuropeptides in Gastroenterology, Florence, Italy. (Fondazione Internazionale Menarini, Piazza del Carmino, 4-20121 Milan, Italy).

6–8 December 1989

Presynaptic Receptors — an Examination of Different Views, New York City, USA. (Conference Department, New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021, U.S.A.)

10–14 December 1989

Sino-Brazilian Symposium on Chemistry and Pharmacology of Natural Products, Rio de Janeiro, Brazil. (Secretariat, Fundacao Oswaldo Cruz, Departmento de Farmacodinamica, Aremida, Brasil, 6365, Rio de Janeiro, Brazil).

3–5 January 1990

British Pharmacological Society Winter Meeting. London, U.K. (Closed meeting for members and guests)

7 February 1990

Royal Society of Chemistry, Free Radicals in Biotechnology and Medicine, London, U.K. (Miss P.E. Hutchinson, The Analytical Division, Royal Society of Chemistry, Burlington House, London W1V 0BN).

26–28 March 1990

Brain Research Association National Meeting, Bristol, U.K. (Dr. I.C. Kilpatrick, Dept. of Pharmacology, The Medical School, University Walk, Bristol BS8 1TD).

1–6 April 1990

6th World Congress on Pain, Adelaide, Australia. (Int. Association for the Study of Pain, 909 NE 43rd Street, Suite 306, Seattle, WA 98105, U.S.A.)

2–4 April 1990

Central and Peripheral Significance of Neuropeptide Y and its Related Peptides, Baltimore, U.S.A. (Conference Department, New York Academy of Sciences, 2 East 63rd Street, New York, NY 10021, U.S.A.).

4–6 April 1990

New Concepts in Anxiety, Castres, France. (Martin Dehaye, Centre de Recherche Pierre Fabre, 17 avenue Jean Moulin, 81100 Castres, France).

18–20 April 1990

British Pharmacological Society Spring Meeting. Sheffield, U.K. (Closed meeting for members and guests)

27–30 May 1990

Heterocycles in Bio-organic Chemistry, Solbacka, Sweden (Secretariat, PO Box 1136, S-11181 Stockholm, Sweden).

29 May–1 June 1990

Fondazione Giovanni Lorenzini Conference on Prostaglandins and Related Compounds, Florence, Italy (Fondazione Giovanni Lorenzini, Via Monte Napoleone, 23, 20121 Milan, Italy).

28–30 June 1990

IUPHAR Satellite — Mediators in Airway Hyperreactivity, The Hague, The Netherlands. (F.P. Nimkamp, Department of Pharmacology, PO Box 80176, 3508 TD Utrecht, Netherlands).

30 June–4 July 1990

6th Southeast Asian/W. Pacific Meeting of Pharmacologists, Hong Kong. (Secretary, Department of Pharmacology, Faculty of Medicine, University of Hong Kong, 5 Sassoon Road, Hong Kong).

27–30 June 1990

International Society for Heart Research XI European Section Meeting, Glasgow, UK. (Professor J.R. Parratt, Dept. of Physiology & Pharmacology, Royal College, University of Strathclyde, Glasgow G1 1XW).

1–6 July 1990

IUPHAR 11th International Congress of Pharmacology, Amsterdam, Netherlands (Contact to be advised).

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