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Characterization of the muscarinic receptor subtype mediating contractions of the guinea-pig uterus

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The muscarinic receptor present on the guinea-pig uterus has been characterized by use of both functional and radioligand binding studies. The contractile responses to carbachol were antagonized by atropine, pirenzepine, methoctramine and hexamethonium with pA_2 values consistent with the presence of an M_2 receptor (i.e. 9.5, 7.0, 8.0 and 3.7 respectively). In binding studies, p*Ki* values were also observed for pirenzepine, methoctramine and AF-DX 116 which were consistent with the presence of a homogeneous M_2 receptor population (i.e. 6.6, 7.9 and 7.1 respectively). The uterus therefore possesses M_2 receptors through which a functional contractile response is mediated and in this respect it is clearly different from other smooth muscles. M_2 receptors in the uterus mediate excitatory responses, an effect in contrast to their action in other tissues.

Introduction It has been proposed that muscarinic receptors exist in three subtypes, denoted as M_1, M_2 and M_3 (Mihm & Wetzel, 1987; Hammer *et al.*, 1987; Doods *et al.*, 1987). The M_1 subtype exhibits a high affinity toward pirenzepine and *o*-methoxy-silahexocyclium (see Eglen & Whiting, 1986 for review; Lambrecht *et al.*, 1988); the M_2 subtype exhibits a high affinity toward methoctramine (Michel & Whiting, 1988) and the M_3 subtype exhibits a high affinity toward hexahydrosiladiphenidol and silahexocyclium (Mutschler *et al.*, 1987). The anatomical location of these subtypes suggest that the cerebral cortex, heart and salivary gland possess a preponderance of M_1, M_2 and M_3 receptors respectively (Doods *et al.*, 1987).

Functional studies have shown that the muscarinic receptor subtype mediating negative inotropic responses differs from that mediating smooth muscle contraction (Eglen & Whiting, 1986). However, this distinction was not apparent in radioligand binding studies (e.g. Nilvebrandt & Sparf, 1988), until it was shown, by use of the selective M_2 antagonist methoctramine, that a heterogeneous population of receptors exists in the ileum (Giraldo *et al.*, 1987; Michel & Whiting, 1988). Approximately 28% of these receptors exhibit a pharmacology similar to the M_3 receptor, with the remaining 72% exhibiting a pharmacology identical to the M_2 receptor (Michel & Whiting, 1988). The contractile responses are mediated through the minor M_3 population, whilst the function of the major M_2 population remains unknown.

In the present study, we have characterized the muscarinic receptor population present in the guinea-pig isolated uterus. This preparation, in contrast to the ileum, possesses a homogeneous population of M_2 receptors and lacks either M_1 or M_3 receptors.

Methods All tissues were isolated from guinea-pigs (female, Dunkin-Hartley, 350 g) pretreated 18 h previously with 0.1 mg kg^{-1} i.p. diethylstilboestrol. Uterine horns were suspended under 1.0 g tension in Sund's physiological salt solution (mM: NaCl 154.0, KCl 5.63, $CaCl_2 6H_2O$ 0.54, $MgCl_2 7H_2O$ 0.95, $NaHCO_3$ 5.95, glucose 2.78) which was gassed with 5% CO_2 in oxygen. The preparations were maintained at 30°C, pH 7.4. After 60 min equilibration, cumulative concentration-response curves were constructed to carbachol with concentrations spaced at 0.5 log intervals. The tissues were washed and, following a further 60 min equilibration, a second curve was constructed in the presence of an antagonist. Only one concentration of antagonist was used on each piece of tissue. The equilibrium dissociation constant was estimated by determination of the pA_2 value (Arunlakshana & Schild, 1959).

Disodium edetate (EDTA) washed membrane fractions were prepared from guinea-pig uterine horns and from rat heart, cerebral cortex and submaxillary gland as described previously (Michel & Whiting, 1988). Binding assays were conducted as previously described (Michel & Whiting, 1988) using a modified Tris-Krebs solution of the following composition (mM): NaCl 144, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgCl_2$ 1.1, (+)-glucose 10, Tris 10: (pH 7.4, 37°C). Muscarinic receptors in uterine, cardiac and glandular preparations were labelled with 0.1 nM [^{3}H]-N-methylscopolamine (NMS) while rat cortical muscarinic receptors were labelled with 0.5 nM [^{3}H]-pirenzepine.

The following drugs were used: carbachol and hexamethonium (Sigma Chemical Co. Ltd), AF-DX 116 ((11[[2-[(diethylamino)methyl]-1-piperidinyl]-acetyl]-5,11-dihydro-6*H*-pyrido[2,3-b][1,4]benzo-

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diazepine 6-)one), methoctramine (Dr R. Clark, Syntex), pirenzepine (Boots plc): [^{3}H]-NMS and [^{3}H]-pirenzepine were obtained from New England Nuclear.

Results In functional studies, the isolated uterus exhibited a contractile response to carbachol ($-\log EC_{50}$ value = 6.83 ± 0.08, mean ± s.e.mean, $n = 8$), which was antagonized in a competitive fashion by atropine ($pA_2 = 9.51 \pm 0.03$, Schild slope = 1.01 ± 0.03, $n = 42$). The responses to carbachol were also antagonized by pirenzepine ($pA_2 = 7.00 \pm 0.02$, Schild slope = 0.99 ± 0.02, $n = 23$), methoctramine ($pA_2 = 8.0 \pm 0.05$, Schild slope = 0.68 ± 0.04, $n = 48$) and hexamethonium ($pA_2 = 3.63 \pm 0.08$, Schild slope = 0.72 ± 0.12, $n = 16$). The antagonism exhibited by methoctramine and hexamethonium was inconsistent with competitive antagonism, since the slope of the Schild plot was significantly ($P < 0.05$) different from unity. The pA_2 values for the above antagonists are consistent with data indicating the presence of an M_2 receptor. The contractile responses to carbachol were not significantly ($P > 0.05$) affected by the presence of tetrodotoxin (1 μM).

In receptor binding assays, muscarinic receptors present in uterine membranes appeared to be homogeneous since the muscarinic antagonists, pirenzepine (M_1), AF-DX 116 (M_2), and methoctramine (M_2), exhibited mass action displacement isotherms (Hill coefficient = 1). The values observed at the uterine binding site were entirely consistent with the presence of an M_2 cardiac type of muscarinic receptor as adjudged by the high affinity of both AF-DX 116 and methoctramine and the low affinity of pirenzepine (Michel & Whiting, 1988). These data are shown in Table 1.

Table 1 p*Ki* values determined from competition radioligand binding studies in various tissues

	AF-DX 116	*Methoctramine*	*Pirenzepine*
Cortex (M_1)	6.6 (0.95)	6.9 (0.94)	7.7 (1.04)
Heart (M_2)	7.0 (1.02)	7.8 (0.97)	6.4 (1.02)
Gland (M_3)	6.0 (1.08)	6.3 (1.03)	6.8 (0.98)
Uterus (M_2)	7.1 (0.94)	7.9 (1.00)	6.6 (0.94)

Values are mean, s.e.mean < 5%, $n = 3$.
The radioligand used was [^{3}H]-NMS except in studies using cortical membranes in which [^{3}H]-pirenzepine was used.

Discussion It has been shown (Giraldo *et al.*, 1987; Roffel *et al.*, 1988; Michel & Whiting, 1988) in ileal and trachael smooth muscle that the muscarinic receptor population is heterogeneous. Approximately 80% of the ileal receptors are M_2 whilst the remaining 20% are M_3. These M_2 and M_3 receptors (see Introduction for reviews of the classification scheme) are pharmacologically identical to those found in cardiac and glandular tissue respectively (Michel & Whiting, 1988). The contractile responses in the ileum and trachea appear to be mediated through the minor, M_3 population (R.M. Eglen unpublished observations).

In the present study, we have characterized the muscarinic receptor present in the uterus. The pA_2 and p*Ki* values from functional and binding studies suggest a homogeneous population of M_2 receptors, through which the contractile responses are mediated. The pA_2 values for pirenzepine, methoctramine and hexamethonium are similar to those reported for M_2 receptors mediating negative inotropic responses (Eglen & Whiting, 1986). The low Schild slopes for methoctramine and hexamethonium indicate deviations from competitive antagonism, a characteristic of these compounds which has also been observed at cardiac M_2 receptors (R.M. Eglen, unpublished observations). The p*Ki* values for AF-DX 116, methoctramine and pirenzepine are similar to those reported at M_2 receptors in cardiac tissue (Michel & Whiting, 1988). The receptor population appears to be homogeneous since the Hill coefficients are not significantly different from unity.

The M_2 receptor present in the guinea-pig uterus provides evidence for an excitatory action mediated through this subtype. The guinea-pig uterus differs from the ileum and trachea in that no M_3 receptors are present. It therefore resembles the rabbit vas deferens in which enhancement of contractile responses to neurogenic stimulation are also mediated through M_2 receptors (Eltze *et al.*, 1988). Taken together, these data illustrate that terminology referring to muscarinic receptor subtypes as cardiac-like, glandular-like or smooth muscle-like is inappropriate. We suggest that, as an alternative to anatomically based classification schemes, that an M_1, M_2 and M_3 scheme be adopted, based upon differential affinities of selective antagonists.

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An A_2-purinoceptor agonist, NECA, potentiates acetylcholine-induced glucagon secretion

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The effect of a stable structural analogue of adenosine, 5'-N-ethylcarboxamidoadenosine (NECA), was studied on glucagon secretion induced by acetylcholine (ACh) in the isolated perfused pancreas of the newborn dog. The perfusion solution contained a physiological concentration of glucose (4.2 mM). In the first set of experiments, ACh (0.5 μM) infused alone for 10 min induced a significant rise of glucagon secretion (370 ± 98%, 4 min after the beginning of infusion). In the second set, NECA (2.2 nM) infused 10 min before ACh administration, had no effect *per se*, but considerably increased the response to ACh (929 ± 262% of basal value within 3 min). So, the more specific A_2 purinoceptor agonist, NECA, potentiated glucagon secretion induced by the cholinoceptor agonist, ACh.

Introduction We have previously shown that the pancreatic A cell has adenosine receptors (P_1-purinoceptors) of the A_2-subtype, the activation of which stimulates glucagon secretion (Chapal *et al.*, 1984; 1985). The A cell is also endowed with cholinoceptors and acetylcholine (ACh) stimulates glucagon release (Alric *et al.*, 1972; Iversen, 1973). On the other hand, ATP is stored with ACh in the nerve endings and co-released with the neurotransmitter (Silinsky, 1975). As it is well known that ATP is rapidly metabolized into adenosine, it is possible that adenosine potentiates ACh-induced glucagon secretion. Our experiments were performed on the isolated perfused pancreas of the newborn dog. The purinoceptor agonist we used was a stable structural analogue of adenosine, more specific for A_2- receptors: 5'-N-ethylcarboxamidoadenosine (NECA).

Methods Four to eight day old dogs weighing 350 to 450 g were anaesthetized with sodium pentobarbitone (60 mg kg^{-1} i.p.). The pancreas was removed according to a technique previously described (Loubatières *et al.*, 1969) for the rat and adapted to the newborn dog. The organ was then transferred into a plastic chamber, maintained at 37.5°C and perfused with Krebs Ringer bicarbonate buffer containing 2 g l^{-1} bovine serum albumin and glucose at a physiological concentration (4.2 mM). The medium was continuously bubbled with a mixture of 95% O_2 and 5% CO_2 so as to maintain the pH between 7.35 and 7.40. The perfusion was performed at a constant pressure; the latter was selected so as to obtain a flow rate of about 4.0 ml min^{-1} at the reference time (45 min). In all experiments a 30 min adaptation period was allowed before taking the first sample. A second sample was taken at 45 min (reference time), allowing determination of basal glucagon output just before any drug administration. For each sampling, pancreatic effluent flow rate was measured and an aliquot of 0.5 ml was immediately frozen in chilled tubes containing 50 μl of a mixture of disodium edetate (EDTA) (32 mM) and aprotinin (Zymofren: 10.000 UKI). Two sets of experiments were performed. In the first one, ACh was applied alone for 10 min from time 55 min. In the second set, the purinoceptor agonist (NECA) was infused from time 45 min for 20 min and ACh was added 10 min after the beginning of NECA infusion. We chose a concentration of NECA (2.2 nM) which was ineffective alone and an ACh concentration (0.5 μM) previously shown to be effective on glucagon secretion (Alric *et al.*, 1972).

Glucagon was measured according to the radioimmunoassay described by Unger *et al.* (1970). We used the BR 124 antibody kindly supplied by the Institut de Biochimie Clinique, Geneva and pork glucagon (Novo) as standard.

NECA was supplied by Byk Gulden Lomberg (Konstanz, F.R.G.) and acetylcholine by Fluka (Buchs, Switzerland).

Our results (means ± s.e.mean) are expressed as percentages of the value obtained at the reference time 45 min (taken as 100%). Glucagon output rates (pg min^{-1}) were calculated by multiplying the

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hormone concentration (pg ml^{-1}) by the pancreatic outflow rate (ml min^{-1}). All data were submitted to analysis of variance using the multiple comparison test of Student-Newman-Keuls (Zar, 1974).

Results *Effects on glucagon secretion* (Figure 1a) When applied alone ACh induced a rapid increase in glucagon output rate. The maximal value reached within 4 min was 370 ± 98% of the reference value (277 ± 74 pg min^{-1} at 45 min). Then the secretion decreased progressively. Five min after stopping ACh infusion, glucagon output rate was similar to the basal one.

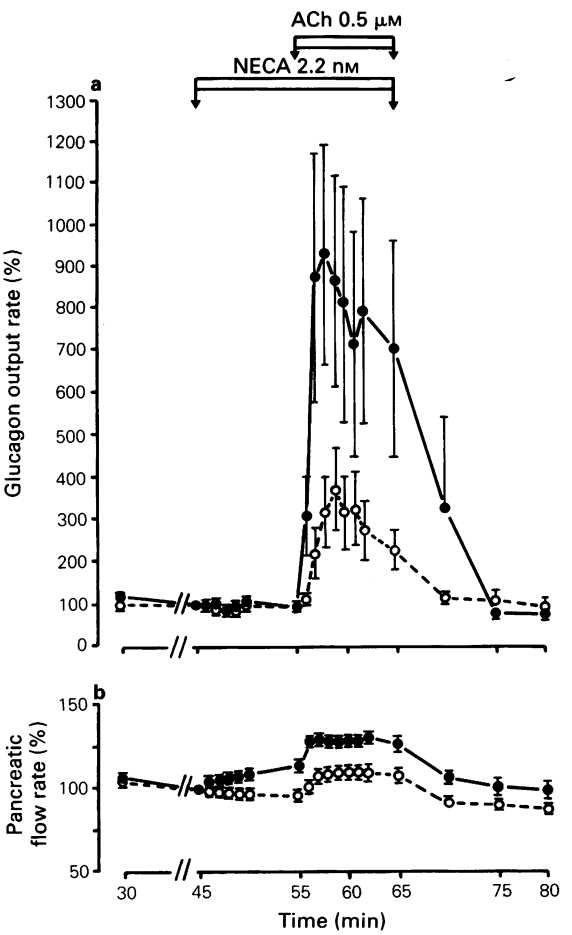

Figure 1 Effects of acetylcholine (ACh) alone (○) or in presence of 5'-N-ethylcarboxamidoadenosine (NECA) (●) on glucagon output rate and pancreatic flow rate. At time 45 min, glucagon output rate (a) and pancreatic flow rate (b) were 277 ± 74 pg min^{-1} and 4.1 ± 0.3 ml min^{-1} for the first set of experiments and 288 ± 92 pg min^{-1} and 3.8 ± 0.2 ml min^{-1} for the second set. Results are given as mean with s.e.mean shown by vertical bars.

The P_1-purinoceptor agonist, NECA, did not itself modify the basal glucagon output rate (288 ± 92 pg min^{-1} at 45 min). When ACh treatment was superimposed at time 55 min, the rise in glucagon output rate reached, from the first min, values significantly higher than those recorded with ACh alone. The maximum occurred within 3 min (929 ± 262% of basal value). Glucagon secretion remained high throughout the combined infusion of ACh and NECA. Five min after stopping the two drugs, glucagon secretion drastically decreased, and at time 75 min, the secretion was comparable to that recorded at the reference time.

Effects on flow rate (Figure 1b). Since cholinoceptor and purinoceptor agonists are known to be effective on blood vessels, we also studied their effects on the pancreatic vascular bed.

Addition of ACh to the perfusion medium induced a small but significant rise in flow rate: from 96% at time 55, the flow rate reached 110% during ACh infusion and decreased immediately after stopping it.

NECA alone progressively increased the flow rate, to 113% just before ACh addition. The addition of ACh further increased the flow rate which immediately reached a plateau at 128% during the combined administration of both drugs. So, the increment due to ACh is similar to that observed in the absence of NECA. The flow rate returned to basal values upon cessation of drug administration.

Discussion Our results clearly demonstrate the potentiation by an A_2-purinoceptor agonist of ACh-stimulated glucagon secretion. In our experimental conditions, the effect of ACh is potentiated by the purinoceptor agonist at a very low concentration (2.2 nM). It may be recalled that NECA is a potent agonist on A_2-receptors of pancreatic glucagon cells; it is 800 times more potent than adenosine (Chapal *et al.*, 1985). The potentiation recorded on glucagon secretion cannot be explained by a modification in flow rate, since NECA and ACh had only additive effects on vessels and the increase in flow rate induced by NECA alone was not followed by a rise in glucagon output. On the other hand it can be observed that pancreatic vessels seem more sensitive to the purinoceptor agonist than pancreatic A cells, since NECA at 2.2 nM was effective on flow rate and not on glucagon secretion.

The potentiation observed on glucagon secretion could be of physiological relevance. As previously mentioned, ATP which is rapidly metabolized into adenosine, is stored in cholinergic synaptic vesicles

and co-released with ACh. Since pancreatic glucagon-secreting A cells of the dog are known to be under vagal control (Kaneto *et al.*, 1974), adenosine could act as a neuromodulator on these cells and amplify the response to the cholinergic transmitter.

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Evidence for an intracellular action of platelet-activating factor in bovine cultured aortic endothelial cells

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Bovine culture endothelial cells (BAECs) generate platelet-activating factor (Paf) following activation by bradykinin (Bk 0.1 nM), the ionophore, A23187 (3 μM), and ATP (10 μM), but Paf is not released from the cells. These stimuli also elicit generation of prostacyclin (PGI_2). The specific and competitive Paf receptor antagonists, WEB 2086 (0.1–1.0 μM) and CV 6209 (0.01–0.1 μM), inhibited Bk-, A23187- and, to a lesser extent, ATP-induced PGI_2 generation but had no effect on basal PGI_2 generation. These data suggest a role for intracellular Paf in signal transduction.

Introduction Platelet-activating factor (Paf) is regarded as a pro-inflammatory mediator (Braquet *et al.*, 1987) that requires release from the cell of origin for its action. Macrophages and polymorphonuclear leukocytes (PMNs) synthesize and release Paf in response to immune complexes, endotoxin and zymosan but these cells also retain a significant portion within the cell. In contrast, endothelial cells synthesize, but do not release Paf in response to bradykinin (Bk), the ionophore A23187, ATP, histamine and thrombin (McIntyre *et al.*, 1985). The function of intracellular Paf has not been identified.

We have observed an inhibitory effect of several Paf receptor antagonists on macrophage prostacyclin (PGI_2) generation in the absence of exogenous Paf (Stewart & Dusting, 1988). This observation, together with extensive data linking exogenous Paf to arachidonic acid mobilization (Braquet *et al.*, 1987), led us to investigate the role of intracellular Paf in eicosanoid generation by endothelial cells since in these cells all the Paf synthesized is retained intracellularly and the stimuli for Paf generation also cause PGI_2 generation. Antagonism of Paf was examined by use of the potent, specific and structurally distinct Paf receptor antagonists, WEB 2086 (Casals-Stenzel *et al.*, 1987) and CV 6209 (Terashita *et al.*, 1987).

Methods Bovine aortic endothelial cells were cultured in monolayers as previously described (Booyse *et al.*, 1975; Dusting *et al.*, 1988) and were grown to confluence in Linbro plastic culture dishes using RPMI 1640 (containing 20% v/v foetal calf serum, 15 mM HEPES, pH 7.4, streptomycin, 100 μg ml^{-1} and penicillin, 100 u ml^{-1}). The medium was exchanged immediately before experiments for one containing 0.25% w/v bovine serum albumin (BSA) in place of foetal calf serum.

Paf receptor antagonists were added 15 min before stimuli and the incubations continued for a further 60 min. The supernatants were harvested for radioimmunoassay (RIA) of the stable PGI_2 product 6-oxo-$PGF_{1\alpha}$ (Salmon, 1978). In experiments in which Paf was assayed, incubations were carried out for 5 min since this period corresponded to the maximum stimulation of Paf which subsequently declined rapidly. The supernatants were assayed for Paf content without further extraction using rabbit washed platelets (Parente & Flower, 1985; Stewart & Dusting, 1988). Paf was extracted from the cell monolayer by addition of 1.0 ml ice-cold 80% ethanol. The extract was evaporated to dryness then reconstituted in Tyrode solution (plus 0.25% BSA, 1.8 mM Ca^{2+}, 10 mM HEPES, pH 7.40) before bioassay.

RPMI 1640 and foetal calf serum were obtained from CSL (Australia) and Flow Laboratories, respectively. Sources of chemicals: ATP; A23187; bovine serum albumin (BSA), grade 5, essentially fatty acid free (Sigma Chemical Co.); hexadecyl platelet-activating factor (Paf, Bachem); 6-oxo-$PGF_{1\alpha}$ (Upjohn); 6-oxo-$PGF_{1\alpha}$ antiserum (Dr J. Salmon, Wellcome Research Laboratories); 6-keto [5,8,9,11,12,14,15 (n)-^{3}H] prostaglandin $F_{1\alpha}$, 170 Ci mmol^{-1} (Amersham); [hexadecyl-1′,2′-^{3}H(N)]-2-acetyl-sn-glyceryl-3-phosphorylcholine, 59.5 Ci mmol^{-1} (New England Nuclear); HEPES (N-2-hydroxy ethylpiperazine-N-2-ethane sulphonic acid, BDH chemicals); CV 6209 (2-[N-acetyl-N-(2-methoxy-3-octadecyl-carbamoyloxy-propoxycarbonyl)amino-methyl]-1-ethylpyridinium chloride), (Takeda Chemical Industries Ltd.); WEB 2086 (3-(4-(2-chlorophenyl)-9-methyl-6H-thieno(3,2-f),(1,2,4)-triazolo, (4,3-a),(1,4)-diazepine-2-yl)-1-(4-morpholinyl)-1-propanone), Boehringer Ingelheim, West Germany.

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Results and Discussion Concentrations of Bk (0.1 nM), A23187 (3 μM), and ATP (10 μM) that were equi-active and submaximal for BAEC generation of 6-oxo-$PGF_{1\alpha}$ were determined from the concentration-response curves for each of these stimuli. In separate experiments, Bk, A23187 and ATP also induced the synthesis, but not release, of Paf whereas unstimulated BAECs did not contain detectable Paf levels (Figure 1). Pre-incubation of BAECs with the Paf receptor antagonists, WEB 2086 (1 μM) and CV 6209 (0.1 μM) caused marked reductions in Bk-stimulated 6-oxo-$PGF_{1\alpha}$, smaller inhibition of A23187 and ATP, but no inhibition of basal 6-oxo-$PGF_{1\alpha}$ generation. These concentrations of WEB 2086 and CV 6209 were maximally effective for inhibition of Bk-stimulated 6-oxo-$PGF_{1\alpha}$ generation and produced 1.63 ± 0.16 and 2.59 ± 0.18 log unit ($n = 6$) rightward shifts of Paf-induced aggregation of rabbit washed platelets, respectively.

The specificity of the Paf receptor antagonism at these concentrations is supported by the failure of either compound to inhibit platelet aggregation induced by a range of stimuli including arachidonic acid, A23187, ADP and collagen (Casals-Stenzel *et al.*, 1987; Terashita *et al.*, 1987). Furthermore, CV 6209 failed to show any direct inhibitory effects on either cyclo-oxygenase or phospholipase A_2 (Terashita *et al.*, 1987).

The extent of the inhibition by the Paf receptor antagonists of PGI_2 generation was clearly dependent on the stimulus used (Figure 1). The lack of inhibition of basal PGI_2 generation suggests that neither Paf receptor antagonist inhibits directly the enzymes involved in PGI_2 synthesis and is consistent with the failure to detect Paf in unstimulated BAECs. The inhibition by the Paf antagonists of PGI_2 generation stimulated by Bk, A23187 and ATP does coincide with the generation of intracellular Paf. The differences in the extent of inhibition between A23187, ATP and Bk may be explained by the distinct mechanisms of BAEC activation: A23187 causes a large and persistent increase in intracellular Ca^{2+}; ATP activates the Na^+/H^+ exchanger (Kitazono *et al.*, 1988); Bk activates phospholipase C (Clark *et al.*, 1986).

Exogenous Paf does not stimulate PGI_2 generation by BAECs (data not shown). However, endothelial cells have a high capacity to metabolize Paf (McIntyre *et al.*, 1985) which may preclude the ability of exogenous Paf to mimic the proposed intracellular action of mobilizing arachidonic acid.

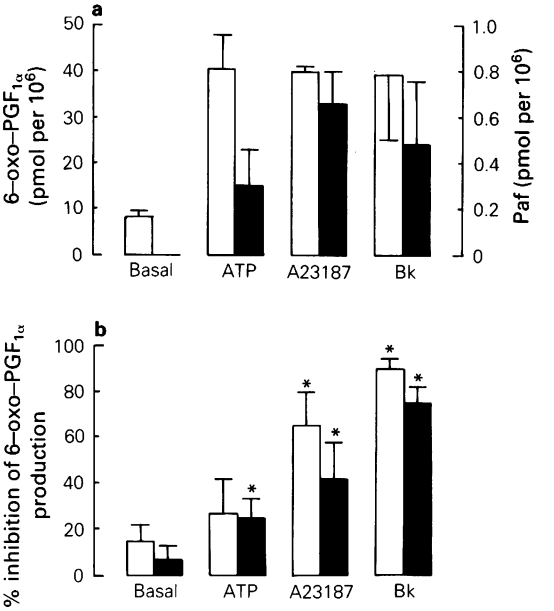

Figure 1 Basal, bradykinin (Bk 0.1 nM), A23187 (3 μM) and ATP (10 μM)-induced generation of 6-oxo-$PGF_{1\alpha}$ (open columns) and Paf (filled columns) by BAECs (a) and inhibition of 6-oxo-$PGF_{1\alpha}$ by the Paf receptor antagonists (b), WEB 2086 (1 μM, open columns) and CV 6209 (0.1 μM, filled columns). The data are presented as the means of 3–8 observations; s.e.mean shown by vertical bars. $^*P < 0.05$, paired t test.

The present data suggest a role for Paf in the signal transduction mechanism for Bk-stimulated PGI_2 generation. The site of intracellular action of Paf has not been identified but recent data suggest that Bk activates phospholipase C in BAECs (Clark *et al.*, 1986) and a link between Paf receptors and phospholipase C activation is well established (Braquet *et al.*, 1987).

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Acute stress and GABAergic function in the rat brain

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1 The function of the γ-aminobutyric acid (GABA)-ergic system in certain areas of the rat brain was investigated after acute (5 min) exposure to immobilization stress.

2 The activities of glutamate decarboxylase and GABA-transaminase, GABA concentrations, GABA turnover *in vivo* and uptake of [^{3}H]-GABA were measured.

3 After 5 min of immobilization stress, GABA concentrations and [^{3}H]-GABA uptake were reduced, and GABA turnover stimulated in the olfactory bulbs. In contrast the uptake of [^{3}H]-GABA was increased in the corpus striatum after 5 min of immobilization stress.

4 None of the parameters measured was significantly altered by acute immobilization stress in the frontal cortex, hippocampus or medio-basal hypothalamus.

5 These findings show that the olfactory bulbs and the corpus striatum are sensitive to the effects of acute stress. Since GABA in the olfactory bulbs is involved in the development of aggression and increased emotional state, it follows that neurochemical changes induced by acute stress might underlie some behavioural manifestations observed after stress.

Introduction

The neurochemistry of stress has been studied widely. The first classical experiments demonstrated alterations in the function of monoaminergic systems in the brain (Bliss *et al.*, 1968; Corrodi *et al.*, 1968).

In recent years the involvement of γ-aminobutyric acid (GABA) in the changes induced by stressful stimuli has also been assessed (Sherman & Gebhart, 1974; Green *et al.*, 1978; Yoneda *et al.*, 1983). The GABAergic system is known to modulate behaviour (Clement *et al.*, 1987) and also to be influenced by environmental stimuli (Da Vanzo *et al.*, 1986). The experimental design and conditions of the environmental stimulus or stress seem to be crucial for the determination of the type of changes which will be induced. Thus, modifications in the GABAergic system have been presented according to the type of stress employed, the duration of the stressful stimulus and the interval of time after the cessation of the stress (Soubrie *et al.*, 1980; Biggio *et al.*, 1981; Skerritt *et al.*, 1981; Havoundjian *et al.*, 1986; Schwartz *et al.*, 1987). Both decreases and increases in brain GABA concentrations have been found after acute stress (Sherman & Gebhart, 1974; Singh *et al.*, 1979). Recently the function of the GABAergic system in the frontal cortex and the corpus striatum of the rat has been shown to respond differently and with different time-courses to the application of cold stress and immobilization stress (Otero Losada, 1988). In the above study changes were observed in the corpus striatum after 1 h of immobilization but were not apparent after more prolonged periods of stress. This raises the possibility that changes might have taken place sooner (i.e.: after less than 1 h) in the corpus striatum and other brain areas.

In order to assess possible rapid changes in the GABAergic system induced by acute stress, the effects of 5 min of immobilization stress upon the GABAergic system in certain brain areas are now described. The areas chosen for study were the frontal cerebral cortex and the corpus striatum, to allow comparisons with the previous study, and the olfactory bulbs, medio basal hypothalamus and hippocampus because previous evidence indicates that the GABAergic system in these areas may be involved in the biochemistry of stress (Da Vanzo *et al.*, 1986; Gilad, 1987).

Methods

Male Wistar rats (190–250 g) were kept on a 12 h light-12 h dark cycle with free access to food and water.

Stress application usually started at 10 h 00 min. For immobilization stress, each animal was placed inside a narrow, cylindrical acrylic tube which was

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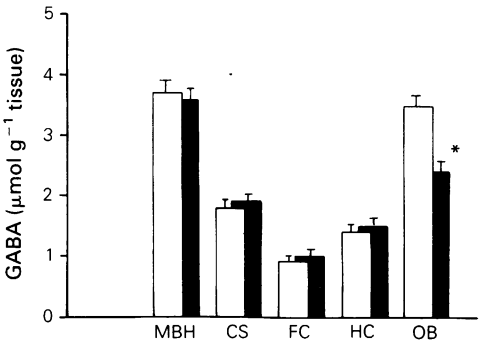

Figure 1 Endogenous γ-aminobutyric acid (GABA) concentrations after 5 min of immobilization stress. Ordinate scale: GABA concentration (μmol g^{-1} tissue) in the medio basal hypothalamus (MBH), corpus striatum (CS), frontal cerebral cortex (FC), hippocampus (HC) and olfactory bulbs (OB) in control (open columns) rats and rats subjected to 5 min of immobilization stress (solid columns). Results are expressed as the mean of at least 4 animals per group and vertical lines show s.e.mean. * $P < 0.05$ compared with control.

perforated for normal breathing and in which only restricted movements were possible. This restriction was applied for 5 min. Control rats were left in their home cages. At the end of the stress period the animals were killed by decapitation.

Enzymatic determinations

The brains were quickly removed and placed on ice. The frontal cerebral cortex, corpus striatum, mediobasal hypothalamus, hippocampus and olfactory bulbs were dissected and homogenized in ice-cold distilled water 1:50 (w/v) to determine the activity of glutamate decarboxylase (GAD,E.C.4.1.1.15), or 1:10 (w/v) for determination of 4-aminobutyrate-2-oxoglutarate aminotransferase (GABA-T,E.C.2.6.1.19).

Glutamate decarboxylase assay

This was performed according to Albers & Brady (1959) and has been described in detail previously (Otero Losada, 1988). Briefly, aliquots of tissue homogenate were incubated in the presence of DL-[1-^{14}C]-glutamic acid (specific activity: 10 nCi μmol^{-1}) 4 mmol l^{-1} (non-saturating concentration). The $^{14}CO_2$ formed was collected and measured by counting radioactivity.

GABA-T assay

The method described by Tunnicliff & Smith (1981) was employed with slight modifications (Otero Losada, 1988). The substrates were GABA 1 mmol l^{-1} (non-saturating concentration) and [1-^{14}C]-α-keto glutaric acid (specific activity 0.44 μCi μmol^{-1}) 0.44 mmol l^{-1} (non-saturating concentration). Excess α-ketoglutarate was extracted with tri-*n*-octylamine (0.2 mol l^{-1} in chloroform) (Sterri & Fonnum, 1978), and the radioactivity in the organic phase was determined by scintillation counting.

GABA concentration

GABA was measured by the method of Lindgren *et al.* (1982) with slight modifications (Van der Heyden & Korf, 1978; Otero Losada, 1988). Tissue homogenates were chromatographed on Dowex 50W × 4 (200–400 mesh) columns and GABA in the eluates was quantified by spectrofluorimetric detection following its condensation with *o*-phthalaldehyde.

Accumulation of GABA after GABA-T inhibition

γ-Vinyl GABA(GVG) is an irreversible and specific inhibitor of GABA-T (Hammond & Wilder, 1985). After GVG administration the accumulation of GABA is proportional to the degree of neuronal activity. Rats were injected with GVG (10 mmol kg^{-1}, i.p.) 3 h before death. GABA concentration was determined as described above.

Uptake of [^{3}H]-GABA

The procedure of Iversen & Kelly (1975) was used. Immediately after decapitation, the tissues were dissected and cut into 100 μm thick slices using a tissue sectioner (Smith & Farquhar Sorvall TC-2) and incubated at 37°C in 5 ml Krebs solution (bubbled with 95% O_2:5% CO_2) for 15 min before the addition of [^{3}H]-GABA (0.4 μmol l^{-1}, specific activity 0.65 Ci mmol^{-1}). In order to block neuronal GABA uptake, blanks were prepared by addition of 2,4-diaminobutyric acid (DABA, 0.2 mmol l^{-1}) at the beginning of the incubation periods. After 5 min of incubation, the slices were washed with ice-cold Krebs, homogenized, and centrifuged (5 min, 10,000 *g*). Aliquots of the supernatants were added to scintillation fluid for radioactivity counting.

Results are expressed as the mean ± s.e.mean and were analysed according to Student's *t* test (Snedecor & Cochran, 1967).

The following drugs were used: protosol (New England Nuclear; NEN), γ-2,3-[^{3}H]-aminobutyric acid (specific activity 29.3 Ci mmol^{-1}, NEN), α-[1-^{14}C]-ketoglutaric acid (sodium salt, sp. activity 53.6 Ci mol^{-1}, NEN), *o*-phthalaldehyde (Sigma), tri-n-octylamine (Sigma), DL-[1-^{14}C]-glutamic acid (specific activity 50.3 mCi mmol^{-1}, NEN); 3-mercaptopropionic acid (Sigma), γ-vinyl-

Table 1 Activity of the γ-aminobutyric acid (GABA)-related enzymes after 5 min of immobilization stress (stress)

	GAD (μmol g^{-1} tissue h^{-1})		*GABA-T* (μmol g^{-1} tissue 30 min^{-1})	
	Control	*Stress*	*Control*	*Stress*
MBH	8.5 ± 0.7	8.3 ± 0.8	12.3 ± 1.1	13.7 ± 1.1
CS	4.5 ± 0.4	3.3 ± 0.3	9.8 ± 0.3	9.9 ± 0.5
FC	2.1 ± 0.2	2.0 ± 0.2	8.7 ± 0.4	8.6 ± 0.5
HC	2.9 ± 0.3	2.7 ± 0.2	10.5 ± 1.0	10.3 ± 0.9
OB	7.7 ± 0.5	7.6 ± 0.6	11.5 ± 0.9	11.7 ± 1.2

Enzyme activities were measured under non-saturating conditions (glutamic acid 4 mmol l^{-1} for glutamate decarboxylase (GAD), GABA 1 mmol l^{-1} and α-ketoglutaric acid 0.44 mmol l^{-1} for 4-aminobutyrate-2-oxo-glutarate amino-transferase (GABA-T) in the following areas: medio basal hypothalamus (MBH), corpus striatum (CS), frontal cerebral cortex (FC), hippocampus (HC) and olfactory bulbs (OB). Results are expressed as the mean ± s.e.mean of 4–6 animals per group.

GABA(GVG) was a gift from Merrell Laboratories; D-penicillamine (Sigma).

Results

At the end of the 5 min period of immobilization stress the animals showed signs of behavioural hyperexcitability, increased motility and sniffing.

As shown in Figure 1 only in the olfactory bulbs was the GABA concentration reduced (31%, $P < 0.05$) by 5 min of immobilization stress.

No changes were observed in the activities of GAD or of GABA-T in any of the areas studied (Table 1), though a tendency for the activity of GAD to be decreased (26%, not statistically significant) was observed in the corpus striatum.

The application of 5 min immobilization stress enhanced the accumulation of GABA after GABA-T inhibition only in the olfactory bulbs (49%, $P < 0.05$), Figure 2).

The neuronal uptake of [^{3}H]-GABA (Figure 3) was reduced by 38% ($P < 0.05$) in the olfactory bulbs and increased by 28% ($P < 0.05$) in the corpus striatum after 5 min immobilization stress. Non-neuronal uptake of [^{3}H]-GABA which represented 15–30% of total uptake in the corpus striatum, medio basal hypothalamus, hippocampus and olfactory bulbs and 40–60% in the frontal cortex, was not modified by the application of stress.

Discussion

The present findings show that the central GABAergic system is affected by a single acute stress (5 min immobilization) only in certain areas of the brain: significant changes were observed in the olfactory bulbs and the corpus striatum, but no differences were found in the frontal cortex, hippocampus or medio basal hypothalamus of immobilized compared to control animals. These highly localised effects might explain the absence of any alteration in GABA concentration and GAD and GABA-T activities after acute forced swimming when measured in the whole brain (Cicardo *et al.*, 1986).

With respect to the lack of an effect in the cerebral cortex, hippocampus and hypothalamus of acute stress, no change in the number of $GABA_A$-receptors has been observed either in the cerebral cortex or the

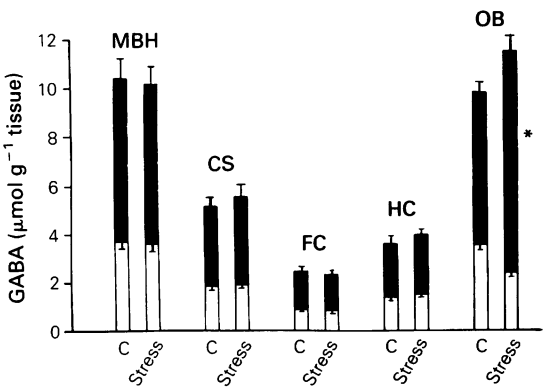

Figure 2 Accumulation of γ-aminobutyric acid (GABA) after inhibition of 4-aminobutyrate-2-oxo-glutarate aminotransferase (GABA-T). Ordinate scale: GABA concentration (μmol g^{-1} tissue). The animals were injected with the GABA-T inhibitor, γ-vinyl GABA (GVG), 10 mmol kg^{-1} i.p. and 3 h later divided into two groups: one group was exposed to 5 min of immobilization stress (Stress) and the other was left in their home cages (control, C). At the end of the 5 min period all the animals were killed by decapitation. The open area of the column represents endogenous GABA concentration and the solid portion GABA accumulated after 3 h of GVG injection. Results are expressed as the mean of 6–8 animals per group and vertical lines show s.e.mean. * $P < 0.05$ compared with control. For abbreviations used see legend of Figure 1.

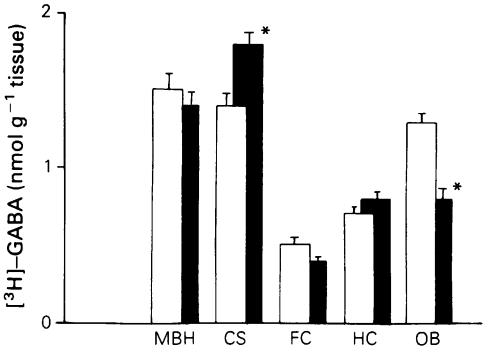

Figure 3 Effect of 5 min of immobilization stress on [^{3}H]-γ-aminobutyric acid ([^{3}H]-GABA) uptake. Ordinate scale: uptake of [^{3}H]-GABA (nmol g^{-1} tissue). Slices (100 μm) of medio basal hypothalamus (MBH), corpus striatum (CS), frontal cerebral cortex (FC), hippocampus (HC) and olfactory bulbs (OB) obtained from control (open columns) or 5 min-immobilized rats (solid columns) were incubated in the presence of [^{3}H]-GABA (0.4 μM, 0.65 Ci mmol^{-1}) for 5 min. Results are expressed as the mean of 4–6 experiments and vertical lines show s.e.mean. * $P < 0.05$ compared with control.

hippocampus after 15 min of forced swimming (Novas *et al.*, 1987) or in the hypothalamus after acute isolation stress (Da Vanzo *et al.*, 1986).

Several factors must be considered in order to interpret correctly the data on GABA turnover, as measured by the accumulation of GABA following GABA-T inhibition. In the context of the experimental conditions, the short duration of the stress stimulus allows only a small contribution to the GABA accumulated after GABA-T inhibition; only enormous changes in GABA turnover can be detected (as appears to be the case for the olfactory bulbs after 5 min immobilization stress). Therefore the real turnover of the neurotransmitter is underestimated and negative results (i.e.: the absence of differences in GABA accumulation in stressed vs control animals) do not necessarily imply that changes have not occurred, rather that they could not be detected. Consequently the present findings would indicate considerable enhancement of the neuronal activity of GABAergic neurones in the olfactory bulbs while they cannot exclude the possibility of undetected changes in other brain areas.

Thus, one of the primary effects of acute stress appears to be the stimulation of GABA turnover in the olfactory bulbs and the reduction in uptake of the neurotransmitter. These factors together with the lack of change in GAD activity would explain the reduction of GABA concentration in this area. A reduction in the number of GABA receptors in the olfactory bulbs after isolation stress has been found previously (Da Vanzo *et al.*, 1986). This could result from an initial increase in the activity of GABAergic neurones in the olfactory bulbs, as suggested by the results.

Lower GABA concentrations have been observed in the olfactory bulbs (and in the corpus striatum) of aggressive mice (Early & Leonard, 1977; Simler *et al.*, 1982; Da Vanzo *et al.*, 1986) and in the olfactory bulbs of aggressive rats (Mandel *et al.*, 1978; 1979a, b; Bolin & Da Vanzo, 1982) in comparison to non-aggressive animals. Local injections of muscimol, DABA or dipropylacetate into the olfactory bulbs has been shown to suppress aggressive behaviour (Mandel *et al.*, 1979a,b), while injection of GABA antagonists enhances aggressiveness (Mandel *et al.*, 1979a). In view of these observations the lowered concentration of GABA found in the olfactory bulbs in the current investigation may be involved in the development of aggression and increased emotional state after stress.

Five min immobilization stress increased the uptake of GABA in the corpus striatum but failed to alter endogenous GABA concentrations. Hence, it is conceivable that the clear tendency to lower GAD activity, although not statistically significant, might have compensated for the increased uptake of GABA and so its endogenous concentration in this area was unchanged. Previously 1 h immobilization stress was shown to reduce GAD activity and GABA concentration in the corpus striatum (Otero Losada, 1988). The increased uptake of GABA after 5 min immobilization stress in the corpus striatum may be associated with the reduction of GAD activity observed after 1 h of immobilization thus reducing the endogenous GABA concentration. The increased uptake of GABA may reduce the synaptic half-life of the neurotransmitter and thus attenuate the inhibitory influence of GABA in the corpus striatum; this would accord with the behavioural hyperexcitability observed in these animals and may be associated with the increased turnover of dopamine observed in the corpus striatum after acute stress (1–10 min of swimming) (Ikeda & Nagatsu, 1985), since GABA has been shown to reduce the frequency of discharge of dopaminergic neurones in this area (Stock *et al.*, 1973; Walters *et al.*, 1973).

In summary, the GABAergic system seems to be differently affected by acute stress according to the brain area studied. The relevance of these regional changes to the neurochemistry of stress requires further research.

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The effects of vasoactive intestinal peptide (VIP) antagonists, and VIP and peptide histidine isoleucine antisera on non-adrenergic, non-cholinergic relaxations of tracheal smooth muscle

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1 The effects of several drugs, including antagonists of vasoactive intestinal peptide (VIP), and antisera to VIP or peptide histidine isoleucine (PHI), on relaxation responses of guinea-pig isolated trachea to electrical field stimulation (EFS) have been examined.

2 β-Adrenoceptor blockade with propranolol only partially blocked the inhibitory response to EFS, but had no effect in tissues from animals pretreated with 6-hydroxydopamine or reserpine.

3 Neither adenosine deaminase, in the presence of dipyridamole, nor the potent adenosine antagonist NPC205 (1,3-*n*-dipropyl-8-(4-hydroxyphenyl)-xanthine) had any effect on the inhibitory response to EFS.

4 The VIP antagonists, [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 and [4-Cl-D-Phe^6, Leu^{17}]-VIP had no effect on the inhibitory response to EFS. Moreover, they were without effect on responses to exogenous VIP or PHI.

5 Overnight incubation with VIP antisera markedly reduced the inhibitory response to EFS. PHI antisera had a similar, but smaller effect.

6 In the presence of a concentration of VIP that is maximal for its relaxant effect, inhibitory responses to electrical stimulation were greatly inhibited.

7 Naloxone and reactive blue 2 each had no effect on inhibitory responses indicating that endogenous opioids and adenosine 5'-triphosphate (ATP) respectively are not involved.

8 The results suggest that VIP and PHI, but not adenosine, contribute to non-adrenergic, non-cholinergic inhibitory nerve responses of guinea-pig trachea. Moreover, the surprising lack of effect of both VIP antagonists on these responses, and in particular, on responses to exogenous VIP, suggests that the receptors mediating VIP-induced tracheal relaxation are different from those that mediate pancreatic secretion.

Introduction

The neurogenic response of airway smooth muscle from several species to electrical field stimulation (EFS) is biphasic, consisting firstly of a predominantly cholinergic contraction, followed by a prolonged relaxation (Coburn & Tomita, 1973; Coleman & Levy, 1974; Ito & Takeda, 1982; Altiere & Diamond, 1984; Taylor *et al.*, 1984; de Jongste *et al.*, 1987). The second phase of the response does not involve exclusively the classical noradrenergic or cholinergic systems (reviewed by Karlsson, 1986), but is mediated, in part, by non-adrenergic, non-cholinergic (NANC) neural mechanisms. This was first demonstrated in guinea-pig trachea (Coburn & Tomita, 1973; Coleman & Levy, 1974), wherein a major component of the response is resistant to atropine, propranolol or chemical sympathectomy with reserpine or 6-hydroxydopamine (6-OHDA).

It has been suggested that the NANC inhibitory component is mediated either by adenosine (Satchell, 1984) or vasoactive intestinal peptide (VIP, Matsuzaki *et al.*, 1980; Ito & Takeda, 1982). However, the

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evidence for either of these neurotransmitter candidates is inconclusive and controversial (Barnes, 1986; Karlsson, 1986). VIP immunoreactive fibres have been localized in the lung of several species (Uddman *et al.*, 1978; Dey *et al.*, 1981; Hakanson *et al.*, 1983). Matsuzaki *et al.* (1980) demonstrated a tetrodotoxin-sensitive release of VIP from guinea-pig trachea during EFS, that the amount released correlated with the degree of relaxation, and that preincubation with VIP antiserum reduced the NANC response. This study has been questioned (Karlsson & Persson, 1983; 1984) in that, firstly, the specificity of the antibodies was not determined, and secondly, that Matsuzaki *et al.* (1980) used the very small relaxations obtained (5–30 mg) in their calculations. Ito & Takeda (1982) found that the relaxation of feline trachea to nerve stimulation was much reduced by partial desensitization to VIP. However, the peptidase α-chymotrypsin has no effect on the NANC relaxation in this preparation (Altiere & Diamond, 1985). It has been suggested (Barnes, 1986; Karlsson, 1986) that another inhibitory substance may be released with VIP during nerve stimulation. For example, VIP and peptide histidine isoleucine (PHI) have identical distribution in the airways (Lundberg *et al.*, 1984), and these peptides, released as cotransmitters, may mediate the NANC inhibitory response (Barnes, 1986).

Conclusive evidence for VIP as a NANC transmitter in the airways has been lacking, largely due to the unavailability of a specific VIP receptor antagonist. Recently, however, two such drugs have been described: [AC-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 has been found to be a VIP antagonist in rat pancreatic membranes (Waelbroek *et al.*, 1985), and [4-Cl-D-Phe^6, Leu^{17}]-VIP a VIP antagonist both of guinea-pig pancreatic amylase secretion, and in colonic epithelial tumour cells (Pandol *et al.*, 1986).

The purpose of the present study was to assess, using several drugs including VIP antagonists, the role of VIP, PHI or adenosine in NANC relaxations to EFS in guinea-pig isolated tracheal smooth muscle. We also examined the effect of antisera specific for VIP or PHI.

Methods

Male, Duncan-Hartley guinea-pigs (350–500 g: Hazelton, Denver, PA) were stunned, exsanguinated and the trachea removed. This was placed in modified Krebs-Henseleit solution (composition mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, glucose 10.0). Following removal of fat and connective tissue, the trachea was slit along its longitudinal axis, and transverse strips consisting of two adjacent cartilage rings were prepared and suspended between two platinum ring electrodes in 10 ml organ chambers containing Krebs solution, maintained at 37°C and gassed with 5% CO_2 in O_2. Preparations were connected to force-displacement transducers for measurement of isometric tension responses. Before each experiment the tissues were equilibrated for 60 min at an initial resting tension of 1.5 g, and washed with fresh solution every 15 min.

Atropine (1 μM) and propranolol (1 μM) were present in the tissue bath except where stated. Rectangular pulses were delivered to the electrodes from an HSE Type 215 stimulator, and frequency-response curves were generated by applying stimuli (50 V, 0.1 ms, 0.1–100 Hz) to the preparations for 30 s. Control frequency-response curves were obtained in each tissue. Where relevant, drugs were added to the bath 10 min before the construction of a second curve. Responses to nerve stimulation are expressed as the mean ± s.e.mean. Concentration-response curves to VIP were obtained by the cumulative addition of the peptide to the bath. The VIP antagonists (10 μM) were then added, and once the tone returned to baseline, a second curve to VIP was constructed.

Antisera to VIP and PHI

For some experiments, tracheal strips were incubated overnight in oxygenated Krebs solution (4°C) containing antiserum either to VIP or PHI. Control tissues were incubated in the same volume of Krebs solution. Experiments were carried out the following day. Following equilibration, a reference contraction to methacholine (MCh, 5 μM) was obtained, NANC responses to EFS were expressed as a % of the maximum relaxation produced by sodium nitroprusside (SNP, 30 μM), added at the end of the experiment.

The VIP antibodies exhibit less than 0.001% cross-reactivity with PHI/PHM and related peptides, and the PHI antibodies exhibit 0.04% cross-reactivity to secretin, 0.03% to VIP and less than 0.01% to other peptides (specificities provided by supplier). In initial experiments, it was found that incubation with the antisera completely abolished responses, not only to EFS, but also to MCh and SNP. Since the antisera contain the preservative merthiolate (0.01%), we thought that this reagent may have been responsible for the toxic effect. In subsequent experiments, therefore, merthiolate was removed by dialysis against Krebs solution. Under these conditions, incubation with antisera had no apparent toxic effects.

Chemical sympathectomy

Guinea-pigs were injected i.p. either with 6-hydroxydopamine (6-OHDA) or reserpine. 6-OHDA

pretreatment consisted of an initial dose of 100 mg kg^{-1} followed by 250 mg kg^{-1} 24 h later (Fedan *et al.*, 1981). The animals were killed 24 h after the second dose. Reserpine (5 mg kg^{-1} i.p.) was given as a single injection 24 h before the experiment (Wakade & Krusz, 1972).

Drugs

Atropine H_2SO_4, propranolol HCl, acetyl-β-methylcholine Cl (MCh), sodium nitroprusside (SNP), tetrodotoxin (TTX), reserpine, 6-OHDA HBr, naloxone HCl, reactive blue 2 (cibacron blue 3GA), porcine VIP, and dipyridamole were obtained from the Sigma Chemical Co., Missouri, U.S.A. PHI was obtained from Bachem Inc, California, U.S.A., and VIP antiserum from Chemicon, California, U.S.A. The VIP antagonists, [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 and [4-Cl-D-Phe^6, Leu^{17}]-VIP, and the PHI antiserum were obtained from Peninsula Laboratories Inc., California, U.S.A. The adenosine receptor antagonist NPC205 (1,3-di-*n*-propyl-8-(4-hydroxyphenyl)xanthine) was synthesized at Nova Pharmaceutical Corporation.

Atropine, propranolol, MCh, SNP, TTX, naloxone, reactive blue 2, [Ac-Tyr^1, D-Phe^2]-(1-29)-NH_2 and [4-Cl-D-Phe^6, Leu^{17}]-VIP were each dissolved in 0.9% w/v NaCl solution (saline). The antisera to VIP and PHI were supplied as lyophilized powders, and were reconstituted in Krebs solution to give concentrations of 1250 pg ml^{-1} and 4000 pg ml^{-1}, respectively. Reserpine was dissolved (0.5 mg ml^{-1}) in ascorbic acid (200%), whereas 6-OHDA (25 mg ml^{-1}) was prepared in ascorbic acid (1%) which had been gassed with oxygen-free N_2 for at least 15 min to prevent oxidation. Dipyridamole was prepared as a 20 mM stock solution in absolute ethanol. NPC205 was prepared as a 1 mM stock solution in water containing 4% ethanol and 2% NaOH. Adenosine deaminase was obtained, dissolved in glycerol, from Boehringer Mannheim. Bath glycerol concentrations did not exceed 15.5 mM.

Results

Responses to electrical field stimulation

During equilibration, tracheal strips generally exhibited gradual, spontaneous increases in basal tone. Responses to EFS consisted of a rapid increase in tension, followed by a more pronounced and prolonged relaxation (Figure 1). Following cessation of stimulation, the tension returned slowly to baseline. After stimulation at a frequency of 50 Hz, the time taken for 50% recovery was 317 ± 34 s (n = 10). The initial, phasic contractile response was abolished by atropine (1 μM), but the relaxation was only partially blocked by propranolol (1 μM; Figures 1 and 2). Higher concentrations of the β-receptor antagonist had no further effect.

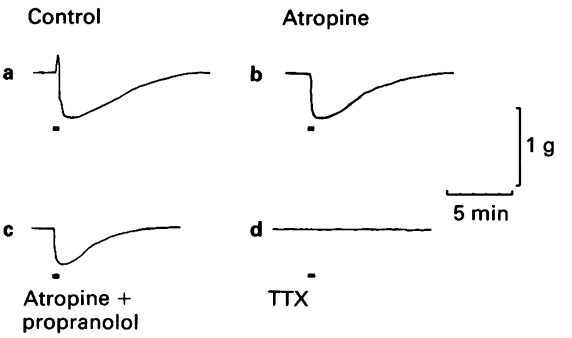

Figure 1 Typical responses of guinea-pig trachea to electrical field stimulation. Stimulation (50 Hz, 0.1 ms) was applied for 30 s, where indicated. Responses were obtained from the same tissue, and show (a) a biphasic, control response, (b) response in the presence of atropine (1 μM), (c) response in the presence of atropine (1 μM) and propanolol (1 μM) and (d) abolition of the response by tetrodotoxin (TTX, 1 μM).

Pretreatment with reserpine resulted in a 38 ± 8% reduction of the maximum inhibitory response, which was not further affected by propranolol (Figure 3). Pretreatment with 6-OHDA had similar

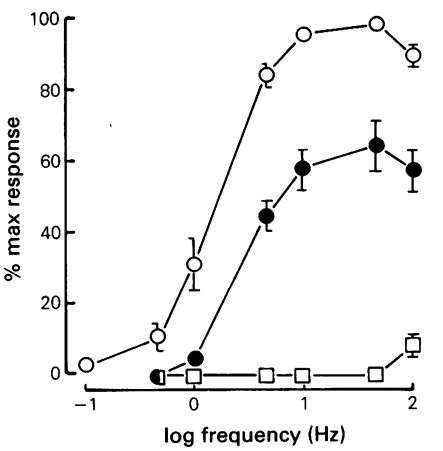

Figure 2 Frequency-response curves for relaxation of guinea-pig trachea induced by electrical field stimulation. Atropine (1 μM) was present throughout. (○) Controls; (●) in the presence of propranolol (1 μM); (□) in the presence of tetrodotoxin (1 μM). Each point is the mean of 8 observations and vertical lines indicate s.e.mean.

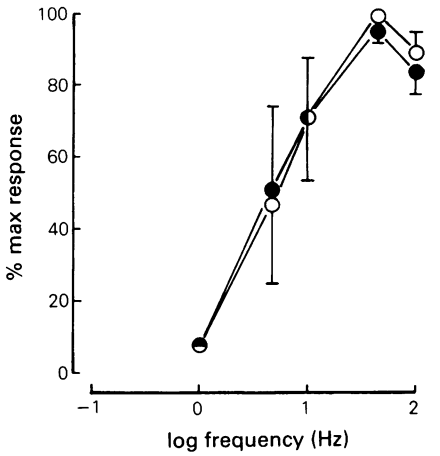

Figure 3 Frequency-response curves of isolated trachea from guinea-pigs pretreated with reserpine (5 mg kg^{-1} i.p., 24 h before the experiment). Atropine (1 μM) was present throughout. (○) Controls; (●) in the presence of propranolol (1 μM). Each point is the mean of 4 observations and vertical lines indicate s.e.mean.

effects to those seen with reserpine. TTX (1 μM) abolished both the excitatory and inhibitory responses at frequencies up to 50 Hz, indicating their neurogenic nature. A small, TTX-insensitive relaxation was observed at 100 Hz (Figure 2), and was probably due to direct electrical stimulation of the smooth muscle.

The role of adenosine

Adenosine deaminase (5 units ml^{-1}), in the presence of dipyridamole (0.5 μM), did not reduce the inhibitory responses to EFS (Figure 4). Since the addition of the adenosine antagonist NPC205 (1 μM) reduced basal tone, tension was returned to the baseline level by the addition of an appropriate concentration of histamine to the bath. In these preparations, NPC205 was without effect on the inhibitory responses (Figure 4).

Effect of reactive blue 2 and naloxone

We also examined the effect of the P_{2y}-purinoceptor antagonist, reactive blue 2 (10 μM) on the NANC relaxation, but this agent was without effect ($n = 4$; data not shown). Similarly, naloxone (10–100 μM) did not reduce the NANC response ($n = 4$, data not shown).

Desensitization to VIP

In order to determine its effect on NANC relaxations, desensitization to VIP was attempted in two ways. Firstly, a low concentration (50 nM) of VIP was applied repeatedly over a period of about three hours. There was, however, no reduction in the response to VIP. Secondly, a high concentration (3 μM) of VIP, which produced a maximal relaxation,

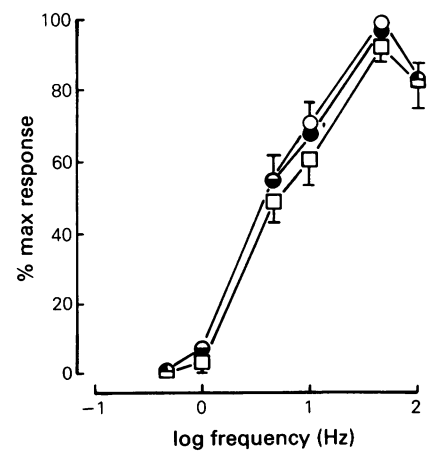

Figure 4 Frequency-response curves for relaxation of guinea-pig trachea induced by electrical field stimulation. Atropine (1 μM) and propranolol (1 μM) were present throughout. (○) Controls, $n = 13$; (●) in the presence of adenosine deaminase (5 units ml^{-1}), $n = 9$; (□) in the presence of NPC205 (1 μM), $n = 4$. Vertical lines indicate s.e.mean.

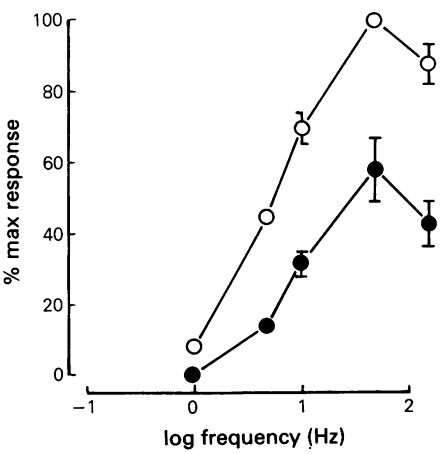

Figure 5 Effect of desensitization to vasoactive intestinal peptide (VIP, 3 μM for 3 h) on electrical field stimulation-induced relaxation of guinea-pig trachea. Atropine (1 μM) and propranolol (1 μM) were present throughout. (○) Controls; (●) in the presence of a maximal concentration of VIP. Each point is the mean of 4 observations and vertical lines indicate s.e.mean.

maintained for over three hours, was applied. By raising the tone of the preparation back to the control level with histamine it was possible to determine the effects of a large concentration of VIP on the inhibitory response to EFS. This procedure resulted in a 40% reduction in magnitude of the inhibitory responses to EFS (Figure 5).

VIP and PHI antisera

Incubation with VIP antiserum markedly reduced NANC responses (Figure 6). Similar, although smaller, effects occurred in tissues incubated with PHI antiserum (Figure 6). Contractions to MCh, and relaxations to SNP were similar in antibody-treated and control preparations. Likewise, NANC responses to EFS (50 Hz) in control, incubated preparations (548 ± 85 mg, $n = 13$) were not different from those obtained in fresh tissues (608 ± 32 mg, $n = 32$).

VIP antagonists

Both VIP antagonists (10 μM), [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 and [4-Cl-D-Phe^6, Leu^{17}]-VIP, elicited small, transient rises in tension. When the tension returned to baseline, in 10–15 min, a second frequency-response curve was obtained. Since the VIP antagonists altered neither the magnitude nor the duration of responses to EFS, we determined their effects on exogenous VIP. Curiously, neither antagonist affected responses to exogenous VIP (Figure 7). In addition, responses to PHI were unaffected by the antagonists ($n = 4$, data not shown).

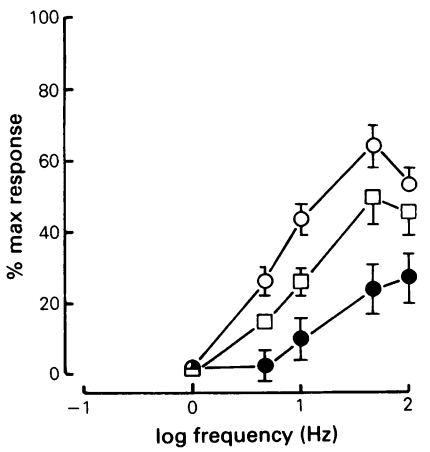

Figure 6 Effect of incubation with antiserum to vasoactive intestinal peptide (VIP, 1250 pg ml^{-1}) or peptide histidine isoleucine (PHI, 4000 pg ml^{-1}) on electrical field stimulation-induced relaxation of guinea-pig trachea. Atropine (1 μM) and propranolol (1 μM) were present throughout. Responses are expressed as a % of the maximum relaxation to sodium nitroprusside (30 μM). (○) Controls, $n = 15$; (●) VIP antiserum, $n = 8$; (□) PHI antiserum, $n = 8$. Vertical lines indicate s.e.mean.

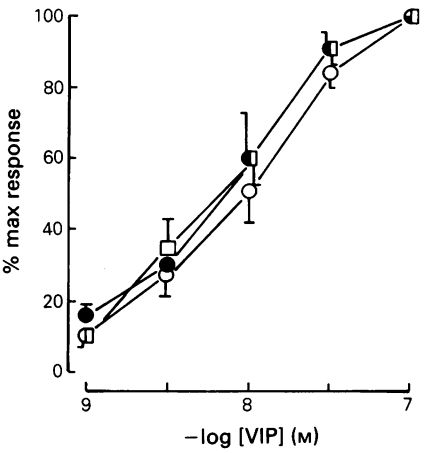

Figure 7 Effect of the vasoactive intestinal peptide (VIP) antagonists [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 (●, 10 μM) and [4-Cl-D-Phe^6, Leu^{17}]-VIP (□, 10 μM) on relaxation responses of guinea-pig trachea to VIP. (○) Controls. Each point is the mean of 5 observations and vertical lines indicate s.e.mean.

Discussion

The biphasic response of guinea-pig trachea to EFS concurs with previous studies in this, and other species, including man (Barnes, 1986; Karlsson, 1986). Similarly, the cholinergic nature of the initial contraction is well-documented. The observation in the present study, that propranolol reduced the magnitude of the relaxation by only 40%, confirms the large NANC component (Coburn & Tomita, 1973; Coleman & Levy, 1974). This is further supported by the observation that in trachea from guinea-pigs chemically sympathectomized with 6-OHDA or reserpine, propranolol has no effect on the inhibitory response to EFS. In human isolated airway smooth muscle the neurogenic inhibitory response does not involve adrenergic nerves, but rather, NANC neural mechanisms appear to account for the total relaxation (Taylor *et al.*, 1984).

Adenosine as the NANC transmitter

A few studies have suggested that in guinea-pig trachea, the NANC inhibitory response to EFS may be mediated by adenosine. For example, the NANC relaxation is augmented by adenosine uptake blocking drugs (Coleman & Levy, 1974), and attenuated by adenosine deaminase (Satchell, 1984). In contrast, the present study failed to demonstrate any effect of the adenosine catabolizing enzyme on the inhibitory response. The reasons for this discrepancy are unknown, but may involve the use of older animals and different tracheal preparations by Satchell (1984). Other studies have failed to demonstrate an effect of the adenosine receptor antagonist theophylline on the NANC relaxation of guinea-pig trachea (Coleman, 1980; Karlsson & Persson, 1984). Similarly, in the present study, the specific adenosine antagonist NPC205 (Schwabe *et al.*, 1985; Kaplita *et al.*, 1986) was without effect on the NANC response. This drug is a potent antagonist of adenosine receptor-mediated relaxation of guinea-pig trachea (Farmer *et al.*, 1988). It is unlikely, therefore, that the NANC neurotransmitter in guinea-pig trachea is adenosine.

ATP as the NANC transmitter

Reactive blue 2 has been described as an antagonist of P_{2y}-purinoceptors, mediating adenosine 5'-triphosphate (ATP)-induced relaxations in several intestinal and vascular preparations (Burnstock & Warland, 1987). In the present study, this agent had no effect on the tracheal NANC relaxation, indicating that ATP acting at P_{2y}-receptors is not involved. Indeed, since the stable analogue, α,β-methylene ATP is inactive in guinea-pig trachea, it has been suggested that there are no receptors for ATP in this tissue, and that relaxant responses to ATP are mediated by its breakdown product adenosine (Christie & Satchell, 1980). Moreover, while ATP-induced tracheal relaxation is virtually abolished by indomethacin, the NANC response is unaffected by the cyclo-oxygenase inhibitor (Karlsson & Persson, 1984). Consequently, ATP is unlikely to be the NANC transmitter in this tissue.

Peptides as the NANC transmitters

Another putative neurotransmitter candidate in guinea-pig airways is VIP, possibly released as a cotransmitter with PHI (Uddman *et al.*, 1978; Matsuzaki *et al.*, 1980; Dey *et al.*, 1981; Barnes, 1986; Karlsson, 1986). The present study confirms previous findings (Venugopalan *et al.*, 1986) that both peptides are potent relaxants of guinea-pig tracheal smooth muscle, VIP being approximately two orders of magnitude more potent than PHI (Lundberg *et al.*, 1984).

In the absence of specific antagonists, alternative experimental approaches are frequently employed to obtain evidence for a putative neurotransmitter candidate. Specific desensitization of cat trachea to VIP, by repeatedly applying the peptide until the response of the preparation was greatly reduced, attenuated the NANC response (Ito & Takeda, 1982). In the present study we attempted, unsuccessfully, to desensitize guinea-pig trachea in a similar manner. We therefore exposed the preparations to a maximum relaxant concentration of VIP, in an attempt to attenuate the response to EFS. This experiment was complicated by the fact that, in the presence of VIP, the trachea remained relaxed for up to 3 h. Therefore, in order to observe further relaxation to EFS, it was necessary to raise the tone with exogenous histamine. Under these conditions, the NANC inhibitory response was reduced by approximately 40%, suggesting that VIP may be involved. It is noteworthy that desensitization to the relaxant effect of VIP in guinea-pig trachea *in vivo* also causes a decrease in the response to NANC nerve stimulation (Venugopalan *et al.*, 1981). It is interesting that, even in the presence of propranolol and a maximally effective concentration of VIP, a major component of the NANC response is still evident. This suggests that a neurotransmitter other than noradrenaline or VIP has a role in the neurogenic relaxation. This other transmitter may be PHI, which has the same distribution in the airways as VIP (Lundberg *et al.*, 1984), and may be released as a cotransmitter with VIP (Barnes, 1986). Conclusive evidence is lacking, however, since we did not determine whether the desensitization to VIP was specific. Furthermore, incubation with PHI antiserum had only a small effect on the magnitude of the NANC relaxation. Conversely, it is possible that another, as yet unknown and perhaps non-peptide, transmitter is involved.

Overnight incubation with VIP-antiserum markedly reduced the magnitude of the NANC inhibitory response of guinea-pig trachea (Matsuzaki *et al.*, 1980), and this was taken to support the notion that VIP is an inhibitory neurotransmitter. As mentioned, however, this study has been criticized (Karlsson & Persson, 1983; 1984), in that inhibition of the NANC response by antiserum was expressed as a percentage of a poorly defined, and very small relaxation. In addition, Matsuzaki and colleagues did not establish the specificity of their antibodies. In the present study we determined the effect of commercially obtained antisera to VIP or PHI on the NANC response. Both antibodies exhibit less than 0.03% cross-reactivity with each other. Furthermore, the

neurogenic relaxations are comparable in magnitude to those obtained by others (Karlsson & Persson, 1984; Taylor *et al.*, 1984).

Preincubation with VIP antiserum caused an approximately 60% reduction in magnitude of the NANC inhibitory response; similar to the 70% reduction obtained by Matsuzaki *et al.* (1980). We also found that PHI antiserum caused only a 20% reduction in the response. It was not possible to examine the effect of VIP and PHI antisera in combination, since mixing the two solutions diluted the VIP antibody to the extent that its effect was markedly reduced. Nevertheless, the separate effects of the antisera strongly imply that VIP in particular, and possibly also PHI, play a role in the NANC inhibitory response to EFS in guinea-pig trachea. That their effects were specific is strengthened further since relaxations to SNP and contractions to MCh were not affected, either by the incubation procedure or by the antisera.

Effect of naloxone

It has been demonstrated that the opiate antagonist naloxone inhibits VIP- and splanchnic nerve-induced catecholamine secretion in rat adrenal medulla (Malhotra & Wakade, 1987a, b). These authors suggested that VIP may be a neurotransmitter in the adrenal medulla, and that naloxone, in the relatively high concentrations used, has a non-specific effect on VIP receptors. Naloxone, however, even at 100 μM, was without effect on the NANC response in guinea-pig trachea. This also suggests that opioid peptides are not involved in the response.

Effect of VIP antagonists

Until recently, conclusive evidence for the role of VIP in NANC neurotransmission has been hindered by the lack of a specific antagonist. However, both [Ac-Tyr^1, D-Phe^2]-GRF(1-29)-NH_2 and [4-Cl-D-Phe^6, Leu^{17}]-VIP have been found to be competitive VIP antagonists in intestinal and pancreatic cells (Waelbroeck *et al.*, 1985; Pandol *et al.*, 1986). Consequently, we sought to determine their effects on the tracheal response to EFS. However, these agents had no effect on the NANC relaxation.

There are several possible explanations for the lack of effect of the antagonists, the most obvious, perhaps, being that VIP is not a NANC transmitter in guinea-pig trachea. However, the results with VIP desensitization, and those with VIP antiserum in the present study, as well as those obtained by Matsuzaki *et al.* (1980), do not support this conclusion. In addition, the putative antagonists were without effect on responsiveness to exogenous VIP (or PHI). Another possibility is that the VIP receptors mediating tracheal relaxation are different from those mediating release of pancreatic amylase or colonic epithelial Cl^- secretion (Pandol *et al.*, 1986). It would be interesting to determine the action of these VIP antagonists in other systems where VIP has an effect. Preliminary data indicate, for example, that [4-Cl-D-Phe^6, Leu^{17}]-VIP (10 μM) displaces specific radiolabelled VIP binding from rat forebrain membranes (P.V. Kaplita, personal communication). Nevertheless, in view of the lack of effect of the antagonists in guinea-pig trachea (despite the marked reduction of the NANC response by VIP antiserum), conclusive evidence for, or against, the NANC transmitter role of VIP, remains equivocal.

In conclusion, the results of the present study provide evidence that at least three neurotransmitters, noradrenaline, VIP and PHI, mediate the neurogenic relaxation in guinea-pig trachea. However, even in preparations desensitized to VIP, or preincubated with specific antisera to VIP or PHI (in the presence of propranolol), a significant component of the NANC response remains. It is conceivable that another neurotransmitter, probably neither adenosine, ATP nor opioid, is also involved in the NANC inhibitory response.

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Effects of peptidases on non-adrenergic, non-cholinergic inhibitory responses of tracheal smooth muscle: a comparison with effects on VIP- and PHI-induced relaxation

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1 The effects of peptidase enzymes on non-adrenergic, non-cholinergic (NANC) inhibitory responses of guinea-pig trachea to electrical field stimulation (EFS), and on relaxations induced by vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI) have been examined.

2 α-Chymotrypsin reduced both the magnitude and, particularly, the duration of the inhibitory response to EFS, whereas papain reduced only the magnitude. Aprotinin, a peptidase inhibitor prevented the effects of α-chymotrypsin but was without effect on papain.

3 α-Chymotrypsin and papain both abolished relaxant responses to exogenous VIP and PHI. The action of α-chymotrypsin was prevented by aprotinin, whereas that of papain was not affected.

4 The peptidases were without effect on concentration-response curves to methacholine or to isoprenaline. It was also observed that, in the absence of the peptidases, aprotinin had no effect on inhibitory responses either to EFS or to exogenous VIP and PHI.

5 It is suggested that neuropeptides, possibly VIP and PHI, released during EFS of guinea-pig trachea, partly mediate NANC relaxations, and that their action may be inhibited by peptidases. However, the lack of effect of aprotonin alone, on responses to EFS, suggests that, if endogenous peptidases are important in terminating the action of neuropeptides, they are resistant to the effect of this particular peptidase inhibitor. It is further suggested that neurogenic relaxation of guinea-pig trachea is also partly mediated by a substance, possibly non-peptide, other than VIP or PHI.

Introduction

It is now recognized that, in addition to the classical neurotransmitters, acetylcholine and noradrenaline, postganglionic nerve endings in most smooth muscle tissues release non-adrenergic, non-cholinergic (NANC) mediators (Daniel, 1985; Burnstock, 1986). NANC inhibitory responses to electrical field stimulation (EFS) in airway smooth muscle from a number of species, including man, have also been demonstrated (Barnes, 1986). The nature of the airway NANC neurotransmitter(s) has not been conclusively elucidated, and probably varies with species studied. In feline (Ito & Takeda, 1982) and guinea-pig (Matsuzaki *et al.*, 1980) airway smooth muscle, a putative candidate is vasoactive intestinal peptide (VIP).

VIP is a potent relaxant of guinea-pig (Venugopalan *et al.*, 1986) and feline (Altiere & Diamond, 1984) airway smooth muscle *in vitro*. The latter study demonstrated that preincubation of cat trachea with the peptidase enzyme α-chymotrypsin abolished the effect of exogenous VIP. Conversely, they also showed that α-chymotrypsin was without effect on the NANC relaxation of this preparation (Altiere & Diamond, 1985), and it was proposed that VIP or related peptides may not act as neurotransmitters in cat airways. It was also considered that peptides resistant to degradation by α-chymotrypsin

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may be responsible for the NANC relaxation in feline trachea. Conversely, canine lung appears not to metabolize VIP (Kitamura *et al.*, 1975), and this may also be the case in the cat.

It has been suggested (Karlsson, 1986) that VIP released during nerve stimulation may act in conjunction with another inhibitory substance. For example, it is known that VIP and peptide histidine isoleucine (PHI), another inhibitory peptide, have identical distribution in the airways (Lundberg *et al.*, 1984). It has been proposed that these peptides, released as cotransmitters, may mediate the NANC inhibitory response of airway smooth muscle (Barnes, 1986).

The present study set out to determine the effects of the peptidases, α-chymotrypsin and papain, on NANC responses of guinea-pig isolated trachea to EFS, and to compare them with effects on responses to exogenous VIP and PHI. In addition, the effect of the peptidase inhibitor aprotinin was examined.

Methods

Male, Duncan-Hartley guinea-pigs (350–500 g: Hazelton, Denver, PA) were used. Preparation of and measurement of isometric tension responses from transverse tracheal strips is described elsewhere (Ellis & Farmer, 1989). Strips were suspended between two platinum ring electrodes in organ baths containing oxygenated, modified Krebs-Henseleit solution at 37°C, and equilibrated for 60 min at an initial resting tension of 1.5 g.

For EFS, rectangular pulses were delivered to the electrodes from a HSE Type 215 stimulator, and frequency-response curves were generated by applying stimuli (50 V, 0.1 ms, 0.1–100 Hz) for 30 s. Control frequency-response curves were obtained in each preparation. Drugs were added to the bath 10 min before the construction of a second curve. Concentration-response curves for VIP or PHI were obtained following their addition cumulatively to the bath. Peptidases were added to the bath 10 min before obtaining a second curve. Where appropriate, aprotinin was added 20 min before the peptidases. Responses are expressed as the mean ± s.e.mean.

Drugs

Atropine H_2SO_4, propranolol HCl, acetyl-β-methylcholine Cl (MCh), isoprenaline HCl, porcine VIP, papain (Type 1V), α-chymotrypsin (Type 1S) and aprotinin were obtained from the Sigma Chemical Co., Missouri, U.S.A. Porcine PHI was obtained from Bachem Inc, California, U.S.A. Isoprenaline was freshly dissolved as a 1 mM stock solution in 0.9% w/v NaCl solution (saline) containing ascorbic acid (0.25%), and dilutions were made in saline. All other agents used were freshly dissolved in saline. Atropine (1 μM) and propranolol (1 μM) were present except where stated.

Results

In the presence of atropine and propranolol, frequency-dependent relaxation responses to EFS were elicited, with the maximum response occurring at 50 Hz. At this frequency, the magnitude of the response was 608 ± 32mg ($n = 32$). Following cessation of stimulation, the tension returned slowly to baseline. After stimulation at 50 Hz, the time taken for 50% recovery was 317 ± 34 s ($n = 10$). Relaxation responses to both VIP and PHI were qualitatively similar, although VIP was more potent (Figure 1).

α-Chymotrypsin (2 units ml^{-1}) had two effects on the inhibitory response to EFS (Figures 2 and 3). Firstly, it decreased the magnitude of the response to about 65% of control (Figure 3a), and, secondly, it markedly reduced the duration of the NANC response (Figure 3b). For example, at a frequency of 50 Hz, the time taken to attain 50% prestimulation baseline was decreased from over 5 min in controls, to approximately 1 min in the presence of the peptidase (Figure 3b). Effects of α-chymotrypsin were prevented completely by preincubation with aprotinin (1.75 u ml^{-1}; Figures 2 and 3). In the presence of α-chymotrypsin, responses to both VIP and PHI, even

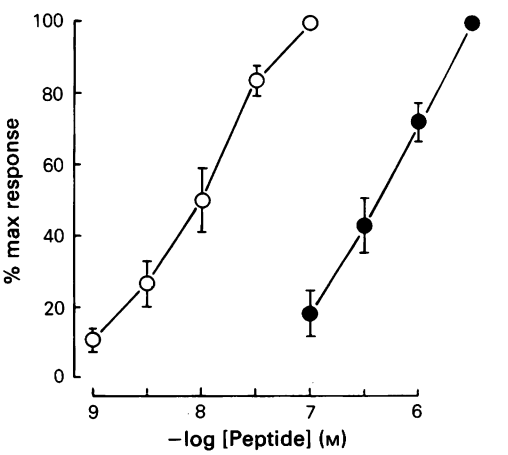

Figure 1 Concentration-response curves to vasoactive intestinal peptide (VIP, ○) and peptide histidine isoleucine (PHI, ●) in guinea-pig trachea. Each point is the mean of 8 observations and vertical lines indicate s.e.mean.

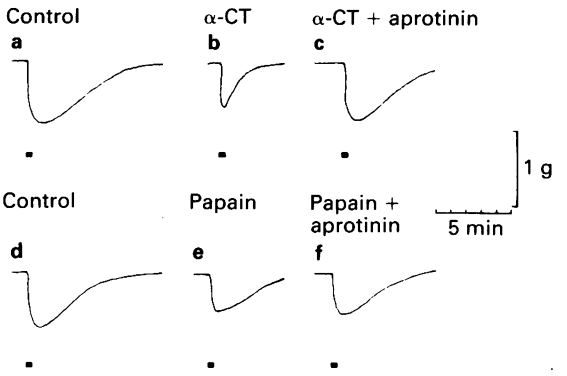

Figure 2 Typical non-adrenergic, non-cholinergic (NANC) relaxations of guinea-pig trachea to electrical field stimulation. Stimulation (50 Hz, 0.1 ms) was applied for 30 s, as indicated by the bars. Atropine (1 μM) and propranolol (1 μM) were present throughout. Responses (a), (b) and (c) were obtained from the same tissue and show, (a) control; (b) the effect of α-chymotrypsin (α-CT, 2 units ml^{-1}) added 10 min before stimulation; (c) inhibition of the effect of α-chymotrypsin by preincubation with aprotinin (1.75 u ml^{-1}, 20 min). Responses (d), (e) and (f) were obtained from the same tissue and show, (d) control; (e) the effect of papain (2 units ml^{-1}) added 10 min before stimulation; (f) inability of aprotinin to inhibit papain.

at high concentrations, were completely abolished, effects which were prevented by preincubation with aprotinin.

Papain (2 units ml^{-1}) decreased the magnitude of the NANC inhibitory response to about 65% of control (Figures 2 and 4a), without affecting its time course (Figure 4b). Aprotonin did not inhibit papain (Figure 4). In addition, papain completely abolished responses to exogenous VIP and PHI, and like its effect on the NANC response, this action of papain was also resistant to prior incubation with aprotinin. Aprotinin alone had no effect on either the magnitude or the duration of the NANC inhibitory responses, or on tracheal responsiveness to VIP or PHI.

In order to ascertain whether the effects of the peptidases on responses to EFS are non-specific, we obtained concentration-response curves to MCh and isoprenaline in the presence and absence of α-chymotrypsin or papain. Atropine and propranolol were absent during these experiments. As can be seen (Figure 5) α-chymotrypsin did not affect responsiveness to either agonist. Similar data were obtained with papain (data not shown). In addition, the inhibitory effects of either of the peptidases on responses to EFS were completely reversible by washing.

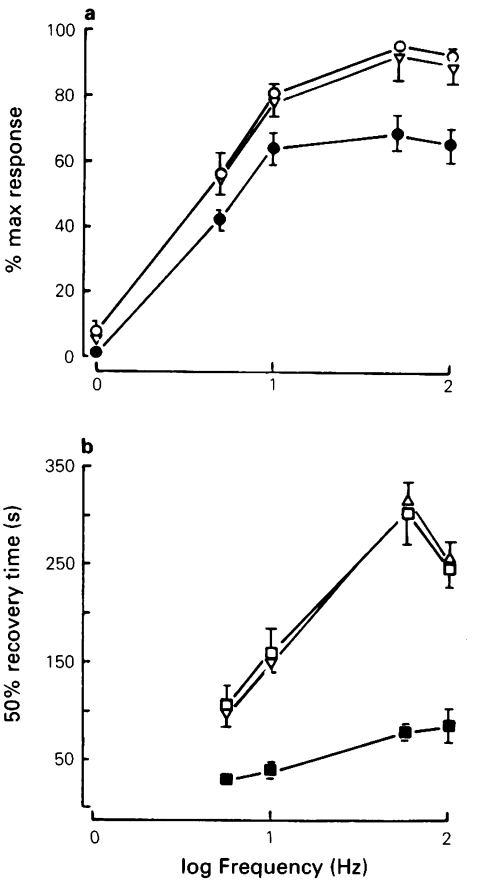

Figure 3 Effects of α-chymotrypsin (2 units ml^{-1}) on NANC relaxations of guinea-pig trachea to electrical field stimulation (50 Hz, 0.1 ms, 30 s), and their reversal by aprotinin (1.75 u ml^{-1}). α-Chymotrypsin was added 10 min before stimulation, and aprotinin, 20 min before α-chymotrypsin. Atropine (1 μM) and propranolol (1 μM) were present throughout. (a) Effect of α-chymotrypsin on magnitude of relaxation: (○) Controls; (●) in presence of α-chymotrypsin; (▽) in presence of α-chymotrypsin and aprotinin. (b) Effect of α-chymotrypsin on time taken for 50% recovery to baseline: (□) Controls; (■) in presence of α-chymotrypsin; (△) in presence of α-chymotrypsin and aprotinin. Each point is the mean of at least 8 observations and vertical lines indicate s.e.mean.

Discussion

In feline trachea, whereas responses to VIP are abolished by α-chymotrypsin (Altiere & Diamond, 1984), the NANC inhibitory response is unaffected (Altiere & Diamond, 1985). This suggested firstly, that VIP,

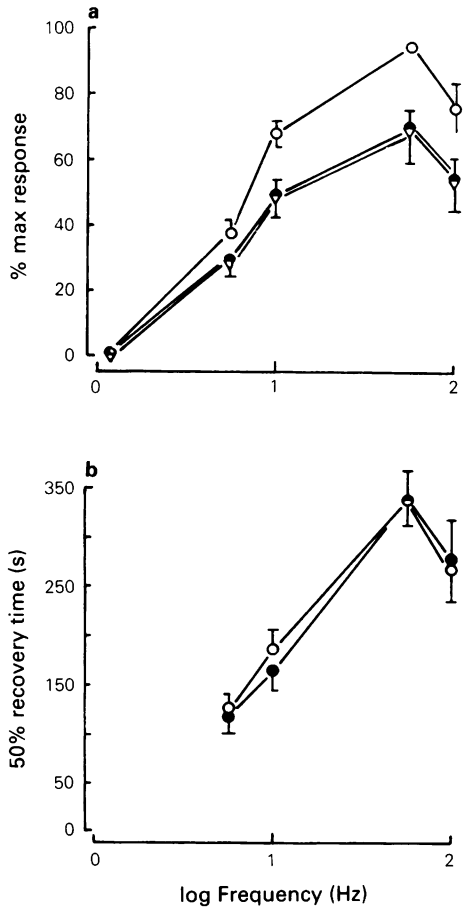

Figure 4 Effects of papain (2 units ml^{-1}) on non-adrenergic, non-cholinergic (NANC) relaxations of guinea-pig trachea to electrical field stimulation (50 Hz, 0.1 ms, 30 s). Papain was added 10 min before stimulation, and aprotinin (1.75 u ml^{-1}), 20 min before papain. Atropine (1 μM) and propranolol (1 μM) were present throughout. (a) Effect of papain on magnitude of relaxation: (○) Controls; (●) in presence of papain; (▽) in presence of papain and aprotinin. (b) Effect of papain on time taken for 50% recovery to baseline: (○) Controls: (●) in presence of papain. Each point is the mean of at least 6 observations and vertical lines indicate s.e.mean.

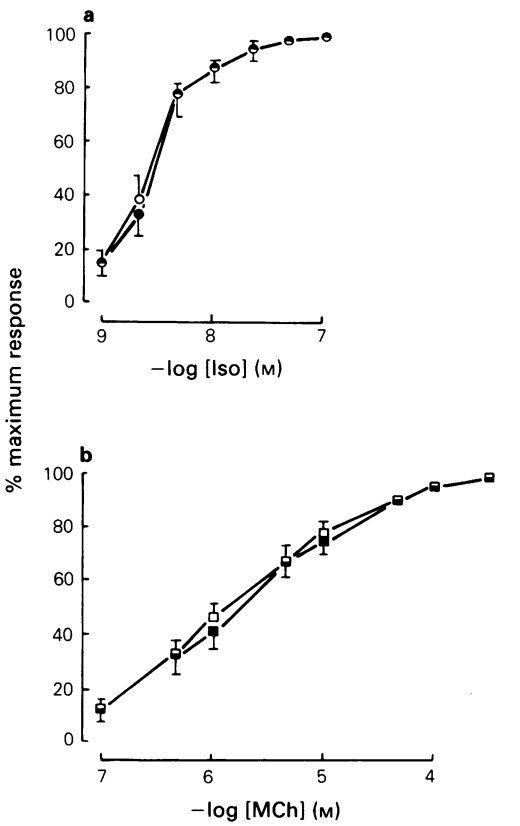

Figure 5 Effect of α-chymotrypsin (2 units ml^{-1}) on guinea-pig tracheal relaxations to isoprenaline (Iso, a) and contractions to methacholine (MCh, b). (a) (○) Control responses to Iso; (●) responses to Iso in presence of α-chymotrypsin; (b) (□) control responses to MCh; (■) responses to MCh in presence of α-chymotrypsin. Each point is the mean of 4 observations and vertical lines indicate s.e.mean.

or related peptides, may not mediate the NANC response or, secondly, if peptides are involved, they may not be susceptible to degradation by α-chymotrypsin. Altiere & Diamond (1984) also showed that a variety of peptidase inhibitors, including aprotinin, did not potentiate cat tracheal responsiveness to VIP, indicating that local enzymatic degradation is not important in inactivation of VIP in this preparation.

Using guinea-pig trachea, we have examined the effect of α-chymotrypsin on neurogenic NANC relaxations and on responses to exogenous VIP, a putative NANC transmitter in this tissue (Matsuzaki *et al.*, 1980; Ellis & Farmer, 1989). In contrast to its action in feline trachea (Altiere & Diamond, 1985), α-chymotrypsin reduced the magnitude and, more pronouncedly, the duration of the NANC response. These effects of α-chymotrypsin were prevented completely by preincubation with the peptidase inhibitor aprotinin. α-Chymotrypsin, which acts at aromatic amino acyl bonds, (Dixon *et al.*, 1979a), also abolished relaxations to VIP and PHI, its effect being prevented by aprotinin. These data suggest that the

NANC inhibitory response in guinea-pig trachea is, at least partly, peptidergic and that it may be mediated by VIP and/or PHI. This is supported further by the observation that papain, another peptidase, also inhibited the NANC response. Moreover, the effects of these peptidases are unlikely to be on effector proteins, since they are completely reversible on washout. In addition, neither peptidase affected tracheal contractions to methacholine or relaxations to isoprenaline.

Aprotinin alone did not influence responses to EFS or to exogenous VIP or PHI. This suggests that neither the NANC transmitter nor the two peptides are inactivated to any appreciable extent by endogenous peptidases such as α-chymotrypsin, trypsin, plasmin or kallikrein, each of which is inhibited by aprotinin (see references in Zynar, 1981). Conversely, papain, which acts at arginine, lysine and glycine residues (Dixon *et al.*, 1979b), also abolished responses to VIP or PHI, an action which was unaffected by aprotinin. Therefore, it is foreseeable that these peptides may be inactivated by airway enzymes that are resistant to the inhibitory effect of aprotinin.

Currently, there is relatively little known concerning the metabolic inactivation of VIP and PHI in the lungs (Bunnett, 1987; Said, 1987). It is noteworthy, however, that relaxations of trachea by VIP can be maintained for several hours (Ellis & Farmer, 1989), indicating that enzymatic degradation, at least *in vitro*, is not a major inactivating pathway for VIP. In canine lung also, there is evidence that VIP is not metabolized (Kitamura *et al.*, 1975). Conversely, relaxation of guinea-pig trachea by VIP is potentiated by phosphoramidon, a neutral peptidase ('enkephalinase') inhibitor (Liu *et al.*, 1987), and it would be interesting to determine the effect of this drug on the tracheal NANC inhibitory response. However, it is unlikely that an opioid peptide is involved, since the NANC response is unaffected by naloxone (Ellis & Farmer, 1989). Our results agree with those obtained with cat trachea (Altiere & Diamond, 1984), in that, in guinea-pig trachea, enzymatic inactivation of VIP does not seem to be an important mechanism. If VIP or PHI do play a role as NANC transmitters in the lung, they may be removed by the circulation and metabolized by blood-borne peptidases.

It is interesting that in rat gastric smooth muscle, α-chymotrypsin produces effects qualitatively similar to those found in guinea-pig trachea in the present study. DeBeurme & Lefebvre (1987) demonstrated recently that while α-chymotrypsin abolished VIP-induced gastric relaxation, it had little effect on the magnitude of the NANC response to EFS, but reduced the duration of the neurogenic response. These authors also showed that, as in the present study, α-chymotrypsin did not affect responsiveness to another mediator, in this case, noradrenaline.

The residual component of the NANC response, in the presence of the peptidases (and propranolol), is unlikely to be mediated by degradation fragments of VIP or PHI, since the activity of the exogenous peptides was abolished, even at high concentrations, by the peptidases. To our knowledge, there are no data on the nature of the degradation products of the action of α-chymotrypsin on VIP, or the biological activity of such peptide fragments. Recently, however, it was demonstrated that α-chymotrypsin-like mast cell chymases cleave VIP, at Tyr^{22}, into two fragments (Caughey *et al.*, 1988). Previous studies on the biological activity, including tracheal relaxation, of various VIP fragments suggests that neither of the resulting fragments produced by chymases would have significant biological activity (Bodanszky *et al.*, 1973; Staun-Olsen *et al.*, 1986).

Another consideration concerning the peptidase-resistant component of the NANC inhibitory response is the possibility that the enzymes, due to their size, may be partially excluded from the synaptic cleft. This is, however, unlikely for the following reasons. Firstly, α-chymotrypsin completely abolishes NANC responses, believed to be mediated by VIP, in dog gastric muscularis mucosa (Angel *et al.*, 1983). Secondly, papain and α-chymotrypsin are similar in size: papain, a single polypeptide chain consisting of 212 amino acid residues, has a molecular weight of 20,700, and α-chymotrypsin, which has 254 amino acid residues in three polypeptide chains, has a molecular weight of 23,000. Yet, these enzymes had qualitatively different effects on the NANC response. Whereas papain and α-chymotrypsin both decreased to the same extent, the maximum magnitude of the NANC response, the latter also markedly reduced the duration of the response. It seems unlikely that these differences could be explained by differential access of the enzymes to the synaptic space. The observation that α-chymotrypsin, but not papain reduced the time course of the NANC response is an interesting one for which we have no explanation at present. The observation that in the presence of α-chymotrypsin, the duration of the response is much reduced suggests that α-chymotrypsin is removing the peptidergic component more rapidly, or more completely, than papain.

If degradation products of VIP or PHI are not responsible for the peptidase-resistant component of the NANC inhibitory response, and if the peptidases employed in the present study diffuse freely to the synaptic cleft, it is possible that another, perhaps non-peptide, substance is released from NANC nerve endings. The nature of this mediator is, at present, unknown but is unlikely to be an opioid peptide, adenosine or ATP (Ellis & Farmer, 1989). Further

studies are therefore necessary to elucidate the nature of the NANC transmitters in the airway.

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The effect of glucocorticosteroids on *in vitro* motility of the ureter of the sheep

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1 The effects of three glucocorticosteroids, hydrocortisone, methylprednisolone and dexamethasone, as well as two non-steroidal anti-inflammatory agents (NSAIDs) indomethacin and diclofenac sodium were tested *in vitro* on the spontaneously rhythmic contracting ureteral preparation of the sheep.

2 The NSAIDs and the steroids methylprednisolone (10^{-7}–10^{-4} M) and dexamethasone (10^{-8}–10^{-4} M), dose-dependently inhibited ureteral motility. Hydrocortisone caused a cessation of spontaneous contractions only at the high concentration of 10^{-4} M.

3 Pretreatment of ureteral strips with the protein synthesis inhibitor cycloheximide (10^{-6} M) abolished the inhibitory action of the corticosteroids on peristalsis, consistent with the suggestion that the action of steroids on the ureter is mediated via the synthesis of the anti-phospholipase protein, lipocortin.

4 The potency of the steroids in descending order was found to be dexamethasone > methylprednisolone > hydrocortisone.

Introduction

A number of non-steroidal anti-inflammatory drugs (NSAIDs) have recently been shown to be efficacious in the clinical management of acute renal colic (Holmlund & Sjödin, 1978; Lundstam *et al.*, 1982). The pain relief produced by these agents may be due to inhibition of prostaglandin synthesis, resulting in intrarenal vasoconstriction, decreased urine production and consequently reduced pelvic pressure (Sjödin & Holmlund, 1982) as well as an analgesic action. The NSAIDs indomethacin and diclofenac sodium (Thulesius & Angelo-Khattar, 1985; Angelo-Khattar *et al.*, 1985) dose-dependently inhibit the intrinsic rhythmic activity of ureteral smooth muscle *in vitro*, ultimately resulting in a complete cessation of motility. This is associated with a reduction in the synthesis of prostaglandin $F_{2\alpha}$, prostacyclin and thromboxane A_2; therefore, the clinical effects can be associated with a spasmolytic action on the ureter (Thulesius *et al.*, 1987).

To date the potential use of glucocorticosteroids in the treatment of ureteral colic has been overlooked. Glucocorticosteroids inhibit the phospholipase A_2 enzyme which catalyses the release of arachidonic acid from membrane phospholipids and as such would also be expected to have a spasmolytic action on the ureter by virtue of a prostaglandin-inhibitory action. The aim of the present study was to ascertain whether steroids block ureteral motility and to compare their efficacy to NSAIDs. The mechanism of steroid action on the isolated ureteral preparation was also investigated. Some results of this study were presented in preliminary form to a meeting of the British Pharmacological Society (Angelo-Khattar & Thulesius, 1987).

Methods

The specimens were obtained from Australian Merino sheep. Kidneys with attached ureters were harvested early in the morning from the local abbatoire. The specimens were put into chilled Krebs-Henseleit solution and transported in thermos flasks to the laboratory. The ureters were dissected free of connective tissue and fat and 4 mm long rings were cut from the proximal part. These preparations were suspended vertically in 10 ml thermostated organ baths filled with Krebs-Henseleit solution at 37°C, bubbled with 95% O_2 and 5% CO_2. The rings were attached to the bottom of the organ bath and the upper end connected with a Bioscience UFI force transducer. Isometric tension was continuously recorded on a Gilson 5/6 five-channel recorder. After suspension of the rings a starting pre-tension of 2 g

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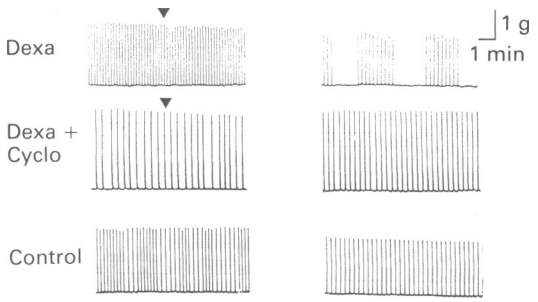

Figure 1 *In vitro* recording of rhythmic contractions of sheep ureteral rings (recording interrupted for 35 min). Upper panel: dexamethasone (10^{-5} M) (Dexa) added at arrowhead. Note intermittent contractions 40 min after drug administration. Middle panel: dexamethasone 10^{-5} M together with cycloheximide 10^{-6} M (Cyclo). Note: no inhibition of rhythmic contractions. Lower panel: control recording without drugs.

was obtained by adjustment with a micrometer screw. When the preparations were allowed to equilibrate, phasic rhythmic contractions started spontaneously. Sixty minutes after mounting when a stable pattern of contractions was established, one drug at a single concentration was added to the bath and the time until peristalsis ceased was measured. This parameter was designated as 'stop time' and used for

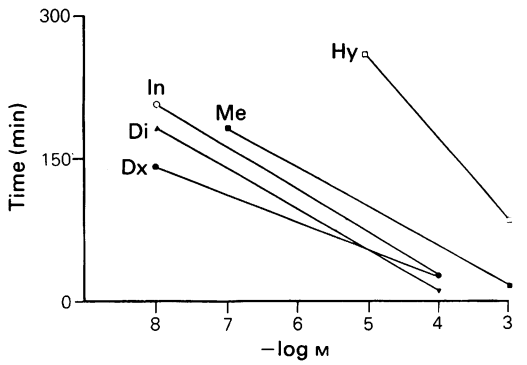

Figure 2 Relationship between concentration of corticosteroids (Hy = hydrocortisone, Me = methylprednisolone, Dx = dexamethasone), non-steroidal anti-inflammatory drugs (In = indomethacin, Di = diclofenac sodium) and time after administration of drug until cessation of phasic contractions = stop time. Graphs are regression lines calculated for 4 doses of each drug: $y = -3.3973-0.224x^{***}$ (for indomethacin); $y = -3.8619-0.224x^{**}$ (for dicloferac sodium); $y = -3.3190-0.032x^{**}$ (for dexamethasone); $y = -2.2755-0.023x^{**}$ (for methylprednisolone); $y = -2.0619-0.011x$ (for hydrocortisone). $^{**}P < 0.01$; $^{***}P < 0.001$.

subsequent assessment of drug effects. Responses were obtained to the NSAIDs indomethacin 10^{-8}–10^{-4} M and diclofenac sodium 10^{-8}–10^{-4} M and to the steroids dexamethasone sodium phosphate 10^{-7}–10^{-4} M, methylprednisolone sodium succinate 10^{-7}–10^{-3} M and hydrocortisone sodium succinate 10^{-5}–10^{-3} M. In addition the effect of dexamethasone (10^{-5} M) after cycloheximide pretreatment (10^{-6} M) was investigated.

Drugs and chemicals

The bathing medium, Krebs-Henseleit solution contained (mM): NaCl 115.3, KCl 4.6, $CaCl_2$ 2.3, $MgSO_4$ 1.2, $NaHCO_3$ 22.1, KH_2PO_4 1.1 and glucose 7.8 and was maintained at pH 7.4.

The following were used: indomethacin (Confortid, 50 mg vials, Dumex A/G, Copenhagen, Denmark); diclofenac Na (Voltaren, Ciba-Geigy Ltd., Basle, Switzerland); dexamethasone as dexamethasone sodium phosphate ampoules (Oradexon, N.V. Organon, OSS, Holland); methylprednisolone (methylprednisolone sodium succinate vials, Solumedrol, Upjohn, Puurs, Belgium); hydrocortisone sodium succinate vials (Solucortef, Upjohn, Puurs, Belgium); cycloheximide (BDH Chemicals Ltd., Poole, England).

Calculations

For each concentration of NSAID and steroid, 6–15 experiments were performed and means calculated. For determination of potency of the individual drugs EC_{50} values were calculated from the dose-effect relationship of stop-times versus log molar concentration calculated by regression analysis based on simple linear regression, $y = a + bx$ where y = stop time in min, x = log dose in mM, a = constant, b = regression coefficient. The method has previously been applied by Bhargava & Thulesius (1987). Mean value ± s.e. of all the data are shown. Standard statistical methods were used for the calculations of means, standard deviation and standard error.

Results

The normal contractile pattern of the sheep ureteral preparation and the effect of the various prostaglandin synthesis inhibitors is shown in Figure 1. After a latency of 10–30 min, phasic rhythmic contractions started spontaneously and could be maintained for 5 h. A decline and ultimately complete cessation of activity was seen earlier in the presence of corticosteroids than in controls. The effect of the two NSAIDs, indomethacin and diclofenac Na, and the

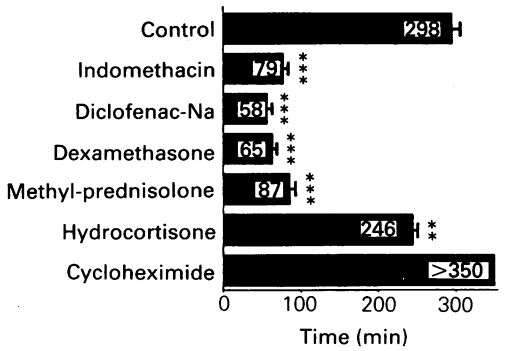

Figure 3 Bar graphs showing mean values for time required for inhibition of spontaneous motility of the sheep ureter (stop times, min) at drug concentration of 10^{-5} M. Control experiments, $n = 10$; indomethacin, $n = 15$; diclofenac Na, $n = 10$; dexamethasone, $n = 8$; methylprednisolone, $n = 8$; hydrocortisone, $n = 6$; cycloheximide, $n = 10$. ***$P < 0.001$; **$P < 0.01$.

three corticosteroids, hydrocortisone, methylprednisolone and dexamethasone, was measured by determining the interval (in min) from drug administration until cessation of rhythmic activity (stop time). Dexamethasone (10^{-8}–10^{-4} M) and methylprednisolone (10^{-7}–10^{-4} M) dose-dependently blocked ureteral motility. Hydrocortisone had an inhibitory action only at the high concentration of 10^{-4} M. Addition of cycloheximide (10^{-6} M) reversed the inhibitory action of the most potent steroid, dexamethasone. Figure 2 shows the relationship between the log molar concentration of the three corticosteroids and two NSAIDs versus stop-time in minutes. The graph was obtained by calculating regression lines for stop-time against molar concentration of the drugs. Figure 3 shows stop times for the control situation, the three corticosteroids, cycloheximide and two NSAIDs at a fixed drug concentration of 10^{-5} M. From this the differential action of the various drugs can be seen. The interesting point in this graph is the fact that cycloheximide itself prolonged the duration of spontaneous ureteral mobility.

Table 1 EC_{50} and relative potency of two non-steroidal anti-inflammatory drugs and three corticosteroids

	EC_{50}	*Relative potency*	n
Indomethacin	$2.1 \pm 2.0 \times 10^{-6}$ M	1	15
Diclofenac Na	$1.1 \pm 0.5 \times 10^{-6}$ M	1.9	10
Dexamethasone	$2.8 \pm 0.6 \times 10^{-6}$ M	0.75	8
Methyl prednisolone	$1.2 \pm 0.4 \times 10^{-5}$ M	0.18	8
Hydrocortisone	$3.8 \times 1.3 \times 10^{-4}$ M	0.008	6

Mean values ± s.e. are given; n = number of experiments.

Discussion

Spontaneous peristaltic contractions are responsible for the transport of urine by the ureter and there is increasing evidence to suggest that this rhythmicity is initiated and maintained by the local release of prostaglandins (Al-Ugaily *et al.*, 1986; Thulesius *et al.*, 1986). Significant amounts of these prostanoids namely $PGF_{2\alpha}$ PGI_2, thromboxane A_2 (TXA_2) were found to be released during ureteral peristalsis and treatment with cyclo-oxygenase blockers was shown to inhibit both ureteral motility (Thulesius & Angelo-Khattar, 1985) and prostaglandin release (Thulesius *et al.*, 1987).

In the present study we have shown that the anti-inflammatory glucocorticoids have an inhibitory action on ureteral smooth muscle similar to that of NSAIDs. This effect was shown to be dose-dependent and complete inhibition of motility with dexamethasone at a high concentration of 10^{-4} M was seen after approximately 24 min which is comparable to that observed for dexamethasone inhibition of prostaglandin release in rabbit coronary microvessel endothelial cells (Rosenbaum *et al.*, 1986). This time lag is indicative of an intranuclear site of action and pretreatment of ureteral strips with cycloheximide, an inhibitor of protein synthesis (Vazquez, 1974) prevented the action of dexamethasone, consistent with the suggestion that steroid action on the ureter is dependent upon the generation of lipocortin, the inhibitory phospholipase modulatory protein (Flower & Blackwell, 1979). The simulation of lipocortin release by steroids has been shown to occur in a number of tissues including rat macrophages (Blackwell *et al.*, 1980), rabbit neutrophils (Hirata *et al.*, 1979) rat renomedullary interstitial cells (Russo-Marie & Duval, 1982) and rabbit coronary microvessel endothelial cells (Rosenbaum *et al.*, 1986). Both the release of arachidonic acid (Hong & Levine, 1976) and histamine (Grosman & Jensen, 1984) are inhibited by glucocorticoids. We have recently reported ultrastructural evidence for the abundant presence of mast cells in the ureter (Al-Ugaily *et al.*, 1988) and although there is no sound explanation as to their possible normal physiological function, it is likely that prostaglandin and histamine release could in part be responsible for the hypermotility and pain associated with ureteral obstruction. Consequently one may speculate that the

addive effects of prostaglandin synthesis inhibition and suppression of histamine release from mast cells by glucocorticoids would make them valuable agents in the treatment of ureteral colic.

The rank order of potency of the steroids was found to be dexamethasone > methylprednisolone > hydrocortisone, which is in agreement with data from the literature (Haynes & Murad, 1985). Furthermore, dexamethasone was found to be similar in potency to the non-steroidal anti-inflammatory agents.

The observation that addition of cycloheximide alone to the organ bath increased spontaneous motility may account for the fact that under basal condition there is a tonic inhibitory influence of corticoids.

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Effects of the bradykinin antagonist B4310 on smooth muscles and blood pressure in the rat, and its enzymatic degradation

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1 Six competitive bradykinin (Bk) antagonists were tested for their agonistic properties on the rat uterus. Five of these peptides showed agonistic effects only at concentrations at least two orders of magnitude higher than those of bradykinin.

2 The antagonistic potency of Lys-Lys-3-Hyp-5,8-Thi-7-DPhe-Bk (B4310) in the rat uterus ($pA_2 = 7.24$) and in the rat duodenum ($pA_2 = 7.31$) was very similar to that determined in an earlier study for the antagonism of the bradykinin-induced stimulation of the trigeminal nerve in the rabbit iris sphincter muscle preparation ($pA_2 = 7.59$).

3 The fall in mean arterial blood pressure induced by i.a. injections of bradykinin was greatly reduced during an i.a. infusion of B4310, but not 10 min thereafter, which indicates a rapid inactivation of B4310 *in vivo*. Bacitracin possibly interferes with the enzymatic cleavage of B4310 but seems to have no effect on the degradation of bradykinin.

4 An i.a. infusion of captopril greatly enhanced the potency of bradykinin in inducing a fall in arterial blood pressure, confirming the important role of angiotensin converting enzyme in the cleavage of bradykinin. However, the design of this experiment did not allow conclusions about the effect of captopril on the degradation of B4310.

5 B4310 incubated with rat lung tissue disappeared from the incubation medium within a few minutes, i.e. as fast as bradykinin, which explains its short duration of action *in vivo*. Captopril partially inhibited the cleavage of both bradykinin and B4310.

6 The present results show that the bradykinin antagonists available at present are useful tools for the investigation of the biological role of bradykinin. However, the susceptibility to enzymatic degradation may limit their usefulness in animal experiments or in clinical studies.

Introduction

Actions of bradykinin have been shown to be inhibited by recently introduced competitive antagonists (Stewart & Vavrek, 1987) in a variety of biological systems such as the rat uterus (Stewart & Vavrek, 1987), the guinea-pig ileum (Stewart & Vavrek, 1987), arteriolar dilatation (Ellis *et al.*, 1987; Barton *et al.*, 1987), venous constriction (Griesbacher & Lembeck, 1987), plasma extravasation (Longridge *et al.*, 1986; Griesbacher & Lembeck, 1987), sensory nerve stimulation (Griesbacher & Lembeck, 1987; Laneuville & Couture, 1987; Steranka *et al.*, 1987; Whalley *et al.*, 1987) and prostaglandin release (Crecelius *et al.*, 1986; Griesbacher & Lembeck, 1987).

In order to obtain additional information about the pharmacological properties of these antagonists we used (1) the rat uterus to quantify not only their antagonistic activities but also their agonistic effects at higher concentrations, (2) the rat duodenum to test the antagonistic action on a smooth muscle preparation which responds to bradykinin with a relaxation and (3) the rat blood pressure to show antagonism against bradykinin in an *in vivo* preparation.

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During passage through the pulmonary circulation, bradykinin is inactivated by several peptidases (Ferreira & Vane, 1967b; Alabaster & Bakhle, 1972; Orawski *et al.*, 1987). Agonistic analogues of bradykinin also have been shown to be broken down in the lung (Stewart & Vavrek, 1987). Angiotensin converting enzyme and carboxypeptidases seem to be major cleaving enzymes responsible for the inactivation of bradykinin (Sheikh & Kaplan, 1986a,b). It has been concluded that the blood pressure lowering effect of angiotensin converting enzyme inhibitors may partly be the result of the protection of circulating bradykinin from enzymatic degradation (Benetos *et al.*, 1986a,b). However, the degradation of bradykinin in plasma or isolated arteries seems to be the result of the action of peptidases different from angiotensin converting enzyme (Marceau *et al.*, 1981; Whalley, 1987).

Earlier experiments indicated that the bradykinin antagonist B4310 has a short duration of action (Griesbacher & Lembeck, 1987). To obtain further information on the inactivation of B4310 *in vivo* we studied the effect of infusions of the peptidase inhibitors bacitracin and captopril on the activity of this bradykinin antagonist. Additionally, the degradation of B4310 by rat lung tissue was determined by bioassay on the rat uterus, and by high performance liquid chromatography (h.p.l.c.).

A preliminary account of the work described in this paper has been presented to the British Pharmacological Society (Lembeck & Griesbacher, 1987).

Methods

Rat uterus

Female Sprague-Dawley rats (180–240 g) were pretreated with diethylstilboestrol (100 $\mu g\,kg^{-1}$, s.c.) 40–44 h before the experiment and their oestrous cycle followed by use of vaginal lavage cytology as described by Traurig *et al.* (1984) and the criteria devised by Long & Evans (1922). Animals in oestrous were killed by cervical dislocation and subsequent exsanguination. The distal parts (1 cm) of the uterine horns were mounted in an organ bath in De Jalón solution (32°C, gassed with 95% O_2 and 5% CO_2) containing 2.8 μM indomethacin and 0.35 μM atropine. A resting tension of 0.5 g was applied to the tissue and contractions recorded isotonically on a pen recorder.

Rat duodenum

Male Sprague-Dawley rats (170–270 g) were killed as above and the proximal part (1.5 cm) of the duodenum was suspended in De Jalón solution containing 0.35 μM atropine and 0.35 μM mepyramine. The resting tension was 1 g and relaxations were recorded isotonically.

Bradykinin was applied to both tissues at intervals of 5 min, the contact time was 1 min. B4310 was added to the organ bath 4 min before the addition of bradykinin. For the determination of pD_2 values the antagonists were applied to the rat uterus in the same way as bradykinin. The values of the responses to the peptides, measured as % of the maximum response to bradykinin, were transformed to logits and were plotted against the logarithm of the concentrations in nM. A regression line was fitted by the least squares method to calculate EC_{50} or pD_2 values. Values for pA_2 and pA_{10} were determined according to Schild (1957).

Rat blood pressure

Female Sprague-Dawley rats (180–280 g) were fasted overnight and pretreated with hyoscine butylbromide (40 $mg\,kg^{-1}$, s.c.) 30 min before anaesthesia with pentobarbitone sodium (50 $mg\,kg^{-1}$, i.p.). The animals were injected with pancuronium bromide (3 $\mu g\,kg^{-1}$, i.v.) and respired artificially. Blood pressure was recorded in a carotid artery with a Statham transducer. The contralateral carotid artery was cannulated for retrograde infusions of B4310 (15 $nmol\,min^{-1}$, 0.15 $ml\,min^{-1}$) and i.a. injections of bradykinin (100 or 25 pmol in a volume of 0.1 ml). After every injection the tubing was rinsed with 0.2 ml of 0.9% NaCl. Infusions of bacitracin (0.2 $mg\,min^{-1}$, 0.1 $ml\,min^{-1}$) or captopril (5 $\mu g\,min^{-1}$, 0.05 $ml\,min^{-1}$) were made into a jugular vein.

Degradation of B4310 by rat lung

Sprague-Dawley rats of either sex (190-280 g) were killed by cervical dislocation. After exsanguination the lung was removed immediately and chopped into slices (30 μM) with a McIlwain tissue chopper: 2 ml of a 10 μM solution of B4310 in Tris buffer was incubated for 15 min with 10 mg of the chopped lung or with the same amount of tissue which had been heated to 96°C for 4 min prior to incubation. In addition, lung slices in Tris buffer alone and B4310 alone were incubated in the same way. After incubation, all preparations were heated to 96°C for 4 min, centrifuged for 15 min at 20000 $m\,s^{-2}$ at 4°C and the supernatant tested for antagonistic potency against bradykinin on the rat uterus.

For h.p.l.c. analysis B4310 and bradykinin were incubated at a concentration of 10 μM in a volume of 1 ml with 50 mg of chopped lung tissue either alone or in combination with captopril (5 μM). Each incubation procedure was repeated three times.

High performance liquid chromatography

H.p.l.c. analyses were performed in a similar manner to that described by Sheikh & Kaplan (1986a,b) with a Beckman gradient system and a Kontron LCD725 UV detector. Samples were applied to a reverse phase column (Waters μBondapak C_{18}, 3.9 × 300 mm), and the separations were carried out with a linear gradient of solvent B (0.05% (v/v) trifluoroacetic acid in acetonitrile) from 10 to 50% over a period of 10 min. Solvent A was 0.05% trifluoroacetic acid in deionized and glass-distilled water. The flow rate was 1.5 ml min^{-1} at room temperature. Products detected at 214 nm were identified by standards of bradykinin and B4310, respectively. Quantification was performed by comparing the peak heights of known amounts of standards with the peak heights of the co-eluting peaks in the incubation samples. The retention times were 8.0 min for bradykinin and 8.4 min for B4310. The sensitivity limit was 100 ng per injected sample (500 μl) for both peptides at a signal-to-noise ratio of 3:1.

Substances

The following substances were used (Thi = β-(2-thienyl)-L-alanine, Hyp = L-4-hydroxyproline): bradykinin (Bk), B4144 (5,8-Thi-7-DPhe-Bk), B4146 (3-Hyp-5,8-Thi-7-DPhe-Bk), B4148 (Lys-Lys-2-Hyp-5,8-Thi-7-DPhe-Bk), B4162 (DArg-3-Hyp-5,8-Thi-7-DPhe-Bk), B4308 (Lys-Lys-2,3-Hyp-5,8-Thi-7-DPhe-Bk), B4310 (Lys-Lys-3-Hyp-5,8-Thi-7-DPhe-Bk), (Dr J.M. Stewart & Dr R.J. Vavrek, Denver, U.S.A.). Stock solutions of all peptides were prepared in a 0.15 M solution of NaCl containing 1 g l^{-1} gelatine and 25 mg l^{-1} cialit (sodium 2-(ethylmercurithio)-benzoxazole-5-carboxylate, Asid-Institut GmbH, F.R.G.) to prevent adsorption to glass and bacterial growth. Atropine (Merck Sharp & Dohme, U.S.A.), mepyramine (Smith Kline & French, U.S.A.), diethylstilboestrol (Serva Feinbiochemie, F.R.G.), indomethacin (Merck Sharp & Dohme, U.S.A.), pentobarbitone sodium (Nembutal, Ceva GmbH, F.R.G.), hyoscine butylbromide (Buscopan, Boehringer Ingelheim, F.R.G.), pancuronium bromide (Pavulon, Organon, Netherlands). Bacitracin (Sandoz GmbH, Austria) and captopril (Squibb Pharma—von Heyden GmbH, Austria) were dissolved and diluted in a 0.15 M solution of NaCl. The composition of the De Jalón solution was (in mM): NaCl 153.9, KCl 5.6, $CaCl_2$ 0.7, $MgCl_2$ 0.1, $NaHCO_3$ 5.0, glucose 3.0. Tris buffer: 0.1 M Tris/HCl in 0.15 M NaCl, pH 7.4.

Statistical analysis

All values represent means ± s.e.mean. Student's *t* test for paired or unpaired comparisons was used as appropriate. Concentration-response curves to bradykinin and bradykinin analogues in the smooth muscle experiments were tested for non-parallelism and non-identity by *t*-tests. The results from the degradation experiments with rat lung were evaluated using the Friedman-test and the Wilcoxon-Wilcox-test.

Results

Agonistic and antagonistic effects in the rat isolated uterus

To determine the agonistic potency of the six bradykinin analogues, concentration-response curves to these peptides were established on the rat uterus. The highest concentration used was 3 μM. All the peptides, except B4308 and B4162, showed agonistic activity (Table 1) with maximum responses equal to those induced by bradykinin. pD_2 values were calculated for these peptides and are shown in Table 1. B4308 and B4162 induced contractions of the rat uterus in the high dose range of 600 to 3000 nM only in 5 and 6 preparations, respectively, of a total of 15 preparations. The highest concentrations produced not more than approximately two thirds (B4308) and one half (B4162) of the maximum contraction induced by bradykinin. However, preparations, in which B4308 and B4162 had no agonistic activity, responded to bradykinin in a concentration-dependent manner. Therefore, the pD_2 values shown in Table 1 for these bradykinin analogues are given in parentheses.

For evaluation of the antagonistic activity of B4310, concentration-response curves to bradykinin were established in the presence of the antagonist in concentrations of 25 to 200 nM. B4310 shifted the

Table 1 Rat uterus: calculated pD_2 values ± 95% confidence intervals and slope of the regression lines for the concentration-response curves for bradykinin and the bradykinin analogues

Peptide	*pD_2*	*Slope*
Bradykinin	8.54 ± 0.12	5.05
B4144	7.26 ± 0.26	6.26***
B4146	6.17 ± 0.08	4.92
B4148	6.41 ± 0.11	3.57***
B4162	(5.53 ± 0.75)	(2.74***)
B4308	(5.76 ± 3.52)	(2.90***)
B4310	5.83 ± 0.08	4.36

n = 5–8. For B4162 and B4308 these parameters were calculated only from those preparations which responded to these peptides (see text), and the results are given in parentheses. Significance of difference in slope compared to bradykinin: ***$P < 0.001$.

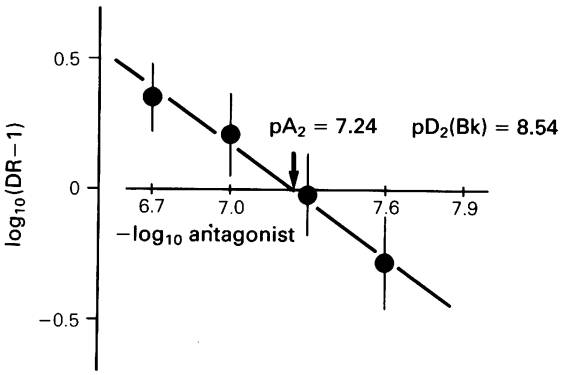

Figure 1 Rat uterus: logarithm of (dose-ratio (DR) minus 1) plotted against negative logarithm of the concentration of B4310 according to Schild (1957). The regression line was calculated by the least squares method. Each point represents mean values, $n = 5$; vertical lines show s.e.mean. $y = 5.4 - 0.71 \times x$; $r = -0.609$.

concentration-response curves to bradykinin to the right in a concentration-dependent manner. No significant differences in the slope of the regression lines or the maximum responses to bradykinin could be found. The ratios of the EC_{50} values for bradykinin in the presence of the antagonist and under control conditions, calculated separately from each of five experiments, were plotted against the negative logarithm of the antagonist concentration and a regression line was fitted by the least squares method (Figure 1). The slope of this regression line was −0.71 which is not significantly different from the theoretical value of −1 for competitive antagonism

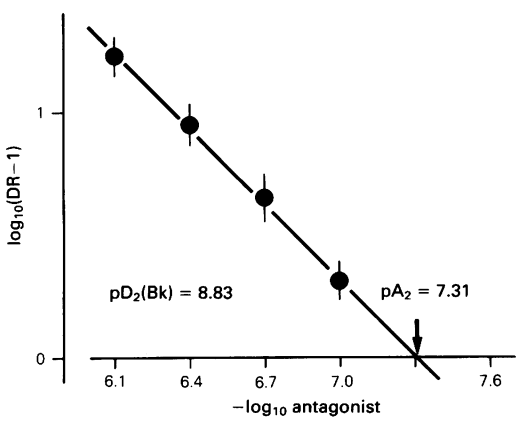

Figure 2 Rat duodenum: Schild-plot for the inhibition of the bradykinin-induced relaxation by B4310. Each point represents mean values, $n = 5$; vertical lines show s.e.mean. $y = 7.46 - 1.02 \times x$; $r = -0.89$.

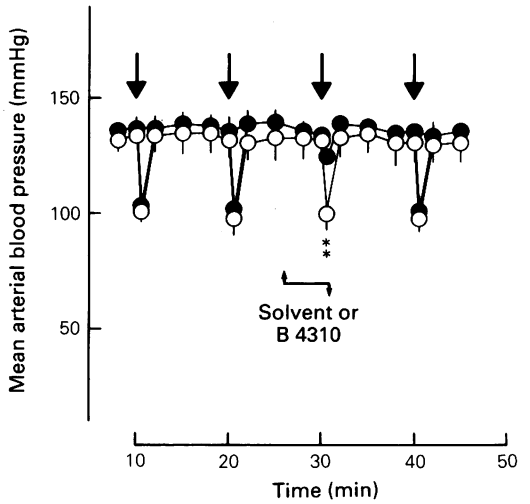

Figure 3 Rat blood pressure: fall in mean arterial pressure induced by 100 pmol bradykinin (Bk) injected i.a. at intervals of 10 min (indicated by arrows). I.a. infusion of B4310 (15 nmol min^{-1}, ●) or 0.15 M NaCl (0.15 ml min^{-1}, ○) began 4 min before the third injection of Bk and lasted for 5 min. Mean values with s.e.mean shown by vertical lines, $n = 6$. Significance of difference between B4310 and saline: $^{**}P < 0.01$.

($\hat{t} = 1.8$, d.f. = 18). A pA_2 of 7.24 and a pA_{10} of 5.90 were calculated for B4310.

Antagonism of the bradykinin-induced relaxations of the rat duodenum

In the experiments on the rat duodenum, B4310 was used in concentrations of 100 to 800 nM. Maximum responses to bradykinin and the slopes of the concentration-response curves to bradykinin were not altered by the antagonist. The shift of the concentration-response curves in the presence of B4310 was evaluated as mentioned above. The Schild-plot is shown in Figure 2. The slope of the regression line was close to unity and the values calculated for pA_2 and pA_{10} were 7.31 and 6.38, respectively.

Inhibition of the bradykinin-induced fall in rat blood pressure

Injections of 0.1 nmol bradykinin into a carotid artery resulted in a fall in mean arterial blood pressure of 30 to 35 mmHg. Repeated injections at intervals of 10 min produced consistent reductions in blood pressure with no desensitization evident. B4310 (15 nmol min^{-1}) was infused i.a. 4 min before the third injection of bradykinin for 5 min (Figure 3).

Control animals received an infusion of a 0.15 M sodium chloride solution. B4310 reduced the bradykinin-induced fall in blood pressure from 35 ± 5 mmHg to 9 ± 1 mmHg, whereas in the saline-infused control animals the fall in blood pressure remained as before. Nine minutes after the end of the infusion the effect of bradykinin was the same in both groups (35 ± 6 mmHg and 33 ± 4 mmHg, respectively).

Effect of bacitracin The effect of the i.a. infusion of B4310 on the fall in arterial blood pressure by 100 pmol bradykinin i.a. was tested during continuous i.v. infusion of bacitracin (0.2 mg min^{-1}) and compared with control animals receiving an i.v. infusion of saline. The inhibition of the effect of bradykinin was more pronounced in the bacitracin-group (4 ± 1 mmHg) than in the saline-group (13 ± 1 mmHg) ($P < 0.01$; Figure 4; 3rd arrow). The next i.a. injection of bradykinin during continuous infusion of bacitracin or saline but without infusion of B4310 resulted in an equal fall in blood pressure in both groups (Figure 4; 4th arrow). The effect of bradykinin was therefore not influenced by bacitracin.

Effect of captopril Infusion of captopril (5 μg min^{-1}) greatly increased the fall in blood pressure induced by the small dose of 25 pmol bradykinin i.a. from 14 ± 1 mmHg to 55 ± 2 mmHg ($P < 0.001$; Figure 5; 3rd arrow). Additional infusion of B4310 reduced the bradykinin-induced fall in blood pressure to 23 ± 2 mmHg ($P < 0.01$; Figure 5; 4th arrow). During control infusions of a 0.15 M solution of sodium chloride instead of B4310, the effect of bradykinin remained as before.

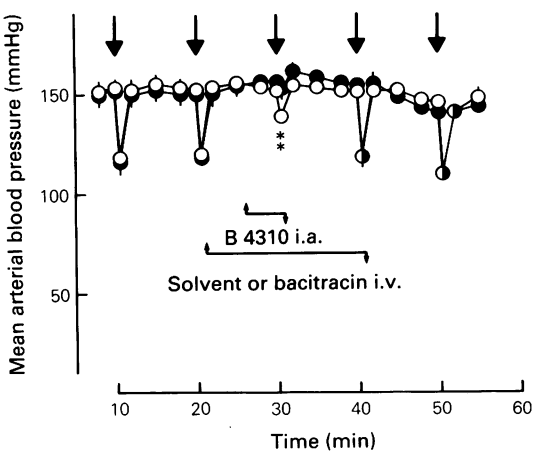

Figure 4 Rat blood pressure: fall in mean arterial pressure induced by 100 pmol bradykinin (Bk) injected i.a. at intervals of 10 min (arrows). Infusion of bacitracin (0.2 mg min^{-1}, i.v., ●) or 0.15 M NaCl (0.1 ml min^{-1}, ○) began 1 min after the second injection of Bk and lasted for 20 min. B4310 was infused in both groups for time indicated. Mean values with s.e.mean shown by vertical lines, $n = 8$. Significance of difference between bacitracin and saline: $^{**}P < 0.01$. Where no s.e.mean is indicated it was smaller than the symbol.

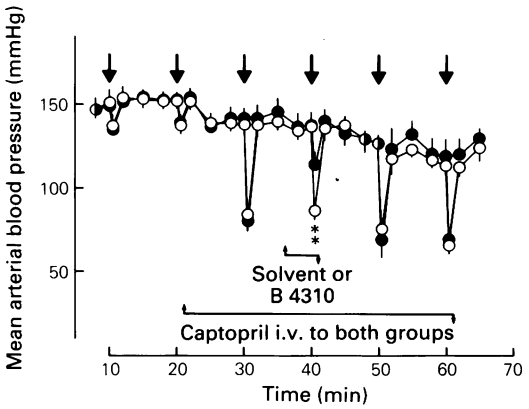

Figure 5 Rat blood pressure: fall in mean arterial pressure induced by i.a. injections of 25 pmol bradykinin (Bk) (arrows). Infusion of captopril (5 μg min^{-1}, i.v.) began 1 min after the second injection of Bk and lasted throughout the experiment (both groups). I.a. infusion of B4310 (15 nmol min^{-1}, ●) or 0.15 M NaCl (0.15 ml min^{-1}, ○) began 4 min before the third injection of Bk and lasted for 5 min. Significance of difference betwen B4310 and saline: $^{**}P < 0.01$. Mean values with s.e.mean shown by vertical lines, $n = 5$.

Degradation of B4310 by rat lung

Determination by bioassay In each rat uterus preparation bradykinin was applied to the tissue in a concentration that produced a contraction of approximately 70% of the maximum effect. B4310, incubated with or without rat lung tissue, was applied to the rat uterus in a volume corresponding to a concentration of 200 nM of unincubated peptide. Incubation with native lung tissue greatly reduced the potency of the antagonist solution to inhibit the action of bradykinin on the rat uterus as compared to incubation of the antagonist without lung tissue ($P < 0.01$, Wilcoxon-Wilcox-test). Inactivation of lung enzymes by heat treatment prevented the degradation of B4310 during the incubation (Table 2). Incubates of native lung tissue alone had no contractile activity on the rat uterus.

Determination by h.p.l.c. Bradykinin and B4310 were incubated for 5, 15 and 45 min with chopped rat lung in the absence or presence of 5 μM captopril (Table 3). Both peptides were destroyed to a large extent within the first 5 min. After 15 min of incubation the two peptides were not detectable any

Table 2 Degradation of B4310 by rat lung: % inhibition of bradykinin-induced contractions of rat uterus by B4310 in a volume corresponding to a concentration of 200 nM of unincubated antagonist

Incubation of B4310	*% inhibition of bradykinin*	
Without tissue	73 ± 8	**
With heat-inactivated lung	68 ± 7	*
With native lung	37 ± 9	

B4310 was incubated for 15 min at 37°C either without tissue, with heat-inactivated lung slices or with native lung tissue. Significance of difference: $*P < 0.05$, $**P < 0.01$ (Wilcoxon-Wilcox-test). Means ± s.e.mean; $n = 8$.

more in the incubates. Captopril inhibited the disappearance of both peptides markedly, but not completely, during the first 15 min of incubation. Thereafter no further degradation could be seen.

Discussion

The discovery of competitive peptide antagonists to bradykinin (Vavrek & Stewart, 1985; Stewart & Vavrek, 1986; 1987; 1988) and the recent development of apparently organ-specific bradykinin antagonists (Stewart, personal communication) prompted further investigation of the pharmacological properties of these peptides (1) to quantify their agonistic compared with their antagonistic potencies, (2) to show antagonism on smooth muscles which respond to bradykinin in different ways, (3) to use an *in vivo* method for the assessment of their activity and duration of action, and (4) to obtain information on enzymatic degradation of the antagonists *in vitro* and *in vivo*. In extension of earlier work (Griesbacher & Lembeck, 1987) only the antagonist B4310 was used, except in the experiments on the rat uterus.

Table 3 Amount of bradykinin (Bk) or B4310 remaining after incubation with chopped rat lung tissue in the presence or absence of 5 μM captopril

	5 min	*15 min*	*45 min*
Bk	27 ± 8	nd	nd
Bk + captopril	64 ± 10	13 ± 5	19 ± 4
B4310	12 ± 3	nd	nd
B4310 + captopril	41 ± 3	8 ± 7	10 ± 1

Determination of bradykinin and B4310 in the incubation medium by h.p.l.c. Means ± s.e.mean; $n = 3$.
nd = not detectable.

Agonistic and antagonistic properties

To assess the agonistic properties of the six bradykinin antagonists we used the rat uterus since this organ is very sensitive to bradykinin (Table 1). B4144 had agonistic effects at concentrations about 20 fold higher than those effective for bradykinin. The rest of the analogues tested in this experiment showed agonistic effects only at concentrations two to three orders of magnitude higher than bradykinin. In the case of B4162 and B4308 some preparations did not react to the peptides at all and B4310 which was used in all other experiments was approximately 500 times less potent in inducing contractions of the rat uterus than bradykinin. It is therefore unlikely that under usual experimental conditions the agonist activity of the bradykinin antagonists would interfere with their antagonistic action.

To assess the antagonistic potency of B4310 in smooth muscle preparations we used the rat uterus and the rat duodenum, two organs which respond to bradykinin in opposite ways: the rat uterus is contracted by bradykinin whereas the rat duodenum responds to bradykinin with a relaxation. The pA_2 values for B4310 determined in these preparations (7.24 and 7.31, respectively) are very close to that determined earlier for the antagonism of the bradykinin-induced stimulation of the trigeminal nerve in the rabbit iris sphincter muscle preparation ($pA_2 = 7.59$; Griesbacher & Lembeck, 1987). An organ-selective specificity of B4310 is therefore rather unlikely.

Degradation of B4310 in vivo *and* in vitro

Stewart & Vavrek (1986) showed that bradykinin antagonists inhibited the blood pressure lowering effect of bradykinin. We used retrograde injections of bradykinin into the carotid artery to assess its vasodilator effect by measuring the rapid and shortlasting fall in arterial blood pressure. It was possible to apply bradykinin repeatedly at a dose of 100 pmol in intervals of 10 min showing that there is no development of tolerance to this dose. The effect only lasted for approximately 1 min, confirming the short half-life of bradykinin in blood, calculated to be less than 30 s in man (Saameli & Eskes, 1962), in the dog (McCarthy *et al.*, 1965) and in the cat (Ferreira & Vane, 1967b). An i.a. infusion of B4310 (15 nmol min^{-1}) almost abolished the effect of the i.a. injection of bradykinin. The full restoration of the action of bradykinin 10 min after the end of the antagonist infusion demonstrated the short duration of the action of B4310.

As it was likely that the antagonistic peptides are cleared in an enzymatically similar manner to bradykinin, the influence of the peptidase inhibitors baci-

tracin and captopril were studied. Bacitracin has been shown to inhibit the cleavage of several different peptides such as TRH and LH-RH (McKelvy *et al.*, 1976), β-endorphin (Patthy *et al.*, 1970) or substance P (Lembeck *et al.*, 1978). However, bacitracin (0.2 mg min^{-1}, i.a.) did not augment the fall in blood pressure induced by bradykinin, thus suggesting that the cleavage of bradykinin is not an effect of bacitracin-sensitive peptidases. The antagonistic potency of B4310 on the other hand appeared to be enhanced, but not prolonged, by bacitracin (Figure 4, 3rd arrow). A possible effect of bacitracin on the enzymatic degradation of B4310 would, however, need more detailed investigation. Bradykinin is largely eliminated from the blood by a single passage through the pulmonary circulation (Ferreira & Vane, 1967a,b; Ryan *et al.*, 1970). Angiotensin converting enzyme seems to play a critical role in the inactivation of bradykinin (Dorer *et al.*, 1974a,b), although other peptidases such as aminopeptidase P may be involved additionally in the lung (Orawski *et al.*, 1987) and in the periphery (Marceau *et al.*, 1981; Whalley, 1987). As the angiotensin converting enzyme inhibitor captopril (Rubin *et al.*, 1978) did not attenuate the breakdown of bradykinin in plasma (Marceau *et al.*, 1981) and isolated arteries (Whalley, 1987) it had been assumed that angiotensin converting enzyme would not contribute to the inactivation of bradykinin in the periphery. In our experiments, however, captopril (5 μg min^{-1}, i.a.) increased the fall in arterial blood pressure induced by an i.a. injection of 25 pmol bradykinin to a size almost three times that of 100 pmol bradykinin in the control experiment (compare 3rd arrow in Figure 5 with 1st and 2nd arrows in Figure 4). This seems to indicate that bradykinin is destroyed very fast in the periphery by an enzyme which is sensitive to captopril, as the action of intra-arterially injected bradykinin is regarded as an immediate action on the arteries before the passage through the lungs (Haddy *et al.*, 1970). Angiotensin converting enzyme is present in vascular endothelial cells (Caldwell *et al.*, 1976; Ryan *et al.*, 1976) and its low potency for inactivation of bradykinin in isolated arteries (Whalley, 1987) might be different from its effect *in vivo*.

B4310 also reduced the effect of bradykinin during the infusion of captopril (Figure 5) as it did in the control experiments (Figure 3), but the design of the experiment did not allow conclusions about the role of angiotensin converting enzyme in the cleavage of B4310. The enzymatic degradation of B4310 was therefore tested *in vitro*. B4310 was incubated with chopped lung tissue and its antagonistic potency was then tested on the bradykinin-induced contractions of the rat uterus. The reduced antagonistic activity of B4310 after incubation with native lung tissue and the inhibition of this inactivation by heat-treatment of the tissue confirms the enzymatic cleavage of B4310 by lung tissue, but does not allow further conclusions (Table 2). Determination by h.p.l.c. of the amount of B4310 and of bradykinin persisting after incubation with chopped lung tissue demonstrated the high degree of degradation of both peptides within the first 5 min of incubation. The cleavage of both peptides was greatly, but not completely, inhibited by captopril, which confirms the contribution of further enzymes in the lung. The fast cleavage of B4310 *in vitro* explains the finding that antagonism of bradykinin actions *in vivo* could only be seen during an infusion of B4310 and the rapid recovery of the bradykinin response after cessation of the infusion.

Our experiments show that the bradykinin antagonists available at present are useful tools for the investigation of the biological role of bradykinin. However, the susceptibility to enzymatic degradation may limit their use in animal experiments or in clinical studies. Therefore long-acting and stable bradykinin antagonists remain to be discovered.

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α_2-Adrenoceptor agonists enhance responses to certain other vasoconstrictor agonists in the rat tail artery

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1 The effects of the α_2-adrenoceptor agonists clonidine, rilmenidine, TL99 and UK14304 on the vasoconstrictor response to sympathetic nerve stimulation and on the concentration-response curves to noradrenaline and phenylephrine were compared in two isolated, perfused vascular tissues: the rat tail artery (which has both postjunctional α_1- and α_2-adrenoceptors), and the rabbit ear artery (in which only α_1-adrenoceptors are present postjunctionally).

2 In the rabbit ear artery, the first observable effect of α_2-adrenoceptor agonists was inhibition of vasoconstrictor responses to sympathetic nerve stimulation. This occurred with concentrations of the α_2-adrenoceptor agonists which were far below those producing vasoconstriction. Responses to noradrenaline were not affected.

3 In contrast, in the rat isolated perfused tail artery, α_2-adrenoceptor agonists, in concentrations that produced no other observable effects, enhanced the vasoconstrictor responses to sympathetic nerve stimulation and to noradrenaline. Much higher concentrations of α_2-adrenoceptor agonists produced vasoconstriction in most preparations and only then reduced the response to sympathetic nerve stimulation. The enhancing effect of α_2-adrenoceptor agonists was blocked by idazoxan, but not by prazosin.

4 Vasoconstrictor responses in the rat tail artery to the relatively selective α_1-adrenoceptor agonist phenylephrine were enhanced by α_2-adrenoceptor agonists. The enhancement of the response to phenylephrine was greater than that to the mixed α_1- and α_2-adrenoceptor agonist noradrenaline.

5 Vasoconstrictor responses in the rat tail artery to vasopressin, ATP and KCl, like those to α_1-adrenoceptor agonists, were enhanced by α_2-adrenoceptor agonists. However, vasoconstrictor responses to 5-hydroxytryptamine and angiotensin II were not enhanced.

Introduction

α_2-Adrenoceptors were first identified on noradrenergic nerve terminals where their activation leads to inhibition of transmitter release (Langer, 1974; Starke & Langer 1979). They were subsequently demonstrated in vascular smooth muscle in which they subserve vasoconstriction, resulting in a pressor response in intact animals (Docherty *et al.*, 1979; Drew & Whiting, 1979; Timmermans *et al.*, 1979; Timmermans & Van Zwieten, 1980), and contraction in isolated preparations of certain blood vessels (Medgett *et al.*, 1981; Holtz *et al.*, 1982; Hicks *et al.*, 1983). Recently a number of authors have shown that postjunctional α_2-adrenoceptors are involved in several physiological functions in various tissues (Berridge *et al.*, 1983; Reid, 1985; Strandhoy, 1985; Skoglund *et al.*, 1987).

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Shepperson (1984) demonstrated that the contraction of the nictitating membrane of the cat evoked by α_1-adrenoceptor agonists was enhanced by predosing with α_2-adrenoceptor agonists. He suggested that, in some tissues, the action of α_2-adrenoceptor agonists may be modulation of the response to α_1-adrenoceptor activation rather than to evoke a discrete response. This was extended by our previous study on the rat tail artery (Xiao *et al.*, 1987b; Xiao & Rand, 1987), which gave rise to the hypothesis that α_2-adrenoceptor agonists do not directly produce vasoconstriction but provide an ancillary drive to the vasoconstrictor stimulus produced by α_1-adrenoceptor agonists.

We have now compared the effects of α_2-adrenoceptor agonists on responses to sympathetic nerve stimulation and α_1-adrenoceptor agonists in two different vascular tissues *in vitro*: the

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rat tail artery, which has postjunctional α_2-adrenoceptors (Weiss *et al.*, 1983; Medgett & Langer, 1984), and the rabbit ear artery, which lacks postjunctional α_2-adrenoceptors (Hieble & Woodward, 1984). We have also studied the effects of α_2-adrenoceptor agonists on responses to various other agents producing vasoconstriction in the rat tail artery.

Methods

The animals used were male Sprague-Dawley rats from our own colony, weighing 250–350 g and New Zealand white rabbits (Tillotside Rabbit Stud, South Australia) of either sex weighing 2–3 kg. The rats were anaesthetized with pentobarbitone (30 mg kg^{-1}, i.p.), and the rabbits were killed by a blow to the neck and exsanguination. Segments of approximately 1 cm long of the rat tail artery and the rabbit central ear artery were dissected out as described by Medgett & Langer (1984, 1986) and de la Lande & Rand (1965), respectively.

The segment of artery was cannulated at the proximal end under a dissecting microscope. The distal end was tied and an opening was made in the wall just below the tie. The segment was mounted vertically, distal end uppermost, under a tension of 0.5 g and perfused, using an LKB peristaltic pump, with a modified Krebs solution at a rate of 4 ml min^{-1}, which has been shown previously to be satisfactory for preparations of both the rabbit ear artery (de la Lande & Rand, 1965) and the rat tail artery (Medgett & Rajanayagam, 1984). The perfusate, after passing through the lumen, was allowed to superfuse the adventitial surface of the vessel. The perfusion pressure was measured with a Statham P23Db pressure transducer and recorded on a Rikadenki chart recorder. Increases in perfusion pressure in mmHg were used to provide a measure of vasoconstrictor responses. Two platinum ring electrodes, 4 mm apart, were located around the proximal end of the artery segments for electrical stimulation of the periarterial sympathetic nerves. The preparations were surrounded by a water jacket maintained at 37°C. A 30 min period of perfusion-superfusion was allowed to elapse before any experimental procedures were carried out.

Stimulation of periarterial nerves was with monophasic square wave pulses of 1 ms duration and supramaximal voltage at a frequency of 2 Hz (delivered from a Scientific & Research Instruments Ltd stimulator). Each period of stimulation was continued until the rise in perfusion pressure had reached the peak level (usually 5–10 s). Vasoconstrictor responses to drugs were obtained by replacing the normal perfusing solution with one containing the required concentration of the agonist, which was subsequently perfused until the response reached a peak value (usually 1–2 min). Depolarization-evoked contractions were obtained by replacing the normal solution with one in which the equivalent amounts of NaCl were replaced by 24–64 mM KCl. Care was taken to limit rises in perfusion pressure to less than 300 mmHg to avoid damage and desensitization of the preparation (Medgett & Rajanayagam, 1984).

Rat tail artery segments were considered to be satisfactory if rises in perfusion pressure of 40 mmHg or more were elicited with stimulation at 2 Hz for 10 s or less. For the rabbit ear artery, the vasoconstrictor effect of noradrenaline was observed to establish that the responsiveness was satisfactory.

The effects of most vasoconstrictor agents were studied by constructing two non-cumulative concentration-response curves; the second was begun 30 min after completing the first. In experiments in which the effects of α_2-adrenoceptor agonists on vasoconstrictor agents were to be assessed, they were added to the perfusing solution after the control responses were established and remained present for the rest of the experiment.

Perfusion solution

The modified Krebs solution had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.45, $NaHCO_3$ 25, KH_2PO_4 1.03, D-glucose 11.1, disodium edetate 0.067. The solution in the supply reservoir was continuously gassed with a mixture of 5% CO_2 in O_2. It passed through a heat-exchanger maintained at 37°C immediately before it entered the perfusion cannula.

Drugs

The following drugs were used: angiotensin II (AII; hypertensin, Ciba-Geigy); adenosine 5'-triphosphate (ATP), sodium salt (Sigma); clonidine hydrochloride (Boehringer Ingelheim); 5-hydroxytryptamine creatinine sulphate complex (5-HT, Sigma); idazoxan hydrochloride (Reckitt & Colman); (−)-noradrenaline hydrochloride (Sigma); rilmenidine (formerly oxaminozoline) phosphate (Servier); phenylephrine hydrochloride (Sigma); prazosin hydrochloride (Pfizer); TL99 (2-(*NN*-dimethyl)amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene, synthesized by Synthelabo, L.E.R.S.); UK14304 (5-bromo-6-[2-imidazoline-2-Yl-aminol]-quinoxaline, Pfizer); vasopressin (Parke-Davis). Stock solutions of noradrenaline, TL99 and 5-hydroxytryptamine were made in distilled water which contained ascorbic acid (0.05 mg ml^{-1}). Other drugs were dissolved in distilled water.

Statistical analysis

Values are given as mean ± s.e.mean and comparisons were by Student's paired or unpaired *t* test, as appropriate. The statistical significance of shifts of concentration-response curves was determined from comparison of the analyses of regression of response on log concentration for the linear portions of the curves; where slopes did not differ, horizontal displacements and their significances were calculated. Probability levels of less than 0.05 were taken as indicating significant differences.

Results

Effects of α_2-adrenoceptor agonists on responses to sympathetic nerve stimulation

In the rabbit ear artery, infusions of clonidine (10 to 300 nM) produced marked concentration-dependent reductions of vasoconstrictor responses to sympathetic nerve stimulation, as illustrated in Figure 1a. With concentrations of 10 and 30 nM, clonidine had no effect on the resting perfusion pressure, but the higher concentrations (100 and 300 nM) produced vasoconstriction; when this occurred, the inhibitory effect was taken from the raised level of perfusion pressure.

In the rat tail artery, infusions of rilmenidine in concentrations that had no direct vasoconstrictor action (0.01 to 3 μM) enhanced the vasoconstrictor responses to sympathetic nerve stimulation significantly (paired *t* test), as illustrated in Figure 1b. When the concentration of rilmenidine was raised to 10 μM, the mean vasoconstrictor response to sympathetic nerve stimulation did not differ significantly from the pre-rilmenidine level. In a few experiments, 10 μM rilmenidine increased the resting perfusion pressure. The highest concentration of rilmenidine used in this series of experiments (30 μM) usually produced vasoconstriction and significantly inhibited the response to sympathetic nerve stimulation. However, in other experiments it was noted that a vasoconstrictor effect only occurred when the concentration of rilmenidine was raised to 100 μM.

Similar patterns of effects to those described above for clonidine on the rabbit ear artery and rilmenidine on the rat tail artery were obtained with the other α_2-adrenoceptor agonists used in this study, but we have not as yet made systematic quantitative comparisons between the various agonists.

Effects of α-adrenoceptor antagonists in the rat tail artery

The relatively selective α_2-adrenoceptor antagonist idazoxan (300 nM) had no effect on the resting perfusion pressure. It reduced vasoconstrictor responses to sympathetic nerve stimulation by 14 ± 8% (n = 10) of the control level, but this was not significant. Idazoxan (300 nM) abolished the enhancement by rilmenidine of the stimulation-induced vasoconstrictor responses, as illustrated in Figure 1b. The direct vasoconstrictor response to the higher concentrations of rilmenidine was not affected by idazoxan. The effect of the higher concentrations of rilmenidine in reducing responses to sympathetic nerve stimulation still occurred in the presence of 300 nM idazoxan (Figure 1b).

The relatively selective α_1-adrenoceptor antagonist prazosin (0.3 nM) markedly reduced vasoconstrictor responses to sympathetic nerve stimulation by 32.8 ± 2.7 (n = 13) of the control level. The enhancement by rilmenidine of the responses to sympathetic nerve stimulation in the presence of prazosin did not

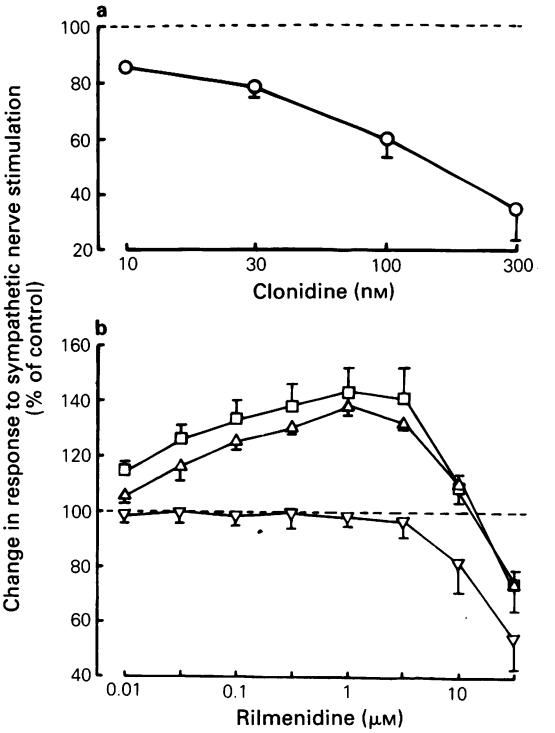

Figure 1 Effects of α_2-adrenoceptor agonists on vasoconstrictor responses to sympathetic nerve stimulation (1 ms pulses at 2 Hz for 10 s at 5 min intervals) in isolated perfused arteries. (a) Effects of clonidine in rabbit ear arteries (n = 5). (b) Effects of rilmenidine in rat tail arteries (□, n = 8), together with its effects in the presence of prazosin (0.3 nM, △, n = 4) or in the presence of idazoxan (300 nM, ▽, n = 4). Symbols indicate mean values and vertical lines show s.e. There were no significant differences between the means for rilmenidine alone and the corresponding means for rilmenidine in the presence of prazosin (*t* test).

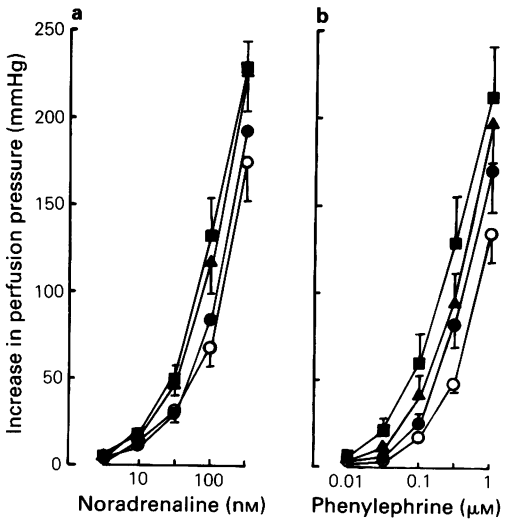

Figure 2 Concentration-response curves for (a) noradrenaline- and (b) phenylephrine-induced vasoconstriction in rat perfused tail arteries. (○) First curves for vasoconstrictor agonist alone (a: $n = 5$; b: $n = 6$). (●), (▲) and (■) second curves in the presence of 10, 100 or 1000 nM rilmenidine, respectively ($n = 6$ for each drug at each concentration). Symbols indicate mean values and vertical lines show s.e.

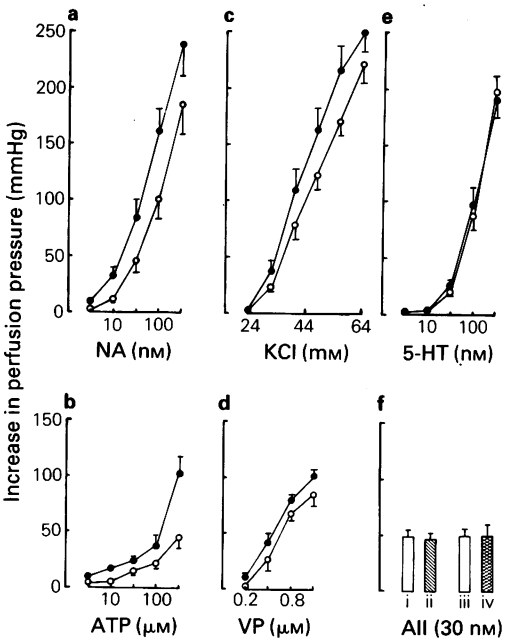

Figure 3 Effects of UK14304 on responses to vasoconstrictor agents in rat isolated perfused tail arteries. (a) Responses to noradrenaline (NA, $n = 6$) in the absence (○) and presence (●) of UK14304 (10 nM). (b–e) Responses to (b) ATP ($n = 4$), (c) KCl ($n = 4$), (d) vasopressin (VP, $n = 5$) and (e) 5-hydroxytryptamine (5-HT, $n = 4$) in absence (○) and presence (●) of UK14304 (30 nM). (f) Responses to 30 nM angiotensin II (AII, $n = 4$): (i) and (iii) are first responses; (ii) and (iv) are second responses in the absence and presence, respectively, of UK14304 (30 nM).

differ significantly from that in its absence (Figure 1b). The vasoconstrictor effect of the higher concentrations of rilmenidine was reduced by prazosin; with 30 μM rilmenidine, 0.3 nM prazosin reduced the vasoconstrictor response to 55 ± 2% ($n = 4$) of the control level. The inhibitory effect of the high concentration of rilmenidine on responses to sympathetic nerve stimulation was not affected by prazosin (Figure 1b).

Qualitatively similar effects of idazoxan and prazosin on the actions of the other α_2-adrenoceptor agonists used in this study have been observed, but no quantitative comparisons have been made.

Effects of α_2-adrenoceptor agonists on responses to noradrenaline and phenylephrine

In the rat tail artery, when two concentration-response curves for either noradrenaline (3–300 nM) or phenylephrine (0.01 to 1 μM) were obtained in succession, the second was virtually superimposable on the first. In the rabbit ear artery, the second concentration-response curve for noradrenaline (3–300 nM) exhibited a slight but not statistically significant time-dependent shift to the left.

When the second concentration-response curve for noradrenaline or phenylephrine in the rat tail artery was obtained in the presence of rilmenidine (0.01–1 μM), it was shifted to the left (Figure 2). The lowest concentration of rilmenidine used (0.01 μM) did not significantly shift the curve for noradrenaline but significantly shifted that for phenylephrine. The other α_2-adrenoceptor agonists used in this study had qualitatively the same effects as rilmenidine on concentration-response curves for noradrenaline and phenylephrine, but a systematic quantitative comparison has not yet been made. However, a comparison of the data in Figures 2a and 3a shows that 1000 nM rilmenidine and 10 nM UK14304 produced about the same shift to the left of the concentration-response curve for noradrenaline.

In the presence of α_2-adrenoceptor agonists at concentrations which significantly shifted the concentration-response curve for noradrenaline to the left in the rat tail artery, there was no significant effect on responses to noradrenaline in the rabbit ear artery.

Effect of α_2-adrenoceptor agonists on response to various types of vasoconstrictor agents in rat tail artery

The effects of α_2-adrenoceptor agonists, exemplified by UK14304, on vasoconstrictor responses to noradrenaline, KCl, 5-hydroxytryptamine, ATP, vasopressin, and angiotensin II are illustrated in Figure 3. UK14304 had no direct vasoconstrictor action at the concentrations used (10 and 30 nM).

The concentration-response curve for noradrenaline (3–300 nM) was significantly shifted to the left by 10 nM UK14304 (Figure 3a), and that for vasopressin (0.2–1.6 μM) was significantly shifted to the left by 30 nM UK14304 (Figure 3d).

Vasoconstrictor responses to ATP (10–300 μM) were significantly enhanced by 30 nM UK14304; the enhancement was greater with the highest concentration than with the lower concentrations of ATP (Figure 3b).

The concentration-response curve for KCl in the presence of 30 nM UK14304 departed significantly from parallelism with the control curve (Figure 3c), but vasoconstrictor responses to 40, 48 and 56 mM KCl were significantly increased (paired t test). The nature of the response to KCl was changed by UK14304: in its absence, there was a rapid initial vasoconstrictor effect followed by a lower secondary phase which slowly declined but, in the presence of UK14304, the initial peak was increased and the secondary phase declined more rapidly (Figure 4).

UK14304 had no effect on the concentration-response curve for 3–300 nM 5-hydroxytryptamine (Figure 3e) or on responses to single doses (30 nM) of angiotensin II (Figure 3f). Concentration-response curves for angiotensin II were not obtained because of the marked tachyphylaxis to its vasoconstrictor action.

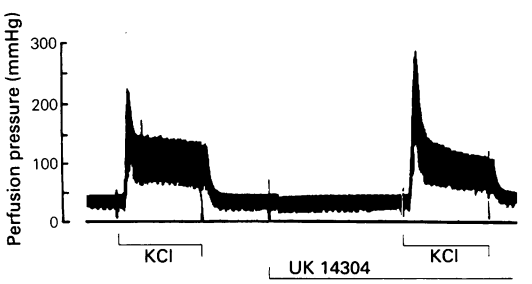

Figure 4 Effect of the addition of UK14304 (30 nmol^{-1}) on vasoconstrictor responses produced by exposure for 5 min to KCl (48 mmol l^{-1}) in a rat isolated perfused tail artery. Vertical axis: perfusion pressure in mmHg. Note that in this experiment the administration of KCl was for a longer period than described in Methods (1–2 min), in order to illustrate more clearly the change in the nature of the response.

Discussion

The aim of the present study was to confirm and extend our previous observations (Xiao & Rand, 1987; Xiao *et al.* 1987a,b), that clonidine and other α_1-adrenoceptor agonists enhanced vasoconstrictor responses elicited by activation of α_1-adrenoceptors in the rat tail artery preparation, and to compare them with those made in the rabbit ear artery preparation, since these two preparations have different populations of postjunctional α-adrenoceptor subtypes (see Introduction).

Vasoconstrictor responses of rabbit ear artery elicited by sympathetic nerve stimulation were reduced by the α_2-adrenoceptor agonist clonidine in concentrations (10–30 nM) far below those producing vasoconstriction (100 and 300 nM). This result is consistent with the findings of Starke *et al.* (1975), using the rabbit pulmonary artery, and Steinsland & Nelson (1975), using the rabbit ear artery; they showed that clonidine inhibited responses to sympathetic nerve stimulation in much lower concentrations than those required to produce vasoconstriction. Both pulmonary and ear arteries from rabbit lack postjunctional α_2-adrenoceptors (Cambridge & Davey, 1980; Hieble & Woodward, 1984), so the only α_2-adrenoceptors on which clonidine can act are prejunctional, thus reducing the release of noradrenaline. The postjunctional α_1-adrenoceptors are only activated by high, non-selective concentrations of clonidine.

Vasoconstrictor responses of rat tail artery elicited by sympathetic nerve stimulation were enhanced by rilmenidine and other α_2-adrenoceptor agonists. Unlike the rabbit ear artery, the rat tail artery has both pre- and postjunctional α_2-adrenoceptors (Weiss *et al.*, 1983; Medgett & Langer, 1984), so it is possible that the enhancing effect of rilmenidine, and other α_2-adrenoceptors agonists, on responses of the rat tail artery to sympathetic nerve stimulation depends on the presence of postjunctional α_2-adrenoceptors. In accord with this, the enhancement of responses to sympathetic nerve stimulation by rilmenidine was abolished by the α_2-adrenoceptor antagonist idazoxan (300 nM) but not by the relatively selective α_1-adrenoceptor antagonist prazosin (0.3 nM).

Taking together the observations made in rabbit ear and rat tail artery preparations, it is possible that the enhancement of vasoconstrictor responses to sympathetic nerve stimulation produced in the latter preparation by α_2-adrenoceptor agonists contains a component attributable to a prejunctional inhibition of noradrenaline release, which partly offsets the

postjunctional augmentation of the contractile response of the vascular smooth muscle. However, we have not yet tested this possibility in experiments in which stimulation-induced release of noradrenaline was measured.

There is a clear dissociation between the direct vasoconstrictor effect of rilmenidine, which was markedly reduced by prazosin but not affected by idazoxan, and its enhancing effect on responses to sympathetic nerve stimulation, which was blocked by idazoxan and not significantly affected by prazosin. It appears, therefore, that its direct vasoconstrictor action is exerted on α_1-adrenoceptors, reflecting loss of selectivity in high concentrations.

The enhancement of responses to sympathetic nerve stimulation by rilmenidine was abolished by a concentration of idazoxan which did not abolish the inhibitory effect of rilmenidine on responses to sympathetic nerve stimulation. A similar finding came from a study in which TL99 was used (Xiao *et al.*, 1987b) and led us to suggest that it indicates a difference between pre- and postjunctional α_2- adrenoceptors. However, it may merely reflect an inadequate competitive blockade by idazoxan of the higher concentration of rilmenidine required for manifestation of the prejunctional inhibitory action.

In rat tail artery, vasoconstrictor responses elicited by noradrenaline and phenylephrine, like those to sympathetic nerve stimulation, were enhanced in the presence of α_2-adrenoceptor agonists. This is consistent with the finding of Shepperson (1984) with the cat nictitating membrane that the contractions evoked by α_1-adrenoceptor agonists were enhanced by pre-dosing with α_2-adrenoceptor agonists. We have demonstrated in this study and elsewhere (Xiao & Rand, 1987; Xiao *et al.*, 1987a,b) that a range of α_2-adrenoceptor agonists markedly enhance vasoconstrictor responses to α_1-adrenoceptor agonists in the rat tail artery in concentrations that are one-hundredth to one-thousandth of the concentrations directly producing vasoconstriction. These findings strongly suggest that the physiological role of postjunctional α_2-adrenoceptors is augmentation of the stimulus arising from activation of α_1-adrenoceptors, rather than direct production of a response.

The enhancement by rilmenidine of responses to noradrenaline was less than that of responses to phenylephrine. A possible explanation for this finding is that a larger proportion of the α_2-adrenoceptors were already activated by noradrenaline than by phenylephrine, since noradrenaline acts on α_1- as well as α_2-adrenoceptors, whereas phenylephrine is a relatively specific α_1-adrenoceptor agonist. Hence an additional α_2-adrenoceptor agonist could be expected to produce a greater effect in the presence of phenylephrine than in the presence of noradrenaline.

If the explanation for the greater enhancement of responses to noradrenaline than those to phenylephrine by rilmenidine is correct, it follows that an agonist acting on both α_1- and α_2-adrenoceptors would be producing a two fold effect; a primary stimulus for the response through the former, and a secondary enhancing effect through the latter. Such a concept has implications for studies on structure-action relationships and kinetic analysis of drug-receptor interactions for drugs acting on α-adrenoceptors. The rat isolated perfused tail artery is not a suitable preparation for exploration of a two fold effect of α-adrenoceptor agonists because large vasoconstrictor responses produce damage and erratic changes in responsiveness, precluding the obtaining of maximal responses and hence of ED_{50} values.

We have not set out systematically to compare α_2-adrenoceptor agonists in respect of their property of enhancing vasoconstrictor responses produced by activating α_1-adrenoceptors. However, the enhancement of responses to noradrenaline was greater with UK14304 than with rilmenidine. The difference may be due to the fact that UK14304 is a full agonist (Grant & Scrutton, 1980; Cambridge, 1981), whereas rilmenidine is a partial agonist (Li & Rand, 1988). Therefore we used UK14304 to determine whether or not α_2-adrenoceptor agonists enhanced vasoconstrictor responses of the rat tail artery produced by agents other than those activating α_1-adrenoceptors.

In rat tail artery, ATP elicited vasoconstrictor responses in concentrations of 3–300 μM. Responses of smooth muscle to ATP are usually due to activation of P_2-purinoceptors (Burnstock *et al.*, 1972; Reilly & Burnstock, 1987). The vasoconstrictor responses to ATP were enhanced by UK14304, especially with the highest concentration of ATP used (300 μM). Vasoconstrictor responses to vasopressin (0.2–1.6 μM) and KCl were also enhanced by UK14304. The increase in the vasoconstrictor responses to KCl produced by UK14304 indicates that α_2-adrenoceptor agonists can enhance not only responses to vasoconstrictor agonists acting on specific receptors, but also the response to a depolarizing agent. Therefore, some common mechanism which links the agents to the vasoconstrictor response may be involved.

A clue to the mechanism is provided by the findings that vasopressin-induced vasoconstriction depends on extracellular calcium and is inhibited by calcium antagonists (Fleckenstein, 1977; Altura & Altura, 1977; Hof, 1985). We have previously found that the enhancing effect of TL99 on vasoconstrictor responses to noradrenaline in the rat tail artery is reduced by diltiazem and abolished in a calcium-free solution (Xiao *et al.*, 1987a). It was suggested, therefore, that α_2-adrenoceptor agonists amplify the vaso-

constrictor responses to contractile agents by augmenting the influx of calcium.

The vasoconstrictor responses to 5-HT and AII were not enhanced by UK14304. The reasons for this are not clear. However, it may be relevant that AII and 5-HT, like α_2-adrenoceptor agonists, also enhance responses to α_1-adrenoceptor agonists (Nicholas, 1969; Medgett *et al.*, 1983; Meehan *et al.*, 1986) and other vasoconstrictor agonists (Van Nueten *et al.*, 1982; Xiao & Rand, 1988) and may have already augmented the influx of calcium to a maximal extent.

It is possible that an endothelium-derived factor contributes as a mediator in the interactions between α_2-adrenoceptor agonists and vasoconstrictor agents that we have observed. It has been established in our laboratory that both rabbit ear and rat tail artery preparations, set up as described in Methods, have a functional endothelium as evidenced by vasodilator responses to acetylcholine which are lost when the endothelium is removed. The enhancing effect of 5-hydroxytryptamine on vasoconstrictor responses to sympathetic nerve stimulation in the rabbit ear artery is not affected by removal of the endothelium, but the enhancement of responses to noradrenaline is attenuated (Meehan *et al.*, 1987). The effect of removal of endothelium on the enhancing effects of α_2-adrenoceptor agonists on vasoconstriction in the rat tail artery has not yet been investigated.

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Measurement of 3-methoxy-4-hydroxyphenylglycol (MHPG) in mouse brain by h.p.l.c. with electrochemical detection, as an index of noradrenaline utilisation and presynaptic α_2-adrenoceptor function

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1 A novel method for measurement of 3-methoxy-4-hydroxyphenylglycol (MHPG) in mouse brain by use of high performance liquid chromatography (h.p.l.c.) with electrochemical detection is described. This technique incorporates an ethyl acetate purification procedure and uses 3-hydroxy-4-methoxyphenylglycol (iso-MHPG) as the internal standard.

2 Inhibition of monoamine oxidase by injection of tranylcypromine (5 and 10 mg kg^{-1}) or pargyline (50 and 100 mg kg^{-1}) markedly decreased brain MHPG concentrations. After injection of the tyrosine hydroxylase inhibitor, α-methyl-*p*-tyrosine (200 mg kg^{-1}), there were time-dependent linear decreases in the concentrations of noradrenaline and MHPG in mouse brain. In addition, a very good correlation ($r = 0.95$, $n = 30$; $P < 0.001$) was found between the concentrations of noradrenaline and MHPG present in the brains of the same mice after α-methyl-*p*-tyrosine treatment.

3 Mouse brain MHPG concentrations were dose-dependently reduced after administration of the α_2-adrenoceptor agonist, clonidine (1–3000 μg kg^{-1}), and elevated by the antagonists, idazoxan (1 and 5 mg kg^{-1}), and yohimbine (1 and 5 mg kg^{-1}). Intracerebroventricular injection of the α_1-adrenoceptor agonist, phenylephrine (5–50 μg) dose-dependently increased MHPG levels. The α_1-adrenoceptor antagonist, prazosin, had no effect at the moderate dose of 1 mg kg^{-1}, but increased MHPG concentrations at 5 mg kg^{-1}. The β-adrenoceptor agonist, clenbuterol (10–1000 μg kg^{-1}) and the antagonist, pindolol (1 and 5 mg kg^{-1}), were both without effect.

4 The decrease in brain MHPG concentrations induced by clonidine (100 μg kg^{-1}) was prevented by prior injection of 1 mg kg^{-1} of idazoxan or yohimbine, but not by prazosin or pindolol.

5 MHPG levels were decreased after administration of the noradrenaline reuptake inhibitor desipramine (5 and 10 mg kg^{-1}) and the non-selective monoamine reuptake inhibitors, sibutramine HCl (BTS 54 524; 1 and 3 mg kg^{-1}) and amitryptyline (5 mg kg^{-1}). However, the selective 5-hydroxytryptamine reuptake inhibitor, zimeldine (5 and 10 mg kg^{-1}), was without effect. Dexamphetamine (1 and 5 mg kg^{-1}) and methamphetamine (1 and 5 mg kg^{-1}) both decreased brain MHPG concentrations in a dose-related fashion.

6 Overall the data show that MHPG can be used as a functional index of both presynaptic α_2-adrenoceptor activity and noradrenaline turnover and utilisation.

Introduction

In the mammalian brain, one major route by which noradrenaline can be metabolised is conversion to 3,4-dihydroxyphenylglycolaldehyde by monoamine oxidase and then to 3,4-dihydroxyphenylglycol (DHPG) by aldehyde reductase. 3-Methoxy-4-hydroxyphenylglycol (MHPG) results from methylation of DHPG by catechol-*O*-methyltransferase and in the rat this exists as the sulphate conjugate, MHPG-SO_4 (Schanberg *et al.*, 1968; Meek & Neff, 1972). In the mouse this final step does not occur and MHPG is only found in the unconjugated form (Ceasar *et al.*, 1974). Sharman (1969) suggested that the formation of DHPG and MHPG may represent the major cerebral transformations of noradrenaline in the mouse.

Noradrenaline release in the brain is under tonic

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inhibitory control by α_2-adrenoceptors which are located on presynaptic noradrenergic nerve terminals (Heepe & Starke, 1985). However, in contrast to the situation in the peripheral nervous system, the overwhelming majority of α_2-adrenoceptors in the brain are located on postsynaptic sites (U'Prichard *et al.*, 1980; Dooley *et al.*, 1983). Consequently, it is not possible to monitor the function of presynaptic α_2-adrenoceptors by use of ligand-receptor binding techniques.

In view of this, a simple method for determination of MHPG in brain tissues by high performance liquid chromatography (h.p.l.c.) with electrochemical detection has been developed based on the method described for measurement of MHPG in plasma by Molyneux & Franklin (1985). The effects of administration of various α- and β-adrenoceptor agonists and antagonists on mouse brain MHPG concentrations were examined to determine whether this noradrenaline metabolite could be used as a monitor of presynaptic α_2-adrenoceptor function. In addition, mice were also given the tyrosine hydroxylase inhibitor, α-methyl-*p*-tyrosine, to discover whether changes in MHPG concentrations reflected noradrenaline turnover.

Methods

Determination of MHPG by h.p.l.c. with electrochemical detection

Analysis of MHPG in brain samples was performed by modification of the technique described by Molyneux & Franklin (1985). Mice were killed and brains (minus cerebellum) were homogenized in 5 volumes (w/v) of ice-cold 0.1 M perchloric acid containing 0.1 μM 3-hydroxy-4-methoxyphenylglycol hemipiperazine (iso-MHPG, internal standard) and 0.4 mM sodium metabisulphite (antioxidant) in a Polytron PT 10–35 disruptor (setting 5–6). Homogenates were centrifuged at 1100 *g* for 15 min at 4°C. The supernatant was then centrifuged at 11,600 *g* for 5 min at room temperature; 1 ml of the resulting supernatant was transferred to a 10 ml glass centrifuge tube containing 5 ml of ethyl acetate and vortex-mixed for 1 min. After centrifugation at 1300 *g* for 10 min at 4°C, the organic phase was transferred to a second 10 ml glass centrifuge tube containing 1 ml of 0.08 M potassium bicarbonate solution. Each tube was then vortex-mixed, centrifuged at 1300 *g* for 10 min at 4°C and the organic phase removed and evaporated to dryness at 30°C under a stream of nitrogen gas. The residues were dissolved in 100 μl of h.p.l.c. mobile phase (0.1 M sodium acetate/citric acid buffer, pH 4.40 containing 8% (v/v) methanol and 4.6 mM 1-octanesulphonic acid sodium salt) and 50 μl was injected onto the column using a WISP 710B autoinjector (Millipore/Waters). The h.p.l.c. system consisted of a DuPont 870 pump set at 0.85 ml min^{-1}, connected to a 5 μm Spherisorb ODS 1 reversed phase analytical column (length 250 × 4.6 mm i.d.) maintained at 45°C and protected by a Brownlee Aquapore RP300 precolumn (length 30 × 4.6 mm i.d.). MHPG and iso-MHPG were detected with a BAS LC-4B amperometric detector set at a potential of +0.75 V versus a Ag/AgCl reference electrode and linked to a DuPont SP 4100 integrator.

Measurement of noradrenaline concentrations in mouse brain by h.p.l.c. with electrochemical detection

For studies comparing the turnover rates of MHPG and noradrenaline in mouse brain after α-methyl-*p*-tyrosine administration, brains minus cerebellum were homogenized in 5 volumes (w/v) of 0.1 M perchloric acid containing 0.1 μM iso-MHPG (internal standard MHPG assay), 0.8 μM isoprenaline (internal standard noradrenaline assay) and 0.4 mM sodium metabisulphite (antioxidant). After centrifugation as described above, 1 ml of the supernatant was used for MHPG determination and 30 μl was injected directly on to the h.p.l.c. system for noradrenaline determination. This comprised a DuPont 870 pump connected via a WISP 712 autoinjector to a 5 μm Hypersil ODS 1 reversed-phase analytical column (length 250 × 4.6 mm i.d.) maintained at 45°C and protected by a Brownlee Aquapore RP 300 precolumn (length 30 × 4.6 mm i.d.). The h.p.l.c. mobile phase was 0.1 M sodium dihydrogen orthophosphate-orthophosphoric acid buffer pH 3.2 containing 16% v/v methanol, 2.8 mM 1-octanesulphonic acid sodium salt and 0.7 mM EDTA. Noradrenaline and isoprenaline were detected using a BAS LC-4B amperometric detector with a TL-5 flow-cell set at a potential of +0.75 V versus a Ag/AgCl reference electrode.

Animals and drug administration procedures

Adult male C57/B1/6 Ola mice (Olac), weighing 15–35 g were used. They were housed in groups of 5 to 10 on a 12 h light/dark cycle (commencing 07 h 00 min) at a temperature of 21°C and 55% humidity. The mice were allowed food and water *ad libitum*.

All drugs were dissolved in 0.9% (w/v) sodium chloride solution (saline), with the exception of prazosin which was dissolved in distilled water. Drugs, with the exception of α-methyl-*p*-tyrosine methyl ester and phenylephrine, were injected intraperitoneally (i.p.) in weight-related doses (0.1 ml 10 g^{-1} body weight). For injection of phenylphrine, mice were first lightly anaesthetized with an air/halothane

mixture and phenylephrine (5–50 μg) was injected intracerebroventricularly (i.c.v.) in 2 μl of saline by use of the stereotaxic apparatus described by Heal (1984). Control mice were injected with saline (2 μl i.c.v.). α-Methyl-*p*-tyrosine (200 mg kg^{-1}) was injected intravenously via the tail veins of mice. This was because accurate estimation of noradrenaline turnover by the method of Brodie *et al.* (1966) requires immediate and complete inhibition of tyrosine hydroxylase in the brain (Costa, 1970).

Turnover parameters were calculated from the formula (see Costa, 1970).

$$\log (NA) = \log (NA_0) - 0.434 k_{NA} t$$

where

NA_0 = concentration of noradrenaline (nmol g^{-1});
NA = concentration of noradrenaline at time 't';
k_{NA} = fractional rate constant.

Drugs and reagents

Drugs were obtained from the following sources: clonidine HCl, 3-methoxy-4-hydroxyphenylglycol hemipiperazine (MHPG), isoprenaline HCl, pindolol, yohimbine HCl, phenylephrine HCl, tranylcypromine HCl, pargyline HCl, α-methyl-*p*-tyrosine methyl ester HCl, methamphetamine HCl (Sigma, Poole); prazosin HCl (Pfizer, Sandwich); idazoxan HCl (Reckitt and Colman, Hull); 3-hydroxy-4-methoxyphenylglycol hemipiperazine (iso-MHPG; kindly donated by Mr M. Franklin, University Department of Psychiatry, Research Unit, Littlemore Hospital, Oxford); desipramine HCl (DMI; Geigy, Horsham); zimeldine $(HCl)_2$ (Astra, Södertälje); amitriptyline HCl, clenbuterol HCl, sibutramine HCl (BTS 54 524; Boots, Nottingham), dexamphetamine SO_4 (Smith, Kline and French, Welwyn Garden City).

Reagents were obtained from the following sources: glacial acetic acid ('AnalaR' grade), perchloric acid ('Aristar' grade), potassium bicarbonate ('AnalaR' grade), sodium metabisulphite ('AnalaR' grade) (BDH, Poole); sodium acetate trihydrate ('HPLC' grade), citric acid ('A.R.' grade), sodium hydroxide ('A.R.' grade), 1-octane sulphonic acid sodium salt ('HPLC' grade) (Fisons, Loughborough); ethyl acetate ('HPLC' grade), methanol ('HPLC' grade) (Rathburn Chemicals, Walkerburn). All water was distilled and deionised before use.

Results

Chromatography

A chromatograph of a standard solution of MHPG and iso-MHPG (both 2.5 ng 100 μl^{-1}), obtained using the h.p.l.c. conditions described in Methods, is shown in Figure 1a. A chromatograph of the MHPG extracted from naive mouse brain is shown for comparison (Figure 1b) and is equivalent to a tissue concentration of 71.5 ng g^{-1} wet weight. The retention times of MHPG and iso-MHPG were 7.00 and 9.30 min, respectively. The ethyl acetate extraction procedure prevented possible interference in the chromatography of MHPG and iso-MHPG by other oxidisable neurochemicals contained in brain tissue samples. However, an unidentified 'late-running' peak sometimes occurred which had a retention time of approximately 37 min. The recoveries ± s.e.mean for the ethyl acetate extractions of MHPG and iso-MHPG were not significantly different and were 27.9 ± 0.4% and 28.2 ± 0.5%, respectively. Voltammographs of MHPG and iso-MHPG were almost identical and were optimum at +0.75 V versus a Ag/AgCl reference electrode. The electrochemical detector responses for MHPG and iso-MHPG were

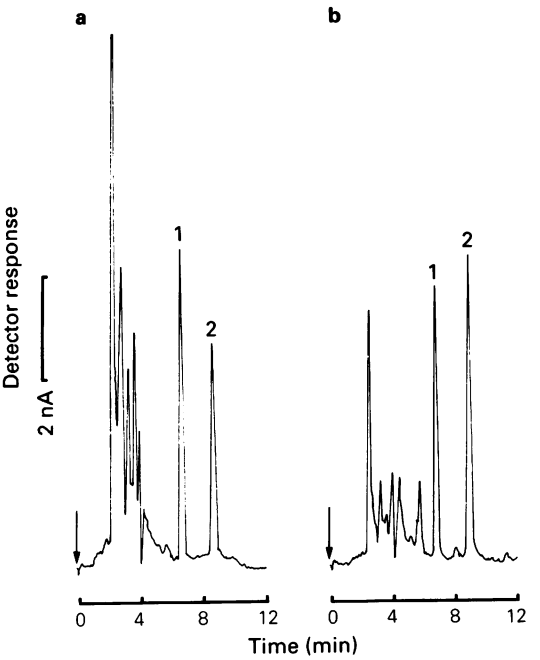

Figure 1 Chromatographs of (a) a standard solution of 3-methoxy-4-hydroxyphenylglycol (MHPG) and 3-hydroxy-4-methoxyphenylglycol (iso-MHPG) and (b) MHPG from the brain of an untreated mouse. The peaks are (1) MHPG and (2) iso-MHPG. (a) A standard solution of 2.5 ng MHPG and iso-MHPG injected in 100 μl of h.p.l.c. eluant. (b) MHPG present in the brain of an untreated mouse using 0.1 μM iso-MHPG as the internal standard for the h.p.l.c. technique. The brain MHPG concentration was 71.5 ng g^{-1} tissue wet weight. The tissue extraction procedure and chromatography technique are fully described in Methods.

Table 1 The effects of monoamine oxidase (MAO) inhibitors, tranylcypromine and pargyline, on 3-methoxy-4-hydroxyphenylglycol (MHPG) concentrations in mouse brain

Drug	*MHPG concentration* (ng g^{-1} tissue wet wt)
Saline (0.25 ml)	73.0 ± 1.6 (6)
Tranylcypromine (5 mg kg^{-1})	12.7 ± 1.1 (6)***
Tranylcypromine (10 mg kg^{-1})	8.5 ± 0.8 (6)***
Saline (0.25 ml)	69.4 ± 2.3 (6)
Pargyline (50 mg kg^{-1})	17.3 ± 0.5 (6)***
Pargyline (100 mg kg^{-1})	20.1 ± 1.0 (6)***

Mice were given an intraperitoneal injection of tranylcypromine (5 or 10 mg kg^{-1}) or pargyline (50 or 100 mg kg^{-1}). Control mice were injected with saline (0.25 ml). Sixty minutes later, mice were killed and brain MHPG concentrations were determined by h.p.l.c. with electrochemical detection. The results ± s.e.mean are presented, with the numbers of mice used given in parentheses. Significantly different from appropriate saline control: *** $P < 0.001$ using Student's unpaired t test.

linear over a wide concentration range (0.5–100 ng injected onto the column). With extracted brain samples the limit of detection was 2.0 ng injected onto the column (signal/noise ratio = 2 : 1).

The effects of monoamine oxidase inhibitors on mouse brain MHPG concentrations

Mouse brain MHPG concentrations were determined 60 min after administration of the monoamine oxidase (MAO) inhibitors, tranylcypromine (5 or 10 mg kg^{-1}) and pargyline (50 or 100 mg kg^{-1}). A pronounced dose-dependent decrease in the brain MHPG level was produced by tranylcypromine (Table 1). The effect of pargyline was maximal at 50 mg kg^{-1} and increasing the dose to 100 mg kg^{-1} did not further reduce the already markedly depleted MHPG concentrations (Table 1).

Effects of α-methyl-p-tyrosine on noradrenaline turnover and MHPG concentrations in mouse brain

After administration of α-methyl-*p*-tyrosine (200 mg kg^{-1}, i.v.), brain concentrations of noradrenaline and MHPG were measured at 0, 60, 120 and 240 min intervals after injection. There was a good correlation between the decline in the log noradrenaline concentration and time ($r = 0.85$, $n = 30$; $P < 0.001$) and this yielded a fractional rate constant (k_{NA}) of 0.11 h^{-1} and a turnover rate of 0.28 nmol g^{-1} wet wt h^{-1} (Figure 2). A time-dependent decline in log MHPG concentration was also observed ($r = 0.93$, $n = 30$; $P < 0.001$) with k_{MHPG} 0.23 h^{-1} and a turnover rate of 0.09 nmol g^{-1} wet wt h^{-1} (Figure 4). There was also a very high correlation ($r = 0.95$, $n = 30$, $P < 0.001$) between the concentrations of noradrenaline and MHPG which were present in the brains of individual mice after α-methyl-*p*-tyrosine administration (Figure 3).

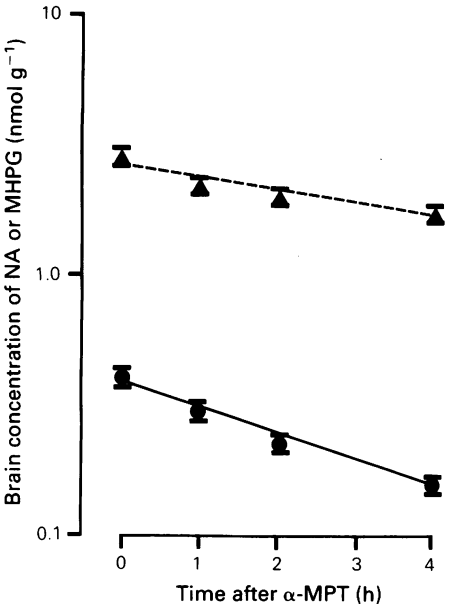

Figure 2 The effects of α-methyl-*p*-tyrosine (α-MPT) administration on the concentrations of noradrenaline (NA) and 3-methoxy-4-hydroxyphenylglycol (MHPG) in mouse brain. Groups of mice were killed at various time intervals after injection of α-methyl-*p*-tyrosine (200 mg kg^{-1}, i.v.) and brain concentrations of noradrenaline and MHPG were determined by separate h.p.l.c. techniques as described in Methods. Results are shown (with s.e.mean indicated by vertical bars) for observations of the decline in noradrenaline (▲) and MHPG (●). Linear correlation coefficients were for noradrenaline: $r = 0.85$, $n = 30$ ($P < 0.001$) and for MHPG: $r = 0.93$, $n = 30$ ($P < 0.001$).

The effects of α- and β-adrenoceptor agonists and antagonists on MHPG concentrations in mouse brain

The effects on brain MHPG concentrations of various α- and β-adrenoceptor agonists and antagonists was determined 60 min after drug administration. The α_2-adrenoceptor agonist clonidine (1–3000 μg kg^{-1}) produced a dose-dependent decrease in mouse brain MHPG concentrations. The maximum reduction of 57% was observed after

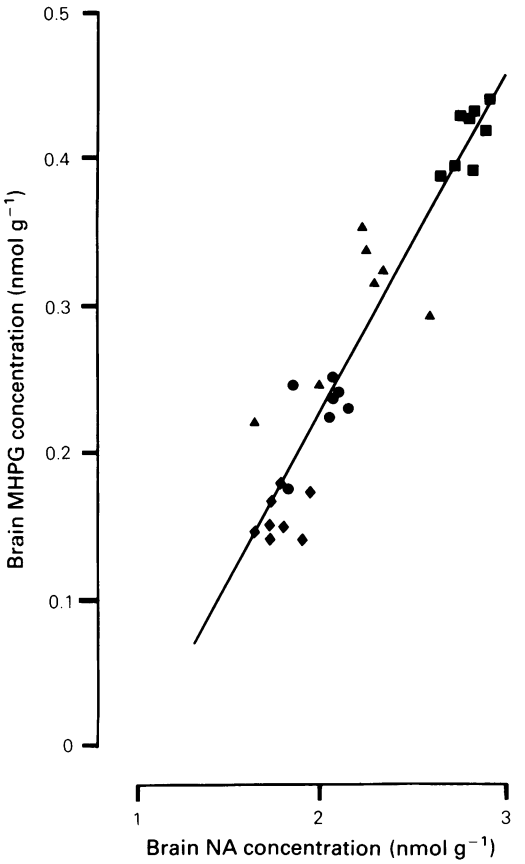

Figure 3 Correlation between the concentrations of noradrenaline (NA) and 3-methoxy-4-hydroxyphenylglycol (MHPG) in mouse brain after administration of α-methyl-*p*-tyrosine. Groups of mice were killed at various time intervals after injection of α-methyl-*p*-tyrosine (200 mg kg^{-1}, i.v.) and noradrenaline and MHPG concentrations were measured in the same animals by the two h.p.l.c. techniques described in Methods. Interval after α-methyl-*p*-tyrosine injection (min): 0 (■); 60 (▲); 120 (●); 240 (◆). Linear correlation $r = 0.94$, $n = 30$ ($P < 0.001$).

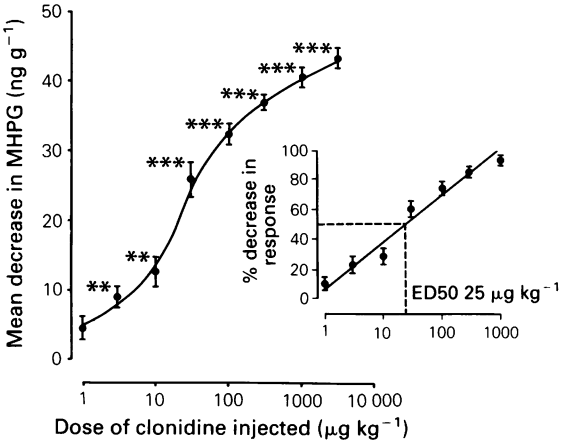

Figure 4 Dose-response relationship for the reduction of brain 3-methoxy-4-hydroxyphenylglycol (MHPG) concentrations by clonidine administration. Brain MHPG concentrations were determined by h.p.l.c. with electrochemical detection 60 min after injection of clonidine (1–3000 μg kg^{-1}) to mice. The inset shows the percentage decrease in response versus the clonidine dose plotted on a log scale. The ED_{50} value is 25 μg kg^{-1}. Results are mean values for 5–6 mice; vertical bars show s.e.mean. The tissue extraction procedure and chromatography technique are fully described in Methods. Significantly different from saline control: ** $P < 0.01$; *** $P < 0.001$.

administration of 3000 μg kg^{-1} and the ED_{50} for this effect was 25 μg kg^{-1} (Figure 4). The α_1-adrenoceptor agonist, phenylephrine (5–50 μg), dose-dependently increased brain MHPG concentrations (Table 2). However, the β-adrenoceptor agonist, clenbuterol (10–1000 μg kg^{-1}), had no effect on MHPG concentrations in mouse brain (Table 2).

Two α_2-adrenoceptor antagonists, idazoxan (1 or 5 mg kg^{-1}) and yohimbine (1 or 5 mg kg^{-1}), both produced dose-related increases in brain MHPG concentrations (Figure 5). The α_1-adrenoceptor antagonist, prazosin, had no effect on MHPG levels

Table 2 The effects of phenylephrine and clenbuterol on 3-methoxy-4-hydroxyphenylglycol (MHPG) concentrations in mouse brain

Drug	*MHPG concentration* (ng g^{-1} tissue wet wt)
Saline (2 μl i.c.v.)	73.2 ± 1.0 (6)
Phenylephrine (5 μg i.c.v.)	106.9 ± 4.3 (6)***
Phenylephrine (25 μg i.c.v.)	159.2 ± 4.5 (6)***
Phenylephrine (50 μg i.c.v.)	174.8 ± 4.3 (6)***
Saline (0.25 ml i.p.)	69.1 ± 1.3 (10)
Clenbuterol (10 μg kg^{-1} i.p.)	69.3 ± 2.1 (6)
Clenbuterol (100 μg kg^{-1} i.p.)	71.7 ± 1.6 (6)
Clenbuterol (1000 μg kg^{-1} i.p.)	67.4 ± 1.7 (6)

Mice were injected intracerebroventricularly with phenylephrine (5–50 μg) or saline (2 μl). In a second series of experiments mice were given an intraperitoneal injection of clenbuterol (10–1000 μg kg^{-1}) or saline (0.25 ml). Sixty minutes later mice were killed and brain MHPG concentrations were determined by h.p.l.c. with electrochemical detection. The results ± s.e.mean are presented, with the number of mice used given in parentheses.

Significantly different from appropriate saline control: *** $P < 0.001$ using Student's unpaired *t* test.

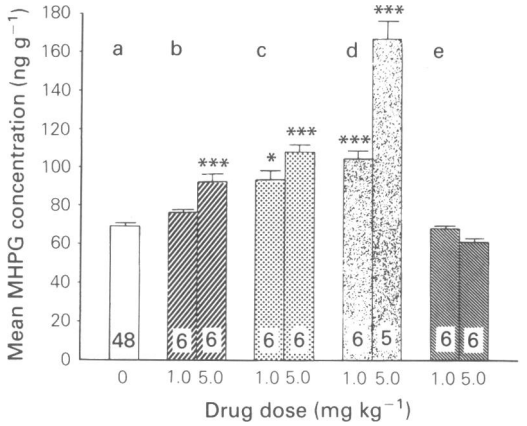

Figure 5 The effect of administration of various α- and β-adrenoceptor antagonists on mouse brain 3-methoxy-4-hydroxyphenylglycol (MHPG) concentrations. Mice were given an intraperitoneal injection of prazosin (1 or 5 mg kg^{-1}, b), idazoxan (1 or 5 mg kg^{-1}, c), yohimbine (1 or 5 mg kg^{-1}, d) or pindolol (1 or 5 mg kg^{-1}, e). Control mice were injected with saline (0.25 ml, a). Sixty minutes later, mice were killed and brain MHPG concentrations were determined by h.p.l.c. with electrochemical detection. The results are presented as MHPG concentration (ng g^{-1} tissue wet wt) with the numbers of mice used shown in each column; vertical bars show s.e.mean. For the sake of clarity saline control groups have been combined. However, data from experimental groups of mice were always compared with their own saline-treated controls which were tested at the same time. Significantly different from appropriate saline controls which were tested at the same time: $^{*}P < 0.05$; $^{***}P < 0.001$ using Student's unpaired t test.

at 1 mg kg^{-1}, but did significantly elevate these at a dose of 5 mg kg^{-1} (Figure 5). The β-adrenoceptor antagonist, pindolol (1 or 5 mg kg^{-1}), had no effect on brain MHPG concentrations at either dose (Figure 5).

The effects of α- and β-adrenoceptor antagonists on the decrease in brain MHPG concentrations induced by clonidine

After 30 min pretreatment with one of the following: prazosin (1 mg kg^{-1}), idazoxan (1 mg kg^{-1}), yohimbine (1 mg kg^{-1}), pindolol (1 mg kg^{-1}) or saline (0.25 ml), mice were injected with clonidine (100 μg kg^{-1}), and were killed after a further 60 min and brain MHPG concentrations were determined.

Clonidine (100 μg kg^{-1}) induced a significant fall in brain MHPG concentrations and this effect was abolished by the α_2-adrenoceptor antagonists, idazoxan (1 mg kg^{-1}) and yohimbine (1 mg kg^{-1}) (Table 3). This reduction was not altered by pretreatment with the α_1-adrenoceptor antagonist, prazosin (1 mg kg^{-1}) or the β-adrenoceptor antagonist, pindolol (1 mg kg^{-1}) (Table 3).

The effects of sibutramine HCl and various other monoamine reuptake inhibitors on brain MHPG concentrations

Mice were injected with sibutramine HCl (1 or 3 mg kg^{-1}), DMI (5 or 10 mg kg^{-1}), amitriptyline (5 or 10 mg kg^{-1}), zimeldine (5 or 10 mg kg^{-1}) or saline (0.25 ml). Sixty minutes later, mice were killed and brain MHPG concentrations were determined.

Table 3 The effects of α- and β-adrenoceptor antagonists on the clonidine-induced decrease in mouse brain 3-methoxy-4-hydroxyphenylglycol (MHPG) concentrations

First injection	*Second injection*	*Brain MHPG concentration* (ng g^{-1} tissue wet wt) ± s.e.
Saline (0.25 ml)	Saline (0.25 ml)	73.5 ± 1.0 (17)
Saline (0.25 ml)	Clonidine (100 μg kg^{-1})	49.7 ± 0.9 (18)***
Prazosin (1 mg kg^{-1})	Clonidine (100 μg kg^{-1})	48.8 ± 1.4 (6)***
Idazoxan (1 mg kg^{-1})	Clonidine (100 μg kg^{-1})	65.4 ± 3.1 (6)†††
Yohimbine (1 mg kg^{-1})	Clonidine (100 μg kg^{-1})	72.1 ± 0.6 (6)†††
Pindolol (1 mg kg^{-1})	Clonidine (100 μg kg^{-1})	43.7 ± 1.7 (6)***

Mice were given an intraperitoneal injection of prazosin (1 mg kg^{-1}), idazoxan (1 mg kg^{-1}), yohimbine (1 mg kg^{-1}) or pindolol (1 mg kg^{-1}). Control mice received saline (0.25 ml). Thirty minutes later, mice were injected with clonidine (100 μg kg^{-1}, i.p.) or saline (0.25 ml, i.p.) and killed after a further 60 min. Brain MHPG concentrations were determined by h.p.l.c. with electrochemical detection. The results ± s.e.mean are presented, with the numbers of mice used given in parentheses. For the sake of clarity saline/saline and saline/clonidine control groups have been combined. However, data from experimental groups of mice were always compared with their own control groups of mice treated with saline/saline or saline/clonidine which were tested at the same time.

Significantly different from appropriate saline/saline control: $^{***}P < 0.001$ using Student's unpaired t test.

Significantly different from appropriate saline/clonidine control: ††† $P < 0.001$ using Student's unpaired t test.

Table 4 The effects of sibutramine HCl and other monoamine reuptake inhibitors on 3-methoxy-4-hydroxyphenylglycol (MHPG) concentrations in mouse brain

Drug	*MHPG concentration* ($ng\,g^{-1}$ tissue wet wt) *Saline control*	*Drug-treated*
Sibutramine HCl ($1\,mg\,kg^{-1}$)	79.2 ± 1.9 (6)	63.0 ± 3.8 (6)**
Sibutramine HCl ($3\,mg\,kg^{-1}$)	79.2 ± 1.9 (6)	52.9 ± 3.5 (6)***
DMI ($5\,mg\,kg^{-1}$)	74.2 ± 2.4 (6)	58.1 ± 2.1 (5)**
DMI ($10\,mg\,kg^{-1}$)	74.2 ± 2.4 (6)	52.9 ± 1.9 (6)***
Amitriptyline ($5\,mg\,kg^{-1}$)	78.6 ± 1.7 (6)	69.0 ± 1.7 (6)**
Amitriptyline ($10\,mg\,kg^{-1}$)	78.6 ± 1.7 (6)	74.8 ± 7.1 (6)
Zimeldine ($5\,mg\,kg^{-1}$)	61.5 ± 3.9 (6)	58.2 ± 2.7 (6)
Zimeldine ($10\,mg\,kg^{-1}$)	61.5 ± 3.9 (6)	58.8 ± 1.9 (6)

Mice were given an intraperitoneal injection of the monoamine reuptake inhibitors: sibutramine HCl (1 or $3\,mg\,kg^{-1}$), desipramine (DMI; 5 or $10\,mg\,kg^{-1}$), amitriptyline (5 or $10\,mg\,kg^{-1}$) or zimeldine (5 or $10\,mg\,kg^{-1}$). Control mice were injected with saline (0.25 ml). Sixty minutes later, mice were killed and brain MHPG concentrations were determined by h.p.l.c. with electrochemical detection. The results ± s.e.mean are presented, with the numbers of mice used given in parentheses.
Significantly different from appropriate saline control group ** $P < 0.01$; *** $P < 0.001$ using Student's unpaired t test.

The non-selective monoamine reuptake inhibitor, sibutramine HCl, induced a dose-related decrease in brain MHPG concentrations (Table 4). The selective noradrenaline reuptake inhibitor, DMI, also caused a dose-related decrease in MHPG levels. Amitriptyline, another non-selective monoamine reuptake inhibitor, decreased brain MHPG concentrations at $5\,mg\,kg^{-1}$, but had no effect at $10\,mg\,kg^{-1}$. The selective 5-HT reuptake inhibitor, zimeldine (5 or $10\,mg\,kg^{-1}$), had no effect on brain MHPG concentrations (Table 4).

The effects of dexamphetamine and methamphetamine on brain MHPG concentrations

Mice were injected with dexamphetamine (1 or $5\,mg\,kg^{-1}$), methamphetamine (1 or $5\,mg\,kg^{-1}$) or saline (0.25 ml). Sixty minutes later, mice were killed and brain MHPG concentrations were determined. Dexamphetamine and methamphetamine both induced dose-related decreases in MHPG concentrations in mouse brain (Table 5).

Discussion

A sensitive and selective method for determination of free MHPG concentrations in mouse brain by use of h.p.l.c. with electrochemical detection is described. This procedure incorporates a simple ethyl acetate extraction procedure. Iso-MHPG was used as the internal standard for this technique and extraction recoveries of MHPG and iso-MHPG were identical. In addition, the electrochemical profiles of MHPG and iso-MHPG were also very similar with maximum oxidation currents being observed at +0.75 V versus a Ag/AgCl reference electrode. There was no chromatographic interference from other oxidisable biological material in brain extracts, although an unidentified late-running peak did sometimes occur with a retention time of approximately 37 min. This procedure was based on the method previously described by Molyneux & Franklin (1985) for measurement of MHPG concentrations

Table 5 The effects of dexamphetamine and methamphetamine on 3-methoxy-4-hydroxyphenylglycol (MHPG) concentrations in mouse brain

Drug	*MHPG concentration* ($ng\,g^{-1}$ tissue wet wt)
Saline (0.25 ml)	73.0 ± 3.4 (6)
Dexamphetamine ($1\,mg\,kg^{-1}$)	63.6 ± 1.7 (6)*
Dexamphetamine ($5\,mg\,kg^{-1}$)	57.2 ± 2.2 (6)**
Saline (0.25 ml)	76.9 ± 1.9 (6)
Methamphetamine ($1\,mg\,kg^{-1}$)	67.2 ± 2.4 (6)*
Methamphetamine ($5\,mg\,kg^{-1}$)	66.2 ± 3.4 (6)*

Mice were given an intraperitoneal injection of dexamphetamine (1 or $5\,mg\,kg^{-1}$) or methamphetamine (1 or $5\,mg\,kg^{-1}$). Control mice were injected with saline (0.25 ml). Sixty minutes later, mice were killed and brain MHPG concentrations were determined by h.p.l.c. with electrochemical detection. The results ± s.e.mean are presented, with the numbers of mice used given in parentheses.
Significantly different from appropriate saline control group: * $P < 0.05$; ** $P < 0.01$ using Student's unpaired t test.

in human plasma samples. Apart from the obvious differences between the tissue matrices involved, the major distinction was the use of an amperometric, rather than a coulometric detector, in the procedure described in this paper. The coulometric detection system has the ability to eliminate background noise from the solvent system and remove potentially interfering substances by complete oxidation or reduction at the first of its two detection electrodes. However, in our hands, no problems were encountered when using the less sophisticated and selective, but more ubiquitous, amperometric detection system.

In an earlier study in this laboratory, an h.p.l.c.-electrochemical detection assay for MHPG determination in mouse brain was developed which used phenyl bonded silica-gel mini-columns for sample purification (Buckett & Diggory, 1985). However, no internal standard was incorporated in this method to correct for differences in recovery. Moreover, iso-MHPG cannot be used as the internal standard for this procedure, because of the marked difference in the recoveries of this internal standard and MHPG (Prow & Heal, unpublished observations).

In brain, noradrenaline is initially metabolized to 3,4-dihydroxyphenylglycolaldehyde by MAO before subsequent conversion to MHPG by aldehyde reductase and catechol-*O*-methyltransferase. When this metabolic route is blocked by administration of the MAO inhibitors, tranylcypromine and pargyline, this produced a marked and rapid decrease in the brain's concentrations of MHPG confirming the earlier reports by Ceasar *et al.* (1974) and Buckett & Diggory (1985).

After inhibition of tyrosine hydroxylase, with α-methyl-*p*-tyrosine, there was a good linear correlation between the reduction of log noradrenaline concentration and time. A similar linear relationship with time was also found for MHPG concentrations in the brain. Although the fractional rate constant for MHPG was approximately twice that for noradrenaline, the turnover rate was only 15% of that of the parent monoamine, reflecting the low brain concentrations of MHPG with respect to noradrenaline. However, a very good correlation was found between the concentration of noradrenaline and MHPG in the brains of individual mice after administration of α-methyl-*p*-tyrosine. These results suggest that MHPG levels in mouse brain may provide a useful index of noradrenaline turnover.

It is well established that noradrenaline release in the brain is under tonic inhibitory control by presynaptic α_2-adrenoceptors located on noradrenaline terminals (Schoffelmeer & Mulder, 1983; Heepe & Starke, 1985). It is suggested that functional release and utilisation of brain noradrenaline is reflected by the formation of MHPG in mice (Ceasar *et al.*, 1974) and MHPG-SO_4 in rats (Meek & Neff, 1972). In this investigation, the α_2-adrenoceptor agonist, clonidine, was found to decrease dose-dependently brain MHPG concentrations in mice and the ED_{50} for this effect was $25\,\mu g\,kg^{-1}$. This agrees with the hypothesis of Andén *et al.* (1976) that, at doses up to $100\,\mu g\,kg^{-1}$, clonidine preferentially stimulates presynaptic α_2-adrenoceptors in the brain. Conversely, administration of the α_2-antagonists, idazoxan and yohimbine, markedly elevated MHPG levels and identical effects have previously been reported for mianserin (Buckett & Diggory, 1985), another drug which is an α_2-adrenoceptor antagonist (Baumann & Maitre, 1977; Pommier *et al.*, 1982). Hence, α_2-adrenoceptor agonists and antagonists produce opposite effects on MHPG formation, in agreement with the hypothesis that noradrenaline release is tonically controlled by presynaptic α_2-adrenoceptors (Schoffelmeer & Mulder, 1983; Heepe & Starke, 1985). The actions of the drugs acting at α_1-adrenoceptors on brain MHPG levels were more complex. Intracerebroventricular administration of the agonist, phenylephrine, produced a marked dose-dependent increase in brain MHPG concentrations. Previously, Heal (1984) demonstrated that central administration of phenylephrine to C57/B1/6 mice caused marked behavioural excitation and increased locomotor activity at these doses and, furthermore, that this response was mediated by at least one other noradrenergic system 'beyond' the α_1-adrenoceptors. Hence, the increase in MHPG concentration which follows phenylephrine administration provides further evidence to confirm that other noradrenaline neurones are involved in the generation of this behavioural syndrome. The α_1-adrenoceptor antagonist, prazosin, had no effect on MHPG concentrations at $1\,mg\,kg^{-1}$, but increased them by 21% at $5\,mg\,kg^{-1}$. This effect was not likely to result from non-selective inhibition of α_2-adrenoceptors, because prazosin has greater than 250 fold α_1/α_2 selectivity in the brains of C57/B1/6 mice (Heal *et al.*, 1987). However, the prazosin dose of $5\,mg\,kg^{-1}$ is greater than that required to abolish α_1-adrenoceptor-mediated activity in these mice (Heal, 1984) and the elevated MHPG levels may result from increased noradrenergic firing which is modulated via a long neuronal feed-back loop.

Sibutramine HCl and amitriptyline, which inhibit noradrenaline and 5-hydroxytryptamine (5-HT) reuptake, and DMI, which is selective for noradrenaline, all reduced levels of MHPG in the brain. This reduction was probably the result of two distinct mechanisms. Firstly, the increased synaptic levels of noradrenaline presynaptic activated α_2-adrenoceptors which decreased noradrenaline release. Secondly, the potent reuptake inhibition prevented noradrenaline from re-entering the presynaptic terminal

for subsequent metabolism by monoamine oxidase (MAO). Amitriptyline and sibutramine HCl were unlikely to have altered brain MHPG concentrations by their effects on 5-HT reuptake, because the selective 5-HT reuptake inhibitor, zimeldine (Ross *et al.*, 1976), had no effect on brain levels of this noradrenaline metabolite. The failure of amitriptyline to decrease MHPG concentrations at the higher dose tested was probably due to inhibition of terminal autoreceptors, because this antidepressant is also a moderately potent α_2-adrenoceptor antagonist (Maggi *et al.*, 1980). By contrast, α_2-adrenoceptor antagonist activity is not possessed by either sibutramine HCl (Heal and Buckett, unpublished observation) or DMI (Maggi *et al.*, 1980). Dexamphetamine and methamphetamine also produced dose-related decreases in MHPG formation. While this may appear to be at variance with their actions on noradrenaline release and function, it has been suggested that the pharmacological effects of amphetamine on noradrenaline are more predominantly the result of reuptake inhibition than release (Von Voigtlander & Moore, 1973; Raiteri *et al.*, 1975).

In rat brain, MHPG mainly exists as the sulphate conjugate (Schanberg *et al.*, 1968; Meek & Neff, 1972) and it has been suggested that this noradrenaline metabolite is a good index of the functional utilisation of noradrenaline (Meek & Neff, 1972). In addition, the inhibitory actions of clonidine on MHPG formation have been widely used as an index of presynaptic α_2-adrenoceptor function (McMillen *et al.*, 1980; Sugrue, 1981; 1982). The results presented in this study support the hypothesis of Ceasar *et al.* (1974) and Buckett & Diggory (1985) that MHPG concentrations in mouse brain reflect noradrenaline utilisation after a wide variety of pharmacological manipulations. Furthermore, the data also indicate that the clonidine-induced decrease in MHPG can be used as a model of presynaptic α_2-adrenoceptor function in mouse brain. Since MHPG, but not MHPG-SO_4, can be simply and sensitively determined by h.p.l.c. with electrochemical detection, this highlights the viability of using mice rather than rats for these types of study.

In conclusion, a simple, sensitive and selective method for measuring free MHPG in mouse brain is described. This noradrenaline metabolite can be used as an index of either noradrenaline utilisation or presynaptic α_2-adrenoceptor function.

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Histamine release in the isolated vascularly perfused stomach of the rat: regulation by autoreceptors

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1 In the isolated vascularly-perfused stomach of the rat, gastrin 1-17 (520 pmol l^{-1}) increased acid output from basal values of 13.7 ± 2.7 to 92.5 ± 11.4 μmol h^{-1} and venous histamine output from 10.1 ± 2.3 to 54.7 ± 7.9 nmol h^{-1} (mean ± s.e.mean).

2 The H_1 receptor agonist 2-methylhistamine (10 μmol l^{-1}) increased acid output to 21.6 ± 2.9 μmol h^{-1} ($P < 0.05$) and reduced basal histamine output to 4.0 ± 0.8 nmol h^{-1} ($P < 0.05$). Gastrin-stimulated acid secretion and vascular histamine output was not significantly affected by 2-methylhistamine (10 μmol l^{-1}).

3 The H_2 receptor agonist, impromidine, dose-dependently increased basal acid secretion, reaching a maximal value of 145.5 ± 11.7 μmol h^{-1} with impromidine (10 μmol l^{-1}), and maximal gastrin-stimulated acid secretion to 167.4 ± 15.1 μmol h^{-1} with impromidine (10 μmol l^{-1}). Impromidine dose-dependently inhibited basal and gastrin-stimulated vascular histamine output.

4 The H_3 receptor agonist **R**-a-methylhistamine, (1 and 10 μmol l^{-1}) minimally increased basal acid secretion. **R**-a-methylhistamine (10 μmol l^{-1}) did not significantly affect maximal gastrin-stimulated acid secretion. Basal and gastrin-stimulated vascular histamine outputs decreased to 4.0 ± 0.8 ($P < 0.05$) and 24.7 ± 4.7 nmol h^{-1} ($P = 0.05$) with **R**-a-methylhistamine (10 μmol l^{-1}).

5 The H_2 receptor antagonist ranitidine (2 μmol l^{-1}) did not inhibit basal acid secretion, but acid outputs with gastrin and all histamine agonists were reduced. Ranitidine did not affect histamine release in the basal state, with gastrin or with any histamine agonist tested.

6 We conclude that gastric histamine release in the rat is regulated via a histamine H_2 receptor sensitive to the histamine agonists tested, but not to ranitidine. It is unlikely that the inhibition of histamine release is secondary to increased gastric acidity.

Introduction

Histamine is recognized as an important intercellular messenger. It is released from histamine-storing cells in several tissues and regulates cellular function via histamine receptors on the effector cell surface. Previous research has led to the discovery of three specific subclasses of histamine receptors designed H_1, H_2 and H_3 receptors (Black *et al.*, 1972; Arrang *et al.*, 1983). The distribution of H_1 and H_2 receptors is very complex (Chand & Eyre, 1975) while so far, H_3 receptors have only been documented in histaminergic neurones.

Histamine is an important mediator substance in the physiological regulation of gastric function. Stimulation of H_1 receptors in the gastric vascular bed induces vasodilatation (Main & Whittle, 1976). The H_2 receptors on the parietal cell mediate the potent acid secretagogue effect of histamine (Black *et al.*, 1972; Soli & Berglindh, 1987), and administration of H_2 receptor antagonists inhibits the acid response to all natural stimuli (Grossman & Konturek, 1974). Until now there have been no studies concerning the presence of H_3 receptors in the gastric mucosa.

The central role of histamine in the regulation of gastric secretion has led to great interest in the mechanism regulating the liberation of this mediator from its mucosal stores. Our studies with the isolated, vascularly perfused stomach of the rat have shown that gastrin induces an immediate and dose-dependent histamine release preceding acid secretion (Sandvik *et al.*, 1987), and furthermore that somatostatin (Sandvik & Waldum, 1988a) and prostaglandin analogues (Sandvik & Waldum, 1988b) inhibit this release. Another interesting aspect of the regulation of gastric histamine release is the possible existence of autoregulatory mechanisms. Previous

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studies have suggested that in the rat, histamine can inhibit its own synthesis and release by a H_2-receptor-mediated feedback mechanism (Håkanson *et al.*, 1977; 1978; Maslinski & Sewing, 1977). While extensive studies have shown that H_3 receptor stimulation inhibits neuronal histamine synthesis and release (Arrang *et al.*, 1987), there are no studies on the role of H_3 receptors in the regulation of gastric histamine release.

We carried out the present study to examine the effects of histamine H_1, H_2 and H_3 agonists on baseline and gastrin-stimulated histamine release in the isolated vascularly perfused stomach of the rat. In this acid-secreting, rat isolated stomach preparation the vascular perfusate is not recirculated, ensuring optimal control of the stimulants applied. Furthermore, the perfusate contains no histamine-storing cells (basophil leukocytes, thrombocytes), and the venous effluent is collected from the portal vein with minimal degradation of histamine. This ensures maximal sensitivity and reproducibility of the assay, with the functional integrity of the stomach preserved.

Methods

The rat isolated vascularly perfused stomachs were prepared as previously described (Kleveland *et al.*, 1986). Briefly, fasted rats weighing 240–260 g (mean 250 g) were anaesthetized with urethane and the stomachs isolated and transferred to an organ bath filled with Krebs and Ringer buffer. The vascular bed was perfused through an arterial catheter with a Krebs and Ringer buffer with an ionized calcium concentration of 1.12 mmol l^{-1}, dextran T70 40 mg ml^{-1}, glucose 5 mmol l^{-1}, pyruvate 5 mmol l^{-1} and 10% (vol/vol) washed ovine erythrocytes. The vascular perfusate was gassed with O_2 (96%) and CO_2 (4%) using a membrane oxygenator, and the stomachs perfused at a rate of 2 ml min^{-1}. The vascular effluent was not recirculated. The gastric lumen was perfused with distilled water (pH 7.0) gassed with 100% O_2. All perfusates and the organ bath were kept at 37°C.

The compounds, 2-methylhistamine (H_1 agonist), impromidine (H_2 agonist) and R-a-methylhistamine (H_3 agonist) were administered from the start of the vascular perfusion. After an initial 'washout' period of 20 min, isobutyl methylxanthine (IBMX) (50 μmol l^{-1}) was added to the vascular perfusate. Baseline acid secretion and histamine release rates were measured with IBMX only. In the stimulation studies, gastrin 1-17 (Gl-17) 520 pmol l^{-1} was added to the vascular perfusion medium after another 20 min and continued for 60 min. One stomach preparation was used as a single experiment, and each drug combination tested on five stomachs.

Sampling and analysis

The vascular and luminal effluents were collected in 10-min fractions. Acid output was determined by titrating each fraction to pH 7.0 with NaOH 1 mmol l^{-1} (Radiometer, Copenhagen, Denmark). The vascular effluent was collected and centrifugated at +4°C, and the supernatant kept frozen at −20°C until analysis for histamine.

The histamine analyses were performed by use of a commercial histamine radioimmunoassay kit (Immunotech, Marseille, France). The kits include tubes coated with monoclonal antibody against acylated histamine, acylating agent and ^{125}I acylated histamine as tracer. The sensitivity is 0.1 nmol l^{-1} histamine, intra-assay variance 6.1%. 2-Methylhistamine, impromidine and R-a-methylhistamine showed histamine crossreactivities of 0.005, 0.005 and 0.002, respectively. Gastrin, ranitidine and IBMX in the concentrations used, did not crossreact with histamine in the RIA.

Calculations

The results are expressed as total acid output and total venous histamine output during the 40–100 min perfusion period. Histamine-like immunoreactivity from the histamine agonists added to the perfusate was subtracted from the measured histamine concentrations when calculating histamine outputs. Differences in acid and histamine outputs were evaluated for significance by the Wilcoxon rank sum test for unpaired samples.

Drugs and solutions

The following drugs were used: isobutyl methylxanthine (Sigma), 2-methylhistamine (Smith, Kline & French), impromidine (Smith, Kline & French), ranitidine (Glaxo), R-a-methylhistamine (J-M Arrang, Paris), gastrin 1–17 (Professor E. Wünch, Munich). All drugs were dissolved in the perfusion buffer, except isobutyl methylxanthine which was first dissolved in 96% ethanol and subsequently diluted in normal saline and the perfusion buffer (final dilution 1 : 860).

Results

Acid secretion

Gastrin 1–17 (520 pmol l^{-1}) significantly ($P < 0.01$) increased acid output from basal (IBMX only) 13.7 ± 2.7 μmol h^{-1} to 92.5 ± 11.4 μmol h^{-1} (mean ± s.e.mean).

The H_1 agonist 2-methylhistamine (10 μmol l^{-1}) increased basal acid output to 21.6 ± 2.9 μmol h^{-1} ($P < 0.05$). Impromidine in the range from

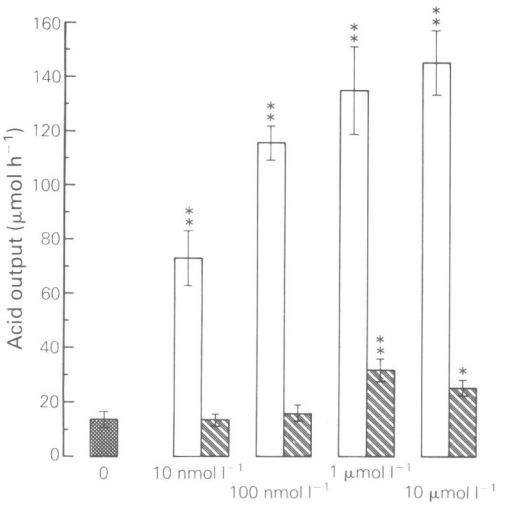

Figure 1 Acid output from rat isolated stomachs, under basal conditions and in response to increasing concentrations of histamine receptor agonist. Open columns impromidine (H_2 agonist), closed columns-**R**-a-methylhistamine (H_3 agonist). Significance of differences from baseline are indicated by * ($P < 0.05$) or ** ($P < 0.01$). Results expressed as mean of five experiments for baseline and each drug concentration; s.e.mean shown by vertical bars.

$10\,nmol\,l^{-1}$ to $10\,\mu mol\,l^{-1}$ dose-dependently increased basal acid output, reaching $145.5 \pm 11.7\,\mu mol\,h^{-1}$ with $10\,\mu mol\,l^{-1}$ (Figure 1). **R**-a-methylhistamine stimulated basal acid secretion, reaching a maximum value of $32.0 \pm 4.2\,\mu mol\,h^{-1}$ with $1\,\mu mol\,l^{-1}$ (Figure 1).

G1–17-stimulated acid output was not significantly changed after $10\,\mu mol\,l^{-1}$ of 2-methylhistamine or **R**-a-methylhistamine (115.0 ± 11.8 and $92.4 \pm 17.4\,\mu mol\,h^{-1}$, respectively). Impromidine increased G1–17-stimulated acid secretion, reaching a maximum value of $167.4 \pm 15.1\,\mu mol\,l^{-1}$ with $10\,\mu mol\,l^{-1}$ concentration (Figure 2).

Ranitidine ($2\,\mu mol\,l^{-1}$) did not affect basal acid output ($8.1 \pm 2.8\,\mu mol\,h^{-1}$). Gastrin-stimulated acid secretion was reduced to $18.7 \pm 6.3\,\mu mol\,h^{-1}$ with this concentration of ranitidine ($P < 0.01$). Ranitidine reduced the acid output stimulated by 2-methylhistamine ($10\,\mu mol\,l^{-1}$) to 14.9 ± 0.5 ($P = 0.05$), and with impromidine ($100\,nmol\,l^{-1}$) to 28.9 ± 0.9 ($P < 0.01$), and with **R**-a-methylhistamine ($10\,\mu mol\,l^{-1}$) to 10.8 ± 1.8 ($P < 0.05$) $\mu mol\,h^{-1}$.

Histamine release

G1–17 induced a prompt release of histamine to the venous effluent, the output increasing from basal 10.1 ± 2.3 to $54.7 \pm 7.9\,nmol\,h^{-1}$ ($P < 0.01$).

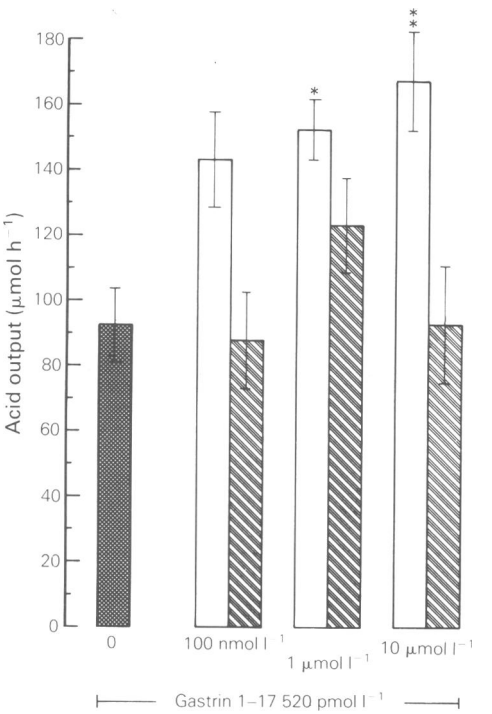

Figure 2 Acid output from rat isolated stomachs, with gastrin 1–17 ($520\,pmol\,l^{-1}$) alone, or in combination with increasing concentrations of histamine receptor agonist. Open columns, impromidine (H_2 agonist); closed columns, **R**-a-methylhistamine (H_3 agonist). Significance of differences from gastrin alone are indicated by *($P < 0.05$) or **($P < 0.01$). Results expressed as mean of five experiments for baseline and each drug concentration, vertical bars show s.e.mean.

2-Methylhistamine ($10\,\mu mol\,l^{-1}$) reduced basal histamine output to $4.0 \pm 0.8\,nmol\,h^{-1}$ ($P < 0.05$). Impromidine dose-dependently inhibited basal histamine output, to a minimum value of $3.7 \pm 0.6\,nmol\,h^{-1}$ with $1\,\mu mol\,l^{-1}$ (Figure 3). **R**-a-methylhistamine depressed basal histamine output to $4.0 \pm 0.9\,nmol\,h^{-1}$ at $10\,\mu mol\,l^{-1}$ ($P = 0.05$) (Figure 3).

During the infusion of 2-methylhistamine ($10\,\mu mol\,l^{-1}$), gastrin-stimulated histamine release was reduced to $23.6 \pm 5.3\,nmol\,h^{-1}$ ($P = 0.05$). Impromidine induced a dose-dependent inhibition of stimulated histamine output, reaching a minimum value of $9.5 \pm 1.0\,nmol\,h^{-1}$ at $10\,\mu mol\,l^{-1}$ (Figure 4). With **R**-a-methylhistamine ($10\,\mu mol\,l^{-1}$), gastrin-stimulated histamine release was reduced to $24.7 \pm 4.7\,nmol\,h^{-1}$ ($P = 0.05$) (Figure 4).

Ranitidine ($2\,\mu mol\,l^{-1}$) did not significantly affect histamine release in the basal state or with gastrin, H_1, H_2 or H_3 agonist, the histamine outputs being

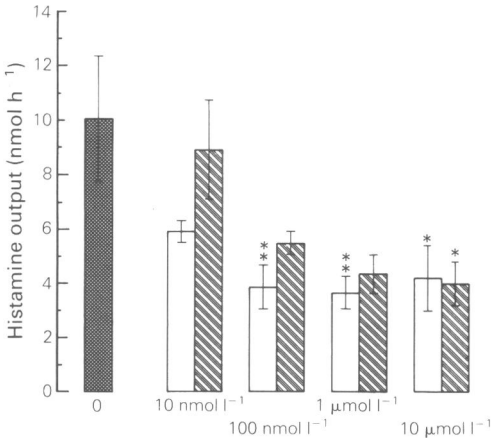

Figure 3 Venous histamine output from rat isolated stomachs, under basal conditions and in response to increasing concentrations of histamine receptor agonist. Open columns, impromidine (H_2 agonist), closed columns, R-a-methylhistamine (H_3 agonist). Significance of differences from baseline are indicated by * ($P < 0.05$) or ** ($P < 0.01$). Results expressed as mean of five experiments for baseline and each drug concentration, vertical bars show s.e.mean.

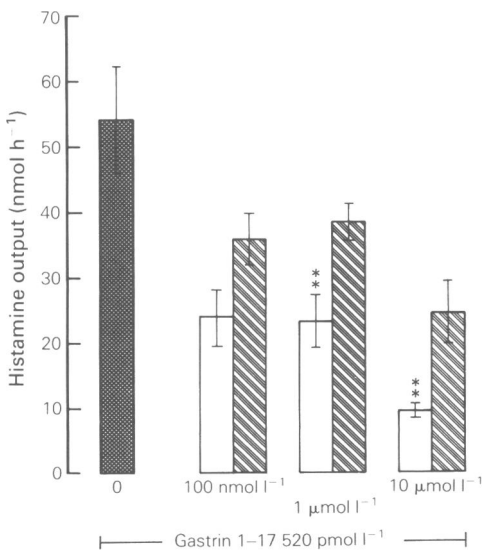

Figure 4 Venous histamine output from rat isolated stomachs, with gastrin 1–17 (520 pmol l^{-1}) alone, or in combination with increasing concentrations histamine receptor agonist. Open bars impromidine (H_2 agonist), closed bars – R-a-methylhistamine (H_3 agonist). Significance of differences from gastrin alone are indicated by ** ($P < 0.01$). Results expressed as mean of five experiments for each drug concentration; vertical bars show s.e.mean.

7.9 ± 1.3, 39.9 ± 5.5, 6.3 ± 0.9, 5.5 ± 1.0 and 4.5 ± 1.0 nmol h^{-1}, respectively.

Discussion

The present study clearly shows that the H_2-agonist impromidine inhibits both baseline and gastrin-stimulated histamine release from the isolated vascularly perfused stomach of the rat. The ECL cell stores most of the histamine present in rat gastric mucosa (Håkanson & Sundler, 1987). The histamine measured in the venous effluent of this stomach preparation therefore most probably originates from those cells. This indicates that autoregulatory histamine H_2 receptors are present on the ECL cell surface. This is in accordance with previous studies (Håkanson *et al.*, 1977; 1978; Maslinski & Sewing, 1977) showing H_2 receptor-mediated inhibition of histamine synthesis (histidine decarboxylase activity) and of histamine mobilization from rat gastric mucosa after pentagastrin infusion.

An additional finding of the present study is that impromidine suppressed histamine output even at concentrations not giving maximal acid secretion. This suggests that the H_2 receptor-mediated suppression of histamine release could be induced by physiological concentrations of free histamine within the gastric mucosa. However, the H_2 antagonist ranitidine in a concentration completely inhibiting gastrin-stimulated acid secretion did not affect venous histamine output. This may suggest that the histamine receptor on the ECL cell is different from the H_2 receptor on the parietal cells, interacting with the H_2 agonist impromidine but not with the H_2 antagonist ranitidine.

The inhibition of histamine release by impromidine could also be secondary to increased acidity of the gastric lumen. Previously we have shown that somatostatin inhibits gastric histamine release (Sandvik & Waldum, 1988a) and the observed inhibition of histamine release could be due to paracrine release of somatostatin induced by acidification. However, the present results make this explanation unlikely since impromidine in increasing concentrations from 100 nmol l^{-1} to 10 μmol l^{-1} given together with gastrin only slightly increased acid output (or lowered gastric pH), and still progressively suppressed histamine release. Furthermore, ranitidine potently inhibited impromidine-induced acid secretion without significantly affecting vascular histamine output. We conclude that the described effect of impromidine on histamine liberation is not due to stimulation of acid secretion.

Histamine H_1 and H_3 receptor agonists as administered in the present study have marginal effects on acid secretion. Furthermore, at high concentrations, these agents suppressed basal vascular histamine

release. We were not able to find effects of H_3 receptor stimulation comparable to the potent suppression of histamine release described in central and peripheral histaminergic neurones (Arrang *et al.*, 1987). This would indicate that the ECL cells do not possess H_3 receptors, and it is unlikely that a significant fraction of the venous histamine comes from gastric histaminergic neurones. The effects of 2-methylhistamine and R-a-methylhistamine on acid secretion and histamine release could be explained by the relative unspecificity of the agents used. The relative H_2 agonist potencies of 2-methylhistamine and R-a-methylhistamine vs. histamine are 0.04 and 0.01, respectively (Schwartz *et al.*, 1986; Arrang *et al.*, 1987). Parietal cells probably possess H_2 receptors only, but 2-methylhistamine and R-a-methylhistamine in the concentrations tested slightly stimulated acid secretion. This suggests that the H_1 and H_3 receptor agonists in the concentrations used show some affinity for H_2 receptors, and this is further supported by the finding that ranitidine inhibited the acid secretagogue effect of those substances. Likewise, the inhibition of histamine release induced by these agents could be a H_2-mediated effect. IBMX sensitizes the rat isolated stomach to the major gastric secretagogues like gastrin, histamine and muscarinic agents, inducing a leftward shift of the dose-response curve. Furthermore, IBMX stimulates gastrin release while the release of histamine and somatostatin is unaffected (Sandvik *et al.*, 1988), and since endogenous gastrin is drained via the venous effluent there are probably no significant indirect effects of IBMX on the parietal and ECL cells in this model. Previous studies from our laboratory have shown that the histamine release from the isolated vascularly perfused stomach of the rat can be stimulated by gastrin (Sandvik *et al.*, 1987), and inhibited by somatostatin (Sandvik & Waldum, 1988a) and misoprostol, a methylated prostaglandin E_1 analogue (Sandvik & Waldum, 1988b). Together with the present results, it is evident that several of the most important substances regulating gastric acid secretion also modulate ECL cell histamine release. This makes endogenous histamine release a probable mechanism of at least some of the effects of these substances on gastric acid secretion in the rat.

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Effects of capsaicin desensitization on the stimulatory effect of kinins, prostaglandins, biogenic amines and various drugs in guinea-pig isolated atria

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1 A simple desensitization protocol was set up using capsaicin and isolated, spontaneously beating atria of guinea-pigs to assess the possible participation of cardiac, capsaicin-sensitive, substance P (SP)- and calcitonin gene-related peptide (CGRP)-containing sensory nerve fibres, in the cardiac stimulatory effects of bradykinin (Bk), kallidin (Kd), 5-hydroxytryptamine (5-HT), histamine, prostaglandin E_2 (PGE_2), prostaglandin E_1 (PGE_1), prostaglandin $F_{2\alpha}$, ($PGF_{2\alpha}$), adrenaline (Ad), glucagon, nicotine and angiotensin II (AII).

2 The positive chronotropic and inotropic effects of Bk, Kd and 5-HT were markedly reduced in capsaicin-desensitized atria compared to control. The percentage inhibition of the chronotropic and inotropic responses to the three agonists seemed to be inversely related to the concentration of agonist used and to vary also with the type of cardiac effect produced by the drug (for Bk the percentage inhibition was: 36–81% (chronotropic effect) and 62–86% (inotropic effect); for Kd: 61–78% (chronotropic effect) and 53–77% (inotropic effect); for 5-HT: 25–66% (chronotropic effect) and 40–64% (inotropic effect)).

3 The positive chronotropic and inotropic effects of histamine, PGE_1, PGE_2, $PGF_{2\alpha}$, glucagon and AII had similar amplitudes in capsaicin-desensitized and control atria.

4 The positive chronotropic and inotropic effects of Ad and nicotine were differentially affected by capsaicin desensitization. The inotropic effects of 7.5×10^{-7} and 7.5×10^{-6} M Ad were reduced by 41 and 27% respectively, in capsaicin-desensitized atria compared to control. The chronotropic effects of 1.54×10^{-5} and 6.17×10^{-5} M nicotine were inhibited by 57 and 26% respectively, by capsaicin desensitization. On the other hand, the chronotropic effect of Ad and the inotropic action of nicotine were of similar amplitude in capsaicin-desensitized and control atria.

5 These results were taken as an indication that a substantial part of the chronotropic and inotropic effects of Bk, Kd or 5-HT in guinea-pig atria, unlike those of histamine, PGE_1, PGE_2 $PGF_{2\alpha}$, glucagon and AII, might be the result of stimulation of capsaicin-sensitive, SP- and CGRP- containing sensory nerve fibres. The slight, differential inhibition of the chronotropic and inotropic effects of Ad and nicotine by capsaicin desensitization suggests a minor contribution by cardiac, capsaicin-sensitive sensory nerve fibres to the effects of nicotine and Ad in guinea-pig atria.

Introduction

Capsaicin (8-methyl-N-vanillyl-6-nonenamide), the active principle of hot red pepper of the plant genus *Capsicum*, has become a useful pharmacological tool to assess the contribution of sensory neurones to various biological phenomena. Several of the actions of capsaicin seem to derive from its unique ability to stimulate small diameter sensory nerve fibres, and to cause a subsequent, prolonged impairment of the mechanism of transmission of nociceptive stimuli (for a review, see Buck & Burks, 1986). Evidence has accumulated in recent years to indicate that the acute effect of capsaicin is to stimulate primary sensory neurones and trigger the release of several neuropeptides, primarily substance P (SP) and calcitonin gene-related peptide (CGRP), from peripheral and central branches of primary sensory neurones (Gamse *et al.*, 1979; Skofitsch *et al.*, 1985; Hua *et al.*, 1985; 1986; Saria *et al.*, 1986). Other studies have shown that chronic treatment of animals with capsaicin produces extensive depletion of primary afferents of their SP and CGRP content (Papka *et al.*, 1981; Gibbins *et al.*, 1985; Lundberg *et al.*, 1985;

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Saito *et al.*, 1986). Capsaicin seems to be markedly specific for SP- and CGRP-containing neurones for, in capsaicin-pretreated guinea-pigs, it causes a depletion of cardiac SP- and CGRP-containing neurones of their SP and CGRP content (Lundberg *et al.*, 1985; Saito *et al.*, 1986; Franco-Cereceda *et al.*, 1987a), without affecting cardiac, adrenergic and vasoactive intestinal polypeptide (VIP)-containing neurones (Papka *et al.*, 1981; Della *et al.*, 1983), or the functional response of guinea-pig atria to field stimulation of cardiac adrenergic nerve fibres or terminals (Saito *et al.*, 1986; 1987).

Capsaicin has previously been shown to exert powerful, positive chronotropic and inotropic effects in isolated, spontaneously beating atria of guinea-pigs (Molnar *et al.*, 1969; Lundberg *et al.*, 1984; Franco-Cereceda & Lundberg, 1985). The precise mechanism of these effects is still unknown, however there is evidence that the effects of capsaicin in guinea-pig hearts are mediated by CGRP released from cardiac capsaicin-sensitive sensory nerves (Franco-Cereceda & Lundberg, 1985; Saito *et al.*, 1986; 1987; Franco-Cereceda *et al.*, 1987a,b; 1988).

We recently showed that guinea-pig atria made unresponsive to capsaicin by capsaicin desensitization also became refractory to stimulation by neurotensin and bradykinin (Bk) (Rioux *et al.*, 1988), two peptides which appear to stimulate cardiac, capsaicin-sensitive sensory nerve fibres in guinea-pigs (Rioux *et al.*, 1986; 1987; Bachelard *et al.*, 1987). These results raise the interesting possibility of using capsaicin desensitization as a tool to assess indirectly the participation of capsaicin-sensitive sensory nerve fibres in the cardiac effects of drugs and natural substances known to affect cardiac functions in mammals.

In the present study we have assessed the influence of capsaicin desensitization under *in vitro* conditions on the cardiac stimulatory effects of naturally occurring substances: Bk, kallidin (Kd), glucagon, angiotensin II (AII), PGE_1, PGE_2, $PGF_{2\alpha}$, 5-HT, histamine, adrenaline (Ad) and nicotine, using the isolated, spontaneously beating atria of guinea-pigs as an experimental model. Bk was included in the present study both as a reference compound and also because our previous study with this peptide was limited to testing a single Bk concentration (Rioux *et al.*, 1988).

Methods

Isolated atria

Albino Hartley guinea-pigs (Charles River Canada Inc.) of either sex weighing between 350–400 g were killed by cervical dislocation followed by sectioning of the carotid arteries. The heart was removed from the animal and placed in oxygenated ($95\%O_2$:$5\%CO_2$), cold (24–25°C) Krebs solution. Both atria were dissected from the ventricles, cleaned of fat and blood and placed under a resting tension of 0.5 g into 12 ml organ baths containing oxygenated Krebs solution maintained at 37°C with a thermostated circulator (Haake model FJ). Under these conditions the atria started beating spontaneously. The beating rate and force of atrial contractions were recorded by means of force displacement (isometric) transducers (Grass FT03) coupled to a Grass polygraph (model 79). The period of tissue equilibration was 30–35 min. Atropine (7.4×10^{-7} M) was added to the Krebs reservoir to prevent the potential interference by acetylcholine released from cardiac vagal neurones with the positive chronotropic and inotropic effects of the various drugs used.

Atrial desensitization

Following the period of equilibration, the atria were made unresponsive (desensitized) to capsaicin by incubation with 1.3×10^{-6} M capsaicin for 30 min. The extent of atrial desensitization to capsaicin was verified by re-exposing the atria once to 1.3×10^{-6} M of capsaicin for 20 min. Time intervals between the first and second capsaicin application were set at 5, 60 and 120 min. Repeated changes of bath fluid were made between the first and second challenge with capsaicin.

Drug testing in capsaicin-desensitized atria

Following exposure to 1.3×10^{-6} M capsaicin (to produce atrial desensitization), the tissues were washed 4 to 5 times, and then allowed to recover for 30 min with washing every 10 min. Thereafter, the tissues were challenged with increasing concentrations of cardiac stimulants (see below), and concentration-response curves were obtained. Only one drug and no more than one or two drug concentrations were tested in a given atrial preparation. This protocol was adopted to limit the duration of each experiment to about 2 h and prevent tissue fatigue. The time of contact between cardiac stimulants and atria was between 5 and 10 min. This time period was found to allow maximal responses to a given drug concentration to be reached. The time interval between two drug applications was set at 30 min. Control atrial responses to cardiac stimulants were obtained in atria incubated for 30 min with capsaicin solvent (0.9% w/v NaCl solution containing ethanol 0.0004% v/v). Capsaicin solvent did not affect the basal beating rate and developed

tension of guinea-pig atria. The volume of drug injections into the organ bath was set at 120 μl.

Drugs, solutions and statistics

The following drugs were used: capsaicin, nicotine, bradykinin triacetate, histamine dihydrochloride, kallidin tetraacetate, 5-hydroxytryptamine creatinine sulphate (5-HT), atropine sulphate, (−)-adrenaline bitartrate, (all from Sigma Chemical Co., St-Louis, MO, U.S.A.); glucagon (Eli Lilly, Indianapolis, IN, U.S.A.), prostaglandin E_1 (PGE_1), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Upjohn, Kalamazoo, MI, U.S.A.), 1-Asp, 5-Ile, -angiotensin II (AII) (Peninsula Labs, Belmont, CA).

Capsaicin, PGE_1, PGE_2 and $PGF_{2\alpha}$ were dissolved in absolute ethanol. Glucagon was dissolved in the diluting solution for injection provided by Eli Lilly Co. It contains glycerin 1.6% v/v with phenol 0.2% w/v, as a preservative; the pH of this solution is about 4.0. Solutions of all drugs were made with 0.9% w/v NaCl solution. Histamine and adrenaline solutions were acidified to pH 4.0 with HCl. Drug concentrations are expressed in mol per litre of the base. The composition of the Krebs solution was (mM): NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.18, $MgSO_4 \cdot 7H_2O$ 1.18, dextrose 11.1, $NaHCO_3$ 25, disodium ethylenediaminetetracetate 0.06.

The statistical significance of differences between responses was evaluated by Student's t test for independent samples and P values of 0.05 or less were considered significant.

Results

Atrial desensitization with capsaicin

The first application of 1.3×10^{-6} M capsaicin for 30 min to isolated, spontaneously beating atria of guinea-pig evoked a marked positive chronotropic and inotropic effect. The kinetics of these effects are illustrated in Figure 1. Note the reversible nature of these effects. The second capsaicin application produced little or no significant change of the basal beating rate and developed tension of atria whether capsaicin was applied 5, 60 or 120 min after washout of the first capsaicin injection into the organ bath. Atria exposed once to 1.3×10^{-6} M capsaicin were thus considered to be desensitized to capsaicin.

Thirty min after ending atrial exposure to capsaicin the mean basal beating rate and developed tension of 50 capsaicin-desensitized atria were 174.8 ± 2.75 beats min^{-1} and 0.24 ± 0.01 g, respectively. Corresponding values in control atria (e.g. atria incubated for 30 min with capsaicin solvent, then washed and allowed to rest with intermittent rinsing over a 30 min period) were 170.2 ± 2.8 beats min^{-1} and 0.25 ± 0.01 g ($n = 50$). The mean values of basal heart rate and developed tension of capsaicin-desensitized atria did not significantly differ from those measured in control atria.

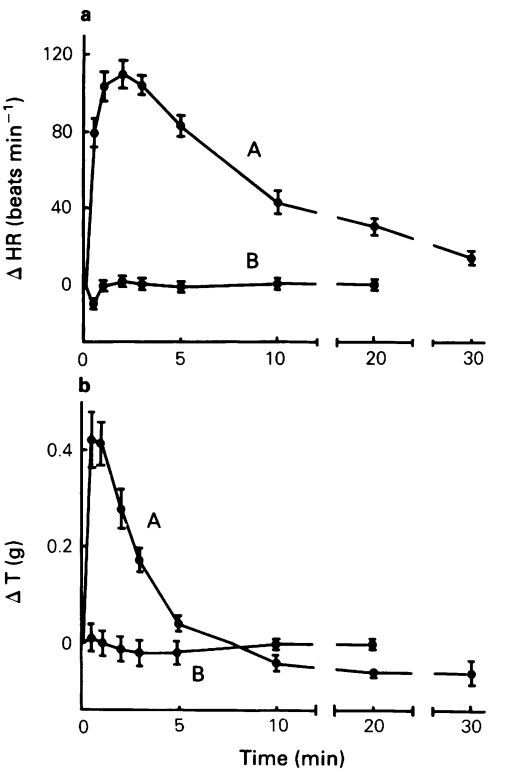

Figure 1 Kinetics of the increase of basal heart rate (ΔHR) (a) and developed tension (in g) (ΔT) (b) elicited by 1.3×10^{-6} M capsaicin in isolated, spontaneously beating atria of guinea-pigs. Curves A were obtained following the first exposure of atria to capsaicin (1.3×10^{-6} M applied for 30 min without tissue washing). Curves B were obtained following a second exposure of atria to capsaicin with a time interval of 120 min between the first and second application. Shorter time intervals (5 and 60 min) between the two capsaicin challenges produced results similar to those illustrated by curves B (curves not shown for the sake of clarity). Note the reversible nature of the chronotropic and inotropic effect of the first capsaicin application and the absence of atrial responses to a second capsaicin application. The points represent the mean of 4–8 determinations and the vertical bars, the standard error of the mean (s.e.mean).

Effects of various cardiac stimulants

The chronotropic and inotropic effects of various natural substances and drugs were measured and compared in control and capsaicin-desensitized atria. The results are shown in Figures 2, 3, 4, 5 and 6. Bradykinin (Bk) (8.06×10^{-9}–8.06×10^{-6} M) and kallidin (Kd) (7.0×10^{-9}–7.0×10^{-6} M) evoked concentration-dependent positive chronotropic and inotropic effects in control and capsaicin-desensitized atria (Figure 2). The chronotropic and inotropic responses to Bk and Kd were markedly inhibited in capsaicin-desensitized atria compared to control. The level of inhibition varied according to both the concentration of agonist used and the type of cardiac effect considered. Percentage inhibition ranged from 36 to 81% and 62 to 86% for Bk chronotropic and inotropic responses, respectively. Corresponding inhibition values for Kd varied between 61 to 78% (chronotropic effect) and 53 to 77% (inotropic effect).

5-HT (1.3×10^{-6}–1.9×10^{-4} M) and histamine (1.36×10^{-7}–1.3×10^{-5} M) caused concentration-dependent positive chronotropic and inotropic effects in both control and capsaicin-desensitized atria (Figure 3). The increases of heart rate and of developed tension evoked by 5-HT were significantly attenuated in capsaicin-desensitized atria compared to control. The level of inhibition of 5-HT-induced chronotropic effects varied between 25 to 66%. Corresponding inhibition values for 5-HT inotropic effects ranged from 40 to 64%. The positive chronotropic and inotropic responses to histamine were of similar amplitude in capsaicin-desensitized and control atria (Figure 3).

PGE_2 (5.7×10^{-10}–2.85×10^{-7} M) and PGE_1 (5.6×10^{-6}–2.8×10^{-7} M) elicited concentration-dependent positive chronotropic and inotropic

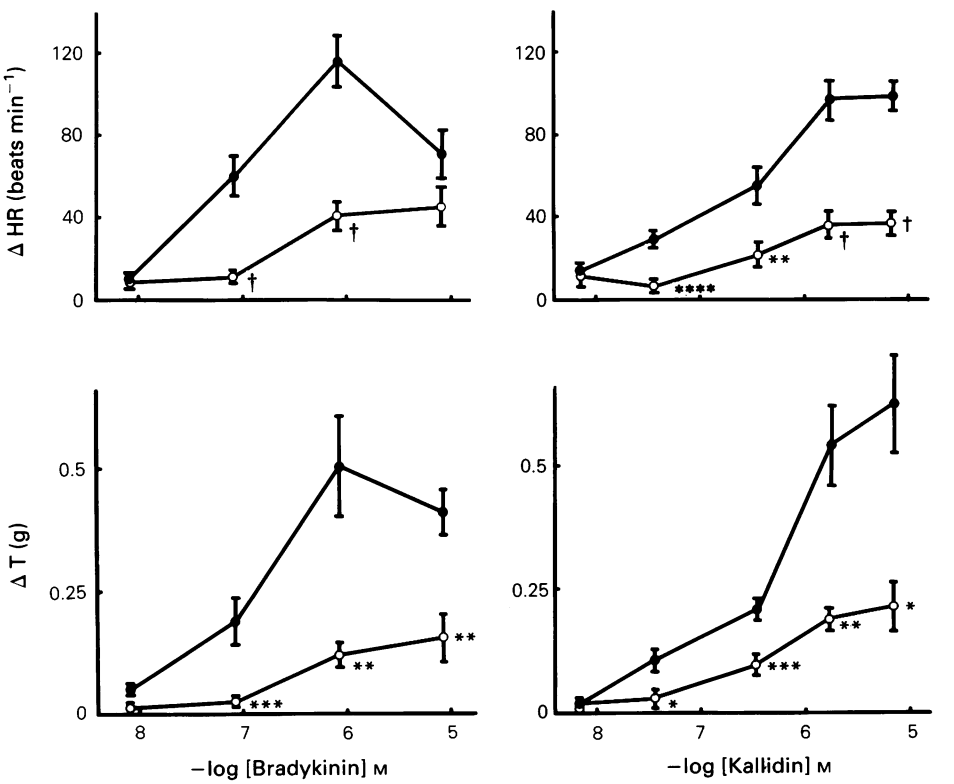

Figure 2 Concentration-response curves for bradykinin (Bk) and kallidin (Kd) in control (●) and capsaicin-desensitized atria (○) derived from guinea-pigs. Note the reduced responses to Bk and Kd in capsaicin-desensitized compared to control atria. The points represent the mean of 4–8 determinations, and the vertical bars the s.e.mean. ΔHR indicates the increase of basal heart rate (expressed as heart beats per min) and ΔT, the increase of basal developed tension (g). * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$; **** $P < 0.005$; † $P < 0.001$, when compared to the appropriate control value.

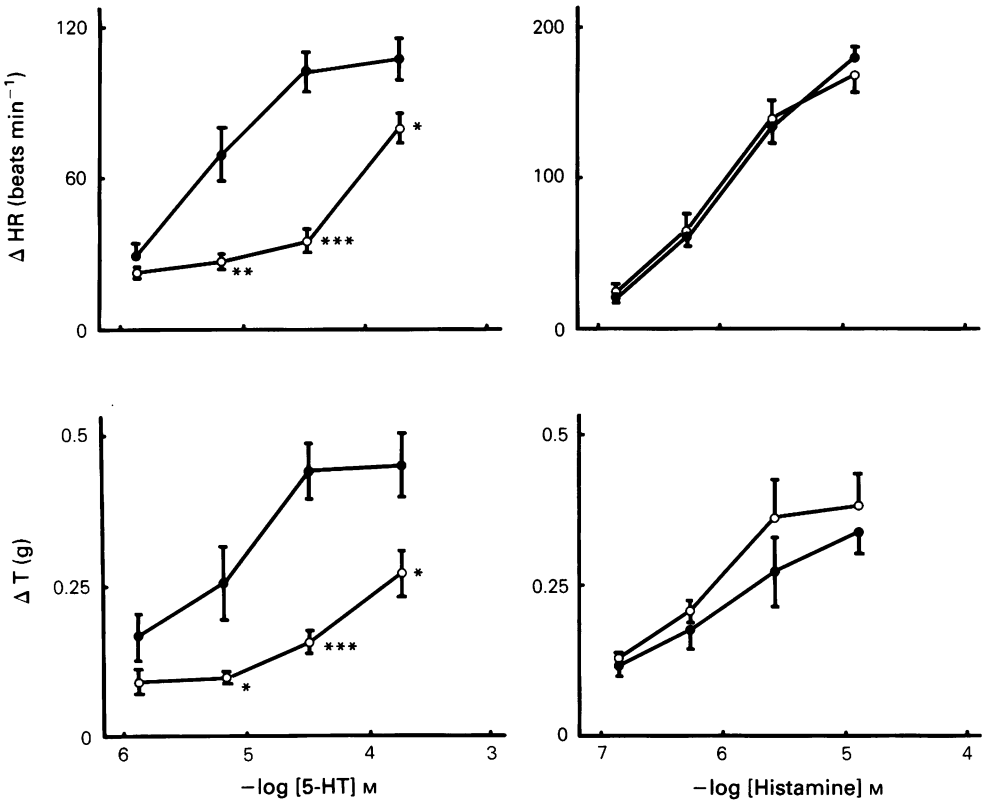

Figure 3 Concentration-response curves for 5-hydroxytryptamine (5-HT) and histamine in control (●) and capsaicin-desensitized (○) atria from guinea-pigs. Note the lower atrial responses to 5-HT in capsaicin-desensitized atria and the absence of effect of capsaicin desensitization on the atrial responses to histamine. The points represent the mean of 4–8 determinations and the vertical bars, the s.e.mean. Other details as in Figure 2. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$ when compared to the appropriate control value.

effects in control and capsaicin-desensitized atria (Figure 4). Both drugs produced cardiac effects of similar amplitude in capsaicin-desensitized and control atria, the only exception being PGE_2 which at a concentration of 2.85×10^{-8} M produced inotropic effects of larger amplitude (+76%) in capsaicin-desensitized than in control atria (Figure 4). Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (2.8×10^{-7} and 2.8×10^{-6} M) also evoked concentration-dependent, positive chronotropic and inotropic effects in control and capsaicin-desensitized atria. Both effects were of similar amplitude in the two groups of atria ($n = 4$ to 7) (data not shown).

Adrenaline (Ad) (1.5×10^{-8}–3×10^{-5} M) and glucagon (5.74×10^{-9}–2.87×10^{-6} M) elicited concentration-dependent positive chronotropic and inotropic effects in control and capsaicin-desensitized atria (Figure 5). The chronotropic effects of Ad and glucagon as well as the positive inotropic responses to glucagon, exhibited similar amplitude in capsaicin-desensitized and control atria. On the other hand the positive inotropic responses to Ad tended to be slightly reduced in capsaicin-desensitized atria compared to control, the level of inhibition varying between 41 and 27% at Ad concentrations of 7.5×10^{-7} and 7.5×10^{-6} M, respectively.

Nicotine (6.17×10^{-6}–3.1×10^{-4} M) and angiotensin II (AII) (4.5×10^{-10}–9×10^{-7} M) evoked concentration-dependent positive chronotropic and inotropic effects in control and capsaicin-desensitized atria (Figure 6). The chronotropic responses to AII, and the inotropic responses to both AII and nicotine, exhibited similar amplitudes in control and capsaicin-desensitized atria. The chronotropic effects of 1.54×10^{-5} and 6.17×10^{-5} M of

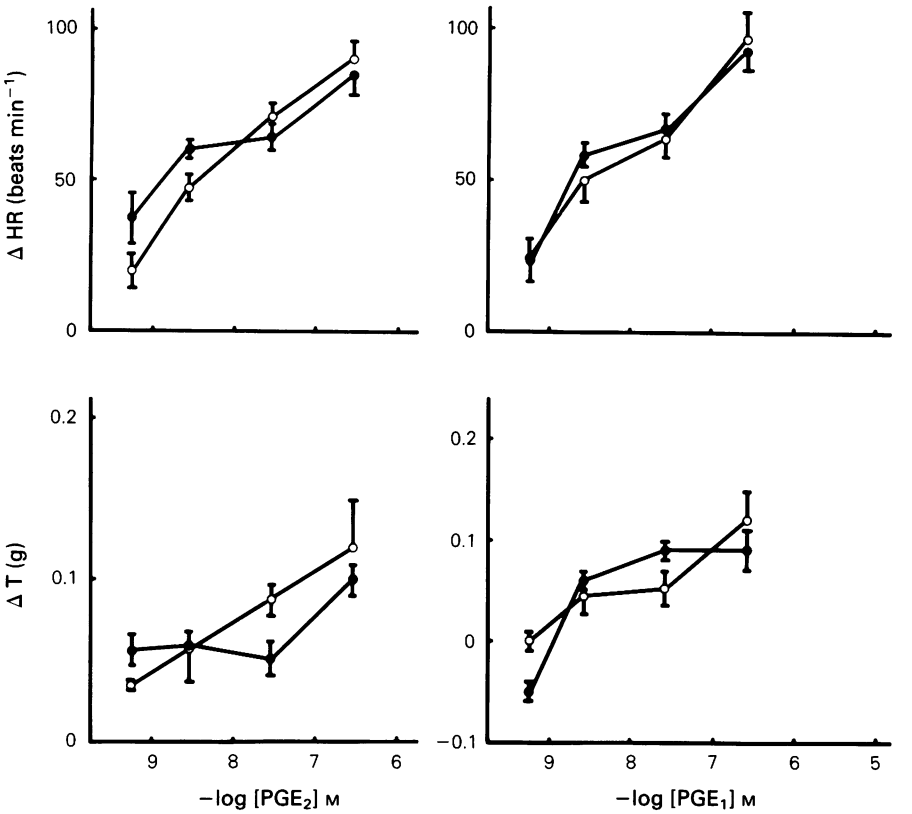

Figure 4 Concentration-response curves for prostaglandin E_2 (PGE_2) and prostaglandin E_1 (PGE_1) in control (●) and capsaicin-desensitized (○) atria from guinea pigs. Note the general lack of effect of capsaicin desensitization on the positive chronotropic and inotropic effects of the two prostaglandins. The points represent the mean of 4 to 12 determinations, and the vertical bars the s.e.mean. Other details as in Figure 2.

nicotine were reduced by 57% and 26% respectively, in capsaicin-desensitized atria compared to control. On the other hand, the maximum concentration of nicotine used (e.g. 3.1×10^{-4} M) evoked positive chronotropic effects of similar amplitude in the two groups of atria (Figure 6).

Discussion

In this paper we show that the positive chronotropic and inotropic effects elicited by 1.3×10^{-6} M capsaicin in guinea-pig isolated atria are gradually reversible within 30 min despite the absence of capsaicin removal from the organ bath. Our results also show that guinea-pig atria exposed once to 1.3×10^{-6} M capsaicin for 30 min become completely refractory (or desensitized) to subsequent capsaicin challenges. The period of tissue desensitization was found to be quite prolonged for the desensitization was still present and undiminished for 120 min after removal of the neurotoxin from the organ bath. These observations are consistent with the results of other workers using similar cardiac preparations (see Introduction). Although the exact mechanism which subserves the positive chronotropic and inotropic effects of capsaicin in guinea-pig atria is still unknown, strong evidence suggests that cardiac capsaicin effects are mediated by CGRP released from cardiac, SP- and CGRP-containing sensory nerve fibres (Franco-Cereceda & Lundberg, 1985; Saito *et al.*, 1986; 1987; Franco-Cereceda *et al.*, 1987a,b; 1988). The mechanism underlying the desensitization produced by this neurotoxin in guinea-pig atria is also unknown. However, given the compelling evidence for the participation of capsaicin-sensitive, SP- and CGRP-containing sensory nerve fibres in the stimulatory effects of capsaicin in the guinea-pig heart or atria (Franco-Cereceda & Lundberg, 1985; Saito *et al.*, 1986; 1987; Miyauchi *et al.*, 1987;

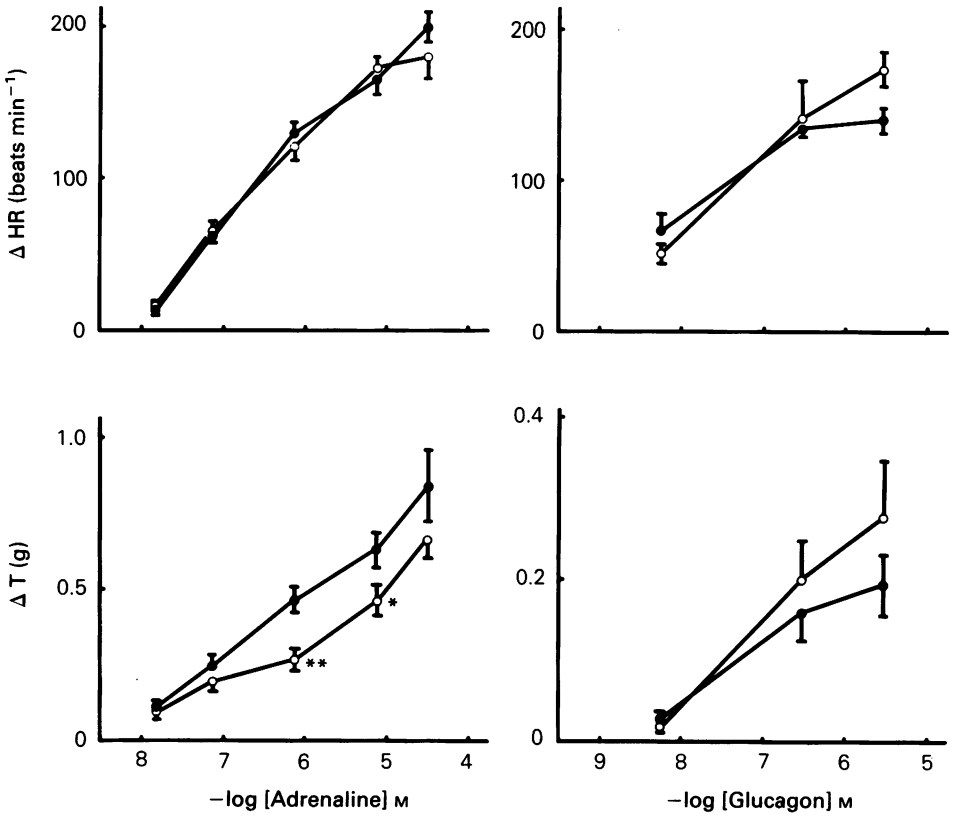

Figure 5 Concentration-response curves for adrenaline (Ad) and glucagon in control (●) and capsaicin-desensitized (○) atria from guinea-pigs. Note the slight reduction of the inotropic effect of 7.5×10^{-7} and 7.5×10^{-6} M Ad in capsaicin-desensitized atria and the absence of modification of the chronotropic effects of Ad and glucagon, and of the inotropic effect of glucagon, by capsaicin desensitization. The points represent the mean of 4–8 determinations, and the vertical bars the s.e.mean. Other details as in Figure 2. * $P < 0.05$; ** $P < 0.02$ when compared to the appropriate control value.

Franco-Cereceda *et al.*, 1987a,b; 1988), it seems logical to hypothesize that the loss of atrial responses to capsaicin following capsaicin desensitization reflects the blockade of SP and CGRP release from atrial, capsaicin-sensitive, SP- and CGRP-containing, sensory nerve fibres. This hypothesis is supported by recent studies showing that capsaicin-induced SP release from cardiac, capsaicin-sensitive sensory nerve fibres in guinea-pig isolated hearts is inhibited, along with the mechanical effects of capsaicin, following the development of tachyphylaxis to capsaicin (Hoover, 1987). Whether SP and CGRP depletion, or failure to repolarize (after a first exposure to capsaicin) of cardiac capsaicin-sensitive, sensory nerve fibres (or endings), is responsible for the persistent loss of cardiac sensitivity to capsaicin is still unknown. Evidence for the involvement of both mechanisms has been provided (Papka *et al.*, 1984; Buck & Burks, 1986; Hoover, 1987; Marsh *et al.*, 1987).

In the present paper we determined the influence of capsaicin desensitization on the positive chronotropic and inotropic effects of Bk, Kd, 5-HT, histamine, PGE_2, PGE_1, $PGF_{2\alpha}$, Ad, glucagon, nicotine and AII, in isolated, spontaneously beating atria of guinea-pigs. With the exception of Ad, glucagon and AII, all the substances included in this study are known to exhibit algesic and/or inflammatory properties (Douglas, 1985; Moncada, 1985). This alone constitutes a good rational basis for considering these substances as potential stimulators of cardiac, capsaicin-sensitive, SP- and CGRP-containing sensory nerve fibres, since capsaicin-sensitive, SP- and CGRP-containing sensory neurones are believed to be pain-conveying nerve fibres (Buck & Burks, 1986). Ad, glucagon and AII

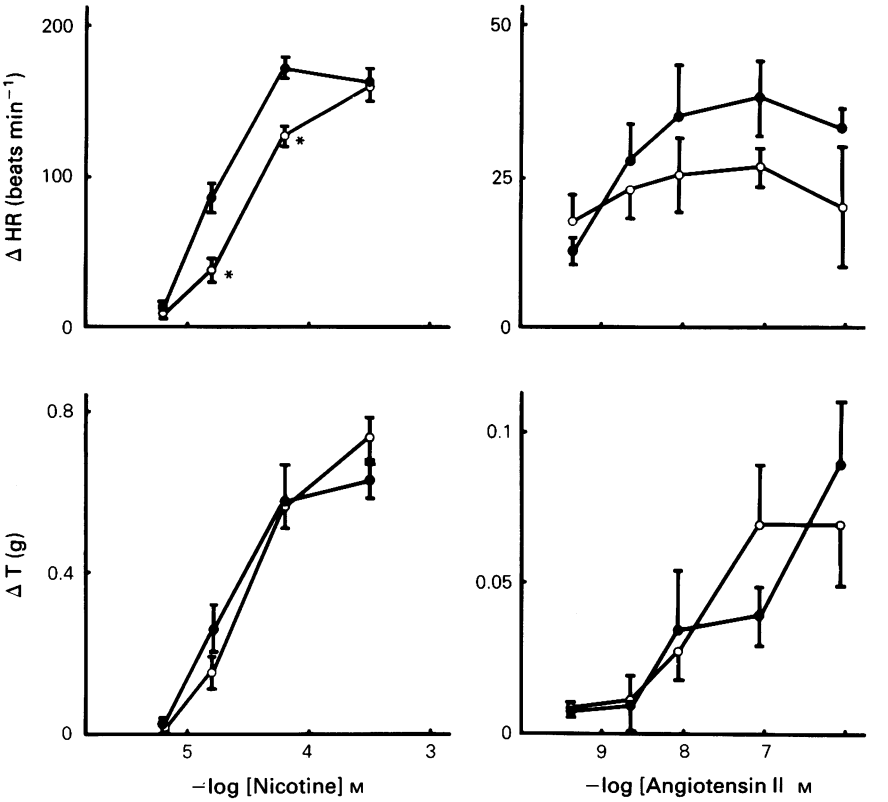

Figure 6 Concentration-response curves for nicotine and angiotensin II (AII) in control (●) and capsaicin-desensitized (○) atria from guinea-pigs. Note the inhibition of the chronotropic effects of 1.54×10^{-5} and 6.17×10^{-5} M nicotine in capsaicin-desensitized atria, and the absence of effect of capsaicin desensitization on the positive chronotropic and inotropic effects of AII, and on the positive inotropic effect of nicotine. The points represent the mean of 4–8 determinations, and the vertical bars the s.e.mean. Other details as in Figure 2. * $P < 0.005$ when compared to the appropriate control value.

were included in this study to serve as reference compounds for all three drugs are known to exert positive chronotropic and inotropic effects in mammalian hearts (Lucchesi, 1968; Regoli *et al.*, 1974), and also because none of them has yet, to our knowledge, been considered as potential stimulators of cardiac, capsaicin-sensitive sensory nerve fibres. The results presented in this paper show that the positive chronotropic and inotropic effects of Bk, Kd and 5-HT are markedly reduced, but not totally abolished, in capsaicin-desensitized atria compared to control. In our opinion, the level of inhibition following capsaicin desensitization of the responses to these substances is sufficiently high to support the suggestion that cardiac, capsaicin-sensitive, SP- and CGRP-containing sensory nerve fibres participate, at least under our experimental conditions, in the chronotropic and inotropic effects of Bk, Kd and 5-HT, possibly via the release of CGRP (see above). Further studies will be needed to substantiate this hypothesis.

As far as the cardiac effects of histamine, PGE_2, PGE_1, $PGF_{2\alpha}$, glucagon and of AII are concerned, we observed no significant difference between capsaicin-desensitized and control atria. These results suggest no involvement of cardiac, capsaicin-sensitive, sensory nerve fibres in the chronotropic and inotropic effects of histamine, PGE_2, PGE_1, $PGF_{2\alpha}$, glucagon and AII, in our experiment model.

The amplitudes of the positive chronotropic effect of nicotine and of the positive inotropic effect of Ad were slightly lower in capsaicin-desensitized than in control atria. These results are somewhat hard to interpret because it is difficult to understand how a chemical mediator (e.g. CGRP) could mediate the chronotropic effect of nicotine in guinea-pig atria

without being also involved in the inotropic effect of nicotine. After all, the chemical mediator should theoretically be released from all atrial, capsaicin-sensitive sensory nerve fibres and come subsequently in contact, not only with pacemaker cells, but also with atrial myocytes. The same type of reasoning also applied to Ad (e.g. its inotropic, but not is chronotropic, effect is reduced in capsaicin-desensitized atria). The precise reason for the differential effect of capsaicin desensitization on the chronotropic and inotropic effect of nicotine or Ad is unknown. However one possible explanation is that the amount of CGRP released by Ad or nicotine is too low to cause chronotropic and/or inotropic effects which are easily distinguishable from those evoked by nicotine or Ad itself (via other mechanisms). That nicotine and catecholamines have, in fact, the ability to stimulate the release of CGRP from cardiac capsaicin-sensitive sensory nerve fibres in guinea-pig hearts is well supported by a recent study by Franco-Cereceda *et al.* (1987b). These authors show that exogenous nicotine and noradrenaline cause a three fold increase of the overflow of CGRP-like immunoreactivity in guinea-pig isolated hearts.

In conclusion the results presented in this report suggest that parts of the chronotropic and inotropic effects of natural substances such as Bk, Kd and 5-HT in guinea-pig isolated atria, in contrast to those evoked by histamine, PGE_1, PGE_2, $PGF_{2\alpha}$, glucagon and AII, might result from the stimulation of cardiac capsaicin-sensitive, SP- and CGRP-containing sensory nerve fibres and be mediated by CGRP released from the latter neural elements. The slight, differential inhibition of the chronotropic and inotropic effects of nicotine and Ad by capsaicin desensitization suggests a minor contribution by cardiac, capsaicin-sensitive sensory fibres to the cardiac effects of nicotine and Ad in guinea-pig atria. Although the physiological meaning of these results is still unknown it is tempting to speculate that endogenous kinins and 5-HT, along with other substances such as neurotensin (Rioux *et al.*, 1988), might behave as natural stimulants of cardiac pain fibres involved in the pain of myocardial ischaemia. The possible involvement of kinins in cardiac pain mechanisms has been emphasized previously (Kimura *et al.*, 1973; Staszewska-Barczak *et al.*, 1976; Lombardi *et al.*, 1982; Kaufman *et al.*, 1984). Further studies involving more direct approaches are needed to substantiate the above hypothesis.

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Electrophysiological estimation of the actions of acetylcholinesterase inhibitors on acetylcholine receptor and cholinesterase in physically isolated *Aplysia* neurones

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1 The actions of representative cholinesterase inhibitors on the acetylcholine responses of physically isolated single neurones from the pedal ganglion of *Aplysia californica* were studied, using electrophysiological techniques and rapid agonist application to analyse both the inhibitory actions on the acetylcholine receptor-channel complex and the degree of inhibition of acetylcholinesterase activity on the same neurone. The inhibitors used were physostigmine, edrophonium and diisopropylfluorophosphate (DFP).

2 When selected neurones were suddenly exposed to 50 μM acetylcholine by a 'concentration clamp' technique a large Na-dependent inward current was initiated, and decayed in the continued presence of acetylcholine without external perfusion. However, if perfusion of the acetylcholine solution was reinitiated the current increased somewhat, indicating that the decay of current was due to some combination of receptor desensitization and local depletion of acetylcholine at the membrane by acetylcholinesterase.

3 With simultaneous application of acetylcholine (50 μM) and physostigmine (0.1 to 100 μM) there was a dose-dependent reduction of peak amplitude of the acetylcholine response. However, physostigmine at low concentrations (0.1 to 10 μM) caused a time-dependent increase in the current amplitude alone with a time- and dose-dependent inhibition of acetylcholinesterase activity. At the highest concentration of physostigmine (100 μM) acetylcholinesterase activity was abolished but the current peak was very depressed. After removal of physostigmine from the bathing solution, the current amplitude decreased toward the control at the two lower concentrations as the inhibitory actions on acetylcholinesterase activity were almost reversible, while at the two higher concentrations (10 and 100 μM) the current increased and the inhibition of acetylcholinesterase remained.

4 When acetylcholine (50 μM) and edrophonium (0.1 to 10 μM) were applied simultaneously, edrophonium caused a dose-dependent increase in the peak amplitude that was correlated with a dose-dependent inhibition of acetylcholinesterase activity. Prolonged exposure to edrophonium did not change the peak amplitude and there was no time-dependent change in the inhibition of acetylcholinesterase activity. At the highest concentration of edrophonium used (100 μM), simultaneous application with acetylcholine augmented the peak amplitude relative to control, but to a lesser extent than 10 μM. Prolonged exposure to the highest concentration of edrophonium caused a time-dependent reduction in the peak amplitude. The effects of edrophonium were quickly reversible after the removal of the drug from the bathing solution.

5 DFP (1 and 10 mM), similar to 100 μM physostigmine, caused a dramatic reduction of the peak current on simultaneous application with acetylcholine. During exposure to DFP the current amplitude and acetylcholinesterase activity were very depressed. After removing DFP from the bathing solution the current amplitude increased to more than the control level after 1 mM DFP, while it did not recover to the control level after 10 mM DFP. The inhibition of acetylcholinesterase activity remained at both concentrations.

6 These results indicate that all three cholinesterase inhibitors have dose-dependent actions both at the acetylcholine receptor-channel complex and at acetylcholinesterase. The methods we have developed may be useful in the evaluation of various cholinesterase inhibitors.

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Introduction

Acetylcholinesterase is an enzyme that degrades acetylcholine and is widely distributed in the nervous system. It is located extracellularly on both neurones and glia (Rotundo, 1987). Because it lowers the concentration of acetylcholine this enzyme has functional effects both on synaptic transmission and on the response of receptive neurones to exogenously applied acetylcholine. In the presence of low concentrations of cholinesterase inhibitors, the peak amplitude of the acetylcholine response is increased (Dreyer *et al.*, 1978), reflecting the inhibition of acetylcholine hydrolysis. However, some anticholinesterases have, in addition to their inhibitory action on the enzyme, direct actions on the acetylcholine receptor-channel complex (Gage & McBurney, 1975; Kordas *et al.*, 1975; Kuba & Tomita, 1975; Magleby & Terrar, 1975).

Recently we have developed an electrophysiological technique that allows the direct estimation of acetylcholinesterase activity on physically (non-enzymatically) isolated single neurones of *Aplysia californica* and *kurodai* (Oyama *et al.*, 1988a). When the solution around a single isolated neurone was rapidly changed from sea water to sea water containing acetylcholine, an inward current was elicited, which peaked and decayed in the continued presence of acetylcholine. A part of the decay is due to receptor desensitization (Katz & Thesleff, 1957). However, restarting the flow of solution around the isolated neurone was found to cause an increase in current but to a value less than the initial peak. If carbachol, an acetylcholine agonist not hydrolyzed by acetylcholinesterase, was used, there was no increase in current when perfusion of the carbachol solution was reinitiated. Thus both receptor desensitization and acetylcholinesterase appear to contribute to the current decay in the presence of acetylcholine. In the absence of flow of the acetylcholine solution, the esterase activity appears to be sufficient to cause a local reduction of the acetylcholine concentration at the membrane. The rise of the current when flow is reinitiated is thus a convenient and rapid electrophysiological indicator of acetylcholinesterase activity, and can be used to monitor the inhibition of the esterase.

In this study, we have examined the actions of representative drugs from three classes of anticholinesterase. Physostigmine (eserine) is a carbamate inhibitor that is reversible, edrophonium is a quaternary ammonium compound that is rapidly active and reversible, while diisopropylfluorophosphate (DFP) is an irreversible alkyl fluorophosphate inhibitor. We have used an electrophysiological monitor of acetylcholine receptor activation and of acetylcholinesterase activity to investigate the relative actions and differences in time course of action of these anticholinesterases.

Methods

The experimental method (internal perfusion with a concentration-clamp technique) was a slight modification of that previously described (Akaike *et al.*, 1986). Experiments were performed on single neurones physically isolated from the pedal ganglia of *Aplysia californica* (Oyama *et al.*, 1988c). Physically isolated neurones were used because the enzymatic treatment for dissociation of neurones removes acetylcholinesterase from their membrane (Kostenko *et al.*, 1974). The ganglion was incubated for 30 min in normal extracellular solution (NES, Table 1) to which 250 $g l^{-1}$ sucrose was added. This treatment caused the neurones to shrink and facilitated the removal of the connective tissue and the top of the capsule, which were carefully removed with fine forceps and a pair of iris scissors. The ganglion was then returned to NES, and the exposed neuronal cell bodies were repeatedly washed by hand pipetting of NES. Individual cell bodies of 120 to 200 μm diameter which were freed from the ganglion were sucked into a pipette with a tip of about 400 μm in diameter, and the axon was cut. These isolated neurones stored in NES remained viable for experiments for up to 2 days after dissociation at room temperature (23 to 24°C).

The suction-pipette technique was employed for internal perfusion and voltage clamping (Lee *et al.*, 1980; Ishizuka *et al.*, 1984). The suction pipette was made from glass capillary of 3 mm inside diameter. The capillary was pulled to a shank length of about 3 mm, the tip of the pipette was cut at an outer diameter of 140 to 160 μm and then fire-polished to give inner and outer diameters of about 30 μm and 100 μm, respectively. The membrane potential was measured through an Ag-AgCl wire in a 3 M KCl-agar plug mounted on the suction pipette holder. The reference electrode (also an Ag-AgCl wire) was placed in a polyethylene catheter which was used for rapid solution changes (Krishtal *et al.*, 1983; Akaike *et al.*, 1986). The resistance between the Ag-AgCl wires was less than 10 kohm when the suction pipette was filled with normal internal solution (NIS: Table 1) and the measurement made in NES. Both electrodes were led to a voltage-clamp circuit (DAGAN 8100). The cell body was drawn on to the suction pipette and held on the tip of the pipette by negative pressure of 300 to 450 mmHg. The membrane inside the pipette either spontaneously ruptured or was broken by hand with a thin Pt-Ir wire. The membrane potential was controlled by a single-electrode voltage-clamp system switching at 10 to

Table 1 Ionic compositions of external and internal solutions (in mM)

Normal external solution (NES)		*Normal internal solution (NIS)*	
NaCl	450	K aspartate	305
KCl	10	KCl	55
$CaCl_2$	10	Mannitol	300
$MgCl_2$	50	EGTA	0.1
HEPES	10	HEPES	20
pH	7.7 to 7.75	pH	7.3

The pH of NES and NIS was adjusted by appropriate amounts of NaOH and Tris base, respectively.

30 kHz and passing current for 25 or 50% of the cycle (Ishizuka *et al.*, 1984). Both current and voltage outputs were monitored on a storage oscilloscope, a digital memory oscilloscope and X-Y recorder.

In order to change the solution around the neurone rapidly, the tip of the suction pipette with the cell body attached was inserted into the polyethylene catheter (a diameter of less than 2 mm) through a pin hole of 1 mm diameter (see Figure 1 of Akaike *et al.*, 1986). The lower end of this catheter was then submerged in fluid in a tissue culture dish (diameter 2.5 cm, depth 1 cm). Negative pressure was applied to the upper end of the catheter to suck the solution from the dish through the catheter and past the neurone. The negative pressure was adjusted to make the rate of flow inside the catheter at least 8 cm per second. The suction was controlled by an electromagnetic valve operated by d.c. The external solution around the cell could be completely changed within 20 ms with this technique, and the usual procedure was to stop the perfusion after 400 or 800 ms of flow. For the purposes of changing solution, the various solutions were put in different dishes, and the dishes were mounted on a circular turntable fixed to a micromanipulator stage. To switch from one solution to another, the dish containing the desired solution was positioned at the end of the catheter by lowering the stage and rotating the turntable, then raising the stage until the tip of the catheter, still containing the previous solution, was again submerged. Suction was then applied to replace the solution in the catheter. The efficacy of the rapid external perfusion was tested in our previous study (Oyama *et al.*, 1988b).

Since the acetylcholinesterase activity of neurones stored in NES seemed to decrease gradually 2 days after isolation, neurones were used within 1 day after dissociation. Only neurones having action potentials of more than 80 mV in amplitude were studied. The neurones isolated from the pedal ganglia have various ionic currents in response to acetylcholine. A population of dark yellow neurones located on the caudal half of the dorsal surface of the pedal ganglion between the pedal connective and pedal nerve showed a large Na-dependent inward current when the cell was voltage-clamped at −60 mV and were used in these studies. Because the neurone was perfused with NES and NIS to make Cl and K equilibrium potentials about −60 and −90 mV, respectively, any K current was outward and any contamination of the inward current carried by Cl was minimized. In the neurones selected for study the inward current elicited by acetylcholine was almost totally eliminated in Na-free solution (NaCl replaced with equimolar Tris Cl). For this study, only neurones that showed a constant amplitude response to 50 μM acetylcholine applied at intervals of 10 min were employed. The efficacy of the internal perfusion for *Aplysia* neurones has also been tested in a previous study (Oyama *et al.*, 1988b).

Figure 1 illustrates the experimental protocol in diagrammatic form. Each acetylcholine application consisted of two periods of solution flow (each lasting 400–800 ms) separated by 15 s. The first solution application led to an inward current that rapidly peaked and then decayed. The second flow 15 s later always gave a second increase of current, but never reached the value of the original peak. After the preparation was stable, a standard protocol was followed in which seven of the above paired flows were given, at 10 min intervals. After the initial acetylcholine control (a), the preparation was washed with NES for 10 min. It was then given paired perfusions with acetylcholine plus inhibitor at one concentration (b), after which it was washed with inhibitor in NES for 10 min. Two more tests with acetylcholine plus inhibitor were given while washing with inhibitor (c, d), after which acetylcholine without inhibitor was tested at the onset of the wash (e) and at 10 (f) and 20 (g) min thereafter. The effects of the anticholinesterases were analysed in terms of the ratio of peak amplitudes of the test as compared to control for the first perfusion (R_p) and the ratio of relative increase in amplitude of the current elicited by a second perfusion after 15 s in the presence of the stationary acetylcholine solution (R_e). The inhibition of acetylcholinesterase should be reflected in an increase in the first peak and a

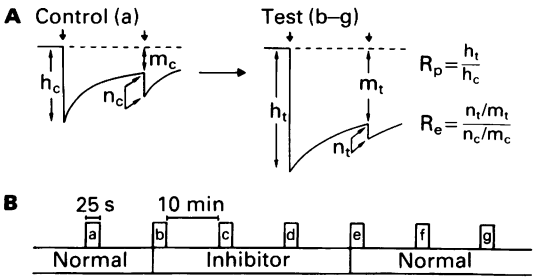

Figure 1 (A) Method for estimating the action of acetylcholinesterase inhibitors on the response elicited by two rapid and time-fixed perfusions of 50 μM acetylcholine. Two arrows over the dotted line (holding current level) indicate the initiation of perfusion, and are separated by 15 s. The perfusion in each case lasted 400 or 800 ms, after which flow was stopped. In this study, we examined the action of acetylcholinesterase inhibitors on the peak amplitude of the first response (h_c and h_t) in control and test responses, and the effect of the inhibitor on the amplitude of response obtained on a second perfusion. The ratio of peaks of the first response, R_p, should increase due to inhibition of acetylcholinesterase, but decrease if the inhibitor has a dominant effect on the acetylcholine receptor or associated ion channel. The second response is quantified by measurement of the maintained current 15 s after the first perfusion (m_c) and the new current generated by the second perfusion (n_c). This new current is an indicator of the remaining cholinesterase activity. Thus the ratios (R_e) of the new to maintained currents, test over control, should become very small as cholinesterase activity is abolished. (B) Experimental protocol. Seven measurements (a) to (g) were performed during each experiment. The interval between each application was 10 min and the period of continuous presence of acetylcholine in each application was 25 s during which time two perfusions were made (a) control in normal external solution (NES; normal). (b) Simultaneous application of acetylcholine with acetylcholinesterase inhibitor (inhibitor). At this time, NES was switched to NES containing acetylcholinesterase inhibitor. After a period of 25 s, the external solution was switched to NES containing inhibitor. This was designed to obtain information concerning the competition at the receptor between acetylcholine and acetylcholinesterase inhibitors. (c) and (d) NES containing acetylcholine and acetylcholinesterase inhibitor was applied to the neurone 10 min after the application of acetylcholine. These procedures were followed to observe the time-dependent actions of the inhibitor. (e) The external solution was switched to the NES containing acetylcholine, and the inhibitor in the external solution was removed from the external solution within 30 ms. After this procedure, the neurone was washed with NES every 30 s. In (f) and (g) acetylcholine was applied to follow recovery from the drug.

decrease in the second, while actions of the inhibitor at the level of the receptor or channel should result in a reduction of the current amplitude, most obvious in the first peak.

All cholinesterase inhibitors were purchased from Sigma. Physostigmine and edrophonium were dissolved in NES just before use. DFP was dissolved in propylene glycol and used as previously described (Kuba *et al.*, 1974). Control applications of the appropriate dilution of propylene glycol in NES had no effect on the R_p and R_e of responses to 50 μM acetylcholine.

Results

Effect of cholinesterase inhibitors at low concentrations

Figure 2 shows the effect of 1 μM physostigmine on the acetylcholine (50 μM) response using the protocol of Figure 1. Simultaneous application of acetylcholine with physostigmine (b) resulted in a slightly reduced peak amplitude, but there was practically no change in the time course of the response. However, after 10 min of treatment with physostigmine (c) the amplitude of the first response was increased, while the ratio (R_e) of increase induced by the second perfusion was decreased. A further small reduction of the second response occurred in the next 10 min (d).

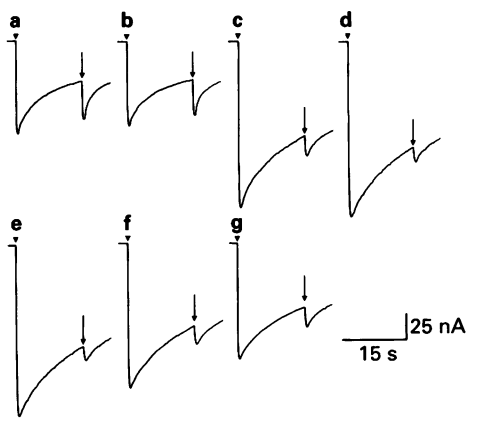

Figure 2 Effect of 1 μM physostigmine on the response elicited by two rapid and time-fixed (400 ms) perfusions of 50 μM acetylcholine. The triangle and arrow indicate the first and second perfusion, respectively. (a) to (g) over each current trace indicate those manipulations described in Figure 1B.

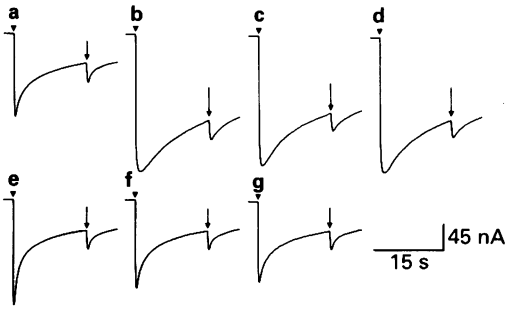

Figure 3 Effect of 1 μM edrophonium on the response elicited by two perfusions of acetylcholine as indicated by the triangle and arrow. (a) and (g) over the current trace indicate those manipulations described in Figure 1B.

After 20 min of washing the response almost reached control values (f and g).

Figure 3 shows the same experiment but with 1 μM edrophonium. Simultaneous application of 50 μM acetylcholine with edrophonium (b) caused an increase in the peak amplitude of the first response and a decrease in the ratio of the second response. Both effects appeared to reach a steady state immediately after the application, as the time course of responses (c) and (d) was very similar to that elicited by simultaneous application (b). Removal of edrophonium from the external solution resulted in quick recovery of the response toward the control (e) and repeated washing of the neurone gave complete recovery of the response (f and g).

Effect of cholinesterase inhibitors at high concentrations

The highest acetylcholinesterase inhibitor concentration employed in this study was 100 μM. Figure 4A shows the effect of 100 μM physostigmine. Simultaneous application of acetylcholine with this concentration of physostigmine decreased the peak amplitude of the first response and diminished the second response (b). Longer exposure further reduced the peak amplitude (c and d). On removal of

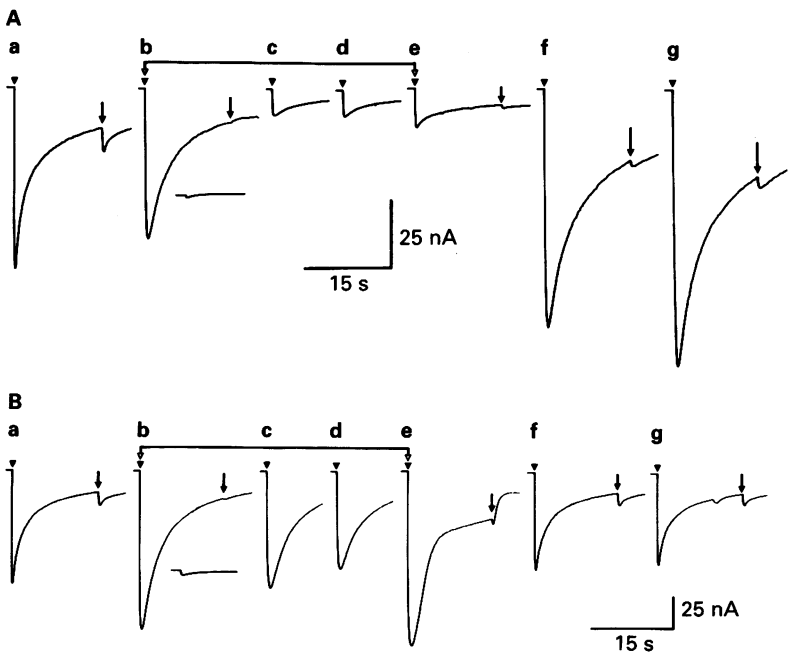

Figure 4 (A) Effect of 100 μM physostigmine on the acetylcholine response. The triangle and black arrow indicate the perfusion of the solution containing acetylcholine. The arrows joined by a bar indicate the period of the presence of physostigmine. The current trace under the current trace of (b) shows the current elicited by 100 μM physostigmine in this neurone. (B) Effect of 100 μM edrophonium on the acetylcholine response. Symbols are the same as in (A). The current trace under the current trace of (b) shows the current elicited by 100 μM edrophonium. The properties of this current have been previously characterized (Oyama *et al.*, 1988b,c).

physostigmine there was a slight recovery (e). After repeated washing the peak amplitude of the first response increased to more than control level but the ratio of the second response was still suppressed (f and g). Figure 4B shows the effect of 100 μM edrophonium. With simultaneous application the peak amplitude of the first response increased and the second response diminished (b). In continued presence of edrophonium the first response was slightly reduced but was still similar in amplitude to the control (c and d). On washing, the first response increased in amplitude and the second recovered slightly (e). With repeated washing there was an almost complete return to control (f and g). Internal perfusion of either edrophonium or physostigmine at 100 μM had no effect on the second response.

Dose-dependency and time course of the action on the first peak current

Figure 5A shows the dose-dependence and the time course of the action on physostigmine (0.1 to 100 μM) on the peak amplitude of the first response. With simultaneous application there was a dose-dependent reduction of peak amplitude of the first response (b). However, 10 min after the start of treatment with 0.1 to 10 μM physostigmine, the response amplitude increased in a dose-dependent fashion. The highest concentration (100 μM) caused a time-dependent decrease in amplitude of the peak of the first response (c and d). Removal of physostigmine from the external solution did not result in a significant immediate change in the amplitude of the response irrespective of the concentration (e). After repeated washing the amplitude of the response decreased at the two lower concentrations (0.1 and 1 μM) while it increased at the two higher concentrations (f and g).

Figure 5B shows the same experiment but with edrophonium (0.1 to 100 μM). Simultaneous application with acetylcholine gave a dose-dependent increase in peak amplitude at concentrations of 0.1 to 10 μM, but the increase in amplitude with 100 μM edrophonium was less than that with 100 μM edrophonium (b). For the three lower concentrations (0.1 to 10 μM) the amplitude of the response did not decrease as compared to that with the initial simultaneous application. However, at the highest concentration (100 μM) the peak amplitude gradually decreased (c and d). With washing, the amplitude of the response at the three lower concentrations was reduced but it increased at the highest concentration (e). These results suggest that at the lower concentrations the primary action of edrophonium is to block acetylcholinesterase while at 100 μM there are also actions on the receptor-channel complex. Repeated

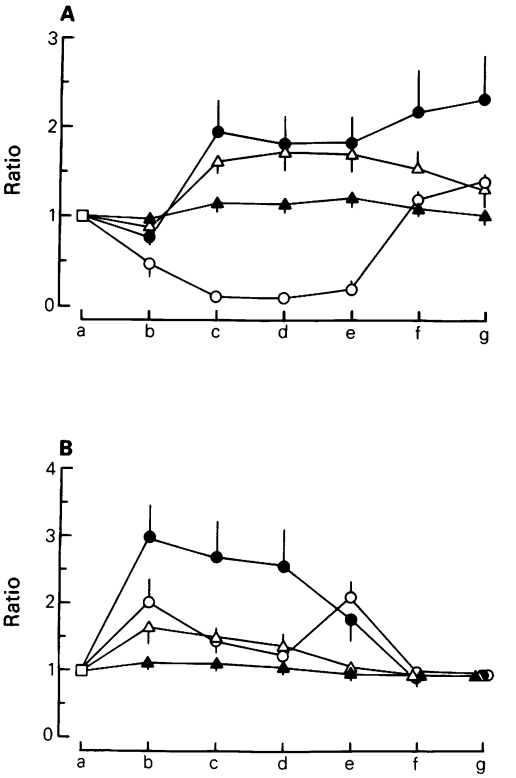

Figure 5 The dose-dependency and time course of the action of acetylcholinesterase inhibitors on the peak amplitude of the first response. (A) The effect of physostigmine (0.1 (▲), 1.0 (△), 10 (●) and 100 (○) μM). The ratio was calculated as R_p of Figure 1A. Each point indicates the mean value and vertical lines s.e. of five experiments. (a) to (g) are those manipulations indicated in Figure 1B. (B) The effect of edrophonium (0.1 (▲), 1.0 (△), 10 (●) and 100 (○) μM). Each point indicates the mean value of ratio calculated with R_p of Figure 1A, and vertical lines show s.e., of five experiments. (a) to (g) are those manipulations indicated in Figure 1B.

washing with NES produced complete recovery with all concentrations (f and g).

Dose-dependence and time course of the acetylcholinesterase activity

Figure 6A shows the dose-dependence and time course of action of physostigmine (0.1 to 100 μM) on the ratio (R_e of Figure 1A) of increase in the response on a second perfusion, which is an indication of remaining acetylcholinesterase activity. Simultaneous application of acetylcholine and physostigmine reduced the ratio of increase in the response

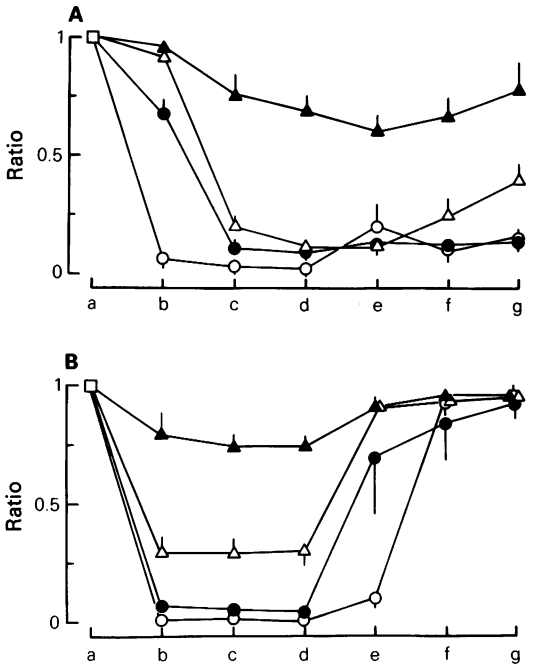

Figure 6 Dose-dependency and time course of the action of acetylcholinesterase inhibitors on the ratio of increase in the second response. Each point indicates the mean value of the ratio calculated as R_e (Figure 1A), and vertical lines show s.e., of five experiments. (a) to (g) are similar to those in Figure 1B. (A) The effect of physostigmine (0.1 (▲), 1.0 (△), 10 (●) and 100 (○) μM). (B) The effect of edrophonium (0.1 (▲), 1.0 (△), 10 (●) and 100 (○) μM).

in a dose-dependent fashion (b). There was a further decrease in the response in the continued presence of physostigmine in a time- and dose-dependent manner (c and d). The time course of action appeared more rapid at higher concentrations. Washing did not immediately affect the second response at all concentrations (e). At the two lower concentrations the effects were reversible with time but at the higher concentration this was not the case (f and g).

Figure 6B shows the same study with edrophonium (0.1 to 100 μM). The action of edrophonium on acetylcholinesterase appeared to reach a steady state immediately after application at all concentrations (b, c and d) and its effect was very dependent on the dose. Upon washout of edrophonium there was a quick recovery at the three lower concentrations but not at the highest concentration (e). Repeated washing of the neurone produced a complete recovery to the control level within 20 min (f and g).

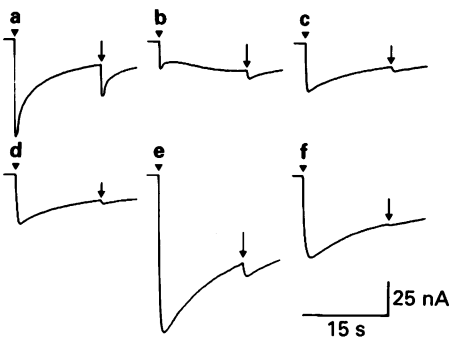

Figure 7 The effect of diisopropylfluorophosphate (DFP) on the response elicited by two rapid and time-fixed (800 ms) perfusions of 50 μM acetylcholine. The concentration of the drug was 1 mM. (a) Control. The triangle and arrow indicate the perfusion. (b) Simultaneous application of acetylcholine with DFP. (c) and (d) 20 and 40 min after the start of DFP application. (e) 20 min after the removal of DFP from the external solution and repeated washing of the neurone. (f) After washing DFP for 40 min, 1 mM edrophonium was applied and reduced both peak amplitude and response to second perfusion.

Effect of DFP, an irreversible anticholinesterase

Figure 7 shows the action of 1 mM DFP on the response elicited by two perfusions of 50 μM acetylcholine. The peak amplitude of the first response was greatly reduced by the simultaneous application of DFP and acetylcholine, and the second response was decreased (b). The mean percentage inhibition of the peak amplitude of the first response was 57.3 ± 4.4% (mean ± s.e. of four experiments). Further exposure to DFP (20 min and 40 min, c and d respectively) did not further decrease the amplitude of the first response; however, the amplitude was still lower than that of the control. During exposure to DFP, the amplitude of the second response was slightly reduced (c and d). The peak amplitude of the first response increased to more than the control level 20 min after removal of DFP from the external solution and repeated washing of the neurone (e). Further increases in the dose of DFP (10 mM) greatly decreased both responses, but did not completely inhibit the response to the second application in two experiments. At this concentration the response did not recover to the control level after washing the neurone for 60 min. Methylsulphonylfluoride, another irreversible cholinesterase inhibitor, had actions similar to those of DFP.

The effect of a high concentration of edrophonium (1 mM) was tested after washing out the DFP for 40 min (f). The peak was suppressed and the small response to the second perfusion was blocked. This

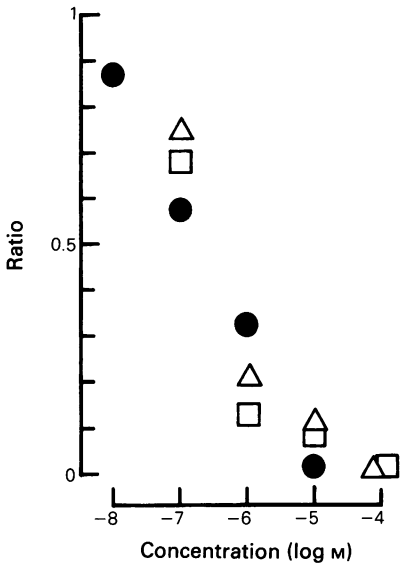

Figure 8 Comparison of the biochemical estimation of inhibitory action of physostigmine on acetylcholinesterase activity with the electrophysiological method described here. (●) Replotted from a previous paper by Carpenter *et al.* (1976). Data from the present electrophysiological studies of cholinesterase activity (△ and □) replotted from (c) and (d) of Figure 6A, respectively.

observation indicates that high concentrations of edrophonium act on the receptor-channel complex.

Discussion

It is reasonable to question whether electrophysiological methods can adequately estimate acetylcholinesterase activity of single neurones. Although we did not conduct biochemical studies on single isolated neurones in parallel with this study, a comparison was made with a previous study of *Aplysia* acetylcholinesterase activity. Figure 8 shows the dose-response plots for the inhibitory action of physostigmine on acetylcholinesterase activity. Biochemical measurement of acetylcholinesterase activity was conducted in ganglionic homogenates of *Aplysia dactylomela* 15 min after the application of physostigmine (filled circles of Figure 8 replotted from Carpenter *et al.*, 1976). In the present study, the activity was estimated 10 and 20 min after the application of physostigmine to single isolated neurones of *Aplysia californica*. In spite of the differences in the method and preparation, the dose-response relation for acetylcholinesterase blockade is similar. Thus it can be concluded that our electrophysiological estimation is adequate. It has the major advantages of being applicable to intact single neurones, which enables the interactions between receptor activation and acetylcholinesterase activity to be studied and quantified. The electrophysiological technique also allows the time course of actions on the same single neurone to be studied.

Effect of physostigmine

The inhibitory action of physostigmine on acetylcholinesterase activity was time- and dose-dependent. While the recovery from the drug action was slight at the two higher concentrations, responses at the two lower concentrations tended to recover within 20 min toward the control level, but never completely. With regard to the action on the peak amplitude of the first response, in spite of complete inhibition of acetylcholinesterase activity with 100 μM physostigmine, the response was greatly suppressed. The simultaneous application of acetylcholine and physostigmine induced a dose-dependent decrease in the peak amplitude of the first response. These results suggest that physostigmine has a rapid and strong inhibitory action on the acetylcholine receptor-channel complex. Some inhibitory effects were previously suggested for this and other carbamate acetylcholinesterase inhibitors (Carpenter *et al.*, 1976; Pascuzzo *et al.*, 1984; Fiekers, 1985; 1986; Slater *et al.*, 1986). Therefore, the amplitude of the response in the presence of physostigmine was modified by the inhibitory action both on acetylcholinesterase activity, resulting in an increase in the response, and on the receptor-channel complex, resulting in a decrease in the response. The onset and recovery from the inhibitory action on the receptor-channel complex was quicker than that on acetylcholinesterase activity. At these concentrations, in spite of repeated washing of the neurone, the inhibition of acetylcholinesterase activity still remained (Figure 5A).

Effect of edrophonium

The increase in amplitude of the first response was well correlated with the inhibitory action of edrophonium (0.1 to 10 μM) on acetylcholinesterase activity (Figures 5B and 6B). This inhibition of acetylcholinesterase reached a steady state immediately after the application and the level of inhibition was dose-dependent (Figure 6B). Thus, edrophonium at less than 10 μM does not appear to affect the acetylcholine receptor-channel complex. At 100 μM edrophonium augmented the peak amplitude, but less than at 10 μM, suggesting that this is the threshold concentration for interaction with the receptor-channel complex. The indicators of edrophonium action on acetylcholinesterase (increase of

the first peak and decrease of the second) were rapidly reversible at the lower concentrations. The effect of edrophonium on the acetylcholine receptor-channel complex was less robust and much less rapid as compared with its inhibitory action on acetylcholinesterase activity.

Differences in the action between the cholinesterase inhibitors

The dose-dependence of the actions of cholinesterase inhibitors was systematically examined only for the two reversible agents, physostigmine and edrophonium. The differences in the action of these inhibitors can be summarized as follows: (1) physostigmine has a prominent inhibitory action on the acetylcholine receptor-channel complex even at low concentrations, while that of edrophonium is very weak and is seen only at concentrations higher than those required for almost complete inhibition of acetylcholinesterase activity. (2) The time course of the inhibitory action of physostigmine on acetylcholinesterase activity is much slower than that of edrophonium and the recovery from the effect of physostigmine is also very slow and incomplete during the 20 min wash, while that of edrophonium is complete within 20 min of wash. (3) The action of DFP is most similar to that of physostigmine both in terms of relative actions at the acetylcholine-channel complex and in terms of time course. However, the actions on the acetylcholine receptor-channel complex were partly reversible, while, as expected, the blockade of acetylcholinesterase was much less so.

Previous studies comparing the inhibitory action of cholinesterase inhibitors on acetylcholinesterase activity have been performed primarily using biochemical methods. Such studies cannot relate the degree of inhibition to the functional acetylcholine response recorded electrophysiologically. Our results show that the electrophysiological estimation of acetylcholinesterase activity can provide useful information about the action of the drugs both on this enzyme and on the acetylcholine receptor-channel complex. They also demonstrate that of the three drugs we studied all affect the acetylcholine receptor-channel complex, in addition to their actions at acetylcholinesterase.

Acetylcholinesterase inhibitors are drugs that have clinically important actions, and are useful in treatment of such diseases as myasthenia gravis and Alzheimer's Disease. A variety of inhibitors of cholinesterase have been used in these treatments, including physostigmine (Christie *et al.*, 1981) and tetrahydroaminoacridine (Summers *et al.*, 1986) for treatment of Alzheimer's patients. Since in these diseases the therapeutic aim is to increase the action of acetylcholine it might be counterproductive to use an acetylcholinesterase inhibitor which also depresses acetylcholine action at the level of the nicotinic cholinoceptor, although the importance of nicotinic receptor activation in the central effects of acetylcholinesterase inhibitors is unclear. Effects of these drugs on muscarinic receptors remain as yet unknown. The methods we have developed may have a utility in the evaluation of various anticholinesterase drugs.

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Contractile effects of substance P and other tachykinins on the mouse isolated distal colon

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1 Substance P (SP), physalaemin, eledoisin and kassinin induced concentration-related contractions of the longitudinal muscle of the mouse distal colon. The responses were not antagonized by atropine (1.5×10^{-7} M), mepyramine (2.5×10^{-7} M), methysergide (5×10^{-7} M), timolol (10^{-6} M), phentolamine (10^{-6} M) or naloxone (4×10^{-7} M). They were enhanced by tetrodotoxin (TTX, 1.5×10^{-7} M). These observations indicate that the contractile responses to the tachykinins result from a direct activation of smooth muscle cells.

2 The contractile activity provoked by SP and physalaemin was inhibited by nifedipine (a Ca^{2+}-entry blocker) and was abolished in Ca^{2+}-free EGTA solution. Such data suggest that the myogenic effects of SP and physalaemin are mainly dependent on their ability to promote Ca^{2+} influx.

3 Eledoisin and kassinin evoked a contractile response in the absence of external Ca^{2+} and their myogenic activity was, to some extent, resistant to the inhibitory effect of nifedipine. This may indicate that an additional process, probably the release of an intracellularly bound Ca^{2+} store, participates in the mechanism by which eledoisin and kassinin contract the mouse distal colon.

4 After desensitization of the mouse distal colon to SP, the contractile activity provoked by SP or physalaemin was totally abolished whilst the responses evoked by eledoisin and kassinin were barely affected. These observations and other experimental findings indirectly support the assumption that the mouse distal colon could possess different tachykinin-binding sites.

Introduction

Substance P (SP) is an undecapeptide belonging to the family of tachykinins. This peptide has been identified in all regions of the gastrointestinal tract of various mammals (Pernow, 1951; Pearse & Polak, 1975; Brodin & Nilsson, 1981; Bartho & Holzer, 1985) and has been shown to exhibit a powerful contractile activity in most intestinal preparations (Pernow, 1983; Bartho & Holzer, 1985). It has been suggested that the contractile responses to SP result either from a direct activation of smooth muscle cells or from a stimulation of intramural neurones (Pernow, 1983; Bartho & Holzer, 1985).

In the large intestine, SP contracts the longitudinal muscle of the guinea-pig transverse colon (Holzer *et al.*, 1981), the muscularis mucosae and the longitudinal muscle of the rat colon (Couture & Regoli, 1982; Bailey & Jordan, 1984), the longitudinal and circular muscle of the rabbit colon (Holzer, 1982) and the guinea-pig taenia caeci (Benham & Bolton, 1983).

As previous investigations led us to propose that the isolated distal colon of the mouse represents a sensitive tool to study the mechanisms by which various agonists and antagonists modulate intestinal tone (Fontaine *et al.*, 1984; Fontaine & Lebrun, 1985; 1988), we decided to analyse the effects of SP on this preparation. Moreover, and in order to gain further insight into the processes involved in the contractile responses to the peptide, the mechanical effects of SP were compared with those evoked by three other tachykinins: physalaemin, eledoisin and kassinin.

Methods

Swiss Webster mice (30–40 g) were stunned and killed by exsanguination. The terminal colon was dissected out and suspended under an initial load of 1 g in an organ bath containing 10 ml of a Krebs solution of the following composition (mM): NaCl 118.1, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 5. Some media contained no $CaCl_2$ (Ca^{2+}-free solution) or were deprived of $CaCl_2$ and enriched with 0.5 mM ethylene glycol-*bis*-(β-aminoethyl-ether)N,N-tetraacetic acid (EGTA) (Ca^{2+}-free EGTA solution). Tetrodotoxin (TTX) (1.5×10^{-7} M) was added to some bathing media in

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order to block the neuronal activity of the muscle preparation (Gershon, 1967). The physiological solutions were maintained at 36 ± 1°C and bubbled continuously with a mixture of O_2 (95%) and CO_2 (5%).

The isometric contractions of the longitudinal muscle were measured with a Grass force-displacement transducer. Preparations were allowed to equilibrate for 60 min before adding drugs. Acetylcholine (ACh, 10^{-7} and 10^{-6} M) was added at the beginning of each experiment to test the reactivity of the preparation. The peptides were left in contact with the tissue for periods of 2–3 min at 15 min intervals. Antagonists were added 10 min before agonists. Some muscle preparations were desensitized to substance P (SP) by addition of 2×10^{-7} M of the peptide.

Concentration-effect curves were constructed for each peptide in the absence and presence of tetrodotoxin (TTX, 1.5×10^{-7} M). The mean maximal contractor response (in g, ±s.e.mean) and the mean EC_{50} (in M, ±s.e.mean) were determined graphically from these experiments. The statistical significance of differences between mean experimental and control data was assessed by use of Student's *t* test.

Drugs

The bathing medium contained, as required: acetylcholine hydrochloride (ACh) (Astra), atropine sulphate (Fluka), eledoisin (Farmitalia Carlo Erba), kassinin (Peninsula), mepyramine maleate (Rhône-Poulenc), methysergide hydrogen maleate (Sandoz), naloxone hydrochloride (Endo), nifedipine (Bayer), phentolamine methane sulphonate (Ciba), physalaemin (Farmitalia Carlo Erba), timolol maleate (Merck Sharp & Dohme), substance P (SP) (Peninsula), tetrodotoxin (TTX) (Calbiochem).

Concentrated solutions (1 mg ml^{-1}) of each peptide were made in distilled water and stored at −20°C in small aliquots which were thawed, diluted in water and used the same day.

Nifedipine was first dissolved in dimethylsulphoxide (DMSO) and further diluted in the experimental medium so that the final concentration of the organic solvent never exceeded 0.1% (v/v) (Fontaine & Lebrun, 1988). Experiments were conducted with minimal light to prevent photodegradation of the dihydropyridine.

Results

Contractile responses to substance P and physalaemin

At concentrations ranging from 10^{-8} to 10^{-5} M, SP and physalaemin induced concentration-related contractions of the mouse distal colon (Figure 1a, b).

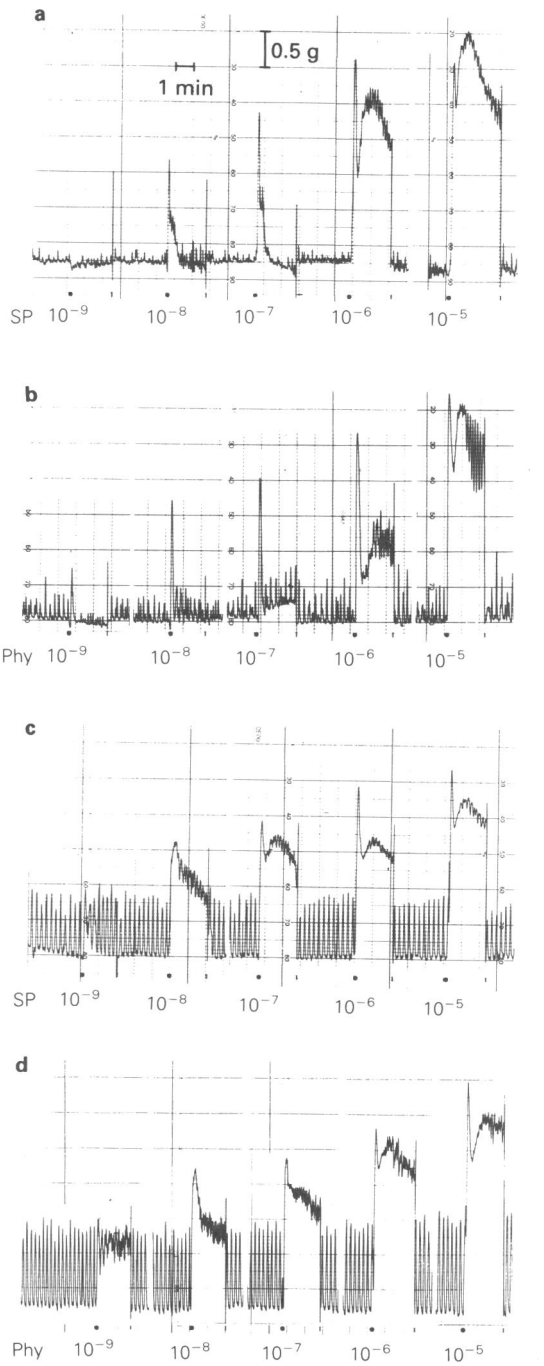

Figure 1 Effects of increasing concentrations of substance P (SP 10^{-9} to 10^{-5} M) (a, c) and physalaemin (Phy, 10^{-9} to 10^{-5} M) (b, d) on the mouse distal colon incubated in the absence (a, b) or presence (c, d) of tetrodotoxin (1.5×10^{-7} M).

Table 1 Maximal tensions and EC_{50} (phasic and tonic components) developed in response to substance P (SP), physalaemin (Phy), eledoisin (El) and kassinin (Ka): concentration-effect curves were constructed in the absence and presence of tetrodotoxin (TTX, 1.5×10^{-7} M)

Line	*Agonist*	*Contractile response*	*TTX* (M)	*Maximal tension* (g)	P	EC_{50} (M)	P
1	SP	Phasic	—	3.1 ± 0.3 (6)		$2.1 \times 10^{-8} \pm 0.5$	
2	SP	Phasic	1.5×10^{-7}	3.2 ± 0.3 (5)	1 vs 2 NS	$1.1 \times 10^{-8} \pm 0.2$	1 vs 2 NS
3	SP	Tonic	—	2.5 ± 0.3 (6)		$3.5 \times 10^{-7} \pm 0.9$	
4	SP	Tonic	1.5×10^{-7}	2.5 ± 0.4 (5)	3 vs 4 NS	$1.0 \times 10^{-8} \pm 0.2$	3 vs 4 $P < 0.01$
5	Phy	Phasic	—	3.4 ± 0.3 (5)		$1.5 \times 10^{-8} \pm 0.3$	
6	Phy	Phasic	1.5×10^{-7}	3.5 ± 0.2 (5)	5 vs 6 NS	$2.0 \times 10^{-8} \pm 0.4$	5 vs 6 NS
7	Phy	Tonic	—	3.0 ± 0.4 (5)		$1.1 \times 10^{-6} \pm 0.4$	
8	Phy	Tonic	1.5×10^{-7}	3.4 ± 0.4 (5)	7 vs 8 NS	$7.0 \times 10^{-8} \pm 0.8$	7 vs 8 $P < 0.05$
9	El	Phasic	—	3.5 ± 0.3 (5)		$3.2 \times 10^{-9} \pm 1.0$	
10	El	Phasic	1.5×10^{-7}	3.6 ± 0.3 (5)	9 vs 10 NS	$4.6 \times 10^{-9} \pm 0.8$	9 vs 10 NS
11	El	Tonic	—	4.0 ± 0.4 (5)		$3.0 \times 10^{-8} \pm 0.4$	
12	El	Tonic	1.5×10^{-7}	4.1 ± 0.2 (5)	11 vs 12 NS	$1.1 \times 10^{-8} \pm 0.3$	11 vs 12 $P < 0.01$
13	Ka	Phasic	—	3.3 ± 0.2 (6)		$1.8 \times 10^{-8} \pm 0.3$	
14	Ka	Phasic	1.5×10^{-7}	3.5 ± 0.3 (6)	13 vs 14 NS	$1.0 \times 10^{-8} \pm 0.2$	13 vs 14 NS
15	Ka	Tonic	—	3.6 ± 0.2 (6)		$2.4 \times 10^{-8} \pm 0.5$	
16	Ka	Tonic	1.5×10^{-7}	3.3 ± 0.2 (6)	15 vs 16 NS	$5.3 \times 10^{-9} \pm 0.1$	15 vs 16 $P < 0.005$

Values are expressed as means ± s.e.mean with the number of individual experiments in parentheses. NS = not significant.

Low concentrations (10^{-8}, 10^{-7} M) mainly provoked phasic contractions whilst high concentrations (10^{-6}, 10^{-5} M) elicited clearly biphasic responses consisting of a fast initial peak (phasic response) rapidly followed by a sustained contraction (tonic response). Maximal tensions and EC_{50} (phasic and tonic components) developed by SP and physalaemin are shown in Table 1.

The contractile responses to SP and physalaemin were reproducible at 15 min intervals and were not modified by atropine ($1{\cdot}5 \times 10^{-7}$ M) methysergide (5×10^{-7} M), mepyramine (2.5×10^{-7} M), phentolamine (10^{-6} M), timolol (10^{-6} M) or naloxone (4×10^{-7} M) ($n = 4$ for each antagonist).

In the presence of tetrodotoxin (TTX, 1.5×10^{-7} M), the spontaneous tone of the longitudinal muscle was increased (Figure c, d) (Fontaine *et al.*, 1984; Fontaine & Lebrun, 1988). When concentration-effect curves to SP and physalaemin were performed in the presence of the toxin, the maximal phasic and tonic responses were not significantly affected but the tonic responses appeared at lower concentrations (Figure 1c, d; Table 1).

Contractile responses to eledoisin and kassinin

Eledoisin and kassinin (10^{-9} and 10^{-8} M to 10^{-6} M) provoked dose-related contractions of the terminal colon (Figure 2a, b). Both tachykinins evoked biphasic contractile activity whose tonic component rapidly exceeded the phasic component. Maximal tensions and EC_{50} (phasic and tonic components) developed by eledoisin and kassinin are shown in Table 1.

The contractile responses to eledoisin and kassinin were reproducible at 15 min intervals and were not modified by atropine (1.5×10^{-7} M), methysergide (5×10^{-7} M), mepyramine (2.5×10^{-7} M), phentolamine (10^{-6} M), timolol (10^{-6} M) or naloxone (4×10^{-7} M) ($n = 4$ for each antagonist). In the presence of TTX (1.5×10^{-7} M) in the medium, the maximal tonic and phasic responses were not modified but the EC_{50} for the tonic responses to eledoisin and kassinin were lower (Figure 2c, d; Table 1).

Effects of nifedipine and Ca^{2+}-free solutions on tachykinin-induced contractions

The effects of increasing concentrations of nifedipine, an organic Ca^{2+}-channel blocker, on SP (10^{-7} M), physalaemin (10^{-7} M), eledoisin (10^{-7} M) and kassinin (10^{-7} M)-induced contractile activity, are illustrated in Figure 3. All the experiments were performed in the presence of TTX (1.5×10^{-7} M). The effects of nifedipine have been quantified on both the phasic and tonic component of the mechanical responses to the tachykinins.

The Ca^{2+}-channel blocker caused a dose-related inhibition of SP and physalaemin-induced contractions (Figure 3a, b). The results further indicate that the tonic component of the contractile responses elicited by SP and physalaemin displays a greater sensitivity towards nifedipine than the phasic component (Figure 3a, b).

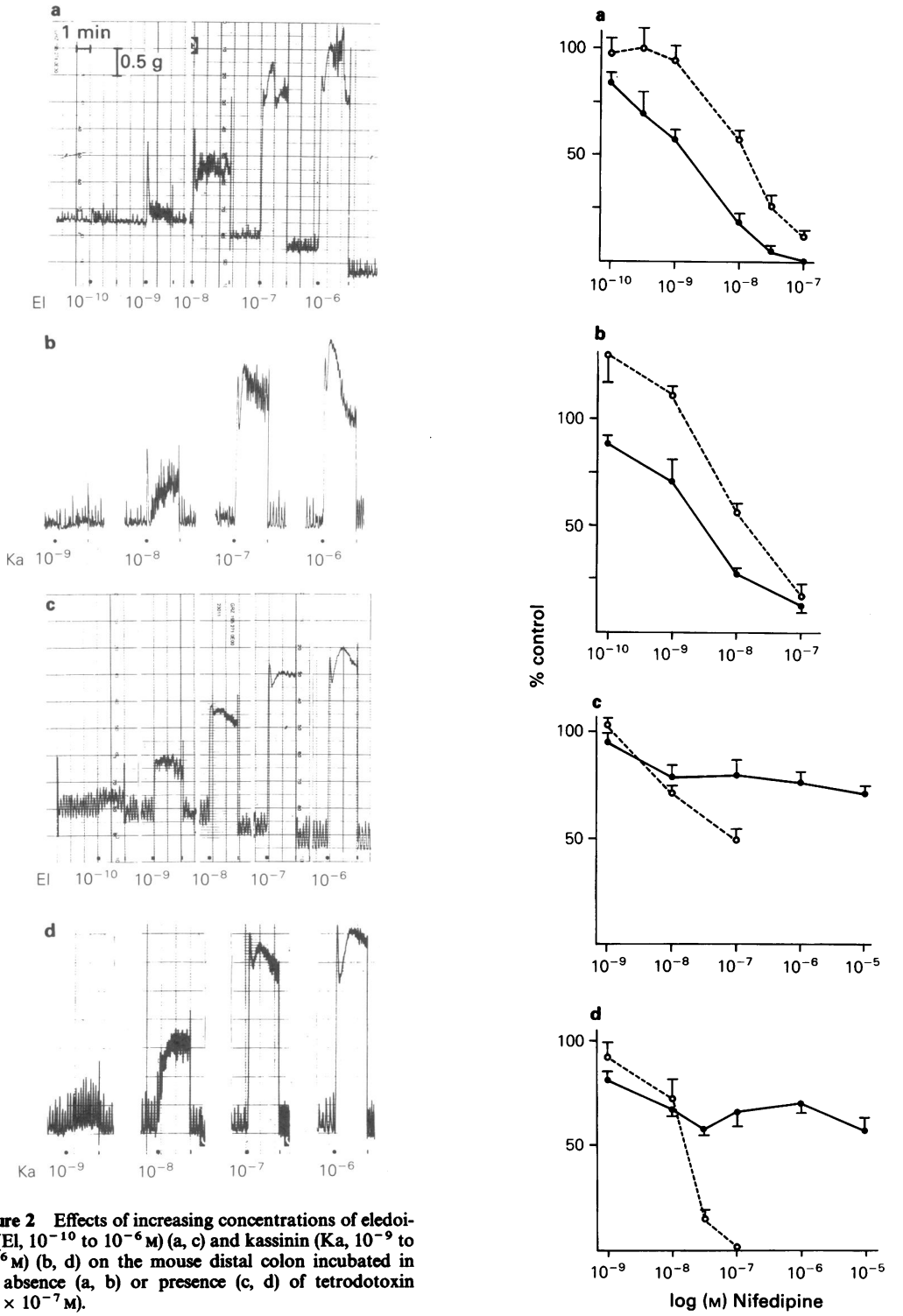

Figure 2 Effects of increasing concentrations of eledoisin (El, 10^{-10} to 10^{-6} M) (a, c) and kassinin (Ka, 10^{-9} to 10^{-6} M) (b, d) on the mouse distal colon incubated in the absence (a, b) or presence (c, d) of tetrodotoxin (1.5×10^{-7} M).

Table 2 Contractile responses to acetylcholine (ACh, 10^{-7} M), substance P (SP, 10^{-8} M), physalaemin (Phy, 10^{-8} M), eledoisin (El, 10^{-8} M) and kassinin (Ka, 10^{-8} M) after desensitization to substance P (SP, 2×10^{-7} M)

Line	*Agonist*	*Contractile response*	*Control tension* (g)	*Tension* (g) *after desensitization to SP*
1	ACh	Phasic	2.6 ± 0.3 (6)	2.5 ± 0.3
2	ACh	Tonic	1.2 ± 0.2 (6)	1.3 ± 0.2
3	SP	Phasic	2.0 ± 0.3 (4)	0.0 ± 0.0
4	Phy	Phasic	2.2 ± 0.3 (4)	0.0 ± 0.0
5	El	Phasic	1.9 ± 0.2 (5)	1.7 ± 0.2
6	El	Tonic	1.2 ± 0.1 (5)	1.1 ± 0.1
7	Ka	Phasic	1.8 ± 0.3 (5)	0.8 ± 0.4
8	Ka	Tonic	1.0 ± 0.1 (5)	0.9 ± 0.1

Values are expressed as means ± s.e.mean with the number of individual experiments in parentheses.

Nifedipine also provoked a dose-related inhibition of the phasic contractile activity evoked by eledoisin and kassinin (Figure 3c, d). The phasic response to kassinin was completely abolished in the presence of 10^{-7} M nifedipine whilst the phasic response to eledoisin still averaged 49.0 ± 5.0% ($n = 5$) of the control value. At higher nifedipine concentrations, the phasic components of the kassinin responses could not be averaged because they were only quantifiable in 3 out of 5 experiments.

On the other hand, the tonic responses to eledoisin and kassinin displayed some resistance to the inhibitory action of the Ca^{2+}-channel blocker (Figure 3c, d). Indeed, in the presence of 10^{-5} M nifedipine in the bathing medium, the tonic component of the eledoisin response and the tonic component of the kassinin response averaged respectively 71.4 ± 2.7% ($n = 5$) and 57.0 ± 5.7% ($n = 4$) of the tonic contractile activity evoked by the agonists in the absence of nifedipine.

Further experiments were conducted on preparations preincubated 20 min with a Ca^{2+}-free or a Ca^{2+}-free EGTA solution containing TTX (1.5×10^{-7} M).

In both conditions, SP (10^{-7} M) and physalaemin (10^{-7} M) failed to contract the mouse distal colon ($n = 4$ in each case).

Figure 3 Concentration-response curves for the inhibitory effect of nifedipine on contractile activity induced by (a) substance P (SP, 10^{-7} M), (b) physalaemin (10^{-7} M), (c) eledoisin (10^{-7} M) and (d) kassinin (10^{-7} M): (○) phasic and (●) tonic component of the responses evoked by the different tachykinins. Results are expressed as percentage (mean ± s.e.mean) of the contractile responses induced by each agonist in the absence of nifedipine ($n = 4$–5). Tetrodotoxin (1.5×10^{-7} M) was present in the physiological solution.

Addition of eledoisin (10^{-7} M) or kassinin (10^{-7} M) to a medium deprived of extracellular calcium induced slight but sustained contractions which averaged respectively 27.6 ± 2.0% ($n = 4$) and 23.3 ± 5.7% ($n = 4$) of those evoked by the agonists in the presence of extracellular calcium. When the medium consisted of Ca^{2+}-free EGTA solution, the sustained contractile effects of eledoisin and kassinin averaged respectively 15.3 ± 1.8% ($n = 4$) and 16.5 ± 5.5% ($n = 4$) of the control responses.

Desensitization of the mouse distal colon to substance P

In order to investigate the involvement of different binding sites in the mechanical activity evoked by tachykinins, experiments were performed after desensitization of the mouse distal colon to SP.

Addition of SP (2×10^{-7} M) markedly enhanced the muscle tone which gradually returned to its initial basal level. A further addition of SP (10^{-8} M) or the addition of physalaemin (10^{-8} M) failed to induce any modification in muscle tone (Figure 4a, b; Table 2).

Preincubation for 5–6 min with SP (2×10^{-7} M) barely affected (no more than a 12% reduction) the biphasic contractile activity evoked by eledoisin (10^{-8} M) and the tonic component of the contractile response to kassinin (10^{-8} M) (Figure 4c, d; Table 2). The phasic component of the kassinin response was abolished in 2 out of 6 experiments (Figure 4d; Table 2).

Finally, the biphasic contractile activity elicited by Ach (10^{-7} M) was not modified after desensitization to SP (2×10^{-7} M) (Table 2).

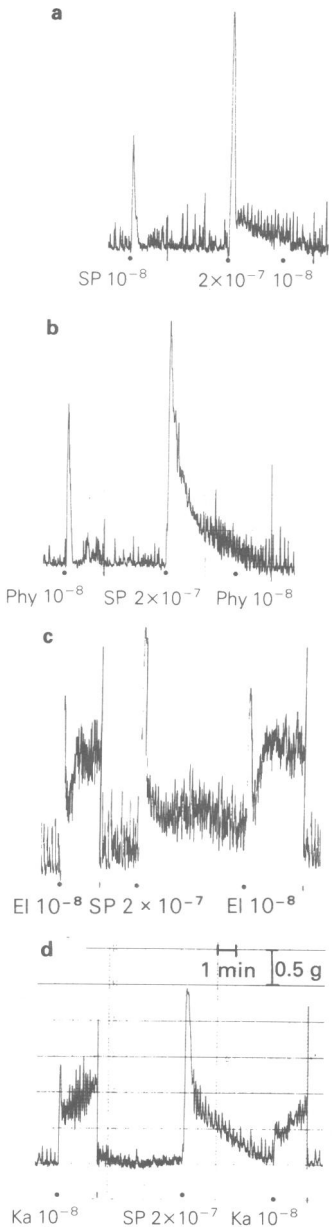

Figure 4 Effects of substance P (SP, 10^{-8} M) (a), physalaemin (Phy, 10^{-8} M) (b), eledoisin (El, 10^{-8} M) (c) and kassinin (Ka, 10^{-8} M) (d) on the mouse distal colon after desensitization to substance P (2×10^{-7} M).

Discussion

The present results clearly indicate that SP, physalaemin, eledoisin and kassinin exert potent myogenic effects on the isolated distal colon of the mouse. The contractile responses to the peptides were not reduced in the presence of tetrodotoxin, atropine, mepyramine, methysergide, timolol, phentolamine or naloxone. Such findings rule out a prejunctional effect of the tachykinins and a role of acetylcholine, histamine, 5-hydroxytryptamine, catecholamines or opioid peptides in the generation of the contractions.

The present data also provide information about the ionic mechanisms underlying the contractile responses to the different tachykinins.

It has been shown that nifedipine, an organic Ca^{2+}-channel blocker, caused a dose-related inhibition of SP and physalaemin-induced contractions. On the other hand, the contractile effects of the two peptides were shown to be markedly reduced in Ca^{2+}-free solution and totally abolished in Ca^{2+}-free EGTA solution. These observations indicate that the contractile effects of SP and physalaemin are highly dependent on extracellular calcium and suggest that the two tachykinins could contract the mouse distal colon mainly by promoting calcium influx.

The results also showed that the phasic component of the contractile responses induced by SP and physalaemin displayed a lower sensitivity towards nifedipine than the tonic component. As the receptor-operated channels have a smaller sensitivity towards Ca^{2+}-channel blockers than the voltage-operated channels, these findings can be interpreted as evidence for the preferential involvement of receptor-operated channels in the phasic component and voltage-operated channels in the tonic component of the contractile activity evoked by SP and physalaemin (Bolton, 1979; Meisheri *et al.*, 1981; Godfraind, 1983). Alternatively, these data are also compatible with the existence of two types of voltage-sensitive channels and with the knowledge that phasic tension can be dependent in part on the release of a bound pool of calcium (Bolton, 1979; Loutzenhiser *et al.*, 1985; Fontaine & Lebrun, 1988; Karaki & Weiss, 1988).

An additional process appears to participate in the mechanism by which eledoisin and kassinin contract the mouse distal colon. Indeed, both peptides evoked a sustained contractile response in the absence of extracellular calcium and the tonic component of their myogenic activity was resistant to the inhibitory effect of high nifedipine concentrations. These results suggest that the residual tachykinin-induced contractions observed in the presence of nifedipine or in the absence of extracellular calcium can be ascribed to the capacity of the agonists to promote the release of an intracellularly bound Ca^{2+} store (Bolton, 1979; Loutzenhiser *et al.*, 1985; Karaki & Weiss, 1988). It is tempting to propose that this intracellular Ca^{2+} translocation could depend on the generation of inositol trisphosphate (IP_3) resulting

from the phosphoinositide hydrolysis provoked by receptor activation (Watson, 1984; Exton, 1985; Somlyo *et al.*, 1985; Khalil *et al.*, 1987; Karaki & Weiss, 1988). However, a sustained contractile activity recorded in the absence of external calcium can also be attributed to the activation of protein kinase C by diacylglycerol released during agonist-induced breakdown of plasma membrane phosphoinositides (Exton, 1985; Khalil *et al.*, 1987; Karaki & Weiss, 1988).

Pharmacological analysis of the contractile responses indirectly supports the assumption that different receptor sub-types could be involved in the mechanical activity provoked by tachykinins. First, the contractile responses evoked by eledoisin or kassinin did not exhibit the same shape as the responses induced by SP and physalaemin. Such a feature cannot be ascribed only to a poor metabolic stability of SP or physalaemin, since nanomolar concentrations of the tachykinins provoked long lasting and sustained contractions in the presence of tetrodotoxin in the bathing medium (Watson, 1983). Second, all four tachykinins were acting in nanomolar concentrations (Watson, 1984; Bartho & Holzer, 1985) and a clear rank order of potencies was not detected (Lee *et al.*, 1982; Pernow, 1983; Bartho & Holzer, 1985). Third, slightly different mechanisms appear to mediate the contractile responses to SP/physalaemin and to eledoisin/kassinin. Last, after desensitization of the mouse distal colon to SP, the biphasic contractile activity provoked by eledoisin or kassinin was barely affected whilst the responses to SP and physalaemin were totally abolished (Lee *et al.*, 1982). Thus, it is tempting to speculate that, like other peripheral tissues, the mouse distal colon could represent a multiple receptor system. According to the present nomenclature, the putative tachykinin binding sites could be labelled NK-1 and NK-2 (Lee *et al.*, 1986; Regoli *et al.*, 1988). Further studies, including radioligand binding assays and the use of selective agonists or antagonists, are nevertheless required to confirm this theory and to identify definitively the receptor sub-types present (Lee *et al.*, 1986; Regoli *et al.*, 1988).

Finally, the observation that tetrodotoxin enhanced the tachykinin-induced contractile activity deserves a short comment. TTX increased the spontaneous activity of the longitudinal muscle and decreased the EC_{50} for the tonic responses to the peptides. A similar action of TTX on the motor effects of SP and kassinin has recently been described in the rat duodenum (Maggi *et al.*, 1986). Such findings are consistent with the view that some neurogenic mechanism decreases the mouse distal colon motility (Fontaine *et al.*, 1984; Maggi *et al.*, 1986; Fontaine & Lebrun, 1988). TTX, by reducing this inhibitory neural influence, would facilitate the excitatory actions of tachykinins. Previous investigations let us to propose that the toxin could interfere with inhibitory nerves causing constant hyperpolarization of the colonic smooth muscle cells (Fontaine & Lebrun, 1988).

In summary, our results clearly indicate that tachykinins exert potent myogenic effects on the mouse distal colon. The contractile effects of SP and physalaemin are highly dependent on Ca^{2+} influx whilst the tonic component of the contractile response to eledoisin and kassinin could be mediated in part by a translocation of intracellular Ca^{2+}. The data also indirectly suggest that the mouse distal colon could be equipped with different tachykinin binding sites.

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Analysis of the hyperpolarizing effect of catecholamines on canine cardiac Purkinje fibres

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1 The hyperpolarization induced by catecholamines on barium-depolarized (0.2–0.8 mM BaCl) canine cardiac Purkinje fibres, *in vitro*, was studied by use of conventional microelectrode recordings of transmembrane electrical potentials.

2 Noradrenaline, adrenaline and isoprenaline hyperpolarized Purkinje fibres in a concentration-dependent manner from a threshold concentration around 5 nM. The three catecholamines were shown to be approximately equipotent. Tachyphylaxis was observed when the interval between catecholamine applications was less than 15 min.

3 Atenolol (10 μM) blocked the hyperpolarization reversibly and theophylline (0.5 mM) potentiated it.

4 Tetrodotoxin (5 μM) did not affect the hyperpolarization induced by isoprenaline. Acetylcholine and histamine, up to 10 μM, were not effective in hyperpolarizing Purkinje fibres.

5 Low extracellular potassium concentrations (zero and 1 mM) did not affect the hyperpolarization, but high extracellular potassium concentrations (10–20 mM), markedly reduced the effect of isoprenaline (100 nM).

6 Reduction of the extracellular sodium concentration produced a roughly proportional reduction in the isoprenaline-induced hyperpolarization. The hyperpolarization was reversibly blocked in 34 mM sodium Tris-Tyrode solution.

7 The hyperpolarization was not reduced in Tyrode solution containing 0.6 mM calcium, but was drastically reduced in zero-calcium Tyrode solution. This effect was reversible.

8 Addition of verapamil (5–10 μM) diminished the hyperpolarization, in a concentration-dependent manner. This effect was partially reversed after washing.

9 Ouabain (0.7–1 μM) significantly reduced the isoprenaline-induced hyperpolarization, but 2,4-dinitrophenol (0.2 mM) did not affect it.

10 Caesium chloride (20 mM) abolished the hyperpolarization. The blockade was only partially reversed upon washing.

11 It is suggested that the hyperpolarization induced by a short exposure to catecholamines is mainly due to an increase in potassium permeability (P_K). A mechanism involving calcium-dependent potassium channels might underlie the increase in P_K.

Introduction

Catecholamines have been shown to affect the resting potential of a great variety of excitable cells *in vitro* in a variable manner. For example, noradrenaline was reported to depolarize sartorius muscle but to hyperpolarize soleus muscle (Horowitz *et al.*, 1985). Noradrenaline and isoprenaline depolarize sheep cardiac Purkinje fibres (Glitsch & Rasch, 1986; Terris *et al.*, 1986), but hyperpolarize canine Purkinje fibres (Gadsby, 1983) and amphibian sympathetic ganglia (Koketsu & Nakamura, 1976). In those preparations in which catecholamines induce hyperpolarization, there has been a tendency to link

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this effect with a possible stimulation of the sodium-potassium pump (see also Eisner, 1986). Other investigations suggest a catecholamine-induced increase in K^+ conductance as the main factor responsible for the hyperpolarization in cardiac cells (Gadsby 1984, for review) and sympathetic ganglia (Smith, 1984a).

In a previous study (Riccioppo Neto & Sperelakis, 1985) it was found that isoprenaline hyperpolarized embryonic chick heart cells that have been depolarized by high concentrations of local anaesthetics/antiarrhythmic agents. The hyperpolarization was not affected by ouabain (1 μM), but was completely blocked by verapamil. The present study analyses the hyperpolarization produced by catecholamines in depolarized (barium-treated) canine Purkinje fibres.

Methods

Adult mongrel dogs (10–15 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.), and their hearts were quickly removed through a lateral thoracotomy. Both ventricles were opened, and various pieces of muscle with attached false tendons were dissected and stored in cool oxygenated Tyrode solution. One piece was mounted in a 10 ml tissue chamber with a Sylgard-lined base and perfused with Tyrode solution aerated with 95% O_2 and 5% CO_2 (pH 7.4). The solution was kept at a temperature of 36 $\pm$ 0.5°C and flowed over the preparation at a rate of 6–7 ml min^{-1}.

The Tyrode solution had the following composition (mM): NaCl 137, $CaCl_2$ 1.8, KCl 4, $MgCl_2$ 0.45, $NaHCO_3$ 12, NaH_2PO_4 0.32 and glucose 5.5. Experiments investigating the influence of various extracellular K^+ concentrations were performed by reducing or increasing the KCl concentration in the Tyrode solution. Low Na^+ solutions were made by isomolar substitution of Na^+ by Tris-HCl, the solutions being aerated with O_2. Low Ca^{2+} solutions always contained compensating concentrations of $MgCl_2$ in order to avoid permanent damage to the membrane due to the reduction of divalent charges in the extracellular medium (Siegelbaum & Tsien, 1980). The great majority of experiments were done in Ba^{2+}-depolarized preparations by the addition of a concentration of $BaCl_2$ (0.2–0.8 mM) sufficient to depolarize Purkinje fibres to a maximum diastolic potential of -62 to -66 mV. In these conditions the fibres fired at a regular rhythm and hyperpolarized more markedly than normally polarized preparations when perfused with catecholamines (see Results). The enhancement of the hyperpolarization after catecholamine addition was the only reason for using $BaCl_2$ as a depolarizing agent. Ascorbic acid (0.1 mM) was added to Tyrode solutions containing catecholamines.

Transmembrane potentials were recorded with conventional glass microelectrodes filled with 3 M KCl having resistances of 10–20 MΩ and displayed, via an input capacity preamplifier (Grass P 16) on an oscilloscope (Tektronix 5113). Oscilloscope traces were photographed on 35 mm film with a camera (Nihon-Kohden PC-3A). Pen-recordings were simultaneously made on a servo-driven potentiometer (E.C.B.). Maximum diastolic potentials (MDP) were measured by hand after enlargement of the film (7 ×). Data are expressed as mean values $\pm$ s.e. mean and comparisons between two means were made by Student's paired t test. P values of less than 0.05 were considered to indicate significant differences.

Drugs used were: acetylcholine chloride (Merck); (−)-adrenaline hydrochloride (Sigma); atenolol hydrochloride (Sigma); caesium chloride (Sigma); 2,4-dinitrophenol (Sigma); histamine hydrochloride (Sigma); (−)-isoprenaline hydrochloride (Sigma); (−)-noradrenaline hydrochloride (Sigma); ouabain octahydrate (Sigma); tetrodotoxin (TTX, Sigma); theophylline (Sigma); verapamil (Knoll). Unless otherwise stated, the results describe the effects of drugs applied during a single stable impalement.

Results

Effects of isoprenaline, adrenaline and noradrenaline on maximum diastolic potential of normally polarized and barium-depolarized Purkinje fibres

Superfusion of canine Purkinje fibres with catecholamines induced hyperpolarization in the great majority of the experiments. The hyperpolarization was more marked in depolarized (barium-treated) than in normally polarized preparations (Figures 1 and 2). Normally polarized and depolarized preparations had MDP of 89.0 $\pm$ 0.9 mV (n = 5) and 63.5 $\pm$ 0.2 mV (n = 56), respectively. The rate of spontaneous discharge was also increased more by catecholamines in depolarized than in normally polarized fibres.

The hyperpolarization was concentration-dependent, attained its peak around 3 min after the beginning of the perfusion and subsided slowly, even in the presence of catecholamines. The development of tachyphylaxis was clearly observed in the first experiments, and a period of 20–25 min of perfusion with drug-free Tyrode solution always separated the addition of catecholamines to avoid this problem.

Isoprenaline (Iso), adrenaline and noradrenaline showed similar potency (Figure 1). In four experiments, atenolol (10 μM) blocked reversibly the hyperpolarization induced by adrenaline (500 nM) and

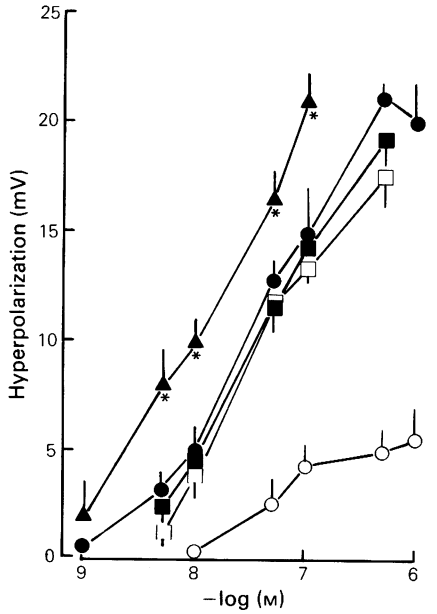

Figure 1 Peak hyperpolarization induced by isoprenaline (Iso) (●), adrenaline (■), noradrenaline (□) on Ba^{2+}-depolarized canine cardiac Purkinje fibres (n between 4 and 8); (▲) effects of Iso on preparations ($n = 3$) previously treated with theophylline (0.5 mM). Open (○): normally polarized preparations ($n = 5$) perfused with Iso. $^{*}P < 0.05$ compared with Ba^{2+}-depolarized, Iso-treated preparations.

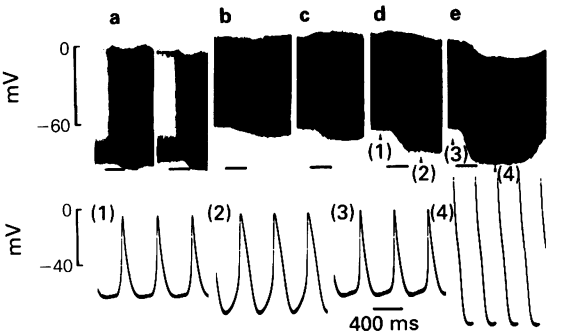

Figure 2 Effects induced by isoprenaline (Iso) perfusion on maximum diastolic potential (a-e) and action potential (1–4) of a spontaneously beating cardiac Purkinje fibre. The preparation was exposed to Iso for periods of only 3 min indicated by the horizontal bars below each trace in the upper panel. In (a), a normally polarized fibre was perfused with two different concentrations of Iso (100 nM and 500 nM) and a time interval of 20 min separated the two records. From (b)–(e) the preparation was depolarized by Ba^{2+} (0.2 mM) and treated with Iso. (b) 5 nM; (c) 10 nM; (d) 100 nM and (e) 500 nM. The pen recording of the upper panel does not reproduce completely the upstroke of the action potential. The lower panel (1–4) shows oscilloscope traces taken in (d) and (e). Time intervals, of 25 min were allowed between Iso additions.

during the blockade, adrenaline did not induce any observable effect that could suggest an eventual action on different α-adrenoceptor sub-types. Isoprenaline, perfused for 3 min, was the catecholamine chosen in the following experiments due to its more specific cardiac β_1-adrenoceptor agonist activity.

In three preparations, prior addition of TTX (5 μM) to the Tyrode solution did not affect the isoprenaline-induced hyperpolarization. In two experiments, superfusion of the preparations with a low-chloride Tyrode solution containing Ba^{2+}, also did not affect the hyperpolarization produced by isoprenaline.

As shown in Figure 1, addition of theophylline (0.5 mM) potentiated the effects of Iso. Theophylline by itself had no effect on the maximum diastolic potentials of the fibres.

In five Ba^{2+}-treated preparations, histamine and acetylcholine were tested up to a concentration of 10 μM without any observable effect.

Influence of the extracellular K^+ concentration

The hyperpolarizing effect of Iso (100 nM) was studied in nine Ba^{2+}-treated preparations in the presence of various extracellular K concentrations ($[K]_o$). Each preparation was tested in control condition ($[K]_o = 4$ mM) and the $[K]_o$ was either increased ($n = 4$) to 15 mM and then to 20 mM, the effect of Iso being tested in these conditions, or decreased ($n = 5$) to 1 mM and then to 0 mM, followed by the addition of the catecholamine in each condition.

As shown in Figure 3, the hyperpolarizing effects of Iso are similar in control and in low K conditions, but are reduced in high $[K]_o$.

Effects of solutions of low Na^+ concentrations on the hyperpolarization induced by isoprenaline

The hyperpolarizing effect of Iso on Ba^{2+}-treated Purkinje fibres was examined in 13 preparations superfused with Tyrode solution containing reduced (Tris-substituted) Na^+ concentrations. After control dose-response curves to Iso had been established, the extracellular sodium concentration ($[Na]_o$) was decreased to 103 mM, 68 mM and 34 mM and new curves obtained in each condition. The MDP changed little after Na^+ substitution (hyperpolarizations of 2–3 mV were found), but the rate of spontaneous discharges decreased gradually and almost disappeared in 34 mM $[Na]_o$. Multiple impalements were made in this set of experiments.

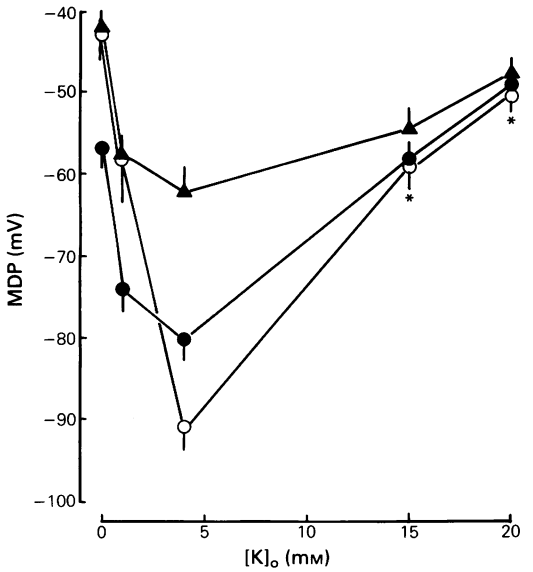

Figure 3 Effects of isoprenaline (Iso) on maximum diastolic potential (MDP) of Purkinje fibres bathed in Tyrode solution containing different extracellular potassium concentrations ($n = 5$ for $[K]_o = 0$ mM and 1 mM; $n = 4$ for $[K]_o = 15$ mM and 20 mM): (○) controls in a given $[K]_o$; (▲) after addition of Ba^{2+} (0.1–0.2 mM) to the Tyrode solutions; (●) addition of Iso (100 nM) to the Ba^{2+}-treated preparations. * P 0.01 compared with preparations treated with Iso in $[K]_o = 4$ mM.

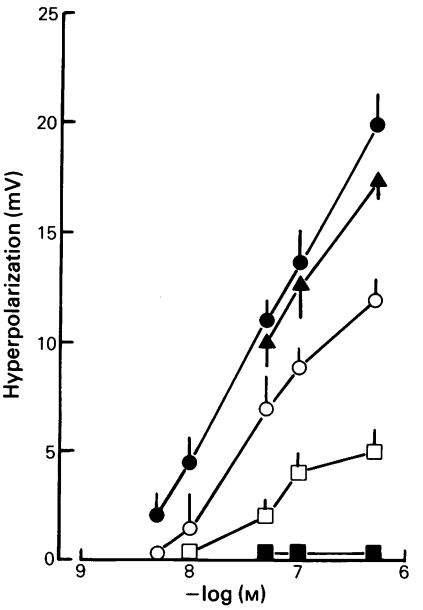

Figure 4 Effects of low Na^+ solutions on the isoprenaline (Iso)-induced hyperpolarizations in Ba^{2+}-treated cardiac Purkinje fibres: (●) control ($n = 13$); (○) 103 mM $[Na]_o$ ($n = 5$); (□) 68 mM $[Na]_o$ ($n = 5$); (■) 34 mM $[Na]_o$ ($n = 3$); (▲) recovery after readmission of normal Na^+ ($n = 8$).

The hyperpolarizing effect of isoprenaline was gradually reduced in 103 mM and in 68 mM $[Na]_o$ and completely abolished in 34 mM, as shown in Figure 4. These effects were totally reversed after readmission of the normal Tyrode solution (Figure 4).

Influence of solutions of low Ca^{2+} concentrations and of verapamil

The hyperpolarizing effects of Iso in seven barium-treated preparations were compared when obtained in control conditions, after reducing Ca^{2+} to 0.6 mM and after omitting calcium from the Tyrode solution (equimolar amounts of Mg^{2+} were added in each condition). It can be seen from Figure 5 that external Ca^{2+} is necessary for the full development of the hyperpolarization.

The addition of verapamil to the bathing solution also decreased, in a concentration-dependent manner, the hyperpolarization produced by Iso on Ba^{2+}-treated Purkinje fibres ($n = 6$; Figure 6). Verapamil was added at an initial concentration of 5 μM, perfused for 20 min and after testing with Iso, its concentration was increased to 10 μM. The blocking effect of the higher concentration of verapamil was only partially reversed after 1 h washing in drug-free Tyrode solution (Figure 6). Three preparations treated with verapamil remained depolarized (around −65 mV) even after 1 h washing with verapamil-free, barium-free Tyrode solution. In these preparations, addition of $CaCl_2$ (0.1 ml; 1 M) directly into the perfusion chamber, promptly restored the resting potential (around −85 mV) and induced spontaneous discharges.

Effects of ouabain and of dinitrophenol

Ouabain (0.7–1.0 μM) perfused for 10 min induced arrhythmic activity and sometimes stopped spontaneous discharges in Ba^{2+}-depolarized Purkinje fibres. In these conditions, Iso (100 nM and 500 nM), produced (six preparations) hyperpolarizations of 8.2 ± 1.3 mV and 12.5 ± 2.2 mV, effects different ($P < 0.05$) from the controls 13.8 ± 3.0 and 18.7 ± 2.6 mV. These effects of ouabain were not completely reversed after 1 h washing.

On the other hand, continuous perfusion of dinitrophenol (DNP, 0.2 mM) did not reduce the Iso-induced hyperpolarization. The application of DNP

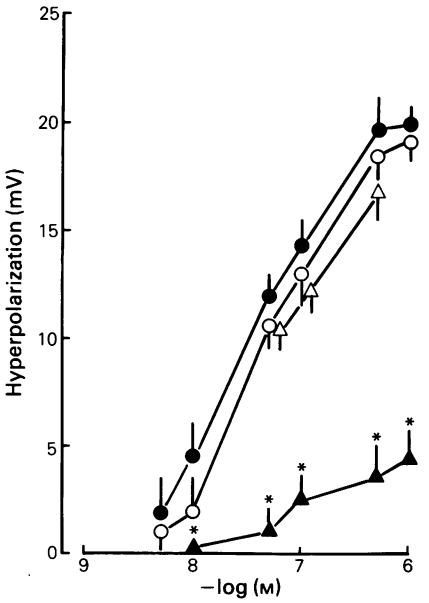

Figure 5 Effects of low Ca^{2+} Tyrode solutions on the hyperpolarization induced by isoprenaline (Iso) in seven Ba^{2+}-depolarized preparations: (●) control in 1.8 mM Ca^{2+}; (○) 0.6 mM Ca^{2+}; (▲) Ca^{2+} omitted from the Tyrode solution; (△) recovery in normal Tyrode solution. * $P = 0.01$ compared to controls in 1.8 mM Ca^{2+}.

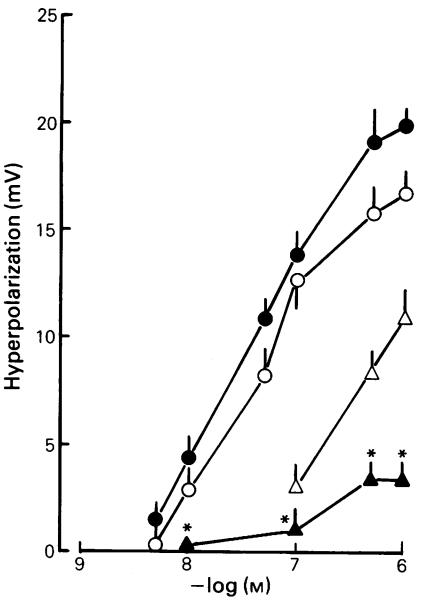

Figure 6 Blocking effects of verapamil on the peak hyperpolarization produced by isoprenaline (Iso) in Ba^{2+}-treated Purkinje fibres ($n = 6$): (●) control; (○) verapamil 5 μM; (▲) 10 μM; (△) partial recovery after 1 h washing in verapamil-free Tyrode solution. * $P = 0.01$ compared with controls before addition of verapamil.

for 15 min usually stopped the spontaneous discharges in Ba^{2+}-treated Purkinje fibres, but the addition of Iso (50 nM and 100 mM) induced hyperpolarizations (14.6 ± 1.8 mV and 17.3 ± 2.2 mV, respectively) similar to those found in control, barium-treated preparations, and induced electrical rhythmic activity in all four preparations tested.

Blocking effect of caesium

Addition of CsCl (20 mM) to the Tyrode solution depolarized four preparations to around −60 mV. The hyperpolarizations induced by 100 nM and 500 nM Iso were completely inhibited in Cs^{+}-treated preparations, and partially recovered their usual amplitudes after 1 h washing in Cs^{+}-free Tyrode solution.

Discussion

Adrenaline, noradrenaline and isoprenaline induced a dose-dependent hyperpolarization in canine cardiac Purkinje fibres which was similar, in many respects, to that observed in cells of the canine coronary sinus (Boyden *et al.*, 1983). The effects appeared in the nanomolar range and their amplitudes reached a maximum of 20–25 mV. Although isoprenaline is generally known as a more potent activator of β-adrenoceptors, the three catecholamines exhibited a somewhat unexpected equipotency in inducing hyperpolarization. Equivalent potencies between them were also found by Boyden *et al.* (1983). Similarly, the half maximal concentrations of isoprenaline and noradrenaline that depolarize sheep cardiac Purkinje fibres are between 1 and 3×10^{-7} M (cf. Glitsch & Rasch, 1986 and Terris *et al.*, 1986). The hyperpolarization was blocked by atenolol and potentiated by theophylline, suggesting that stimulation of β_1-adrenoceptors and enhanced levels of cyclic AMP are involved in its development. The phenomenon under study exhibits some specificity difficult to explain since histamine (which also enhances cyclic AMP levels in cardiac muscle) and acetylcholine were not able to induce it even at high concentrations. It is not known to what extent the barium concentration used to depolarize the Purkinje fibres might have affected the responses to these agents.

Two main mechanisms (which are not mutually exclusive) have been proposed to explain the hyperpolarization induced by catecholamines on cardiac

tissues: (a) stimulation of the Na^+-K^+ pump to generate more outward current (Trautwein & Schmidt, 1960; Vassalle & Barnabei, 1971; Wasserstrom *et al.*, 1982; Lee & Vassalle, 1983; Désilets & Baumgarten, 1986); (b) increase in the K^+ permeability (Boyden *et al.*, 1983; Gadsby, 1983). The present experiments, although not decisive in relation to these hypotheses are in favour of a catecholamine-induced increase in K^+ permeability, perhaps involving Ca^{2+} activated K^+ channels.

The results obtained under conditions in which the $[K]_o$ was varied between zero and 20 mM were not in favour of a catecholamine-induced increase in the electrogenic pump activity. It is well known that removal of external K^+ inhibits the sodium pump activity in cardiac tissues (Deitmer & Ellis, 1978; Eisner & Lederer, 1979; Gadsby & Cranefield, 1979). Zero $[K]_o$, however, did not reduce Iso-induced hyperpolarization, which agrees with the results of Boyden *et al.* (1983). On the other hand, an increase in $[K]_o$ up to 20 mM drastically reduced the hyperpolarization produced by Iso but it would not be expected to block electrogenic Na^+ pumping. It should be pointed out that, under no circumstances, did the catecholamine hyperpolarization reach a value more negative than the calculated equilibrium potential for K^+ (E_K); this also argues against a participation of an electrogenic Na^+ pump (Rasmussen *et al.*, 1984; 1986). In isolated myocardial cells from the guinea-pig, it has been shown that depolarization by 20 mM $[K]_o$ does not reduce electrogenic pumping (Gadsby *et al.*, 1985). However, a reduction in the magnitude of the hyperpolarization would be expected to occur, if it were due to outward *K* current flowing in conditions of high $[K]_o$, in which E_K would be less negative.

A decrease in $[Na]_o$, theoretically, should diminish the thermodynamic load to the sodium pump (Weer, 1984) and, perhaps, potentiate its possible stimulation by catecholamines. The contrary was observed, namely, low $[Na]_o$ diminished and blocked the hyperpolarization induced by isoprenaline.

The results obtained in low Ca^{2+} solutions suggest that a Ca^{2+}-dependent phenomenon underlies the hyperpolarization induced by catecholamines. Since an inwardly directed Ca^{2+} current would depolarize the fibre, it is reasonable to assume that a K^+ current flowing outward is responsible for the hyperpolarization. It is well established that β-adrenoceptor agonists cause an increase in transmembrane Ca^{2+} currents (see Reuter, 1983 and Tsien, 1983, for reviews). Calcium-dependent K^+ currents have been described in many cell types (see Schwarz & Passow, 1983, for a review) including sheep cardiac Purkinje fibres (Isenberg, 1977a). As with previous observations in embryonic chick hearts (Riccioppo Neto & Sperelakis, 1985), the slow channel blocker, verapamil, at a high concentration (10 μM), blocked the hyperpolarization evoked by isoprenaline. This finding reinforces the hypothesis of a Ca^{2+}-dependent phenomenon and might help in understanding the puzzling depolarization of different preparations which follows *in vitro* superfusion with verapamil (Rosen *et al.*, 1974; Shigenobu *et al.*, 1974; Wit & Cranefield, 1974).

It could be argued that the outward hyperpolarizing current is also Na^+-dependent since it was blocked in low sodium solutions. Our results showed that the fast Na^+ channels are not necessarily implicated since the hyperpolarization was not affected by tetrodotoxin. It seems that a certain amount of external sodium is necessary in order to assure proper functioning of voltage-dependent Ca^{2+} channels, since the Ca^{2+}-dependent slow responses induced in depolarized heart muscle by catecholamines and methylxanthines disappeared in low (30 mM) $[Na]_o$ (Schneider & Sperelakis, 1975). It was speculated that in certain types of Ca^{2+}-conducting channels, Na^+ ions must be bound externally in the membrane for adequate gating. However, studies in dialysed ventricular cells have recently shown that the Ca^{2+} channel current was not significantly changed when Tris replaced external Na^+ (Matsuda & Noma, 1984). A sodium-dependent Iso-activated inward current has been recently described in single guinea-pig ventricular myocytes (Egan *et al.*, 1987). This current was resistant to strophanthidin, and unaffected by caesium, calcium-channel blockers or zero Ca^{2+}. This current also provokes depolarization and is probably different from the one described in the present study. The explanation for the effect of low sodium solutions on the isoprenaline-induced hyperpolarization is therefore not known.

Cardiac glycosides have been shown to abolish the catecholamine-induced increase in cation pumping through the cardiac membrane (Lee & Vassalle, 1983; Pecker *et al.*, 1986), but a short exposure (2 min) to acetylstrophanthidin did not affect the hyperpolarization induced by catecholamines in canine coronary sinus (Boyden *et al.*, 1983) and Purkinje fibres (Gadsby, 1983). Ouabain also did not reduce the Iso-induced hyperpolarization in cultured embryonic chick heart cells (Riccioppo Neto & Sperelakis, 1985). In the present experiments ouabain significantly reduced the hyperpolarization. On the other hand, DNP, a well-known sodium pump inhibitor in cardiac tissues (Kleinfeld *et al.*, 1961), which has been shown to block the hyperpolarization caused by high-frequency stimulation of sheep cardiac Purkinje fibres (and, therefore, assumed to be electrogenic Na^+ extrusion) (Vassalle, 1970), did not reduce the Iso-induced hyperpolar-

ization. A similar finding was obtained with adrenaline and DNP by Trautwein & Schmidt (1960). These discrepancies might have been caused by the so-called 'secondary effects', due to longer exposures of cardiac tissues to ouabain (Aronson *et al.*, 1973), which include: depressed delayed rectification; blockade of the repolarization phase of the action potential (Isenberg & Trautwein, 1974), suppression of slow responses in guinea-pig heart muscle (Schneider & Sperelakis, 1976) and possibly an increase in passive Na^+ permeability (Robbins, 1977). It should be emphasized that any non-specific decrease in membrane resistance under the influence of ouabain, as observed in rat diaphragm muscle fibres (Kuba & Nohmi, 1987), could concomitantly decrease the measured peak of hyperpolarization, without necessarily reflecting reduction in an outward current. On the other hand, as pointed out by Gadsby (1984), pump stimulation by catecholamines might be indirect, due to extracellular K^+ accumulation caused by the increase in K^+ permeability. This hypothetical mechanism has recently received experimental support in relation to the hyperpolarizing effects of acetylcholine on bull-frog atrial muscle fibres (Hasuo *et al.*, 1988). If we assume that such a mechanism is also contributing to the Iso-induced hyperpolarization, ouabain would be expected partially to reduce this phenomenon, as observed in the present experiments. The contribution of an augmented electrogenic Na^+ extrusion to the observed hyperpolarization is difficult to imply from the present study.

The experiments in which caesium was used to block Ca^{2+}-dependent K^+ channels (Isenberg, 1976; 1977b; Hille, 1986), reinforce the suggestion that the Iso-induced hyperpolarization observed might be caused by an increase in K^+ permeability secondary to a rise in $[Ca]_i$.

It would be of interest to test other preparations such as nervous tissues, skeletal muscle etc, in which catecholamines have shown hyperpolarizing effects (Smith, 1984b; Kuba & Nohmi, 1987), to determine whether they are also Ca^{2+}-dependent phenomena.

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Involvement of 5-HT_2 receptors in the behaviours produced by intrathecal administration of selected 5-HT agonists and the TRH analogue (CG 3509) to rats

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1 The behavioural effects of the intrathecal injection of a thyrotrophin-releasing hormone (TRH) analogue L-orotyl-L-histidyl-prolineamide (CG 3509, 0.5 μg), the non-selective 5-HT_1 and 5-HT_2 receptor agonist 5-methoxy-N,N'-dimethyltryptamine (5-MeODMT, 2–100 μg) and the selective 5-HT_2 receptor agonist 2,5-dimethoxy-α,4-dimethyl-benzene ethamine hydrochloride (DOM, 2-25 μg) were compared with the response of systemically administered 5-MeODMT (2 mg kg^{-1}, i.p.) in rats, to establish whether the agonist-induced behaviours were mediated by bulbospinal 5-HT_1 or 5-HT_2 receptors.

2 Intrathecal injection of 5-MeODMT or DOM produced dose-related back muscle contractions (a previously undocumented behaviour) and wet-dog shakes which were both markedly attenuated by ritanserin pretreatment (1 mg kg^{-1}, i.p.) indicating the involvement of 5-HT_2 receptors. In contrast, reciprocal forepaw treading, flat body posture and Straub-tail were evoked by 5-MeODMT but not by DOM indicating that these behaviours were not produced by 5-HT_2 receptor activation alone. However, as ritanserin pretreatment reduced the reciprocal forepaw treading induced by intrathecal 5-MeODMT, this behaviour may be facilitated by 5-HT_2 receptor activation.

3 Intrathecal 5,7-dihydroxytryptamine (5,7-DHT, 2 × 150 μg) treatment decreased thoraco-lumbar spinal cord 5-HT (−95%) and potentiated the back muscle contractions produced by intrathecal DOM injection without altering the wet-dog shake behaviour. None of the components of the 5-HT syndrome produced by 5-MeODMT (2 mg kg^{-1}, i.p.), with the exception of a small increase in wet-dog shakes, was significantly altered by intrathecal 5,7-DHT (which reduced thoraco-lumbar spinal cord 5-HT by 84%). Taken together these data suggest that the only 5-HT agonist-induced behaviour mediated by the activation of 5-HT_2 receptors located postsynaptic to bulbospinal 5-hydroxytryptaminergic (5-HTergic) neurones was back muscle contractions.

4 The wet-dog shake and forepaw licking behaviors produced by intrathecal CG 3509 (0.5 μg) were attenuated when ritanserin was administered intrathecally 30 min before, but not when it was given at the same time as CG 3509 and neither behaviour was altered by intrathecal 5,7-DHT. This suggests that bulbospinal 5-HTergic neurones are not involved in the production of these TRH analogue-induced behaviours and that the 5-HT_2 receptors which mediate these behaviours are not located in the spinal cord.

Introduction

The systemic administration of drugs which increase synaptic levels of 5-hydroxytryptamine (5-HT) or agonists which act on postsynaptic 5-HT receptors produces a well-defined behavioural syndrome including reciprocal forepaw treading, hindlimb abduction, lateral head weaving, Straub tail, wet-dog shakes and hyperactivity in the rodent (Grahame-Smith, 1971; Jacobs, 1976; Deakin & Green, 1978) which all have clear motor components and can be

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used to assess 5-hydroxytryptaminergic (5-HTergic) neuronal function. The neuronal substrates which mediate these behaviours (except for hindlimb abduction) are thought to be the pontine and medullary raphe nuclei and their synaptic contacts in the lower brain stem and spinal cord (Jacobs & Klemfuss, 1975; Jacobs 1976; Bédard & Pycock, 1977; McCall & Aghajanian, 1979) though the localisation of the 5-HT receptor sub-types involved has not been established.

The tripeptide thyrotrophin-releasing hormone (TRH) is also present in high concentrations in the ventral horn of the spinal cord of a number of experimental animals (Hökfelt *et al.*, 1975; Kardon *et al.*, 1977; Lechan *et al.*, 1984; Fone *et al.*, 1987a) and man (Bennett *et al.*, 1986), where it co-exists with 5-HT and the undecapeptide substance P in descending medullary raphe nerve terminals (Johansson *et al.*, 1981; Pelletier *et al.*, 1981; Gilbert *et al.*, 1982). All three neurotransmitters have been localised, by electronmicroscopy, within boutons making close synaptic contact with ventral horn motoneurones (Atsumi *et al.*, 1985; Ulfhake *et al.*, 1987) consistent with the proposal that these neurones regulate spinal motor output. When applied iontophoretically to spinal motoneurones TRH has similar electrophysiological effects to substance P and 5-HT, facilitating the activation of motoneurones produced by the excitatory amino acids glutamate or aspartate (White, 1985). In addition, the intrathecal administration of TRH or TRH analogues produces clearly defined motor behaviours, including a dose-related wet-dog shake and marked forepaw-licking (Fone *et al.*, 1987b; 1988; Johnson *et al.*, 1988), which resemble components of the 5-HT-syndrome. Furthermore, Barbeau & Bédard (1981) reported that TRH, like 5-HT, increases spontaneous EMG activity in the hindlimb of chronic spinalised rats and as these effects were blocked by the 5-HT antagonist cyproheptadine, they suggested that TRH may act on 5-HT receptors situated on motoneurones or interneurones in the lumbar spinal cord of the rat. An alternative explanation for these findings, however, is that TRH releases 5-HT from bulbospinal nerve terminals.

The present study therefore, firstly determined whether any of the behaviours evoked by intrathecal administration of the non-selective 5-HT_1 and 5-HT_2 receptor agonist 5-methoxy-N,N'-dimethyltryptamine (5-MeODMT) or the selective 5-HT_2 receptor agonist 2,5-dimethoxy-α,4-dimethyl-benzene ethamine hydrochloride (DOM), involved activation of spinal 5-HT receptors. Secondly we investigated whether the behaviours produced by the intrathecal injection of the TRH analogue CG 3509 (L-orotyl-L-histidyl-prolineamide) were also mediated via the same spinal 5-HT receptors.

Methods

Intrathecal cannulation and behavioural recording techniques

An intrathecal cannula was implanted in male Wistar rats under sodium methohexitone anaesthesia (60 mg kg^{-1}, i.p.) so that the caudal tip of the cannula was at the thoraco-lumbar junction of the spinal cord (Yaksh & Rudy, 1976; Fone *et al.*, 1987b). Following a seven day recovery period, rats were placed in a sound-proof chamber, to which they had previously been habituated, and the number of back muscle contractions, the number of wet-dog shakes and the time spent forepaw-licking following the intrathecal injection of saline (10 μl + 20 μl wash-in), 5-MeODMT, DOM or CG 3509 were recorded for 30 min using a camera and a video-recorder, as described previously (Fone *et al.*, 1987b). In addition, in those rats receiving i.p. or intrathecal injections of 5-MeODMT or DOM, lateral head weaving, reciprocal forepaw treading, flat body posture and Straub-tail were scored on a scale of 0–3 (0 absent, 1 present < 50% of the time, 2 present > 50% of the time and 3 present all of the time), each behaviour being monitored for 20 s once every 2 min with i.p. injections or once every min with intrathecal injections. In addition, the number of turns through 90° and wet-dog shakes were counted for 20 min following the i.p. injection of 5-MeODMT.

Drug administration

In one group of rats (A in Table 1) CG 3509 (0.5 μg, $n = 7$) was administered intrathecally at four day intervals 30 min after the injection of (a) vehicle i.p. (0.04 M lactic acid in 5% dextrose on day 7), (b) ritanserin (1 mg kg^{-1}, i.p., on day 11), (c) ritanserin 5 μg intrathecally (on day 19) and (d) vehicle (0.04 M lactic acid in 5% dextrose) given intrathecally (on day 23). Ritanserin (5 μg) was also given intrathecally at the same time as CG 3509 (0.5 μg, on day 15). A second group of rats (B in Table 1, $n = 8$) received intrathecal injections, at four day intervals, of saline or 5-MeODMT (2, 10, 25, 50 and 100 μg) and four days later 5-MeODMT (25 μg) was re-administered 30 min after ritanserin (1 mg kg^{-1}, i.p.). A third group of rats (C in Table 1, $n = 7$) were given saline and DOM (2, 10 and 25 μg) intrathecally at four day intervals and the response to DOM (10 μg) was re-examined 30 min after ritanserin (1 mg kg^{-1}, i.p.).

In two further groups of rats (D in Table 1), the behavioural response to the intrathecal injection of CG 3509 (0.5 μg) was compared two days before and ten days after vehicle (10 μl, 5.68 mM ascorbate in 0.154 M saline, $n = 5$) or 5,7-dihydroxytryptamine (5,7-DHT, 2 × 150 μg, $n = 6$) which were both given

Table 1 Protocol for drug administration

Days	*7*	*9*	*11*	*13*	*15*	*17*	*19*	*21*	*23*	*25*	*27*	*31*
Group A	CG 3509 vehicle (i.p.)		CG 3509 ritanserin (i.p.)		CG 3509 ritanserin (i.t.)		CG 3509 ritanserin (i.t.)		CG 3509 vehicle (i.t.)			
Group B	Saline		5-MeODMT		5-MeODMT		5-MeODMT		5-MeODMT		5-MeODMT	5-MeODMT ritanserin (i.p.)
Group C	Saline		DOM		DOM*		DOM		DOM ritanserin (i.p.)			
Group D	5-MeODMT	CG 3509	5,7-DHT*	5,7-DHT*			5-MeODMT	CG 3509				
Group E	DOM	5,7-DHT	5,7-DHT				DOM					

For each group of rats (A to E, $n = 7$–11), the behavioural response to the test drug (on the upper line) was examined with the pretreatments listed (on the lower line). In groups B and C the different doses of 5-MeODMT and DOM respectively, were given in a randomised order. For further details of the doses of each drug used see the methods. * Five rats in group D received vehicle instead of 5,7-DHT.
5,MeODMT = 5-methoxy-N,N'-dimethyltryptamine; DOM = 2,5-dimethoxy-α,4-dimethyl-benzene ethamine HCl; 5,7-DHT = 5,7-dihydroxytryptamine; CG 3509 = L-orotyl-L-histidyl-prolineamide.

intrathecally 11 and 13 days after surgery and 1 h after desipramine (25 mg kg^{-1}, i.p.). Furthermore, in the same rats, the behavioural response in the first 20 min following i.p. injection of the non-selective 5-HT$_1$ and 5-HT$_2$ agonist 5-MeODMT (2 mg kg^{-1}, i.p.) was assessed four days before and eight days after treatment with vehicle or 5,7-DHT. In a separate group of rats (E in Table 1, $n = 7$), the behavioural response to DOM (10 μg intrathecally) was also compared two days before and ten days after 5,7-DHT treatment (2 × 150 μg administered as detailed above).

Biochemical assays

In animals given 5,7-DHT (see above) the thoracolumbar spinal cord, brain stem and hypothalamus were dissected (on the same day as the last behavioural test), extracted and maintained at −80°C prior to assay. Levels of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) were determined by high performance liquid chromatography with electrochemical detection (h.p.l.c.-e.d.) and TRH and substance P levels were measured by well characterized radioimmunoassays (RIAs) as previously described in detail (Fone *et al.*, 1987a).

Drugs

L-Orotyl-L-histidyl-prolineamide (CG 3509, Grunenthal GMBH), 5-methoxy-N,N'-dimethyltryptamine hydrochloride (5-MeODMT), 5,7-dihydroxytryptamine creatinine sulphate (Sigma) and 2,5-dimethoxy-α,4-dimethyl-benzene ethamine hydrochloride (DOM provided by Janssen) were dissolved in saline (0.154 M sodium chloride). Ritanserin (Janssen) was dissolved in 0.04 M lactic acid in dextrose.

Analysis of results

All behavioural experiments were performed using a blind protocol as previously described (Fone *et al.*, 1987b). Values given are mean ± s.e.mean and Student's *t* test was used for statistical analysis unless otherwise indicated in the text.

Results

Behaviours produced by intrathecal or intraperitoneal 5-MeODMT administration

Within 2 min of intrathecal injection, 5-MeODMT (2–100 μg) produced wet-dog shakes and readily quantifiable, brief, contractions of the muscles of the

back without accompanying hindlimb movements; subsequently referred to as 'back muscle contractions'. As shown in Figure 1, both the number of wet-dog shakes and the number of back muscle contractions recorded in the 30 min post-injection period were linearly correlated to $\log_{10}$ dose 5-MeODMT (within the range of 1–50 μg, but did not increase further with 100 μg 5-MeODMT). With all but the lowest dose (back muscle contractions) or the 2 and 10 μg doses (wet-dog shakes) of 5-MeODMT the incidence of both behaviours was significantly greater than observed with saline. Intrathecal 5-MeODMT also caused components of the 5-HT-syndrome (including flat body posture, reciprocal forepaw treading, and Straub tail) which although not pronounced, increased in a dose-related manner, however, lateral head weaving was not evoked by this route of administration (Table 2). Ritanserin (1 mg kg^{-1}, i.p.) pretreatment significantly attenuated the number of wet-dog shakes and back muscle contractions and the amount of reciprocal forepaw treading produced by intrathecal 5-MeODMT, although none of the other components of the 5-HT-syndrome were affected by this treatment (Figure 2 and Table 2). Intrathecal 5-MeODMT also caused scratching of the torso and caudally directed bites (as reported by Hylden & Wilcox, 1983; Larson & Wilcox, 1984), but these were not quantified in the present study.

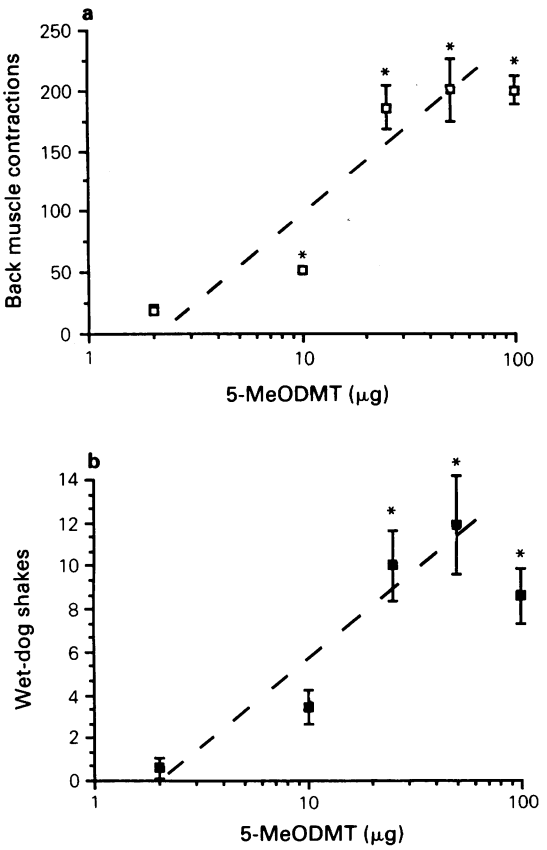

Figure 1 The total number (mean ± s.e.mean, $n = 8$) of (a) back muscle contractions and (b) wet-dog shakes produced in 30 min following the intrathecal administration of 5-methoxy-N,N'-dimethyltryptamine (5-MeODMT, 2-100 μg) was significantly different ($P < 0.05$, ANOVAR) from the effect of saline (7 ± 3 and 1 ± 1 in 30 min, respectively) with all but the lowest dose (back muscle contractions) and the 2 and 10 μg dose (wet-dog shakes). The $\log_{10}$ dose-responses were both linearly correlated ($r = 0.959$ (a) and 0.929 (b), $P < 0.01$) from 1 to 50 μg, according to the method of least squares.

The systemic administration of 5-MeODMT (2 mg kg^{-1}, i.p.), which had previously been shown to evoke a submaximal behavioural response (Nisbet & Marsden, 1984), produced lateral head weaving, flat body posture, hindlimb abduction, reciprocal forepaw treading, wet-dog shakes and hyperlocomotion (assessed by counting the number of turns) but not back muscle contractions as observed after intrathecal injection of 5-MeODMT. With the exception of a small, but significant increase in the number of wet-dog shakes, none of these behaviours produced by i.p. 5-MeODMT were significantly altered by intrathecal 5,7-DHT pretreatment (Table 3), although the apparent reduction in flat body posture following 5,7-DHT treatment only just failed to reach significance.

Behaviours produced by intrathecal administration of DOM

Figure 3 shows that the intrathecal injection of DOM (2–25 μg) caused back muscle contractions and wet-dog shakes, both of which were significantly

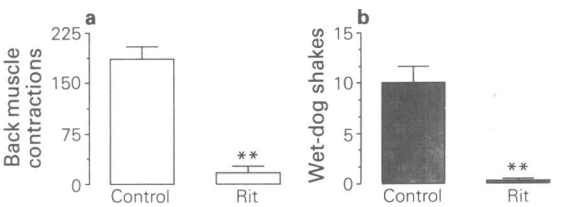

Figure 2 Effect of ritanserin (Rit, 1 mg kg^{-1} i.p., −30 min) pretreatment on the total number (mean with s.e.mean shown by vertical bars, $n = 8$) of back muscle contractions (a) and wet-dog shakes (b) produced in 30 min following the intrathecal injection of 5-methoxy-N,N'-dimethyltryptamine (25 μg, $n = 8$). ** $P < 0.001$, Student's t test.

Table 2 Behavioural effect of intrathecal 5-methoxy-N,N'-dimethyltryptamine (5-MeODMT)

5-MeODMT dose (μg)	*0*	*2*	*10*	*25*	*50*	*100*	*25 plus Rit*
Flat body posture	1.0 (0–4)	0.0 (0–0)	4.6 (0–36)	14.7* (1–34)	21.8* (0–32)	16.4** (4–44)	12.6 (2–31)
Reciprocal forepaw treading	0.3 (0–1)	0.5 (0–2)	0.5 (0–3)	3.3* (2–6)	7.4* (5–9)	6.0* (3–8)	1.0† (0–3)
Straub-tail	1.0 (0–3)	1.3 (0–6)	2.9* (0–6)	2.9* (0–6)	3.1* (1–6)	9.1* (1–17)	1.1 (0–3)
Lateral head weaving	3.8 (1–6)	3.9 (2–6)	2.6 (1–6)	2.4 (0–6)	3.8 (2–6)	2.8 (0–8)	4.9 (2–8)

Scoring procedures are described in the Methods. The maximum score per behaviour = 60, values given are mean (range) from eight animals. * $P < 0.05$, ** $P < 0.01$ Wilcoxon rank test from the effect of saline following significant Kruskal-Wallis test and † $P < 0.01$ from the effect of 5-MeODMT (25 μg). 25 plus Rit = 5-MeODMT (25 μg) given 30 min after ritanserin (1 mg kg^{-1}, i.p.).

greater than the effect in saline. Both behaviours appeared to increase in a dose-related manner and were virtually abolished by ritanserin pretreatment (1 mg kg^{-1}, i.p., Figure 3). All doses of DOM also produced an immediate and marked extension of the hindlegs together with chewing and licking of the hindlegs and abdomen. In addition some yawning was observed with the highest dose of DOM. However, no tail elevation, flat body posture, lateral head weaving or reciprocal forepaw treading were evident with any dose of DOM (average behavioural scores for 25 μg DOM being 0, 1, 6 and 1 respectively, from a possible maximum of 60). Neither was there any evidence of hyperlocomotion when compared with saline.

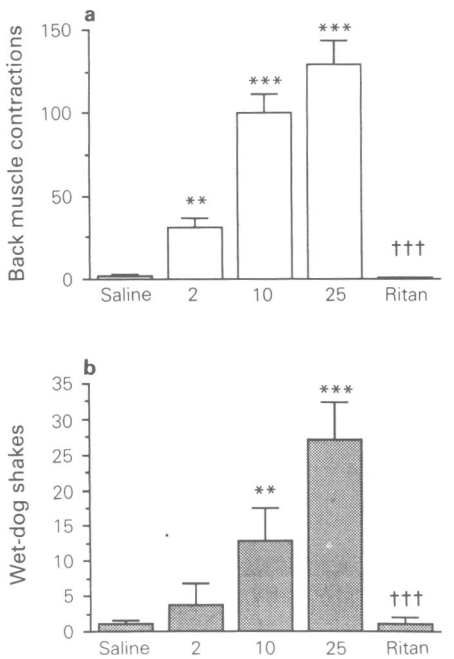

Figure 3 A comparison of the total number (mean ± s.e.mean, $n = 7$) of back muscle contractions (a) and wet-dog shakes (b) produced by the intrathecal injection of saline or 2,5-dimethoxy-α,4-dimethylbenzene ethamine HCl (DOM) (2,10 and 25 μg) given alone with the response to DOM (25 μg) given 30 min after ritanserin (Ritan, 1 mg kg^{-1}, i.p.). ** $P < 0.001$ and *** $P < 0.0001$, ANOVAR from rats given saline and ††† $P < 0.0001$ from rats given DOM (25 μg) alone.

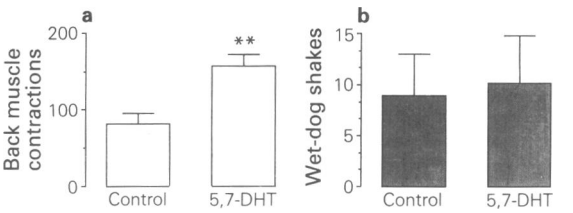

Figure 4 Comparison of the number of back muscle contractions (a) and wet-dog shakes (b) produced in 30 min by intrathecal 2,5-dimethoxy-α,4-dimethylbenzene ethamine HCl (DOM, 10 μg) administration two days before (Control) with that evoked ten days after intrathecal injection of 5,7-dihydroxytryptamine 5,7-DHT (2 × 150 μg, $n = 6$). ** $P < 0.01$, Student's t test from the control response.

Intrathecal pretreatment with 5,7-DHT, which significantly reduced thoraco-lumbar 5-HT levels (95% below that in vehicle-treated controls), significantly enhanced the number of back muscle contractions evoked by intrathecal DOM administration (from 82 ± 14 to 157 ± 16 in 30 min with 10 μg DOM, $P < 0.01$) without altering the wet-dog shake response (Figure 4).

Behaviours produced by intrathecal CG 3509 administration

The intrathecal injection of CG 3509 (0.5 μg) produced marked wet-dog shake and forepaw-licking

Table 3 Effect of intrathecal 5,7-dihydroxytryptamine (5,7-DHT) on the behavioural response to 5-methoxy-N,N'-dimethyltryptamine (5-MeODMT, 2 mg kg^{-1}, i.p.)

Treatment	*Vehicle* ($n = 5$) *before*	*Vehicle* ($n = 5$) *after*	*5,7-DHT* ($n = 6$) *before*	*5,7-DHT* ($n = 6$) *after*
Wet-dog shakes	2 ± 2	1 ± 1	1 ± 1	6 ± 2*
Number of turns	78 ± 15	89 ± 17	60 ± 8	59 ± 20
Flat body posture	11.6 (10–14)	10.4 (0–17)	9.5 (9–16)	7.5 (5–10)
Forepaw treading	5.6 (3–10)	3.2 (0–6)	3.3 (2–5)	2.2 (1–5)
Head weaving	7.8 (6–10)	9.4 (7–12)	9.5 (9–10)	7.5 (5–10)
Hindlimb abduction	12.4 (10–16)	15.6 (13–21)	13.2 (9–18)	14.7 (15–20)

The number of wet-dog shakes and turns through 90° were counted (mean ± s.e.mean) and the other behaviours were scored mean (range) as described in the Methods, the maximum score being 30 for each behaviour. * $P < 0.05$ Student's t test from before 5,7-DHT treatment.

There was no significant difference in the behavioural score within the groups (before and after either treatment, Wilcoxan Rank test) nor between vehicle and 5,7-DHT groups after treatment (Mann Whitney U test).

behaviours, with the peak effect at 6–9 min as reported previously (Fone *et al.*, 1987b; Johnson *et al.*, 1988) but unlike intrathecal 5-HT agonist administration it did not produce any back muscle contractions. The wet-dog shakes and forepaw-licking behaviours induced by CG 3509 were attenuated by a 30 min intrathecal pretreatment with ritanserin and wet-dog shakes were also reduced by i.p. ritanserin pretreatment (Figure 5), but neither behaviour was significantly reduced when ritanserin was given intrathecally at the same time as CG 3509 (Figure 5).

Both the wet-dog shakes and forepaw-licking behaviours induced by CG 3509 were unaltered by intrathecal injections of vehicle or 5,7-DHT (Figure 6a and b).

Effect of 5,7-DHT on brain and spinal cord 5-HT, TRH and substance P levels

Intrathecal 5,7-DHT administration markedly depleted 5-HT, TRH and substance P levels in the thoraco-lumbar ventral horn (reduced by 84%, 96% and 59%, respectively, from levels in vehicle-treated controls; Figure 7), but levels were unchanged in the hypothalamus, and only TRH levels were significantly increased in the brainstem (Figure 7). 5,7-DHT treatment also caused a small decrease in the 5-HT and TRH levels in the dorsal horn of the spinal cord and although substance P levels appeared to be reduced in this region, compared with vehicle-treated controls, this did not reach significance (Figure 7).

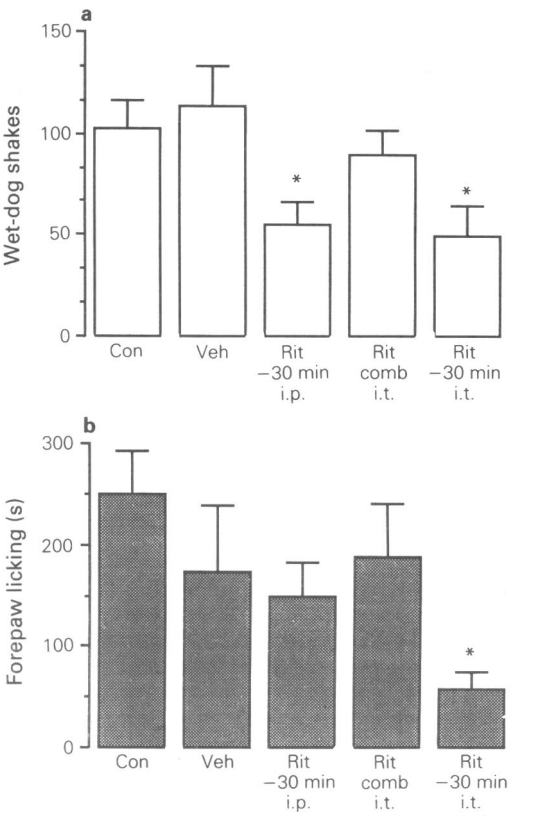

Figure 5 Effect of the pretreatment with either vehicle (Veh) intrathecally, ritanserin 1 mg kg^{-1} i.p. (Rit −30 min, i.p.) or ritanserin 5 μg intrathecally given at the same time (Rit comb i.t.) or 30 min before (Rit −30 min i.t.) CG 3509, on the wet-dog shake (a) and the forepaw-licking (b) behaviours (mean with s.e.mean shown by vertical lines over 30 min, $n = 8$) produced by the intrathecal administration of 0.5 μg CG 3509 alone (Con). * $P < 0.02$, Student's t test from the appropriate control.

Discussion

Intrathecal administration of the 5-HT agonist, 5-MeODMT produced the characteristic 5-HT behavioural syndrome previously observed following systemic administration of 5-HT agonists or drugs

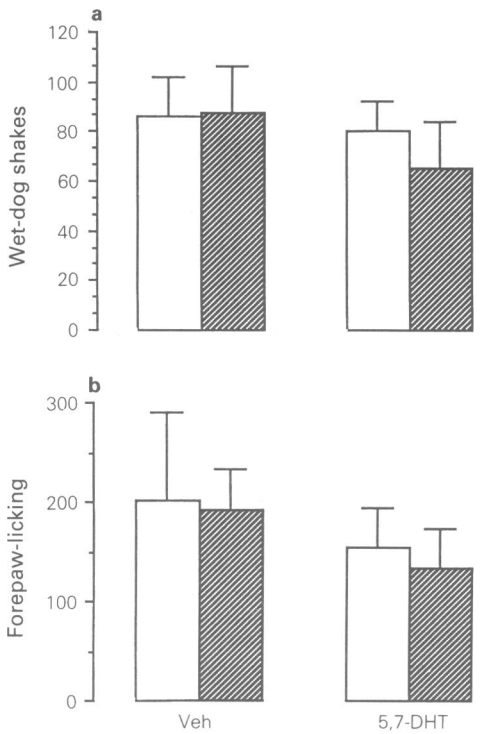

Figure 6 A comparison of the wet-dog shake (a) and the forepaw-licking (b) behaviours evoked in 30 min following intrathecal CG 3509 (0.5 μg) administration given two days before (open columns) and ten days after (filled columns) treatment with vehicle (10 μl, 5.68 mM ascorbate in 0.154 M saline, $n = 5$) or 5,7-dihydroxytryptamine (5,7-DHT, 2 × 150 μg intrathecally, $n = 6$).

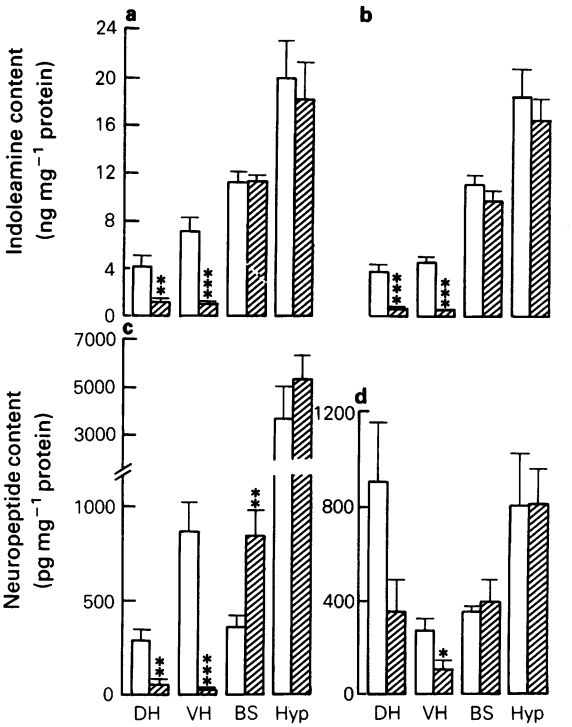

Figure 7 The effect of vehicle (open columns, $n = 5$) and 5,7-dihydroxytryptamine (hatched columns, 2 × 150 μg intrathecally, $n = 6$) treatment on (a) 5-hydroxytryptamine, (b) 5-hydroxyindoleacetic acid, (c) thyrotrophin-releasing hormone and (d) substance P levels (mean with s.e.mean shown by vertical bars) in the thoraco-lumbar dorsal (DH) and ventral (VH) horns, brain stem (BS) and hypothalamus (Hyp). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; Student's t test from vehicle-treated controls.

which increase synaptic 5-HT levels (Grahame-Smith, 1971; Jacobs, 1976), with the exception that lateral head weaving was not discernable. In addition, intrathecal 5-MeODMT caused marked, dose-related back muscle contractions, a characteristic and readily quantifiable motor response which does not appear to have been described previously. The close similarity of the dose-response curves for the wet-dog shakes and back muscle contractions produced by intrathecal 5-MeODMT is consistent with the idea that these behaviours are mediated by a common 5-HT receptor subtype. Since both these behaviours and the reciprocal forepaw treading evoked by intrathecal 5-MeODMT were reduced by pretreatment with the selective 5-HT$_2$ receptor antagonist ritanserin, 5-HT$_2$ receptors are implicated in these three behaviours. Furthermore, intrathecal injection of the selective 5-HT$_2$ receptor agonist DOM (Glennon, 1987) also produced wet-dog shakes and back muscle contractions which were both attenuated by ritanserin, while none of the other 5-MeODMT-induced behaviours (including reciprocal forepaw treading) were observed. Taken together these results suggest that 5-HT$_2$ receptor activation may facilitate reciprocal forepaw treading but activation of this receptor alone is not sufficient to elicit this behaviour. In addition, as back muscle contractions were only evoked when 5-HT agonists were given intrathecally, and were not seen following the systemic administration of 5-MeODMT, this behaviour is likely to be mediated by spinal 5-HT$_2$ receptors.

Previous studies suggest that while 5-HT-induced wet-dog shakes are inhibited in a dose-related manner by 5-HT$_2$ receptor antagonists such as ketanserin and pirenpirone (Yap & Taylor, 1983; Koshikawa *et al.*, 1985) and are produced by the systemic administration of the 5-HT$_2$ receptor agonist

1 - (2,5 - dimethyl - 4 - iodophenyl) - 2 - aminopropane (Heaton *et al.*, 1988), the other components of the 5-HT-syndrome are largely mediated by 5-HT_1-like receptors (Lucki *et al.*, 1984; Goodwin & Green, 1985; Tricklebank *et al.*, 1985; Kennett & Curzon, 1988). For this reason the presence of another 5-HT_2 receptor-mediated behaviour, namely the back muscle contractions described in the present study, provides a very useful tool to investigate further the neuropharmacology of spinal 5-HT.

In the present study, intrathecal 5,7-DHT administration depleted 5-HT (−84%), TRH (−96%) and substance P (−59%) from the ventral horn of the spinal cord where the indoleamine and neuropeptides co-exist (Johansson *et al.*, 1981; Pelletier *et al.*, 1981; Gilbert *et al.*, 1982). With the exception of an elevation in brainstem TRH, previously reported following i.c.v. 5,7-DHT (Manaker *et al.*, 1985), intrathecal 5,7-DHT injection had no effect on indoleamine or peptide levels in the hypothalamus or brainstem. The increase in brainstem TRH probably results from 5,7-DHT causing degeneration of 5-HT-peptidergic terminals in the ventral horn of the spinal cord without affecting the brainstem perikarya (Towle *et al.*, 1984), so preventing nerve terminal synthesis of 5-HT without reducing the neuronal cell body synthesis of TRH which consequently accumulates in the brainstem soma. The lack of effect of 5,7-DHT on substance P levels in the brainstem can not at present be explained. Within the ventral horn postsynaptic supersensitivity to 5-HT agonists would be expected following such a marked depletion of 5-HT, since similar lesions of the 5-HTergic system produce a parallel increase in the 5-MeODMT-induced head twitch response and the number of cortical 5-HT_2 receptors in mice (Heal *et al.*, 1985). As 5,7-DHT treatment, in the present study, markedly enhanced the DOM-induced back muscle contractions, this behaviour would appear to be mediated by activation of spinally-located 5-HT_2 receptors. In contrast, neither the wet-dog shake response to DOM nor any of the components of the 5-HT-syndrome induced by 5-MeODMT (apart from a small increase in the number of wet-dog shakes) were altered by 5,7-DHT treatment. The present results therefore suggest that bulbospinal 5-HT-ergic neurones are not involved in the lateral head weaving, flat body posture, hindlimb abduction, reciprocal forepaw treading, hyperactivity (number of turns) or wet-dog shakes produced by 5-HT agonists. In comparison, Deakin & Green (1978) showed that microinjection of 5,7-DHT into the rostral cervical spinal cord (C_1 and C_2) decreased spinal 5-HT levels by 70% and significantly enhanced the head weaving, Straub-tail and hindlimb abduction produced by 5-MeODMT ($2\,mg\,kg^{-1}$, i.p.). However, the neurotoxin may have spread rostrally from the latter injection site so destroying 5-HT neurones not affected by the more caudal intrathecal injection procedure used in the current study. In agreement with this proposal, i.c.v. 5,7-DHT has been shown to enhance 5-MeODMT-induced hindlimb abduction and reciprocal forepaw treading (Nisbet & Marsden, 1984) and injections of 5,7-DHT into the most caudal thoracic segment (T_{13}) of the rat, only increased the Straub-tail and not the hindlimb abduction, head weaving or reciprocal forepaw treading produced by i.p. 5-MeODMT (Dickinson *et al.*, 1984).

Intrathecal administration of the TRH analogue CG 3509 produced wet-dog shake and forepaw-licking behaviours as previously described (Fone *et al.*, 1987b; Johnson *et al.*, 1988), but did not produce back muscle contractions as would be expected if TRH directly activates spinal 5-HT_2 receptors. Both the wet-dog shake and forepaw-licking behaviours were attenuated by a 30 min intrathecal pretreatment with ritanserin although neither response was altered when ritanserin was given intrathecally at the same time as CG 3509. We have previously shown that in the first 9 min following intrathecal administration of radiolabelled TRH or TRH analogues the radioactivity remains principally confined to the spinal cord (Fone *et al.*, 1987b; Johnson *et al.*, 1988). Since 30 min pretreatment with ritanserin was required to obtain antagonism of the TRH analogue-induced behaviours, the 5-HT_2 receptors involved in these effects may be located rostral to the spinal cord.

The degeneration of bulbospinal 5-HT neurones (which contain TRH) produced by 5,7-DHT increases spinal TRH receptor number according to binding studies (Sharif *et al.*, 1983; Ogawa *et al.*, 1985) and augments the facilitation of spinal reflexes normally evoked by TRH (Barbeau & Bédard, 1981; Clarke & Stirk, 1983). Thus several lines of evidence suggest that the intrathecal 5,7-DHT treatment, used in the present study, would induce up-regulation of both 5-HT and TRH receptors in the spinal cord. Despite this, intrathecal 5,7-DHT did not affect the wet-dog shakes or the forepaw-licking produced by intrathecal TRH analogue administration, supporting the proposal that these behaviours are not mediated by bulbospinal 5-HTergic neurones. In accordance with this proposal previous work suggests that the brain area involved in mediating 5-HT- and TRH-induced wet-dog shake behaviour is in the medial brainstem, between the colliculi and the anterior commissure, as lesions in this region reduce the shaking produced by 5-hydroxytryptophan (5-HTP) and water immersion (Wei *et al.*, 1973; Bédard & Pycock, 1977; Wei, 1981), while micro-injection of TRH into the medial brainstem (Wei, 1981) or the area of the locus coeruleus (Kalivas & Horita, 1981; Webster *et al.*, 1982)

caused vigorous wet-dog shakes. Since intrathecal 5,7-DHT significantly enhanced the 5-MeODMT-but had no effect on the TRH analogue-induced shaking, the 5-HT- and TRH-induced wet-dog shakes may be mediated by separate 5-HTergic pathways. In agreement with this idea, Drust & Connor (1983) found that while 5-HT-induced wet dog shakes were blocked by the 5-HT antagonist methysergide and enhanced by 5,7-DHT, neither treatment effected the TRH-induced shaking.

In summary, both i.p. and intrathecal injection of the non-selective 5-HT agonist 5-MeODMT produced components of the 5-HT-syndrome, but back muscle contractions were only observed with intrathecal injections and so may be dependent upon marked stimulation of spinal 5-HT receptors, while lateral head weaving appears to be mediated by more rostral neurones as it was not evoked by this route of administration. 5-HT$_2$ receptors may be involved in the wet-dog shakes and back muscle contractions evoked by the 5-HT agonists 5-MeODMT or DOM, as these behaviours were attenuated by ritanserin. Intrathecal 5,7-DHT treatment, which would be expected to increase 5-HT and TRH receptors in the spinal cord, was without effect on the wet-dog shakes and forepaw-licking behaviours produced by intrathecal CG 3509, or the lateral head weaving, flat body posture, hindlimb abduction, reciprocal forepaw treading or hyperlocomotion produced by i.p. injection of 5-MeODMT, indicating that spinal 5-HT and TRH receptors are not involved in mediating these behaviours. Spinal cord 5-HT$_2$ receptors located postsynaptically in relation to bulbospinal 5-HTergic nerve terminals are implicated in 5-HT-agonist induced back muscle contractions, since 5,7-DHT pretreatment significantly enhanced this behaviour. However, the wet-dog shake behaviour produced by TRH and 5-HT agonists may not be mediated by the same spinal pathways as they were differentially affected by 5,7-DHT.

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Effect of phorbol ester and pertussis toxin on the enhancement of noradrenaline release by angiotensin II in mouse atria

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1 Mouse atria were incubated with [^{3}H]-noradrenaline, and the outflow of radioactivity due to electrical field stimulation (5 Hz, 60 s) was used as an index of noradrenaline release. Angiotensin II (0.01 and 0.1 μM) significantly enhanced the stimulation-induced (S-I) outflow of radioactivity.

2 Phorbol 12-myristate 13-acetate (0.001, 0.03, 0.1 and 1.0 μM), a protein kinase C activating phorbol ester, significantly enhanced the S-I outflow of radioactivity. When angiotensin II (0.1 μM) was present with the concentration of phorbol 12-myristate 13-acetate that was maximally effective in increasing the S-I outflow (0.1 μM), the enhancement of S-I outflow produced by angiotensin II was maintained.

3 Polymyxin B (70 μM), an inhibitor of protein kinase C, significantly inhibited the S-I outflow. Polymyxin B also inhibited the enhancement of the S-I outflow produced by angiotensin II (0.1 μM).

4 In another series of experiments mice were injected with pertussis toxin (1.5 μg per mouse), 4 days before their atria were removed. The effectiveness of pertussis toxin pretreatment was determined indirectly using carbachol. Carbachol caused a concentration-dependent fall in both the rate and force of beating of isolated spontaneously beating atria from mice pretreated with vehicle. This effect of carbachol was not seen with atria from mice pretreated with pertussis toxin.

5 Pertussis toxin pretreatment did not alter the enhancement of the S-I outflow of radioactivity produced by angiotensin II (0.01 and 0.1 μM).

6 These results suggest that angiotensin II receptor modulation of noradrenaline release is not mediated through either a pertussis toxin sensitive guanine nucleotide-binding protein or activation of protein kinase C.

Introduction

Phospholipase C is a membrane-bound enzyme which can be stimulated by agents acting through a variety of receptors including α_1-adrenoceptors, muscarinic cholinoceptors and angiotensin II receptors (Nabika *et al.*, 1985; Fisher & Agranoff, 1987). These receptors are linked to phospholipase C by a family of guanine-nucleotide binding proteins, so called G proteins (Berridge, 1987; Gillman, 1987). Phospholipase C activation may be implicitly involved in receptor-response coupling. Phospholipase C can hydrolyse membrane phosphatidylinositol bisphosphate, with the generation of two 'second messengers', inositol 1,4,5 trisphosphate and diacylglycerol (Berridge, 1987). Inositol 1,4,5 trisphosphate releases Ca^{2+} from intracellular stores, whereas diacylglycerol can activate a calcium/phospholipid-dependent protein kinase, protein kinase C (Nishizuka, 1986; Berridge, 1987). Protein kinase C appears to regulate various cell functions by phosphorylation of specific proteins (see Nishizuka, 1986; Niedel & Blackshear, 1986).

Activation of prejunctional receptors on sympathetic nerve endings results in either the inhibition or enhancement of noradrenaline release (see Starke, 1977; Rand *et al.*, 1980). Activation of phospholipase C might be involved in the effects of some of these facilitatory receptors, since increased intracellular Ca^{2+} due to inositol 1,4,5 trisphosphate-induced release of Ca^{2+} would probably increase noradrenaline release. Furthermore, activation of protein kinase C by phorbol esters enhances noradrenaline release (Wakade *et al.*, 1985; Shuntoh & Tanaka, 1986; Ishac & De Luca, 1988; Musgrave & Majewski, 1988).

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There are three prejunctional receptors, activation of which results in enhancement of action potential-induced noradrenaline release: prejunctional β-adrenoceptors (Majewski, 1983), prejunctional adrenocorticotrophic hormone (ACTH) receptors (Göthert & Hentrich, 1984; Costa & Majewski, 1988) and prejunctional angiotensin II receptors (see Starke, 1977; Zimmerman, 1981). There is reasonable evidence that the prejunctional β-adrenoceptors (Johnston *et al.*, 1987; Costa & Majewski, 1988) and prejunctional ACTH receptors (Göthert & Hentrich, 1984; Costa & Majewski, 1988) operate through the adenylate cyclase/adenosine 3':5'-cyclic monophosphate (cyclic AMP) second messenger pathway. However prejunctional angiotensin II receptors do not appear to be linked to adenylate cyclase (Costa & Majewski, 1988). A more likely possibility for prejunctional angiotensin II receptors is that they are linked to the phospholipase C second messenger pathway, as has been suggested for angiotensin II receptors in other cells (Nabika *et al.*, 1985; Fisher & Agranoff, 1987).

The aim of the present study in mouse atria was to determine if angiotensin II enhancement of noradrenaline release is due to activation of the phospholipase C pathway. The most direct approach would be to use inhibitors of phospholipase C. However, there are no suitable inhibitors of phospholipase C to test this hypothesis. For example, two commonly used phospholipase C inhibitors, mepacrine and neomycin, have a number of other actions including inhibition of calcium channels (mepacrine: Baba *et al.*, 1983; neomycin: Hughes *et al.*, 1988). Therefore, we approached the problem indirectly. Firstly, the effect of activators and inhibitors of protein kinase C on angiotensin II enhancement of noradrenaline release was assessed. Secondly, the effects of pertussis toxin, which has been shown to inactivate the guanine-nucleotide binding protein which links angiotensin II receptors to phospholipase C (Pfeilschifter & Bauer, 1986; Gaul *et al.*, 1988) were determined.

Methods

Stimulation-induced outflow of radioactivity from mouse atria

Random bred Swiss White mice (17–38 g) were stunned, decapitated and their atria rapidly dissected free. The atria were then suspended between a stainless steel tissue hook and a force transducer in an organ bath containing Krebs-Henseleit solution maintained at 37°C and bubbled with a mixture of 5% CO_2, 95% O_2. Isolated atria were incubated with [^{3}H]-noradrenaline (2.9 μCi ml^{-1}, 0.2 μM) for 20 min and then washed repeatedly for 60 min in [^{3}H]-noradrenaline-free Krebs-Henseleit solution. A priming stimulation (5 Hz, 30 s) was delivered 30 min after washing began and the atria were then placed under a basal tension of 0.1 g. When washing was completed, the atria were bathed with 4 ml of Krebs-Henseleit solution for 3 min periods for a total of 54 min. At 9 and 39 min after the start of this procedure the atria were stimulated through a pair of platinum electrodes (5 Hz, 60 s, square wave pulses 15 V cm^{-1}, 1 ms duration). The effect of drugs, except for polymyxin B, on the stimulation-induced outflow of radioactivity was determined by adding them to the bathing solution 15 min before the second stimulation.

In some experiments the second stimulation was performed 45 min after the first (at 54 min) and polymyxin B was added to the bathing solution 30 min before the second stimulation. The longer incubation time has been found to be necessary for optimal inhibition of protein kinase C activity by polymyxin B (Allgaier & Hertting, 1986).

The spontaneous outflow of radioactivity was taken as the mean radioactive content of the bathing solution during the 3 min period immediately before and the 3 min period commencing 6 min after the start of stimulation. The stimulation-induced (S-I) component of the outflow of radioactivity was calculated by subtracting the mean spontaneous radioactive outflow from the radioactive content of each of the two 3 min samples collected immediately after the start of stimulation, and the resulting values summed. This value was then expressed as a fraction of the tissue content of radioactivity at the time of stimulation (the fractional S-I outflow).

At the end of the experiment the atria were dissolved in 2 ml Soluene (Packard Instruments). The radioactivity present in the bathing solution and atria was determined by liquid scintillation counting after the solutions were mixed with 10 ml Picofluor (Packard Instruments). Corrections for counting efficiency were made by automatic external standardization.

Pertussis toxin pretreatment

Mice were injected with purified pertussis toxin (1.5 μg protein per mouse delivered in 0.1 ml lysine/glycerol buffer pH 7.5) or vehicle (0.1 ml lysine/glycerol buffer) through the tail vein. After 4 days the mice were stunned, decapitated and the atria rapidly dissected free and suspended in Krebs-Henseleit solution as described above. The basal tension applied to the suspended atria was 0.1 g. The negative inotropic and chronotropic effects of carbachol on the spontaneously beating atria were determined by adding increasing concentrations of carbachol to the

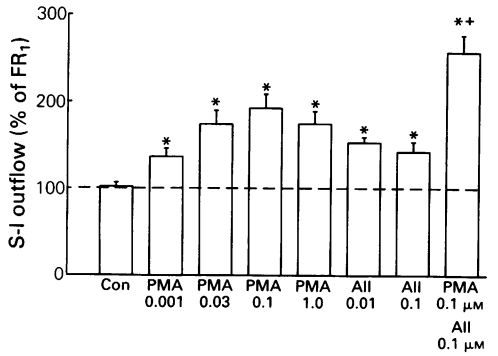

Figure 1 The effect of angiotensin II and phorbol ester on the fractional stimulation-induced (S-I) outflow of radioactivity from mouse atria incubated with [^{3}H]-noradrenaline. There were two periods of stimulation (5 Hz for 60 s each) 30 min apart, angiotensin II (AII, 0.01 and 0.1 μM) and phorbol 12-myristate 13-acetate (PMA, 0.001, 0.03, 0.1 and 1.0 μM) were present from 15 min before the second stimulation. The fractional S-I outflow of radioactivity in the second period of stimulation (FR_2) is expressed as a percentage of that in the first (FR_1). The absolute value of FR_1 as a percentage of total tissue radioactivity was $1.25 \pm 0.07\%$ ($n = 65$). The columns represent means and the vertical bars s.e.means. * Significantly different from control (Con, $P < 0.05$, Scheffé's test after analysis of variance). The facilitatory effect of the combination of angiotensin II and phorbol 12-myristate 13-acetate was significantly greater than that produced by either drug alone ($P < 0.05$, Scheffé's test after two way analysis of variance) but not significantly different from the simple addition of the individual effects of the drugs ($^+P > 0.05$, two way analysis of variance). The number of experiments in each group is given in Table 1.

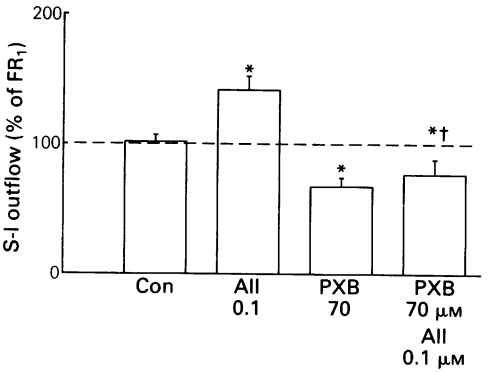

Figure 2 The effect of polymyxin B on the enhancement of fractional stimulation-induced (S-I) outflow of radioactivity by angiotensin II from mouse atria incubated with [^{3}H]-noradrenaline. There were two periods of stimulation (5 Hz for 60 s each) 45 min apart, polymyxin B (PXB 70 μM) was present from 30 min and angiotensin II (AII, 0.1 μM) was present from 15 min before the second stimulation. The fractional S-I outflow of radioactivity in the second period of stimulation (FR_2) is expressed as a percentage of that in the first (FR_1). The columns represent means and the vertical bars s.e.means. * Significantly different from control (Con, $P < 0.05$, Scheffé's test after analysis of variance). † Significant inhibition compared to angiotensin II in the absence of polymyxin B ($P < 0.05$ two way analysis of variance). The number of experiments in each group is given in Table 1.

organ bath by the cumulative method. After determination of the concentration-response curve to carbachol the atria were washed in Krebs-Henseleit solution and then incubated with [^{3}H]-noradrenaline as described above.

Statistical analysis

Values are given as mean ± s.e.mean. The results were analysed by use of Student's unpaired t test or two way analysis of variance as indicated. After analysis of variance, differences between individual means were determined by Scheffé's multiple range test (Winer, 1962). A probability of a type 1 error of less than 5% ($P < 0.05$) was taken to indicate statistical significance.

Buffer and saline solutions

The Krebs-Henseleit solution contained (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.45, $NaHCO_3$ 25.0, KH_2PO_4 1.03, D-(+)-glucose 11.1, disodium EDTA 0.067 and ascorbic acid 0.14.

The lysine/glycerol buffer contained (mM): NaCl 250, $NaHCO_3$ 25, L-lysine 10. The reagents were initially dissolved in deionized water and the pH adjusted to 7.5, then glycerol was added in a 1:1 ratio to give the above concentrations.

Phosphate buffered saline (pH 7.4) contained (mM): Na_2HPO_4 60, NaCl 145 and 0.2 g gelatine 100 ml^{-1}.

Drugs

Drugs used were: (−)-7,8-[^{3}H]-noradrenaline (Amersham; U.K.), angiotensin II (val^5-angiotensin II, Sigma; U.S.A.), carbamyl choline chloride (carbachol, Sigma; U.S.A.), phorbol 12-myristate 13-acetate (Sigma; U.S.A.) and polymyxin B (Sigma; U.S.A.). Stock solutions of angiotensin II were made up in phosphate buffered saline pH 7.4, stock solutions of phorbol 12-myristate 13-acetate were made up in dimethyl sulphoxide and both stocks were kept at −20°C. Drugs were further diluted in Krebs-Henseleit solution.

Table 1 Effect of drugs and pertussis toxin pretreatment on the spontaneous outflow of radioactivity from mouse atria incubated with [^{3}H]-noradrenaline

Drugs	*Concentration* (μM)	R_2/R_1 (%)	n
S_1 to S_2 interval 30 min			
Control		83 ± 1	5
AII	0.01	87 ± 3	7
AII	0.1	85 ± 2	6
PMA	0.001	84 ± 3	6
PMA	0.03	88 ± 1	5
PMA	0.1	87 ± 2	6
PMA	1.0	88 ± 3	5
AII +	0.1		
PMA	0.1	85 ± 3	4
S_1 to S_2 interval 45 min			
Control		80 ± 2	10
Polymyxin B	70	*93 ± 5	5
AII	0.1	79 ± 3	5
AII +	0.1		
Polymyxin B	70	*100 ± 6	5
S_1 to S_2 interval 30 min, pertussis toxin pretreatment			
Control		88 ± 3	4
AII	0.01	83 ± 1	5
AII	0.1	89 ± 1	4

There were two periods of stimulation either 30 or 45 min apart. The spontaneous outflow of radioactivity at the second stimulation (R_2) was expressed as a percentage of that at the first stimulation (R_1). See Methods for calculation details. Drugs were present 15 min before the second stimulation except polymyxin B, which was present 30 min before the second stimulation. Means and s.e.means are shown, n represents the number of experiments. * $P < 0.05$ Student's unpaired t test. Phorbol 12-myristate 13-acetate, PMA; angiotensin II, AII.

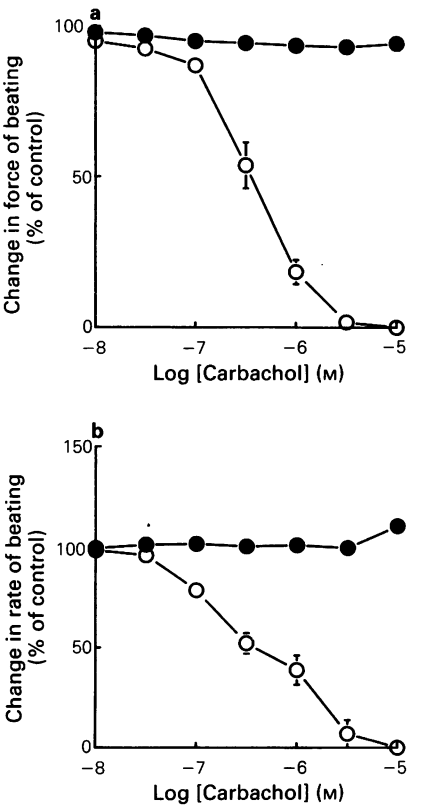

Figure 3 Effect of i.v. pertussis toxin pretreatment (1.5 μg protein per mouse) on the carbachol-induced decrease in (a) rate and (b) force of beating of isolated, spontaneously beating, mouse atria. Means and s.e.means (vertical lines) are shown for vehicle-pretreated mice (○, $n = 8$) and pertussis toxin-pretreated mice (●, $n = 12$). Where s.e.mean bars are absent they are smaller than the symbol size.

Pertussis toxin (155 μg protein ml^{-1} lysine/glycerol buffer) was a generous gift of Dr P. Marley, Commonwealth Serum Laboratories, Parkville, Australia and was stored at −20°C. The toxin was judged pure and homogeneous after bioassay and SDS gel electrophoresis (Marley *et al.*, 1985).

Results

Effect of phorbol ester on the enhancement of stimulation-induced outflow by angiotensin II

Angiotensin II (0.01 and 0.1 μM) significantly enhanced the S-I outflow of radioactivity from mouse atria incubated with [^{3}H]-noradrenaline (Figure 1). Phorbol 12-myristate 13-acetate (PMA), a protein kinase C activating phorbol ester (Ashendel, 1985), significantly enhanced the S-I outflow of radioactivity in a concentration-dependent manner (Figure 1). The significant enhancement of S-I outflow of radioactivity produced by angiotensin II (0.1 μM) was additive with that produced by the maximally effective concentration of PMA (0.1 μM, Figure 1).

Polymyxin B (70 μM), a putative selective inhibitor of protein kinase C (Mazzei *et al.*, 1982), by itself reduced the S-I outflow of radioactivity (Figure 2). Polymyxin B (70 μM) significantly inhibited the enhancement of S-I outflow of radioactivity produced by angiotensin II (0.1 μM, Figure 2).

None of the above drugs affected spontaneous outflow of radioactivity except polymyxin B (Table 1).

Effect of pertussis toxin

The spontaneous rates of beating of atria isolated from vehicle (396 ± 34, $n = 8$)-and pertussis toxin (438 ± 16, $n = 12$)-pretreated mice were not significantly different ($P > 0.1$, unpaired t test). Similarly the spontaneous force of beating of atria isolated from vehicle (0.47 ± 0.04 mN, $n = 8$)- and pertussis toxin (0.50 ± 0.05 mN, $n = 12$)-pretreated mice were not significantly different ($P > 0.1$, unpaired t test).

Carbachol (0.01–10 μM) produced a concentration-dependent decrease in force and rate of beating in vehicle-treated mice (Figure 3). However, in spontaneously beating atria from mice pretreated with pertussis toxin (1.5 μg protein per mouse) carbachol had no effect on either parameter.

There were no significant differences in the fractional S-I outflow of radioactivity from atria of vehicle and pertussis toxin pretreated mice (see Figures 1 and 4 legends, $P > 0.05$, Student's unpaired t test). Angiotensin II (0.01 and 0.1 μM) produced a significant enhancement of S-I outflow of radioactivity from atria of untreated mice (Figure 4),

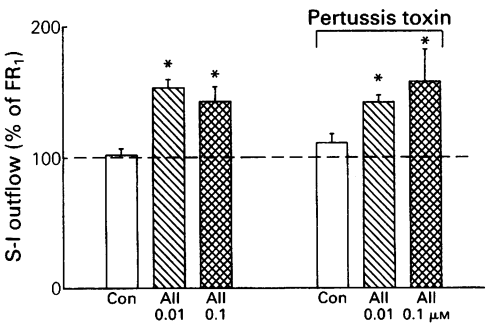

Figure 4 The effect of pretreatment of mice with pertussis toxin on the enhancement of fractional stimulation-induced (S-I) outflow of radioactivity produced by angiotensin II from mouse atria incubated with [^{3}H]-noradrenaline. There were two periods of stimulation (5 Hz for 60 s each) 30 min apart, angiotensin II (AII, 0.01 and 0.1 μM) was present 15 min before the second stimulation. The fractional S-I outflow of radioactivity in the second period (FR_2) of stimulation is expressed as a percentage of that in the first (FR_1). The absolute FR_1 value as a percentage of total tissue radioactivity for pertussis toxin pretreated mice was $1.38 \pm 0.13\%$ ($n = 13$). The columns represent means and the vertical bars s.e.means. * Significantly different from control (Con, $P < 0.05$, Scheffé's test after two way analysis of variance). The number of experiments in each group is given in Table 1.

and this enhancement of S-I outflow of radioactivity produced by angiotensin II was not affected by pertussis toxin pretreatment (Figure 4).

Discussion

The present study was conducted in mouse atria previously incubated with [^{3}H]-noradrenaline and the stimulation-induced (S-I) outflow of radioactivity was used as an index of noradrenaline release (see Johnston *et al.*, 1987). Angiotensin II significantly enhanced S-I outflow of radioactivity. In a previous study in mouse atria this enhancement of release by angiotensin II was blocked by saralasin (Rajanayagam *et al.*, 1989), suggesting that the effect is mediated by angiotensin II receptors. These observations have been made in a wide variety of sympathetically innervated tissues (see reviews by Starke, 1977; Zimmerman, 1981) and suggest that sympathetic nerve endings possess prejunctional facilitatory angiotensin II receptors.

The question arises as to the mechanism of action of angiotensin II in enhancing noradrenaline release. A distinct possibility is that it involves the phospholipase C second messenger system (Fisher & Agranoff, 1987). One arm of the phospholipase C system involves activation of protein kinase C (Nishizuka, 1986; Berridge, 1987) and in some tissues, such as rat ventricular myocytes, there is direct biochemical evidence that protein kinase C is involved in the effects of angiotensin II (Dösemeci *et al.*, 1988). In the present study a protein kinase C activating phorbol ester, PMA (Ashendel, 1985), caused significant enhancement of S-I outflow of radioactivity in mouse atria. This finding is consistent with other observations that phorbol esters increase S-I noradrenaline release from sympathetic neurones (Wakade *et al.*, 1985; Shuntoh & Tanaka, 1985; Ishac & De Luca, 1988; Musgrave & Majewski, 1989) and suggests that protein kinase C is involved in the facilitation of noradrenaline release. If the protein kinase C pathway is maximally activated by PMA then agents acting through this pathway should not enhance noradrenaline release. However, when noradrenaline release was increased by a maximally-effective concentration of PMA (0.1 μM), angiotensin II in the presence of PMA still enhanced the S-I outflow of radioactivity and its effect was additive with that of PMA. This suggests that angiotensin II does not produce enhancement of noradrenaline release through activation of protein kinase C.

Polymyxin B has been shown to be a selective inhibitor of protein kinase C (Mazzei *et al.*, 1982). Polymyxin B (100 μM) has been found to inhibit transmitter noradrenaline release and the facilitatory

effect of phorbol dibutyrate on noradrenaline release from rabbit hippocampal slices and this was attributed to an effect on protein kinase C (Allgaier & Hertting, 1986). In the present study polymyxin B (70 μM) was used since it blocked the facilitatory effect of PMA on noradrenaline release in mouse atria (Musgrave & Majewski, 1989). By itself polymyxin B inhibited the S-I outflow of radioactivity. Furthermore, polymyxin B strongly inhibited the enhancement of the S-I outflow of radioactivity produced by angiotensin II. At first sight this suggests that angiotensin II facilitation of noradrenaline release does involve protein kinase C. This is the direct opposite of the conclusion reached from the interaction between PMA and angiotensin II discussed above.

In another study also using mouse atria we seriously questioned the specificity of polymyxin B for protein kinase C (Musgrave & Majewski, 1989). It was found that polymyxin B, in a concentration-dependent manner (7–70 μM) reduced the facilitatory effect of PMA on stimulation-induced noradrenaline release (Musgrave & Majewski, 1989). Although this may be evidence for polymyxin B acting through protein kinase C, polymyxin B (7–70 μM) also reduced the facilitatory effect of 8-bromo-3′:5′-cyclic adenosine monophosphate (8Br cyclic AMP) on noradrenaline release in the same concentration-dependent manner. These results with 8Br cyclic AMP are especially pertinent since this agent is known to act through protein kinase A (Beebe *et al.*, 1984). Thus polymyxin B appears to be non-protein kinase C selective. In further support of this, polymyxin B also inhibited the noradrenaline release-enhancing effects of a variety of unrelated agents in mouse atria: idazoxan, tetraethylammonium, high frequency (10 Hz) but not low frequency (5 Hz) field stimulation (Musgrave & Majewski, 1989). The mechanism of action of polymyxin B in this regard is unclear. For example, although polymyxin B blocked the release-enhancing effect of idazoxan (which increases release by blocking α_2-autoreceptors, see Starke, 1977), it did not prevent the inhibitory effect of the α_2-adrenoceptor agonist clonidine on noradrenaline release (Musgrave & Majewski, 1989). This suggests that polymyxin B was blocking only release-enhancing effects and not the prejunctional α_2-adrenoceptor mechanism itself. In view of these findings we doubt that the ability of polymyxin B to block the facilitatory effect of angiotensin II on noradrenaline release is necessarily evidence for an involvement of protein kinase C in prejunctional angiotensin II effects.

Angiotensin II receptors have been shown to be linked to phospholipase C through a guanine nucleotide-binding protein which can be inactivated by pertussis toxin (Pfeilschifter & Bauer, 1986; Gaul *et al.*, 1988). Pertussis toxin was therefore used to determine if this was also true in sympathetic nerves. First, atria from mice pretreated with pertussis toxin were assessed for the effectiveness of the toxin treatment by examining the cardiac effects of the muscarinic cholinoceptor agonist carbachol. Pretreatment of mice with pertussis toxin abolished the fall in rate of beating and force of beating produced by carbachol in spontaneously beating atria. As the effects of carbachol on force and rate of beating are mediated through a pertussis toxin-sensitive guanosine 5′-triphosphate (GTP)-binding protein (Endoh *et al.*, 1985; Martin *et al.*, 1985; Pfaffinger *et al.*, 1985), this suggests that the toxin treatment was effective.

The enhancement of S-I outflow of radioactivity by angiotensin II was maintained in atria from mice pretreated with pertussis toxin when compared to results in atria from untreated mice. This suggests that if angiotensin II receptors are linked to phospholipase C, the GTP-binding protein involved is not pertussis toxin-sensitive. This does not necessarily imply that prejunctional angiotensin II receptors are not linked to phospholipase C, only that a pertussis toxin-sensitive GTP-binding protein is not involved. In cultured mesangial cells (Pfeilschifter & Bauer, 1986) and cultured smooth muscle cells (Gaul *et al.*, 1988) of the rat the stimulation of phospholipase C by angiotensin II was inhibited by pertussis toxin. However, studies in rat hepatocytes (Johnson & Garrison, 1987) and anterior pituitary cells (Bokaert *et al.*, 1988) demonstrated that angiotensin II receptor-mediated stimulation of phospholipase C was unaffected by pertussis toxin. These results suggest that the pertussis toxin sensitivity of the GTP-binding protein linking angiotensin II receptors to phospholipase C varies between tissues.

Taken together, the above results suggest that if angiotensin II modulates noradrenaline release from sympathetic nerves through the phospholipase C second messenger system, then the angiotensin II receptor is not linked to phospholipase C through a pertussis toxin-sensitive GTP-binding protein. Further, these results indicate that the diacylglycerol/protein kinase C arm of this pathway is unlikely to be involved in angiotensin II receptor-mediated facilitation of noradrenaline release from sympathetic nerves. The present study does not rule out the possible involvement of the other arm of the phospholipase C pathway, inositol trisphosphate generation.

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Swim-stress-induced antinociception in young rats

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1 Opioid and non-opioid mechanisms have been implicated in the phenomenon of stress-induced antinociception in adult rodents. We have studied stress-induced antinociception in developing rats and characterized differences in the neurochemical basis of this effect in pre- and post-weanling animals.

2 Twenty and 25 day old rats were stressed using warm water (20°C) swimming for 3 or 10 min periods and antinociception was assessed by the tail immersion test (50°C).

3 A 3 min swim in 20 and 25 day old rats produced marked antinociception which was blocked by naloxone, Mr 1452, 16-methyl cyprenorphine and levallorphan but not Mr 1453 or N-methyl levallorphan. The δ-opioid receptor antagonist ICI 174,864 attenuated stress-induced antinociception in 25 day old rats but was without effect in 20 day old animals.

4 A 10 min swim in 25 day old rats produced antinociception which was non-opioid in nature. In contrast, antinociception was not observed in 20 day old rats after a 10 min swim-stress.

5 Pretreatment of animals with dexamethasone blocked 3 min swim-stress antinociception in 20 and 25 day old animals but had no effect on antinociception induced by a 10 min swim.

6 Swim-stress-induced antinociception can be observed in young rats and dissociated into opioid and non-opioid types dependent on the duration of swimming stress. The non-opioid type appears to develop more slowly and cannot be observed in preweanling rats. The opioid type is a predominantly μ-receptor phenomenon in preweanling animals but δ-receptor components are observable in postweanling rats.

Introduction

There is a substantial literature which demonstrates the involvement of endogenous opioid peptides in the control of stress in both experimental animals and in man. One phenomenon which has been extensively studied in adult rodents is that of stress-induced antinociception (SIA). For example, stressors such as footshock (Madden *et al.*, 1977), swimming (Bodnar *et al.*, 1978a), 2-deoxy-D-glucose treatment (Bodnar *et al.*, 1978b), food deprivation (McGivern *et al.*, 1979), and immobilisation (Amir & Amit, 1978) have all been shown to produce antinociception in the adult rat. The receptor systems involved in SIA are stressor-dependent and opioid and/or non-opioid mechanisms have been implicated (see Bodnar, 1984). There are relatively few studies of SIA in developing animals but these indicate that this effect can be observed in the neonate (Bardo *et al.*, 1981; Hamm & Knisely, 1984; 1987; Kehoe & Blass, 1986). We describe here studies on SIA in 20 and 25 day old rats using warm water swimming as the stress procedure, and demonstrate that the nature of this response is markedly different at these postnatal ages.

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Methods

Animals and experimental conditions

Wistar albino rats (University of Surrey strain) of either sex were used in all experiments and maintained at a constant temperature (21 ± 1°C) and light/dark cycle (lights on at 07 h 00 min). Litters were culled to 8 pups per dam at birth and where possible cross-fostered. Neonates were weaned at

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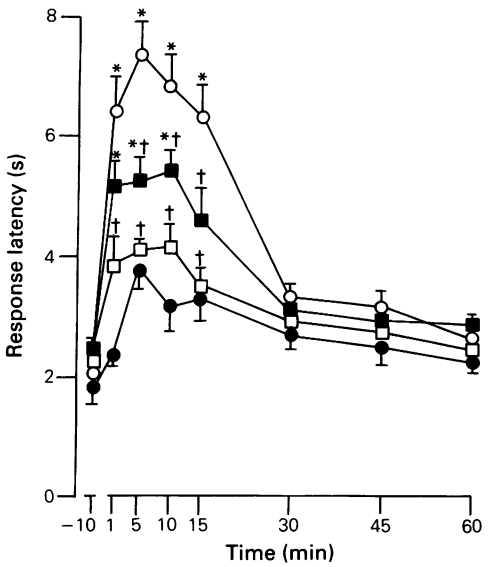

Figure 1 Effect of naloxone on antinociception induced by a 3 min swim in 20 day old rats. Values represent means and vertical lines s.e.mean for groups of 10 animals. (●) Saline injected, unstressed; (○) saline injected, swim-stressed; (■) naloxone ($1\,mg\,kg^{-1}$), swim-stressed; (□) naloxone ($10\,mg\,kg^{-1}$), swim-stressed. Significant differences from non-stressed controls are indicated by $*P < 0.05$ and from swim-stressed controls by $\dagger P < 0.05$.

day 21. All experimental procedures were carried out in a quiet, windowless, air-conditioned laboratory and animals equilibrated to these conditions for 2 h before experimentation. Twenty day old pups remained with the mother, except during drug administration, swimming and antinociceptive testing, to minimize stress due to maternal deprivation. Each series of determinations represents measurements made on five separate litters on at least two separate days to avoid inter-litter and inter-day variation. All procedures were carried out between 13 h 00 min and 17 h 00 min.

Swim-stress procedures and antinociceptive testing

Each litter was divided into four treatment groups. One group received 0.9% saline i.p. and remained in the home cage as a control group. The other 3 groups were injected i.p. with saline or appropriate drug (using a blind procedure and a dose volume of $0.1\,ml\,20\,g^{-1}$) and 10 min later placed individually in 20°C water for 3 or 10 min. The water was contained in 2 litre beakers (14 cm in diameter; 12 or 20 cm deep for 20 and 25 day old pups, respectively). At the end of the swimming period pups were gently dried before antinociceptive testing. For studies with dexamethasone, rats were injected 24 h ($0.4\,mg\,kg^{-1}$) and 1 h ($0.2\,mg\,kg^{-1}$) before swimming as described by Lewis *et al.* (1980). Rectal temperatures were measured before drug administration and after swimming stress using an Edale temperature probe.

Antinociception was assessed by the tail immersion test (50°C) as described by Kitchen *et al.* (1984) for neonatal rats. Nociceptive responses were measured immediately before drug administration and 1, 5, 10, 15, 30, 45 and 60 min following the swim-stress. Treatment group means were statistically compared using analysis of variance and Dunnett's test.

Drugs

Dexamethasone was purchased from Sigma Chemical Co., U.K. The following were gifts: naloxone hydrochloride (Du Pont Pharmaceuticals); 16-methyl cyprenorphine (M8008; Reckitt & Colman); Mr 1452/3 ((+)/(−)-5,9-α-dimethyl-2-(3-furylmethyl) -2'-hydroxy-6,7-benzomorphan methane sulphonate; Boehringer Ingelheim); levallorphan (Roche Products); N-methyl levallorphan (Sanofi Research); ICI 174,864 (N,N-bisallyl-Tyr-Aib-Aib-Phe-Leu-OH, Aib = α-amino-isobutyric acid; I.C.I. Pharmaceuticals). All drugs were dissolved in saline with the exception of ICI 174,864 which was suspended in saline and diluted with an equivalent volume of 0.1 M L-arginine (Sigma) and 16-methyl cyprenorphine which was dissolved in 0.1 M HCl and neutralised with $NaHCO_3$.

Results

Nociceptive latencies in 20 and 25 day old rats not subjected to swimming stress showed small but significant increases after intraperitoneal injection of saline (Figures 1–6). This effect was most marked 15 min after injection and thereafter nociceptive latencies fell to pre-injection values. The doses of the opioid antagonists levallorphan, N-methyl levallorphan, naloxone, Mr 1452/3, 16-methyl cyprenorphine and ICI 174,864 used in the current study had no effect on nociceptive latencies in unstressed 20 or 25 day old rats (data not shown). In addition, no overt behavioural effects were observed for any of these antagonists.

A 3 min swim at 20°C in both 20 and 25 day old rats produced marked antinociception which became evident 1 min after the swimming period and persisted for at least 15 min. At both ages this effect was significantly attenuated by naloxone (1 and $10\,mg\,kg^{-1}$) (Figures 1 and 2). In 20 day old rats antinociception induced by a 3 min swimming period

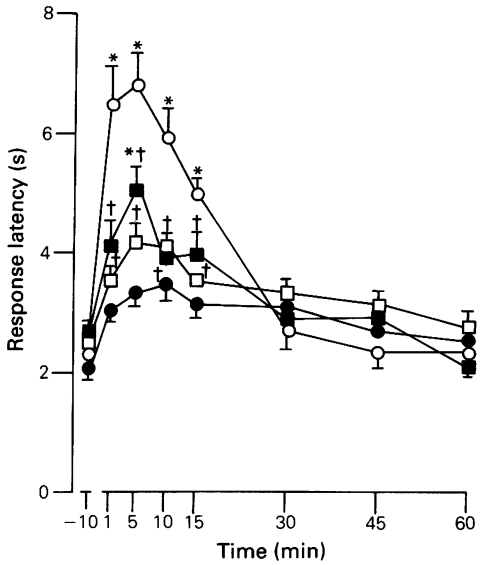

Figure 2 Effect of naloxone on antinociception induced by a 3 min swim in 25 day old rats. Values represent means and vertical bars s.e.mean for groups of 10 animals. (●) Saline injected, unstressed; (○) saline injected, swim-stressed; (■) naloxone (1 mg kg^{-1}), swim-stressed; (□) naloxone (10 mg kg^{-1}), swim-stressed. Significant differences from non-stressed controls are indicated by * $P < 0.05$ and from swim-stressed controls by † $P < 0.05$.

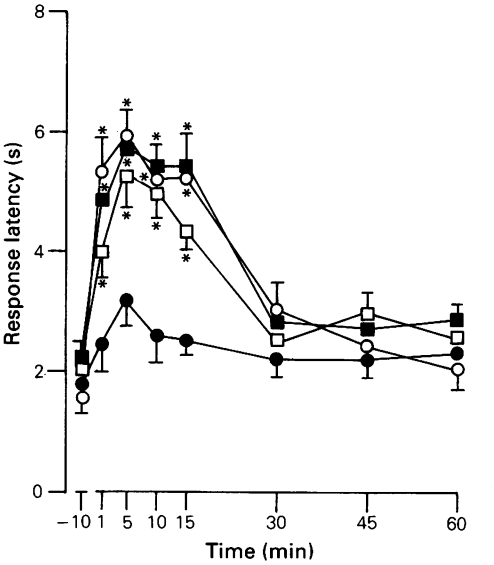

Figure 3 Effect of ICI 174,864 on antinociception induced by a 3 min swim in 20 day old rats. Values represent means and vertical lines s.e.mean for groups of 10 animals. (●) Vehicle injected, unstressed; (○) vehicle injected, swim-stressed; (■) ICI 174,864 (1 mg kg^{-1}), swim-stressed; (□) ICI 174,864 (10 mg kg^{-1}), swim-stressed. Significant differences from non-stressed controls are shown by * $P < 0.05$.

Table 1 Effects of various opioid antagonists on peak antinociceptive effects of 3 min swimming stress in 20 day old rats

Drug treatment	*Tail immersion latencies* (s)
Vehicle control	5.8 ± 0.3
Levallorphan (10 mg kg^{-1})	3.4 ± 0.3*
N-methyl levallorphan (10 mg kg^{-1})	5.3 ± 0.3
Vehicle control	6.5 ± 0.5
Mr 1452 (10 mg kg^{-1})	3.2 ± 0.3*
Mr 1453 (10 mg kg^{-1})	5.6 ± 0.4
Vehicle control	5.5 ± 0.3
16-methyl cyprenorphine (1 mg kg^{-1})	3.5 ± 0.3*
16-methyl cyprenorphine (10 mg kg^{-1})	3.0 ± 0.2*

Results are expressed as mean values ± s.e.mean for groups of 10 animals. Peak effects represent measurements 5 min after swimming stress. The time course profile of antagonism, where observed, was similar to that shown for naloxone (Figure 1). Significant differences from swim-stressed controls are denoted by * $P < 0.05$.

was blocked by Mr 1452 (10 mg kg^{-1}) but not its stereoisomer Mr 1453 (Table 1). In addition, 16-methyl cyprenorphine (1 and 10 mg kg^{-1}) and levallorphan (10 mg kg^{-1}) blocked swim-stress-induced antinociception in 20 day old rats but a 10 mg kg^{-1} dose of the quaternary derivative N-methyl levallorphan was ineffective (Table 1). The δ-receptor antagonist ICI 174,864 (1 and 10 mg kg^{-1}) significantly reduced 3 min swim-stress antinociception in 25-day old rats but exhibited no antagonism in 20 day old animals (Figures 3 and 4).

Twenty day old rats subjected to a 10 min swimming period showed no increase in nociceptive latencies up to 1 h after swimming (Figure 5). In contrast, 25 day old rats exhibited antinociception after a 10 min swim, an effect not antagonized by up to 10 mg kg^{-1} naloxone (Figure 6) or Mr 1452 (data not shown).

Pretreatment of animals with dexamethasone blocked 3 min swim-stress antinociception in 20 and 25 day old animals but was without effect upon antinociception induced by a 10 min swimming period in 25 day old rats (Table 2). Dexamethasone treatment *per se* was without effect on nociceptive latencies.

Marked hypothermia was observed immediately after swimming stress in both 20 day (1 min post

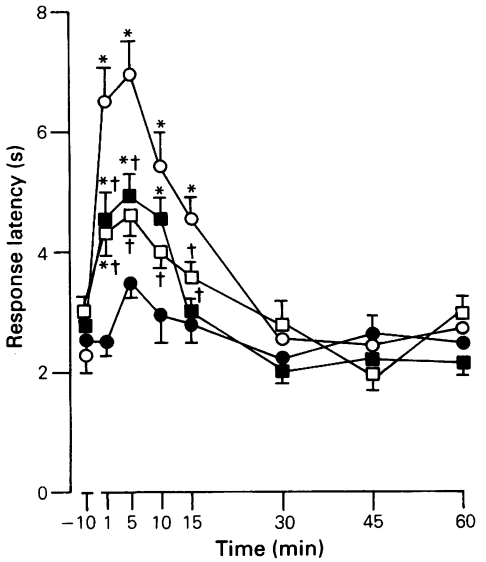

Figure 4 Effect of ICI 174,864 on antinociception induced by a 3 min swim in 25 day old rats. Values represent means and vertical lines s.e.mean for groups of 10 animals. (●) Vehicle injected, unstressed; (○) vehicle injected, swim-stressed; (■) ICI 174,864 (1 mg kg^{-1}), swim-stressed; (□) ICI 174,864 (10 mg kg^{-1}), swim-stressed. Significant differences from non-stressed controls are indicated by * $P < 0.05$ and from swim-stressed controls by † $P < 0.05$.

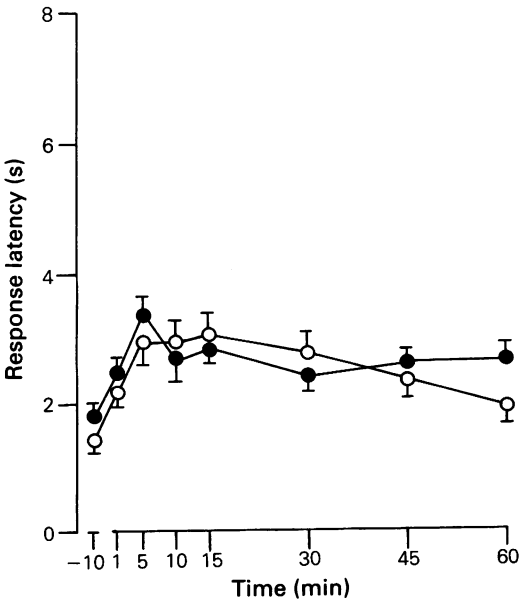

Figure 5 Effect of a 10 min swim on tail immersion latencies in 20 day old rats. Values represent means and vertical lines s.e.mean for groups of 10 animals. (●) Saline injected, unstressed; (○) saline injected, swim-stressed.

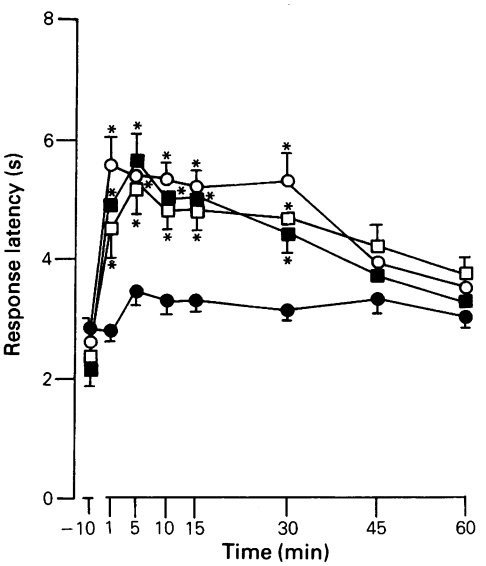

Figure 6 Effect of naloxone on antinociception induced by a 10 min swim in 25 day old rats. Values represent means and vertical lines s.e.mean for groups of 10 animals. (●) Saline injected, unstressed; (○) saline injected, swim-stressed; (■) naloxone (1 mg kg^{-1}), swim-stressed; (□) naloxone (10 mg kg^{-1}), swim-stressed. Significant differences from non-stressed controls are shown by * $P < 0.05$.

swim: 36.8 ± 0.2 vs 28.2 ± 0.2°C (3 min); 37.1 ± 0.1 vs 23.7 ± 0.2°C (10 min)) and 25 day old rats (1 min post swim: 37.8 ± 0.2 vs 31.5 ± 0.9°C (3 min); 37.8 ± 0.1 vs 24.6 ± 0.6°C (10 min)). This response,

Table 2 Effect of dexamethasone treatment on peak antinociceptive effects of 3 and 10 min swimming stress in 20 and 25 day old rats

	Tail immersion latencies (s)	
Age/stress	*Control*	*Dexamethasone treated*
20d/unstressed	2.9 ± 0.2	3.0 ± 0.3
20d/3 min swim	5.5 ± 0.3*	3.8 ± 0.2*†
25d/unstressed	3.4 ± 0.3	3.1 ± 0.4
25d/3 min swim	6.7 ± 0.5*	4.2 ± 0.3†
25d/unstressed	3.6 ± 0.2	3.3 ± 0.2
25d/10 min swim	5.5 ± 0.2*	5.3 ± 0.2*

Results are expressed as mean values ± s.e.mean for groups of 10 animals. Peak effects represent measurements 5 min after swimming stress. The time course profile of antagonism, where observed, was similar to that shown for naloxone (Figures 1 and 2). Significant differences from non-stressed controls are indicated by * $P < 0.05$ and from swim-stressed controls by † $P < 0.05$.

which was not accompanied by pronounced behavioural depression, persisted for 30 and 45 min respectively for 3 and 10 min swims in 20 day old animals but for 25 day old rats the duration of hypothermia was shorter (15 and 30 min respectively for 3 and 10 min swims). Naloxone at 1 and 10 mg kg^{-1} did not significantly alter the hypothermic effects of swimming (data not shown).

Discussion

Short warm water swims in our study produced SIA in 20 and 25 day old rats confirming other ontogenetic studies using maternal deprivation (Kehoe & Blass, 1986), electric shock (Hamm & Knisely, 1984) and cold water swimming (Hamm & Knisely, 1987) as stressors. The temperature and duration of swimming has been shown in adult rats to alter markedly the neurochemical basis of the SIA. In the adult, short swimming periods in warm water are sensitive to naltrexone indicating opioid receptor mediated antinociception, whereas longer or cold water swims are insensitive to opioid antagonists (Terman *et al.*, 1986). The reversal of short warm water swim SIA in 20 and 25 day old rats by the opioid antagonists naloxone and Mr 1452 but not Mr 1453 demonstrates that this phenomenon, as in the adult, is opioid receptor-mediated and also that it exhibits stereoselectivity. The relatively high concentration of naloxone required to antagonize completely this SIA might suggest the involvement of non-μ opioid receptor subtypes. It is, however, unlikely that κ- or δ-sites are involved in SIA in 20 day old rats since 16-methyl cyprenorphine which has a low affinity for κ-receptors (Smith, 1987) completely blocks this response and ICI 174,864, a selective δ-antagonist (Cotton *et al.*, 1984) is without effect in this age group. However, it should be pointed out that there do appear to be neurochemical dissimilarities in the SIA produced by short swims at 20 and 25 days of age, since the latter is antagonized by the δ-antagonist ICI 174,864. This suggests that δ-receptors may be important in this phenomenon at later postnatal ages and accords with the delayed development of this receptor subtype (McDowell & Kitchen, 1986) and with the work of others who have shown antagonism of SIA by ICI 174,864 in mice (Hart *et al.*, 1985). Nevertheless most lines of evidence, including antagonism of SIA by levallorphan, suggest that μ-sites may play a role in this antinociceptive response. Moreover, the lack of effect of the quaternary derivative N-methyl levallorphan which does not pass the blood brain barrier and which acts as a peripheral opioid antagonist *in vivo* (Dragonetti *et al.*, 1983; personal observations), confirms the central nature of swimming SIA in young rats. This contrasts with results in the adult showing that quaternary naloxone significantly reduces continuous footshock stress indicating a peripheral component (Chance & Nelson, 1986).

The possibility that the observed SIA does not truly reflect a reduced pain perception and is secondary to alterations in, for example, thermoregulation, was assessed by monitoring rectal temperature. Like others we were able to dissociate the hypothermic and antinociceptive effects of swim stress (Bodnar *et al.*, 1978c; Terman *et al.*, 1986) and, moreover, the hypothermia was unaffected by naloxone. It therefore appears that true SIA can be observed in young rats.

As in adult rats, a naloxone-resistant SIA can be observed by longer swimming in 25 day old rats, effects which have also been observed with increasing stress severity using footshock (Terman *et al.*, 1984). As in adults, this antinociception is not antagonized by dexamethasone, in contrast to the shorter swimming SIA, suggesting involvement of the hypothalamus-pituitary-adrenal axis only in the opioid-mediated SIA (Lewis *et al.*, 1980). We were unable to observe antinociception in 20 day old animals subjected to a 10 min swim, suggesting that the neurochemical basis for non-opioid SIA does not develop until after the third postnatal week.

None of the opioid antagonists *per se* produced hyperalgesia in these young animals, an effect which has been obtained for naloxone in some studies in adult rats (Jacob & Ramabadran, 1977). In addition there was no indication of any μ-agonist activity which has been observed with the δ-antagonist ICI 174,864 and its metabolite product in isolated tissue studies (Cohen *et al.*, 1986).

In conclusion, swim SIA can be observed in 20 day and 25 day old rats. Short swims produce opioid-mediated antinociception, which appears to be predominantly a μ-receptor phenomenon but shows a δ-receptor component at the later age. Longer swims produce a non-opioid antinociception only at the older age suggesting that non-opioid pain modulatory systems develop more slowly than those dependent on endogenous opioids.

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Development of cholinergic nerve transmission in the chick oesophagus

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1 The onset and development of cholinergic mechanisms in the smooth muscle of the chick oesophagus were studied by estimating the changes in mechanical response and biochemical parameters between 9 days of incubation and 7 days after hatching.

2 Transmural and vagal nerve stimulation first evoked contraction in the oesophagus at 10 days and 11 days of incubation, respectively. These contractions were inhibited by atropine (1–2 μM) and potentiated by physostigmine (0.2 μM). On the other hand, hexamethonium (200 μM) had an inhibitory effect on vagal nerve stimulation but not on transmural nerve stimulation.

3 The relative amplitude of contraction induced by both vagal nerve and transmural stimulations compared to high K^+ (80 mM)-induced contractions, progressively increased with age in embryos up to 19 days of incubation.

4 The activity of choline acetyltransferase (ChAT), an enzyme synthesizing acetylcholine (ACh), also gradually increased in the oesophagus during the period from 9 days to 19 days of incubation, which was similar to the change in the nerve-mediated contraction. On the other hand, the cholinesterase activity reached a maximum at 13 days of incubation and decreased until 7 days after hatching.

5 The contractile response to ACh and binding sites of [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) were observed in the oesophagus at 9 days of incubation. The maximum response produced by ACh (300 μM) tended to be greater in early stages (9–13 days of incubation) than in later stages. The sensitivity estimated from pD_2 values increased up to 15 days of incubation. During the embryonic period, the number of muscarinic receptors estimated from the binding of [^{3}H]-QNB changed very little.

6 These results suggest that in the chick oesophagus, extrinsic and intrinsic cholinergic innervation start to function at 10 days and 11 days of incubation, respectively and continue to develop progressively up to the time of hatching. It seems likely that the functional and biochemical maturation of receptive mechanisms on the smooth muscle precede those of cholinergic innervation.

Introduction

The enteric ganglion cells in the intrinsic plexus are derived from the neural crest in the early embryonic stage (Le Douarin & Teillet, 1973) and differentiate into many types of neurones in the gut wall (cholinergic: Le Douarin, 1980; 5-hydroxytryptaminergic: Epstein *et al.*, 1980; peptidergic: Epstein *et al.*, 1983). The functional significance of these neurones is not fully understood. There are few reports available concerning the onset of functional innervation in the gastrointestinal tract. It was reported that these neurones begin to function in sequential manner and that the intrinsic neurone which functions first is cholinergic and excitatory in nature (Gershon & Thompson, 1973; Miyazaki *et al.*, 1982; Ito *et al.*, 1988). In the earliest stage at which the response to nerve stimulation appears,

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both the nerve elements and the smooth muscle cells were observed to be morphologically immature (Gershon & Thompson, 1973).

Morphological studies on the enteric nervous system indicated that the maturation of ganglion cells and synapses occurs slowly during the embryonic period (Bennett & Cobb, 1969; Daikoku *et al.*, 1975; Gershon *et al.*, 1981; Lolova, 1983a, b), suggesting that quantitative and qualitative changes in functional innervation may also take place during this period. Little is known concerning the functional maturation of cholinergic innervation of the smooth muscle in the course of embryonic development after the first appearance of the innervation.

In the chick oesophagus, autonomic innervation was reported to be predominantly parasympathetic cholinergic (Bowman & Everett, 1964; Ohashi & Ohga, 1967; Ohashi, 1971) as an inhibitory innervation has not been observed. If this is the case, the study of the development of cholinergic innervation would be somewhat simplified. Also, the responses to stimulation of extrinsic parasympathetic and intramural nerves can be subjected to a pharmacological analysis simultaneously (Everett, 1966).

Therefore, we examined the change of responses to nerve stimulation and to acetylcholine (ACh) in the oesophagus isolated with the vagus nerve from chick embryos, to determine the time of onset and maturation of cholinergic neuro-effector transmission in the course of embryonic development. In parallel with the functional study, biochemical studies on cholinergic enzymes and muscarinic receptors were performed.

Methods

Fertilized eggs and hatched chicks (Shaber Star Cross) were used. The eggs were maintained in a humidified incubator at 38 ± 0.5°C until they were used in the experiments. The age of the chicks which usually hatched at 21 days of incubation was designated as 0 days at hatching. The chicks examined were from 9 days of incubation to 7 days after hatching.

Organ bath study

A segment of oesophagus upward from the crop, about 0.5–1.5 cm long, was isolated together with or without the right vagus nerve and adhering jugular vein from the embryos or from the chicks; the latter had been killed by stunning. These preparations were placed either in a 20 ml organ bath containing Krebs solution (to test drug action) or in a 2 ml bath perfused at a rate of 2 ml min^{-1} with the solution (to test nerve stimulation). The composition of Krebs solution was as follows (mM): NaCl 118.0, KCl 4.75, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.5. The solution was saturated with a mixture of 95% O_2 and 5% CO_2 (pH 7.4) and kept at 37 ± 1°C. The oesophageal segment was allowed to equilibrate for 45–60 min before the start of any experimental procedure. The mechanical response of the oesophagus was recorded by means of an isotonic transducer (Nihon Kohden, TD-112S) and a pen writing recorder (Nihon Kohden, RJG-3002; Nihon Denshikagaku, U-228).

Because of the difficulty in dissecting the vagus nerve from other tissues, the vagus nerve was stimulated together with the jugular vein and glossopharyngeal nerve by use of a suction electrode (0.5–1 mm diameter) as described by Seno *et al.* (1978). Transmural nerve stimulation was applied through parallel Ag–AgCl electrodes (1×14 mm, separated by 6 mm) placed at either side of the oesophageal segment. Transmural stimulation was carried out in the presence of hexamethonium (C_6, 200 μM) in order to restrict the stimulation to the postganglionic nerves (Everett, 1966). Trains of rectangular pulses (0.5 ms for vagal stimulation or 1 ms for transmural stimulation, supramaximal voltage) at various frequencies (0.1–20 Hz) were delivered for 10 s by an electrical stimulator (Nihon Kohden, SEN-7013). Drugs were applied to the organ bath directly with a pipette or as constant infusion streams.

The tension applied to the preparation was 10–20 mg at 9–11 days of incubation; 35–45 mg at 13 days of incubation; 100–120 mg at 15 days of incubation; 200–250 mg at 17 days of incubation; 450–500 mg at 19 days of incubation and 1–2 g at 21 days of incubation to 2 days after hatching. As the amplitude of the responses induced by drugs and nerve stimulation depend on the length of preparations, all responses were expressed as a percentage of the response caused by the application of potassium chloride (high K^+, 80 mM).

Enzyme assay

After blotting, weighing and cutting into small pieces with scissors, the oesophagus was homogenized in 50 mM Na–K phosphate buffer (pH 7·4) containing 0.5% Triton X-100 with Polytron for 30 s. The homogenate was filtered through a nylon cloth (120 μm mesh) and was centrifuged for 15 min at 11,000 g at 0°C. The supernatant was used for enzyme assays. Protein in the homogenates and supernatant samples was measured according to Lowry *et al.* (1951) with bovine serum albumin used as a standard.

Choline acetyltransferase (ChAT) activity was assayed according to the radiochemical methods of

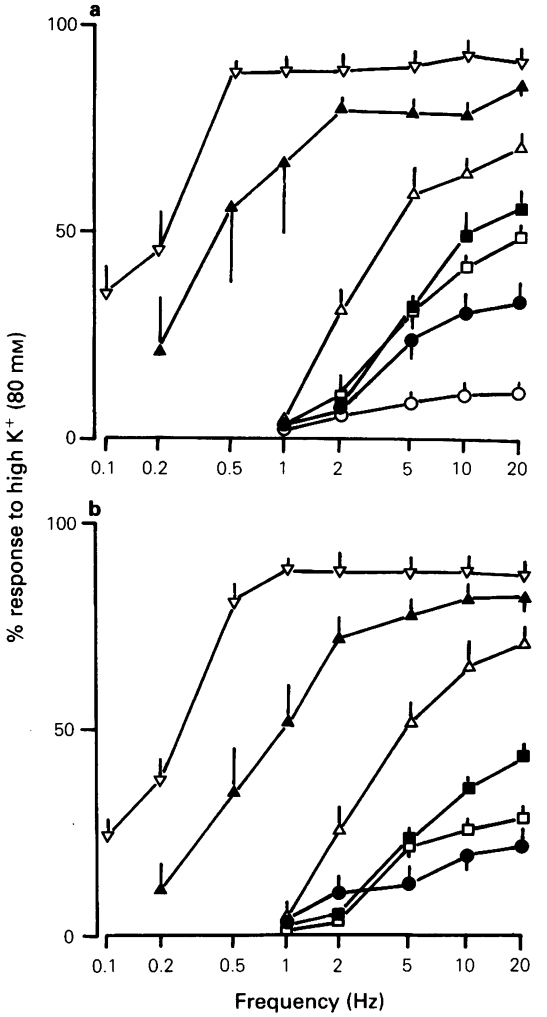

Figure 1 Relationships between frequency of electrical stimulation and amplitude of contractile response in the chick oesophagus at different ages. (a) Transmural nerve stimulation in the presence of hexamethonium (C_6, 200 μM). (b) Vagal nerve stimulation. Ordinate scale: the relative amplitude of responses expressed as a percentage of high K^+ (80 mM)-induced contraction. Abscissa scale: frequency of electrical stimulation for 10 s on a logarithmic scale. Each point shows the mean (n = 4–10) amplitude of the response of the preparation at 10 days (○), 11 days (●), 13 days (□), 15 days (■), 17 days (△), 19 days (▲) and 21 days (▽) of incubation. Vertical lines show s.e.mean.

Fonnum (1975): 90 μl of substrate solution containing 0.2 mM [^{14}C]-acetyl CoA (5 mCi mmol^{-1}), 50 mM sodium phosphate buffer (pH 7.4), 300 mM NaCl, 15 mM EDTA, 0.1 mM physostigmine and 10 mM choline chloride were added to 10 μl of the sample in a centrifuge tube. After the mixture was incubated at 37°C for 20 min, the reaction was stopped by adding ice-cold 10 mM phosphate buffer containing 0.2 mM ACh. Labelled ACh was extracted with ethylbutylketone containing sodium tetraphenylboron and its radioactivity was counted in a liquid scintillation counter. Cholinesterase (ChE) activity was measured according to Chiappinelli *et al.* (1976). The final concentrations of the incubation mixture were: 2 mM [^{14}C]-ACh (0.25 mCi mmol^{-1}), 50 mM sodium phosphate buffer (pH 7.2). Buffered substrate solution (90 μl) was added to each 10 μl sample. After incubation for 30 min at 37°C, [^{14}C]-ACh was removed with ethylbutylketone containing 15 mg sodium tetraphenylboron. The radioactivity of the [^{14}C]-acetate produced was counted.

Radioligand binding assay

The isolated oesophagus was homogenized in cold 50 mM phosphate buffer solution (pH 7.4). The homogenate was filtered through nylon mesh and the filtrate was diluted to an appropriate protein concentration with the buffer. Binding of [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) to muscarinic receptors in the homogenate was measured according to the method of Yamamura & Snyder (1974). The homogenate, containing 100–140 μg protein, was incubated with [^{3}H]-QNB (43.3 Ci mmol^{-1}) at 25°C for 60 min. The reaction was stopped by an addition of 4 ml of ice-cold buffer. Then, the mixture was passed through a filter (Whatman GF/C) and washed with 3 × 4 ml of the buffer. The radioactivity remaining on the filter was measured at about 40% efficiency. Specific binding was expressed as the differences between the binding in the presence and absence of atropine (10 μM). A Scatchard plot was derived from the data which were analysed by the least squares method. The binding of [^{3}H]-QNB was linearly related to the amount of protein in the incubation mixture up to approximately 300 μg.

Drugs

Drugs used and their source were as follows; acetylcholine chloride (ACh; Tokyo Kasei), atropine sulphate (Wako), physostigmine sulphate (physostigmine, Wako), 1-1-dimethyl 4-phenyl piperazinium iodide (DMPP, Tokyo Kasei), hexamethonium hydrochloride (C6; Tokyo Kasei), phentolamine mesylate (Ciba), tetrodotoxin (TTX, Sigma), [^{14}C]-ACh, [^{14}C]-acetyl CoA (Amersham) and [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB, Amersham).

Table 1 The effects of drugs on the responses of chick oesophagus induced by transmural (TMS) and vagal nerve stimulation (VS) at different ages

Age	*E10*	*E11*	*E13*	*E15*	*E17*	*E19*	*E21*
TMS (10 Hz)							
Control	13.5 ± 1.8 (7)	29.2 ± 3.1 (8)	40.8 ± 2.4 (11)	52.9 ± 3.3 (11)	67.4 ± 3.9 (7)	81.2 ± 3.2 (4)	93.0 ± 3.8 (4)
Atropine	0 (7)	15.8 ± 4.1 (7)	29.3 ± 4.3 (11)	20.0 ± 1.9 (11)	18.0 ± 3.9 (7)	15.1 ± 9.0 (4)	53.5 ± 8.8 (4)
Physostigmine	20.2 ± 1.6 (7)	42.6 ± 4.9 (5)	88.4 ± 4.0 (3)	—	—	—	—
VS (10 Hz)							
Control	0 (7)	16.1 ± 3.0 (10)	26.1 ± 1.5 (14)	36.8 ± 2.0 (11)	66.5 ± 3.3 (10)	83.6 ± 2.9 (8)	87.6 ± 2.5 (5)
Atropine	—	0 (6)	9.3 ± 3.5 (6)	18.0 ± 6.8 (4)	7.3 ± 2.7 (4)	25.7 ± 8.2 (6)	31.4 ± 4.4 (5)
C_6	—	3.3 ± 2.5 (4)	6.3 ± 2.4 (11)	6.6 ± 3.1 (9)	31.9 ± 6.0 (8)	83.6 ± 2.9 (4)	86.4 ± 3.1 (5)

The amplitude of the nerve-induced response obtained in the absence or presence of atropine (1–2 μM), physostigmine (0.2 μM) or hexamethonium (C_6, 200 μM) is expressed as the percentage of the response to high K^+ (80 mM). E represents the age of incubation in days. Values in parentheses indicate the number of samples.

Statistical analysis

All results were expressed as mean ± s.e.mean. The difference between the mean values for data were evaluated by Student's *t* test and a *P*-value of 0.05 or less was considered statistically significant.

Results

Mechanical responses to transmural and vagus nerve stimulation during development

Transmural nerve stimulation (10 Hz) for 10 s in the presence of C_6 (200 μM) did not elicit any response in the oesophagus at 9 days of incubation ($n = 7$). However, the application of high K^+ (80 mM) and ACh (20 μM) caused a contractile response, showing that the smooth muscle of the oesophagus can contract at this stage. The contractile response to transmural stimulation was first observed in all the preparation at 10 days of incubation and its amplitude increased depending on the stimulus frequency (Figure 1a). The stimulation of the vagus nerve first produced a small contraction in the preparation after 11 days of incubation (Figure 1b). In these early stages, the responses to both types of nerve stimulation persisted after the cessation of the stimulation (10 Hz) and were long-lasting (about 2 min, Figure 2a). The application of TTX (0.78 μM) to the perfusing solution abolished these contractile responses to both types of nerve stimulation.

The degree of contractile response induced by transmural or vagal nerve stimulation at different ages was estimated by comparison with the contraction caused by high K^+ (80 mM), which was used as an index of maximal contractile activity for each preparation. The relative amplitude of responses to transmural and vagal stimulations increased gradually up to 19 days or 21 days of incubation (Figure

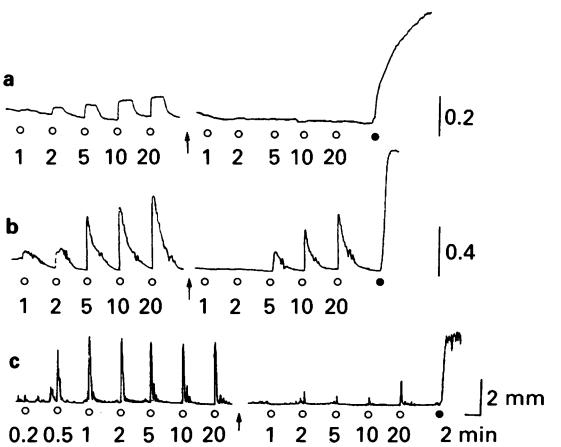

Figure 2 Effects of atropine on the responses to transmural nerve stimulation in developing chick oesophagus at different ages. Transmural nerve stimulation (○) was applied for 10 s to the oesophagus at 10 days of incubation in the presence of physostigmine (0.2 μM, a), 13 days of incubation (b) and 19 days of incubation (c). Arrows (↑) show the application of atropine (1 or 2 μM). Numbers associated with symbols refer to stimulus frequencies (Hz). After the end of stimulation, the preparation was perfused with high K^+ (80 mM, ●) solution.

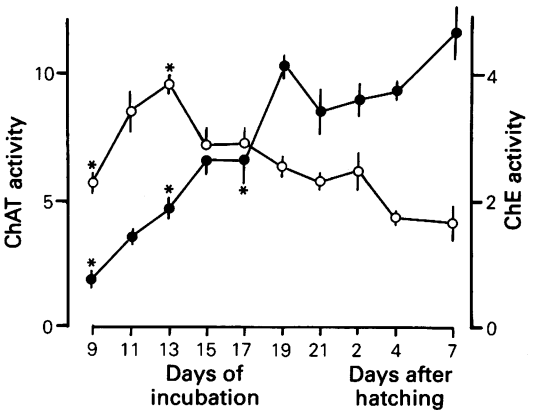

Figure 3 Developmental changes in activities of choline acetyltransferase (ChAT) and cholinesterase (ChE) in chick oesophagus. Ordinate scales: ChAT activity (nmol synthesized ACh $h^{-1} mg^{-1}$ protein, left) and ChE activity (μmol hydrolyzed ACh $h^{-1} mg^{-1}$ protein, right). Abscissa scale: developmental age (days). Each point shows the mean ($n = 3$–5) of ChAT (●) and ChE (○) activity in the oesophagus at a different age. Vertical lines show s.e.mean. * Significant difference ($P < 0.05$) from that of preceding age.

1 and Table 1). The response to both forms of stimulation in preparations between 10 and 17 days of incubation appeared at frequencies of 1 or 2 Hz and reached a maximum at 10 or 20 Hz. After 19 days of incubation, the nerve-mediated contraction appeared at a lower frequency (0.1 or 0.2 Hz) and its maximal response was obtained at 1 or 2 Hz (Figure 1). The duration of responses to both forms of nerve stimulation shortened from about 2 min at 10–13 days of incubation to about 15 s in duration after 17 days of incubation (Figure 2).

Thus, responses to transmural and vagal nerve stimulation were observed first at 10 days and 11 days of incubation, respectively and the amplitude of these responses increased and the duration decreased with age during the embryonic period.

Pharmacological analysis of the responses to nerve stimulation

The response to transmural stimulation at 10 days of incubation was abolished by atropine (0.2–1 μM, Figure 2a) even in the presence of physostigmine (0.2 μM, $n = 7$). After that age, atropine (1–2 μM) significantly inhibited the amplitude of the contractions but did not completely block them, especially at relatively high frequencies (5–10 Hz, Figure 2b and c, Table 1). On the other hand, atropine almost always blocked the response to lower frequencies (less than 2 Hz) at all stages of development. The ratio of the atropine-resistant component to the atropine-sensitive one in the response to transmural stimulation at 10 Hz tended to be somewhat higher in the preparations at 11 and 13 days of incubation (Figure 2b, Table 1). The atropine-resistant contraction was also observed in the response to stimulation of the vagus nerve (Table 1). Although the response to both forms of nerve stimulation gradually increased up to 19 days of incubation, the atropine-resistant responses at 10 Hz changed little during this period (Table 1). These responses to both types of nerve stimulation in the presence of atropine were not affected by an α-adrenoceptor blocker phentolamine (2.7 μM) but were blocked completely by TTX (0.78 μM), thus indicating the response was of neural origin.

After treatment with physostigmine (0.2 μM) for 30 min, the preparations at 10 to 13 days of incubation responded to transmural stimulation at lower frequencies (0.2 Hz) and the amplitude of the response at each frequency was also increased. The effect was somewhat less at 10 days and 11 days of incubation than at 13 days of incubation (Table 1). In the preparations at 13 days of incubation, the response to transmural stimulation (10 Hz) was much augmented in amplitude by physostigmine ($223.7 \pm 20.6\%$, $n = 3$).

At 11 days of incubation, when the response to vagus nerve stimulation was first detected, treatment with the ganglionic blocking agent, C_6 (200 μM) for 20 min caused blockade or extreme inhibition (to $16 \pm 10\%$ of the control, $n = 4$) of the response to vagus nerve stimulation at 10 Hz (Table 1), suggesting that the response was mediated by excitation of preganglionic nerves at this stage. The effect of C_6 decreased after 15 days of incubation. On the 21st day of incubation, only responses at the low frequency (0.1–2 Hz) were sensitive to C_6.

These findings suggest that the contractile responses to transmural and vagal nerve stimulation were mainly mediated by excitation of intramural cholinergic nerves, and that ganglionic transmission appeared at 11 days of incubation.

The developmental change of ChAT and ChE activity in the chick oesophagus

ChAT and ChE activity were measured in the oesophagus at various ages to investigate whether the increase of the response induced by nerve stimulation was related to the biochemical maturation of the cholinergic innervation. Low activity of ChAT, an enzyme synthesizing ACh, was already detected in the oesophagus at 9 days of incubation (1.90 ± 0.19 nmol ACh synthesized $h^{-1} mg^{-1}$ protein). ChAT activity, thereafter, increased progressively and was five times greater at 19 days of

incubation (10.39 ± 0.40) than it was at 9 days of incubation (Figure 3). ChAT activity decreased at the time of hatching, followed by a slight increase until 7 days after hatching. In contrast to ChAT activity, a relatively high value of ChE activity (2.28 ± 0.18 μmol ACh hydrolyzed $h^{-1} mg^{-1}$ protein) was measured even at 9 days of incubation (Figure 3). This activity reached a peak value (3.84 ± 0.15) at 13 days of incubation and thereafter decreased progressively up to 7 days after hatching (1.67 ± 0.39). The increase in ChAT activity but not ChE activity was consistent with the results of the nerve-induced response, suggesting that the developmental increase of neuronal responses was in part due to the biochemical maturation of the nerve.

Responses to ACh in the developing chick oesophagus

ACh (20 μM) caused contraction in the oesophagus even at 9 days of incubation. The contractile response to ACh was blocked by atropine (1 μM, $n = 4$), suggesting that the response was mediated by muscarinic receptors. From the cumulative dose-response curve to ACh (0.1–300 μM) in each oesophagus, the sensitivity (pD_2 value) of the oesophagus to ACh and the maximum contractile response were estimated at each developmental age (Figure 4). The mean pD_2 values for ACh increased significantly during the period between 9 days of incubation (5.01 ± 0.10, $n = 4$) and 15 days of incubation (5.71 ± 0.12, $n = 7$). The mean pD_2 values, thereafter, changed very little up to 2 days after hatching (5.98 ± 0.11, $n = 3$, Figure 4). On the other hand, as shown in Figure 4, the maximum response obtained to ACh (300 μM) was greater in the early stages (9–13 days of incubation) than in later stages. However, in contrast to the maximum response to transmural stimulation (10 Hz), the maximum response to ACh was relatively constant and was about the same as the high K^+ (80 mM)-induced contractions throughout development.

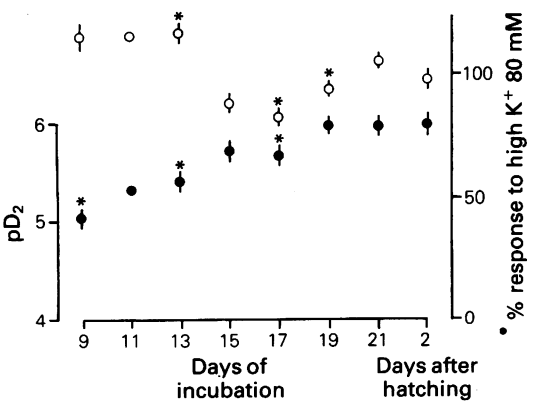

Figure 4 Developmental changes in maximum contractile response and sensitivity (pD_2 value) to acetylcholine (ACh) in the chick oesophagus. Ordinate scales: pD_2 value (left) and maximum response (right) to ACh, expressed as a percentage of high K^+ (80 mM)-induced response. Abscissa scale: developmental age (days). Each point shows the mean ($n = 3$–9) of pD_2 value (●) and maximum response (○), estimated from cumulative dose-response curves in each oesophagus at a different age. Vertical lines show s.e.mean. * Significant difference ($P < 0.05$) from that of the preceding age.

The binding site of [^{3}H]-QNB in the developing oesophagus

The binding of [^{3}H]-QNB to homogenates was saturable with high affinity and the Scatchard plot derived from the specific binding data indicated a straight line with a correlation coefficient of 0.974 ± 0.005 ($n = 13$), suggesting a single binding site. The specific binding site of [^{3}H]-QNB was observed in the preparations at 9 days of incubation and its maximum binding sites (B_{max}) per mg protein, estimated from the Scatchard plot, were not significantly different ($P > 0.05$) at 15 days of incubation (Figure 5a). The B_{max} value increased slightly at 21 days of incubation and a maximal increase was observed in preparations at 6–8 days after hatching. This suggests that the number of binding sites for [^{3}H]-QNB per mg protein of oesophagus changed very little at least during the embryonic period. The apparent dissociation constant (K_d value) of [^{3}H]-QNB did not differ significantly at different developmental ages except for that at 9 days of incubation. The pD_2 value at 9 days of incubation was higher than at other ages (Figure 5b).

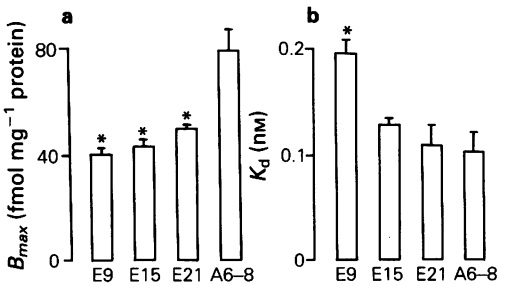

Figure 5 The maximum binding sites (B_{max}) and apparent dissociation constant (K_d) for [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) in the chick oesophagus at different ages. Ordinate scales: B_{max} (fmol mg^{-1} protein, a) and K_d (nM, b) of [^{3}H]-QNB binding. E indicates the age in days of incubation and A indicates the age in days after hatching. Each column shows the mean ($n = 3$–4) of B_{max} (a) and K_d (b) obtained from Scatchard analysis of [^{3}H]-QNB binding to homogenates of chick oesophagus at different ages. Vertical bars show s.e.mean. * Significant difference ($P < 0.05$) from that of the preceding age.

Discussion

The contractile response to transmural nerve stimulation was first observed at 10 days of incubation. The response seemed to be mediated mainly by intramural cholinergic nerves as it was sensitive to atropine and physostigmine, but not C_6. These observations suggest that the intrinsic cholinergic nerve starts to function in the chick oesophagus as early as 10 days of incubation. The early appearance of functional cholinergic innervation to the smooth muscles of the chick oesophagus is consistent with the observation in the rabbit ileum (Gershon & Thompson, 1973), rat ileum (Miyazaki *et al.*, 1982) and rat stomach (Ito *et al.*, 1988). The C_6- and atropine-sensitive response elicited by vagal nerve stimulation in the chick oesophagus appeared a day later than that induced by transmural nerve stimulation, suggesting that the oesophagus could accept the input from the vagus nerve at 11 days of incubation and that ganglionic transmission also develops immediately after neuro-effector transmission is established. These findings are consistent with observations on the chick embryonic heart in which cardiac inhibitory responses to vagal and intramural nerve stimulation were detected simultaneously on the 12th day of incubation (Pappano & Löffelholz, 1974).

The atropine-resistant component of the neuronal response may be due to intrinsic non-cholinergic excitatory nerves. Hassan (1969) and Bartlet (1974) have observed that an atropine-resistant contraction was induced by vagal stimulation in the young chick oesophagus. Immunohistochemical studies (Saffrey *et al.*, 1982; Epstein *et al.*, 1983) indicated the appearance of nerve cells and nerve fibres containing many peptides postulated as neurotransmitters in chick gut at early stages of development. The non-cholinergic excitatory intrinsic nerves, whose transmitter is not known may also develop simultaneously with cholinergic nerves. The time course of maturation of the non-cholinergic innervation may be different from that of cholinergic innervation, as suggested by the finding that the amplitude of the atropine-resistant response did not increase during the embryonic period.

The amplitude of the maximal neuronal response increased during the embryonic period, while the maximum response induced by ACh remained largely unchanged during this period, suggesting that the functional maturation of cholinergic innervation occurs at this time. The increase in magnitude of the neuronal response may be related in part to the change in the amount of releasable ACh. This supposition was supported by the findings that the activity of the enzyme synthesizing ACh, ChAT, increased gradually up to 19 days of incubation which is in parallel with the change in neuronal response and that the amplitude of the maximum response induced by nerve stimulation was also potentiated by physostigmine. In morphological studies, it was observed that the differentiation and maturation of intramural ganglia and their nerve fibres innervating smooth muscle layers occurred during prenatal periods (Bennett & Cobb, 1969; Daikoku *et al.*, 1975; Gershon *et al.*, 1981; Lolova, 1983a, b). Thus, the cholinergic neuro-effector transmission may continue to develop during embryonic periods after the start of functional innervation. The long-lasting responses to nerve stimulation, characteristic in the early stages did not seem to be caused by the absence of the mechanism for degradation of released ACh, because high activity of ChE was already observed in the oesophagus even at early stages.

The contractile response induced by ACh and the considerable number of binding sites for [^{3}H]-QNB which were observed in the preparation at 9 days of incubation when nerve stimulation was ineffective, suggest that the appearance of a response to the transmitter preceded that of functional cholinergic innervation. It has been reported (see Romanoff, 1960) that smooth muscle layers become recognizable in the developing chick oesophagus at about 9 days of incubation. In the intestine of the rabbit foetus, immature smooth muscle (probably myoblasts) were reported to be able to contract (Gershon & Thompson, 1973). In accordance with these morphological observations, the smooth muscles of chick oesophagus seem to acquire functional muscarinic receptors in the immature state.

The maximum response induced by ACh did not change extensively in the course of the embryonic period, nor was there a marked change in the maximum binding sites for [^{3}H]-QNB. It is likely that the population of muscarinic receptors on the smooth muscle did not change during the embryonic period, although the homogenate of chick oesophagus used in receptor binding experiments contained a heterogeneous population of cell fragments. The sensitivity of the oesophageal smooth muscle to ACh increased up to 15 days of incubation. It seems unlikely that the increase in the sensitivity was due to the change in the activity of ChE, because there was not always a correlation between both changes. The developmental increase in sensitivity to ACh is consistent with observations in the rat stomach (Ito *et al.*, 1988) but not with other observations (human ileum; Boréus, 1967; guinea-pig ileum; Boréus & McMurphy, 1971; rat ileum; Miyazaki *et al.*, 1982) in which there was no significant change in sensitivity to ACh. The difference may be due to species and organ differences.

In summary, it is suggested that cholinergic nerve function continues to mature progressively in the chick oesophagus. The onset and maturation of the

receptor mechanisms on smooth muscle seem to precede those of cholinergic innervation. This was also supported by the differential development between the activity of the cholinergic enzyme, ChAT and muscarinic receptor populations during the embryonic period.

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Nedocromil sodium inhibits the A23187- and opsonized zymosan-induced leukotriene formation by human eosinophils but not by human neutrophils

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1 Inflammatory cells such as eosinophils and neutrophils are thought to contribute actively to the pathogenesis of asthma by the release of bronchoconstrictor mediators including leukotrienes. Previous studies have revealed the almost exclusive synthesis of leukotriene C_4 (LTC_4) by human eosinophils and of leukotriene B_4 (LTB_4), 20-OH-LTB_4 and the non-enzymatically formed LTB_4-isomers by neutrophils when stimulated *in vitro* with the calcium ionophore A23187 or opsonized zymosan (OZ). In this study we have investigated whether nedocromil sodium, a new anti-asthma drug, was capable of inhibiting A23187- and OZ-induced leukotriene formation by these cells.

2 Nedocromil sodium inhibited A23187- and OZ-induced LTC_4 formation by eosinophils in a concentration-dependent manner (mean IC_{30} for A23187: 5.6×10^{-5} M; mean IC_{30} for OZ: 6.3×10^{-5} M), whereas it did not inhibit A23187- and OZ-induced LTB_4 formation by neutrophils.

3 Extension of the preincubation time of the cells with the drug did not alter the observed inhibitory capacity. The optimal preincubation time was 5 min.

4 The *in vitro* inhibition of LTC_4 formation by eosinophils by nedocromil sodium may be a valuable property of this drug in the treatment of asthma.

Introduction

When allergic asthmatic individuals are challenged with allergen, the early phase asthmatic attack occurring within 30 min after contact with the allergen may be followed by a late phase bronchoconstriction which takes place 4–6 h after challenge (Durham & Kay, 1985; de Monchy, 1986). This late phase asthmatic reaction develops without further contact with the allergen. The cellular events taking place during the late phase response are considered highly relevant to the pathogenesis of asthma (Durham & Kay, 1985; de Monchy, 1986). Bronchoalveolar lavage (BAL) studies to assess the cellular changes during this response have shown that inflammatory cells, particularly eosinophils, infiltrate the bronchioli at the beginning of the reaction (de Monchy *et al.*, 1985; Metzger *et al.*, 1985; Diaz *et al.*, 1987). There is evidence suggesting the involvement of the sulphidopeptide leukotrienes in the late phase bronchoconstriction (Verhagen & Bruijnzeel, 1985; Bruijnzeel *et al.*, 1986). This idea has been further substantiated by the observation that eosinophils specifically synthesize the potent bronchoconstrictor leukotriene C_4 (LTC_4) upon *in vitro* challenge with a wide variety of stimuli (Verhagen *et al.*, 1984a; Bruijnzeel *et al.*, 1985a, b; 1987b). In the case of neutrophils these stimuli almost exclusively induce the formation of the strongly chemotactic compound leukotriene B_4 (LTB_4), 20-OH-LTB_4 and the non-enzymatically formed LTB_4-isomers.

Since nedocromil sodium is capable of inhibiting allergen-induced early and late phase asthmatic responses when inhaled before allergen challenge (Youngchaiyud & Lee, 1986; Dahl & Pedersen, 1986; Abraham *et al.*, 1987), we have investigated the effect of this drug on leukotriene formation by eosinophils and neutrophils isolated from human peripheral blood.

Methods

Purification of granulocytes

Granulocytes were isolated as follows. Platelet-rich plasma was removed after centrifugation of citrated blood (15 min, $275\,g_{max}$, room temperature). After collection of the buffy coat, granulocytes were separated from lymphocytes and monocytes by centrifugation on Ficoll-Paque (20 min, $1000\,g_{max}$, room

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temperature). The remaining erythrocytes were removed by ammonium chloride lysis at 0°C and subsequent centrifugation. Thereafter the cells were regenerated at 37°C in minimum essential medium/10% foetal calf serum for 30 min. After centrifugation the mixed granulocyte preparation was resuspended in buffer A (phosphate-buffered saline/trisodium citrate/human pasteurized plasma protein = 80/10/10, v/v/v) at a concentration of 75×10^6 cells ml^{-1}.

Purification of eosinophils and neutrophils

Method A Eosinophils and neutrophils were separated from each other by two subsequent centrifugations of a mixed granulocyte preparation over isotonic Percoll solutions with densities of 1.082 and 1.085 $g\,ml^{-1}$. One millilitre of mixed granulocytes (containing 75×10^6 cells) was carefully layered on 4 ml of an isotonic Percoll solution (density: 1.082 $g\,ml^{-1}$) and centrifuged (10 min, $1000\,g_{max}$, room temperature). Neutrophils (over 98% pure) migrated to the top of the 1.082 $g\,ml^{-1}$ Percoll solution, whereas the eosinophils and some neutrophils were present as a small pellet on the bottom. After resuspension of the eosinophil pellets in a 1.085 $g\,ml^{-1}$ Percoll solution (cell concentration $\leqslant 75 \times 10^6$ cells per 4 ml) 4 ml of this suspension was layered on 1 ml of a Percoll solution with a density of 1.100 $g\,ml^{-1}$. After centrifugation (10 min, $1000\,g_{max}$, room temperature), eosinophils (over 90% pure) were concentrated at the interface of both Percoll layers. This isolation procedure was used for the purification of neutrophils from normal individuals and also for the purification of eosinophils and neutrophils from individuals with moderate eosinophilia (e.g. allergic asthmatics). Since the isolation of eosinophils from normal individuals with this method was often disappointing, we developed an alternative isolation method (method B).

Method B The mixed granulocyte preparation (containing 75×10^6 cells ml^{-1} buffer A) was first incubated with FMLP (10 nM) in buffer A for 10 min at 37°C before 1 ml of cell suspension was layered on 4 ml of an isotonic Percoll solution (density: 1.082 $g\,ml^{-1}$). To prevent contamination of the eosinophils with cell debris and remaining erythrocytes, 1 ml of Percoll (density: 1.100 $g\,ml^{-1}$) was brought under the Percoll layer of 1.082 $g\,ml^{-1}$. After centrifugation (10 min, $1000\,g_{max}$, room temperature) eosinophils with a purity of over 85% were present at the interface of both Percoll layers. These eosinophils were collected, washed with buffer A and resuspended in Dulbecco's salt solution.

The density of the eosinophils obtained by method B was identical with the density of the eosinophils obtained by method A (i.e. 1.085 $g\,ml^{-1}$: see Koenderman *et al.*, 1988). Furthermore, the use of FMLP at a concentration of 10 nM did not influence the LTC_4 generation by eosinophils. Eosinophils from the same normal individuals isolated according to methods A and B synthesized similar amounts of LTC_4 upon challenge with A23187 (10 μM): 32 ± 6 ng per 10^6 eosinophils (method A) versus 26 ± 3 ng per 10^6 eosinophils (method B) or with opsonized zymosan (OZ, 0.5 mg ml^{-1}): 30 ± 3 ng per 10^6 eosinophils (method A) versus 36 ± 6 ng per 10^6 eosinophils (method B) (mean $\pm$ s.e.mean, $n = 6$).

Incubation procedure

Purified eosinophils and neutrophils were suspended in Dulbecco's salt solution (pH 7.4) at a concentration of 1×10^6 cells ml^{-1}, preincubated at 37°C for the indicated period of time and then stimulated with A23187 or OZ. Eosinophils were stimulated with A23187 for 20 min or with OZ for 60 min. Neutrophils were stimulated with A23187 for 5 min or with OZ for 15 min. Each experiment was performed in the presence of 1 mM $CaCl_2$ and 5 mM reduced glutathione. Reactions were stopped by the addition of an equal volume of ice-cold radioimmunoassay (RIA) kit buffer to the incubation mixture. The cells were then sedimented (20 min, $2000\,g_{max}$, 4°C) and the supernatants collected and stored at −70°C until analysis (within 1–2 months after the incubation).

Analysis of leukotriene formation

LTC_4 formation was analysed by means of a commercially available RIA (New England Nuclear, Boston, MA, U.S.A.). LTB_4 formation was also analysed by means of a commercially available RIA (Amersham, Buckinghamshire, U.K.). Leukotriene formation was occasionally analysed by reversed phase-h.p.l.c. (Verhagen *et al.*, 1984b). Leukotriene formation was expressed in ng per 10^6 cells.

Statistical analysis

Statistical analysis was performed by use of Student's *t* test for paired data.

Materials

Calcium ionophore A23187, reduced glutathione, N-formyl-methionyl-leucyl-phenylalanine (FMLP) and zymosan A were purchased from Sigma (St. Louis, MO, U.S.A.). Ficoll-Paque (1.077 $g\,ml^{-1}$) and Percoll (1.129 $g\,ml^{-1}$) were obtained from Pharmacia (Uppsala, Sweden). Nedocromil sodium was provided by Fisons plc (Loughborough, U.K.) All other materials were reagent grade. Opsonized zymosan (OZ) was prepared as previously described (Goldstein *et al.*, 1975). Human blood was obtained from healthy volunteers at the Red Cross Bloodbank

Foundation (Utrecht) and from extrinsic allergic asthmatic patients visiting our Outpatient Department. These patients were classified as described elsewhere (Bruijnzeel *et al.*, 1987a).

Results

Leukotriene formation by eosinophils and neutrophils from normal individuals and allergic asthmatics

The optimal conditions for A23187- and OZ-induced leukotriene formation by both eosinophils and neutrophils were determined in previous studies (Bruijnzeel *et al.*, 1985a, b). Under these conditions (A23187 10 μM; incubation time: 20 min for eosinophils/5 min for neutrophils; OZ 2.5 mg ml^{-1}; incubation time: 60 min for eosinophils/15 min for neutrophils) eosinophils and neutrophils of normal individuals and allergic asthmatic individuals did not synthesize statistically significantly different amounts of leukotrienes (see Table 1). Since no differences were observed between leukotriene formation of eosinophils and neutrophils from normal individuals and allergic asthmatic individuals when cells with identical density were stimulated, it was decided to study only cells from normal individuals in subsequent experiments.

A23187 and opsonized zymosan concentration ranges

LTC_4 formation by eosinophils The concentration-dependency of A23187- and OZ-induced LTC_4 formation by eosinophils and the inhibitory effects of nedocromil sodium (10 μM) are shown in Figure 1. These figures suggest that at a concentration of 5 μM A23187 and of 0.5 mg ml^{-1} OZ almost optimal inhibition by nedocromil sodium of induced LTC_4 formation occurs.

LTB_4 formation by neutrophils The concentration-dependency of A23187- and OZ-induced LTB_4 formation by neutrophils is shown in Figure 2.

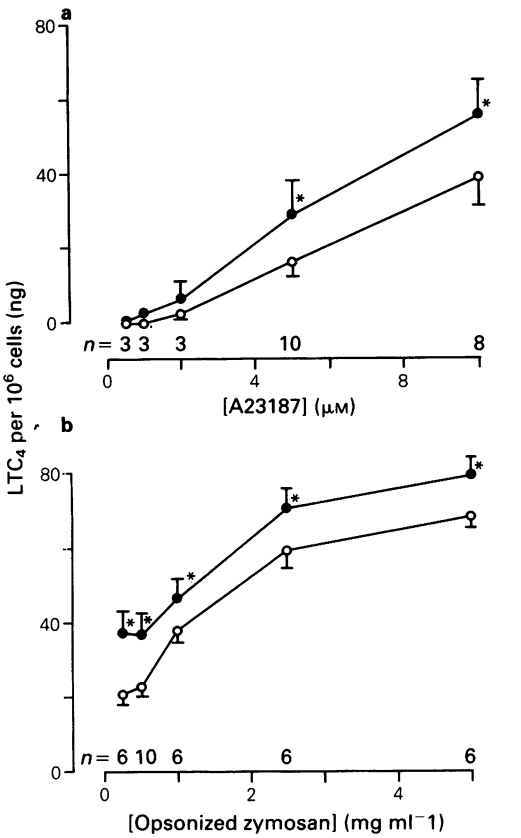

Figure 1 Concentration-dependency of A23187 (a)- and opsonized zymosan (b)-induced leukotriene C_4 (LTC_4) formation by purified human eosinophils in the absence (●) or presence (○) of nedocromil sodium (10 μM). The cells were preincubated for 5 min with the drug before stimulation. *$P < 0.05$, Student's t test for paired data. Points shown are mean values and vertical lines indicate s.e.mean.

Table 1 A23187 (10 μM)- and opsonized zymosan (OZ, 2.5 mg ml^{-1})-induced leukotriene C_4 (LTC_4) formation (ng per 10^6 cells) by eosinophils and leukotriene B_4 formation (ng per 10^6 cells) by neutrophils from normal individuals (N) and allergic asthmatic individuals (A)

Cell type	*Stimulus*	*Time* (min)	*LTC_4 or LTB_4 formation* N	A
Eosinophils	A23187	20	57 ± 12[a]	36 ± 7[b]
	OZ	60	40 ± 8	46 ± 15
Neutrophils	A23187	5	16 ± 4[c]	11 ± 4[d]
	OZ	15	7 ± 1	6 ± 2

Results shown are means ± s.e.mean (n = 5).
Eosinophils and neutrophils of the same density for both N and A were studied.
Cell purities: [a]86 ± 3%, [b]97 ± 3%, [c]96 ± 1%, [d]95 ± 1%.
No statistically significant differences between N and A were observed (Student's t test for paired data).

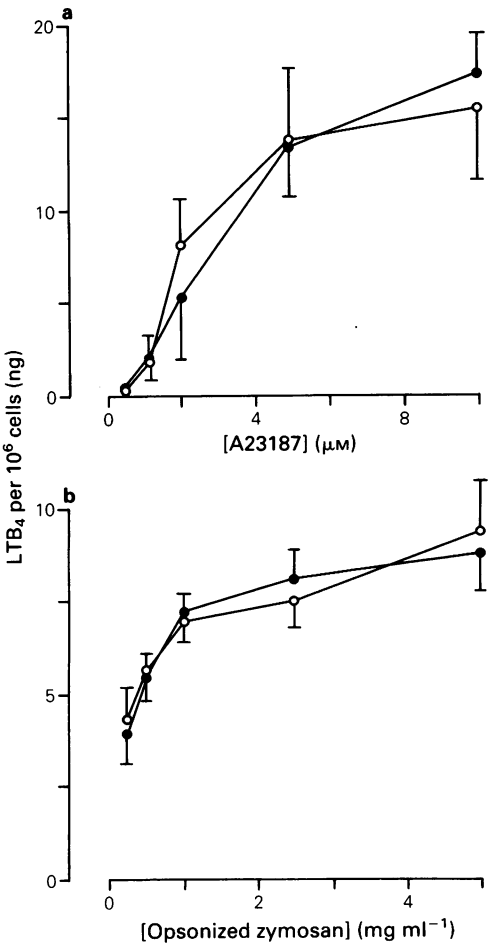

Figure 2 Concentration-dependency of A23187 (a)- and opsonized zymosan (b)-induced leukotriene B_4 (LTB_4) formation by purified human neutrophils in the absence (●) or the presence (○) of nedocromil sodium (10 μM). The cells were preincubated for 5 min with the drug before stimulation. Points shown are mean values ($n = 3$) and vertical lines indicate s.e.mean.

Nedocromil sodium (10 μM) did not inhibit induced LTB_4 formation.

Nedocromil sodium concentration range

LTC_4 formation by eosinophils Cells were preincubated for 5 min with nedocromil sodium at various concentrations. A23187 (5 μM)- and OZ (0.5 mg ml^{-1})-induced LTC_4 formation by eosinophils was inhibited by nedocromil sodium in a concentration-dependent manner (Figure 3). The mean concentration of nedocromil sodium (IC_{30}) required to give 30% inhibition of LTC_4 release induced by A23187 was 5.6×10^{-5} M ($n = 7$). Similarly, the IC_{30} for OZ-induced LTC_4 release was 6.3×10^{-5} M ($n = 7$).

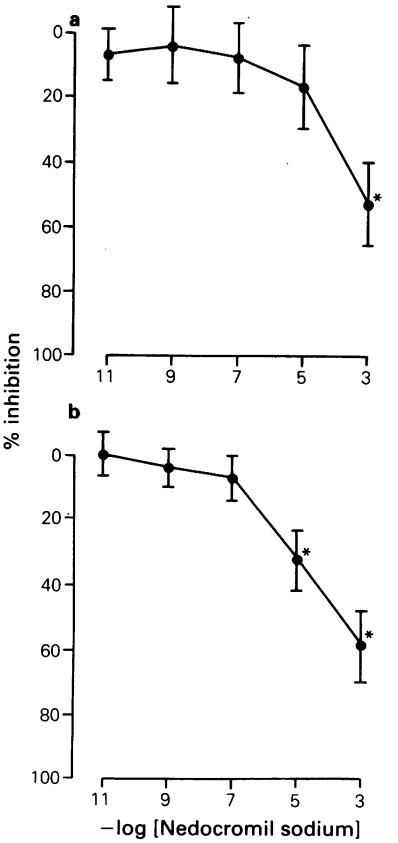

Figure 3 Inhibition of A23187 (5 μM) (a)- and opsonized zymosan (0.5 mg ml^{-1}) (b)-induced leukotriene C_4 (LTC_4) formation by purified human eosinophils at various concentrations of nedocromil sodium. The cells were preincubated for 5 min with the drug before stimulation. The incubation without nedocromil sodium was taken as the 100% value. Points shown are mean values ($n = 7$) and vertical lines indicate s.e.mean. * $P < 0.05$, Student's t test for paired data.

LTB_4 formation by neutrophils A23187 (5 μM)- and OZ (0.5 mg ml^{-1})-induced LTB_4 formation was not inhibited by nedocromil sodium at concentrations of 10^{-9} M–10^{-3} M.

Variation of the preincubation time of the cells with nedocromil sodium

To exclude any effect of duration of preincubation time with nedocromil sodium, the preincubation time was extended from 5 to 30 min. After preincubation for 10 min with nedocromil sodium (10^{-3} M) the following inhibition percentages were obtained:

A23187 (5 μM); 62 ± 12%, OZ (0.5 mg ml^{-1}); 60 ± 21% (mean ± s.e.mean, $n = 4$). After preincubation for 30 min with the same nedocromil sodium concentration the following inhibition percentages were obtained: A23187 (5 μM); 61 ± 14%, OZ (0.5 mg ml^{-1}); 50 ± 23% (mean ± s.e.mean, $n = 3$). In the case of induced LTB_4 formation by neutrophils, no inhibition was obtained by increasing the preincubation time with the drug. These results suggest that a preincubation time of 5 min is sufficient for optimal inhibition of A23187- and OZ-induced LTC_4 formation by eosinophils.

Discussion

We have shown that nedocromil sodium inhibits A23187- and OZ-induced LTC_4 formation by human eosinophils in a concentration-dependent way. In contrast, LTB_4 formation by human neutrophils induced by the same stimuli was not inhibited by this drug. Nedocromil sodium has been demonstrated to inhibit *Ascaris* antigen- and anti-IgE-induced LTC_4 formation by bronchoalveolar mast cells from *Ascaris suum* infected macaque monkeys (Wells *et al.*, 1986). However, A23187- and OZ-induced LTB_4 formation by alveolar macrophages from normal and asthmatic individuals (A23187 induced LTB_4 formation only) were not inhibited by the compound, although there was a tendency towards inhibition of A23187-induced LTD_4 formation (Damon *et al.*, 1986). Here, the mean IC_{30} value obtained for inhibition of A23187-induced LTC_4 formation by eosinophils is one order of magnitude greater than that found for inhibition of LTC_4 formation by bronchoalveolar mast cells from the macaque monkey, but is the same order of magnitude as that obtained for inhibition of LTD_4 formation by human alveolar macrophages. This may be due to potency differences between the stimuli used.

The inhibition by nedocromil sodium of LTC_4 formation by eosinophils, but not of LTB_4 formation by neutrophils, led us to speculate that nedocromil sodium may inhibit glutathione-S-transferase activity in eosinophils, leading to the formation of non-enzymatically formed LTB_4-isomers. However, reversed phase-h.p.l.c. analysis of the pooled samples remaining after RIA-analysis showed a complete absence of non-enzymatically formed LTB_4-isomers. Since it has been found that nedocromil sodium induces phosphorylation of a 78000 molecular weight mast cell protein which is likely to be involved in the termination of mediator release (Wells & Mann, 1983; Theoharides *et al.*, 1980), it is tempting to suggest as an alternative hypothesis that phosphorylation of such a protein takes place in eosinophils but not in neutrophils. Further studies are required for investigate this possibility.

The finding that LTC_4 formation by eosinophils may be inhibited by nedocromil sodium indicates that this drug acts not only on mast cells but also on secondary inflammatory cells. Since eosinophilia in the lung is one of the characteristic features of asthma, it is of value that topically applied drugs such as nedocromil sodium can prevent formation of the potent bronchoconstrictor compound LTC_4 by this cell.

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N-methylhydroxylamine inhibits and M&B 22948 potentiates relaxations of the mouse anococcygeus to non-adrenergic, non-cholinergic field stimulation and to nitrovasodilator drugs

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1 The effects of N-methylhydroxylamine (NMH) and of M&B 22948 on relaxations of the mouse anococcygeus to non-adrenergic, non-cholinergic (NANC) field stimulation and to a number of smooth muscle relaxant drugs were investigated.

2 Relaxations to NANC field stimulation (10 Hz; 60 s train) were reversibly blocked by NMH (1–5 mM), which also caused weak, transient reductions of carbachol (50 μM)-induced tone. N,N-dimethylhydroxylamine (2 mM) and hydroxylamine (5 μM) reduced tone to the same extent as NMH, but neither produced any inhibition of NANC relaxations.

3 M&B 22948 10 μM, which by itself reduced tone by 12%, potentiated submaximal but not maximal relaxations to NANC field stimulation; overall the log frequency–response curve was displaced to the left by a factor of 2.

4 Sodium nitroprusside (0.01–1 μM), hydroxylamine (0.5–100 μM), and nitric oxide (2–200 μM) all relaxed carbachol-induced tone; relaxations to submaximal concentrations of these nitrovasodilators were reduced in the presence of 2 mM NMH, and potentiated in the presence of 10 μM M&B 22948.

5 Neither NMH (2 mM) nor M&B 22948 (10 μM) affected relaxations induced by submaximal concentrations of vasoactive intestinal peptide (VIP; 1 μM), papaverine (10 μM), 3-isobutyl-1-methylxanthine (10 μM), or 8-bromo-cyclic guanosine monophosphate (100 μM); relaxations to adenosine 5'-triphosphate (ATP, 2 mM) were unaffected by M&B 22948, but were potentiated by NMH.

6 The selective inhibition by NMH, and potentiation by M&B 22948, of NANC and nitrovasodilator-induced relaxations of the mouse anococcygeus suggests that the NANC transmitter is neither VIP nor ATP, but resembles the nitrovasodilator drugs in its mode of action. The NANC transmission system is therefore similar to that recently described in the bovine retractor penis.

Introduction

The neurotransmission system mediating non-adrenergic, non-cholinergic (NANC) relaxations of the anococcygeus muscle remains unidentified. Since the original description of the phenomenon by Gillespie (1972) three substances have emerged as possible transmitter candidates; adenosine 5'-triphosphate (ATP; Burnstock *et al.*, 1978), vasoactive intestinal peptide (VIP; Gibson & Tucker, 1982; Hunter *et al.*, 1984; Larson *et al.*, 1985), and an extract from rat anococcygeus and bovine retractor penis (inhibitory factor; Gillespie & Martin, 1980; Bowman & Gillespie, 1982). However, final identification of the NANC transmitter has been hampered by a lack of drugs that selectively inhibit, or selectively potentiate, the neurotransmission system. Experiments using putative antagonists of VIP (Carvajal *et al.*, 1986) or ATP (Gibson & Tucker, 1982; Sneddon *et al.*, 1982) have

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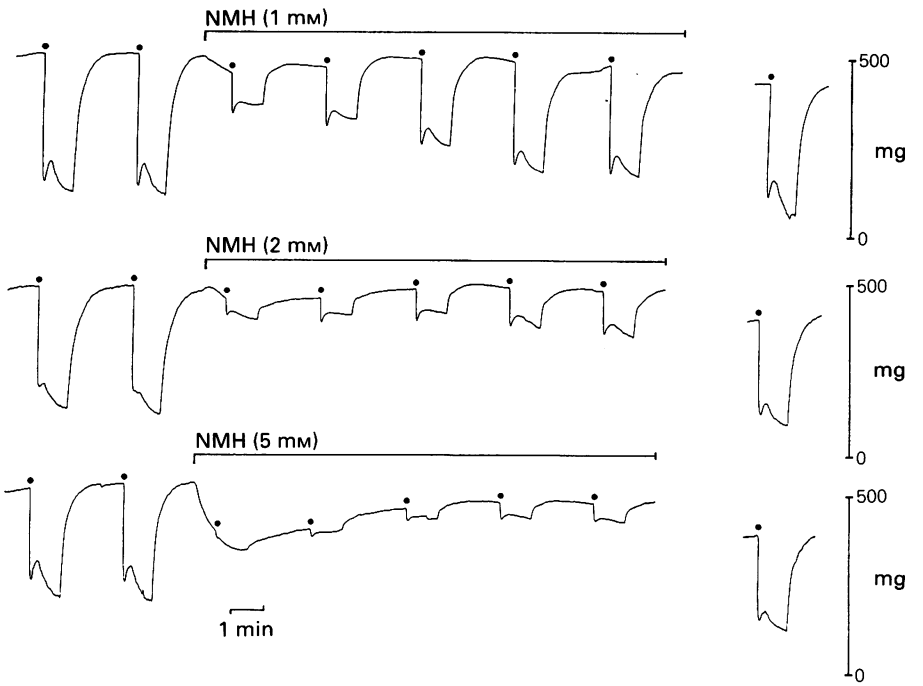

Figure 1 The effect of three concentrations of N-methylhydroxylamine (NMH) on relaxations of mouse anococcygeus muscles to repeated trains of non-adrenergic, non-cholinergic (NANC) field stimulation (●; 10 Hz; 60 s train). Muscle tone was raised with 50 μM carbachol. NMH produced transient relaxation and marked inhibition of NANC responses. The extreme right hand trace in each panel shows that the inhibitory effect of NMH was easily reversed 20 min after washout.

given inconclusive results, since the antagonists inhibited neither NANC-induced relaxations nor those induced by the appropriate agonist. Recently, however, Bowman & Drummond (1984, 1985) reported that N-methylhydroxylamine (NMH) blocked, and M&B 22948 potentiated, relaxations of the bovine retractor penis to inhibitory factor, NANC field stimulation, and sodium nitroprusside (SNP). These results suggested that the inhibitory factor (or a closely related substance) might be the NANC transmitter of the retractor penis, and that the actions of the transmitter could be mimicked by nitrovasodilator drugs; subsequent studies have revealed that the inhibitory factor may resemble the nitrovasodilators chemically and, like these drugs, may induce smooth muscle relaxation via formation of nitric oxide (NO) with consequent activation of guanylate cyclase (Martin *et al.*, 1988; Gillespie & Sheng, 1988). Since NANC transmission in the anococcygeus shows some similarities to that in the retractor penis (Bowman & Gillespie, 1982), it seemed possible that NMH and M&B 22948 might prove useful in the characterization of NANC transmission in the anococcygeus and, in particular, might provide the means to differentiate among the three main transmitter candidates. Thus, in the present study, we have investigated the effects of NMH, and of M&B 22948, on NANC transmission in the mouse anococcygeus and compared these with their effects on a number of smooth muscle relaxant drugs.

Methods

Male mice (LACA strain; 25–35 g) were killed by stunning and exsanguination. The paired anococcygeus muscles were dissected out and set up in series, joined by the ventral bar, in 1 ml glass organ baths containing Krebs-bicarbonate solution (mM: NaCl 118.1, KCl 4.7, $MgSO_4$ 1.0, KH_2PO_4 1.0, $CaCl_2$ 2.5, $NaHCO_3$ 25.0 and glucose 11.1) which was maintained at 37°C and gassed continuously with 95% O_2:5% CO_2. A resting tension of 200–400 mg was placed on the tissue and changes in tension recorded with a Grass FTO3 force-displacement transducer attached to a Graphtec Linearecorder (WR 3101). Muscles were allowed to equilibrate for 45 min before beginning the experiment.

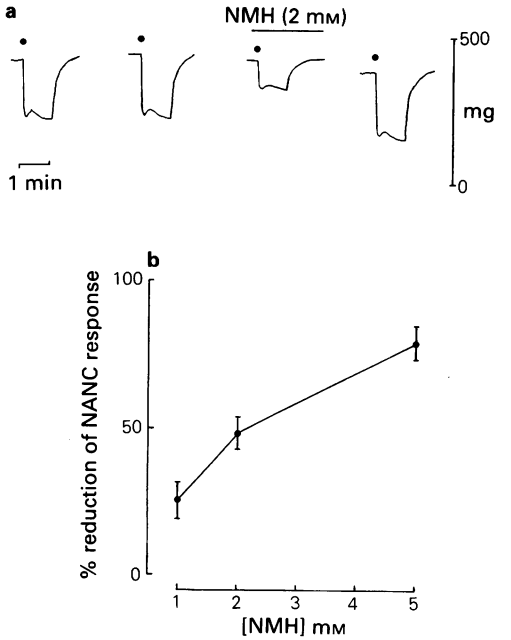

Figure 2 (a) The effect of N-methylhydroxylamine (NMH) on relaxations of a mouse anococcygeus muscle to a single train of non-adrenergic, non-cholinergic (NANC) field stimulation (●; 10 Hz; 60 s train): 3 min before each train muscle tone was raised with 50 μM carbachol, which was then washed out after each train. The muscle was allowed to rest for 20 min before tone was raised again. NMH (added 10 min before the train of field stimulation) markedly reduced the NANC response, this effect being easily reversed by washout. (b) Concentration-response curve for the inhibitory effect of NMH on NANC-induced relaxations of the mouse anococcygeus. The experimental protocol was the same as that outlined in (a). Each point represents the mean from at least 6 individual muscle preparations; s.e. shown by vertical bars.

Field stimulation was applied by two parallel platinum electrodes running down either side of the tissue (Gibson & Wedmore, 1981); these were attached to a Grass S48 stimulator (1 ms pulse width; 70 V). To observe NANC relaxations to field stimulation in the anococcygeus it is necessary to raise muscle tone and to negate contractions due to stimulation of sympathetic nerve endings. Tone was raised with 50 μM carbachol in all experiments; sympathetic responses were prevented by including 1 μM phentolamine in the Krebs solution and by pre-incubating each muscle with 30 μM guanethidine for 10 min, during the 45 min equilibration period. All muscles used in this study were treated with these sympatholytic drugs; when relaxations to ATP were to be investigated the Krebs solution also contained 2.8 μM indomethacin (Gibson & Wedmore, 1981).

To record relaxations to drugs, tone was first raised with 50 μM carbachol. When carbachol-induced tone had reached a plateau (usually within 3 min) the relaxant drug was added to the organ bath; the response was calculated as the peak % reduction of carbachol-tone occurring within 4 min of adding the relaxant drug. Both carbachol and relaxant were then washed out of the organ bath, and the muscle allowed to rest for 20 min before the tone was raised again.

All drugs were dissolved in distilled water, except M&B 22948 which was dissolved in 20% triethanolamine, and indomethacin which was dissolved in 0.5% Na_2CO_3 (to give 10 mM stock solutions). Nitric oxide solution was prepared by bubbling 10 ml of NO gas through 25 ml distilled water (4°C; previously bubbled with N_2 for 10 min) in a sealed glass vacuum flask. The organ bath concentrations of NO given in this paper were calculated by assuming that this method of preparation produced a saturated solution of 3 mM NO (Mellor, 1928; Shikano *et al.*, 1987), and therefore they should be considered only as an approximation of the true values.

Drugs used during this study were: adenosine 5'-triphosphate (disodium salt; Sigma); 8-bromo-cyclic guanosine monophosphate (Sigma); carbachol (BDH); N,N-dimethylhydroxylamine (Aldrich); guanethidine sulphate (Ciba); hydroxylamine hydrochloride (Sigma); indomethacin (Sigma); 3-isobutyl-1-methylxanthine (Sigma); M&B 22948 (2-O-propoxyphenyl-8-azapurin-6-one; May & Baker); N-methylhydroxylamine hydrochloride (Sigma); nitric oxide (99%; BDH); papaverine hydrochloride (Hopkin & Williams); phentolamine mesylate (Ciba); sodium nitroprusside (Sigma); vasoactive intestinal peptide (Sigma).

Statistical analysis was by Student's *t* test.

Results

The effect of N-methylhydroxylamine on NANC relaxations

As found previously (Gibson & Yu, 1983), 60 s trains of field stimulation (10 Hz) produced biphasic NANC relaxations of the mouse anococcygeus muscle (Figure 1). Addition of NMH (1–5 mM) between successive trains of field stimulation produced two distinct effects. First, NMH by itself produced reductions of carbachol-induced tone; these relaxations were concentration-related but were transient, tone returning to the original, carbachol-induced level within 10 min (Figure 1). Secondly, NMH inhibited NANC-induced relaxations; this

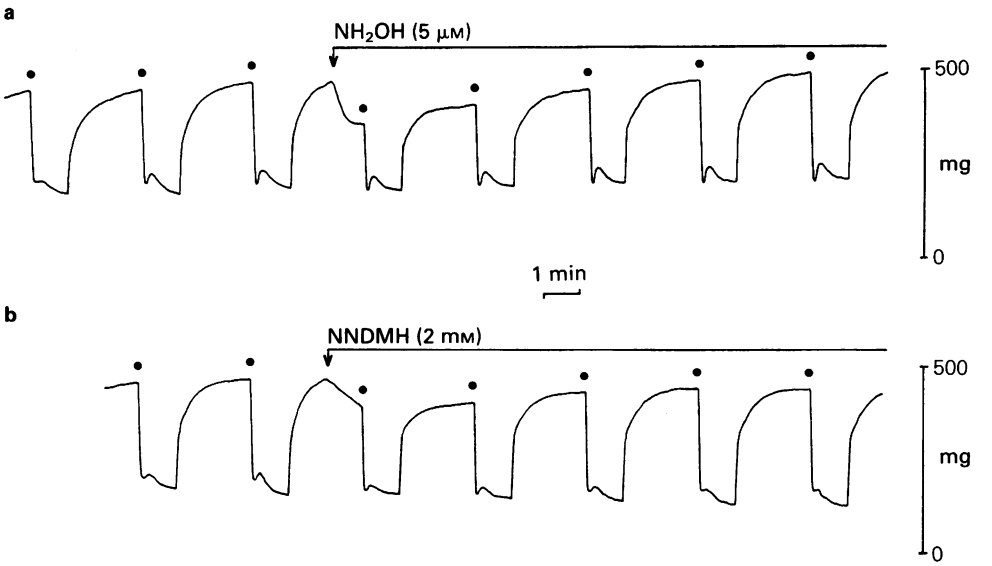

Figure 3 The effect of hydroxylamine (NH_2OH, a) and of N,N-dimethyl-hydroxylamine (NNDMH, b) on relaxations of mouse anococcygeus muscles to repeated trains of non-adrenergic, non-cholinergic (NANC) field stimulation (●; 10 Hz; 60 s train). Muscle tone was raised with 50 μM carbachol. Both drugs caused transient relaxations, but neither inhibited NANC responses.

effect was most marked immediately after application of NMH, but after 10 min the level of inhibition had reached a nearly steady-state, 2 mM NMH reducing NANC relaxations by about 50% (Figure 1). Since it was possible that the partial recovery of the NANC response observed during the first 10 min might have been due to the repeated trains of field stimulation, we carried out experiments such as that illustrated in Figure 2a. Here the muscles received only one train of field stimulation on each occasion that tone was raised, after which the carbachol was washed out of the bath; the muscle was then allowed to rest for 20 min before tone was raised again. Under these conditions, 2 mM NMH (added 10 min before the next train of field stimulation) again caused a 50% reduction of the NANC response (Figure 2a). By use of this protocol, the concentration-response relationship for inhibition of NANC relaxations by NMH was determined (Figure 2b).

The inhibitory effect of NMH was easily reversed by washout (Figures 1 and 2a). However, after washout it was noted that contractile responses to carbachol were often reduced (by about 10%); for this reason, in all experiments described in this paper each tissue received only one exposure to NMH.

As a comparison, we studied the effects of two other compounds, closely related chemically to NMH. N,N-dimethylhydroxylamine (1–5 mM) produced transient relaxations, similar to those of NMH; hydroxylamine (0.5–100 μM) was a much more potent relaxant and in later experiments it was included as an example of a nitrovasodilator drug (see Figure 5), as it has been in other studies (Waldman & Murad, 1987). However, concentrations of N,N-dimethyl-hydroxylamine (2 mM) and hydroxylamine (5 μM) which reduced tone to the same extent as NMH (2 mM), produced no inhibition of NANC relaxations (Figure 3).

The effect of M&B 22948 on NANC relaxations

By itself, M&B 22948 (1–100 μM) produced concentration-related relaxations of carbachol-induced tone (Figure 4a); unlike those to NMH, relaxations to M&B 22948 were sustained; to study the effects of M&B 22948 on NANC responses it was therefore necessary to choose a concentration which by itself produced only a small relaxation. M&B 22948 10 μM, which reduced tone by 12% (Figure 4a) was chosen.

In the presence of 10 μM M&B 22948, the log-frequency-relaxation curve was shifted to the left by a factor of 2 (Figure 4b); NANC relaxations to lower frequencies of field stimulation (0.5, 1, 2, and 5 Hz) were significantly potentiated by M&B 22948, but those to higher frequencies (10 and 20 Hz) were unaffected.

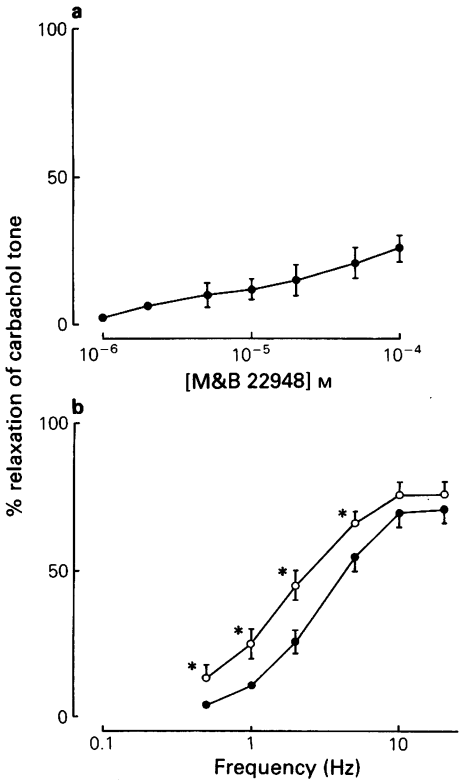

Figure 4 (a) Concentration-response curve for M&B 22948-induced relaxations of the mouse anococcygeus. Muscle tone was raised with 50 μM carbachol. Each point represents the mean from at least 6 individual muscle preparations; s.e. shown by vertical bars. (b) Frequency-response curves for non-adrenergic, non-cholinergic (NANC; 60 s train) relaxations of the mouse anococcygeus in the absence (●) and presence (○) of 10 μM M&B 22948. Muscle tone was raised with 50 μM carbachol. Each point represents the mean from at least 6 individual muscle preparations; s.e. shown by vertical bars. * $P < 0.05$: value significantly different from that in the absence of M&B 22948. M&B 22948 potentiated relaxations to lower frequencies of NANC stimulation but the maximum relaxation was unaltered.

The effect of N-methylhydroxylamine and M&B 22948 on relaxations to nitrovasodilator drugs

The nitrovasodilator drugs, SNP (0.01–1 μM), hydroxylamine (0.5–100 μM), and NO (2–200 μM) produced concentration-related relaxations of carbachol-induced tone (Figure 5). The effects of 2 mM NMH and 10 μM M&B 22948 were studied on concentrations of each relaxant which reduced tone by between 30–70%. NMH reduced (Figure 6a) and M&B 22948 potentiated (Figure 6b) relaxations in

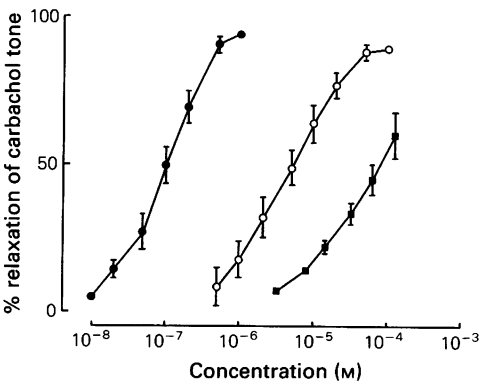

Figure 5 Concentration-response curves for relaxations of the mouse anococcygeus in response to sodium nitroprusside (●), hydroxylamine (○) and nitric oxide (■). Muscle tone was raised with 50 μM carbachol. Each point represents the mean from at least 6 individual muscle preparations; s.e. shown by vertical bars.

response to SNP (0.1 μM), hydroxylamine (10 μM), and NO (30 μM with NMH; 8 μM with M&B 22948).

The effect of N-methylhydroxylamine and M&B 22948 on relaxations to non-nitrovasodilator drugs

Concentration-response curves for relaxations to VIP (0.1–5 μM), papaverine (1–100 μM), 3-isobutyl-1-methylxanthine (IBMX; 1–100 μM), 8-bromo-cyclic guanosine monophosphate (8Br-cyclic GMP; 10–500 μM), and ATP (0.5–10 mM) are shown in Figure 7; again, the effects of NMH and M&B 22948 were studied on suitable submaximal concentrations of each relaxant. Responses to VIP (1 μM), papaverine (10 μM), IBMX (10 μM), and 8Br-cyclic GMP (100 μM) were unaffected by either 2 mM NMH (Figure 8a) or 10 μM M&B 22948 (Figure 8b); those to ATP (2 mM) were unaffected by M&B 22948 but, unusually, were significantly potentiated by NMH.

Discussion

Bowman & Drummond (1985) pointed out that NMH was far from ideal as an antagonist of NANC responses in the bovine retractor penis since, by itself, it produced marked fluctuations of muscle tone; this was also true in the mouse anococcygeus and, in addition, NMH tended to reduce subsequent sensitivity to carbachol. Despite these problems, NMH proved to be relatively selective, inhibiting

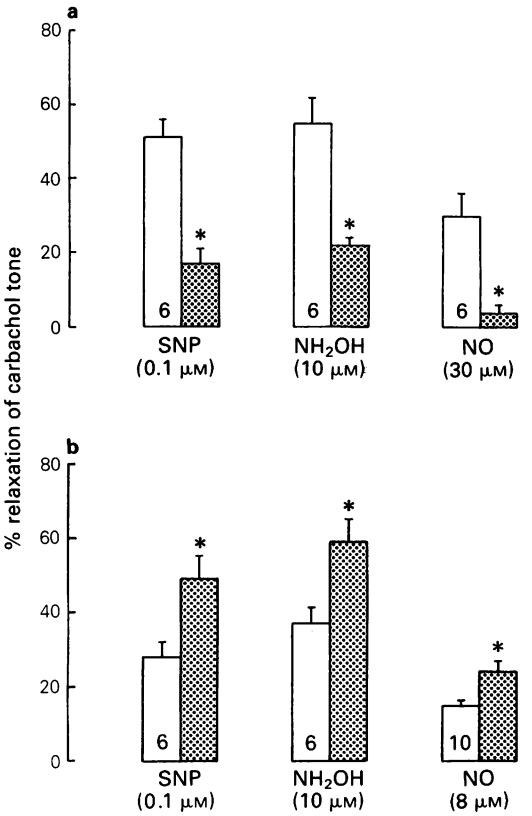

Figure 6 Histograms showing the effect of N-methylhydroxylamine (2 mM, a) and of M&B 22948 (10 μM, b) on relaxations of the mouse anococcygeus to submaximal concentrations of sodium nitroprusside (SNP), hydroxylamine (NH_2OH) and nitric oxide (NO). The columns represent the % relaxation of carbachol (50 μM)-induced tone (mean with s.e. shown by vertical bars) observed in the absence (open columns) and presence (stippled columns) of either NMH or M&B 22948. The figure within the open column gives the number of muscle preparations studied with each relaxant. * $P < 0.05$: value significantly different from corresponding open column. NMH inhibited and M&B 22948 potentiated relaxations to all three nitrovasodilators.

relaxations to NANC stimulation and to nitrovasodilators but not those to a variety of non-nitrovasodilator smooth muscle relaxants. Similarly M&B 22948 produced a selective potentiation of NANC and nitrovasodilator-induced relaxations. This selectivity of action of NMH and M&B 22948 allows several conclusions to be drawn about the nature of NANC transmission in the mouse anococcygeus and, in particular, about the three main transmitter candidates; VIP, ATP, and inhibitory factor.

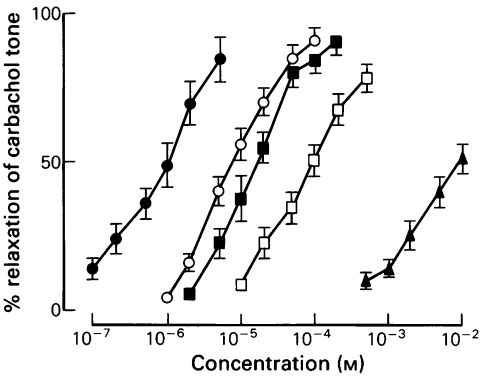

Figure 7 Concentration-response curves for relaxations of the mouse anococcygeus in response to vasoactive intestinal peptide (●), papaverine (○), 3-isobutyl-1-methylxanthine (■), 8-bromo-cyclic guanosine monophosphate (□), and adenosine 5'-triphosphate (▲). Muscle tone was raised with 50 μM carbachol. Each point represents the mean from at least 6 individual muscle preparations; s.e. shown by vertical bars.

VIP has been detected by immunocytochemistry and by radioimmunoassay in anococcygeus muscles from several species (Gibson & Tucker, 1982; Hunter *et al.*, 1984; Larson *et al.*, 1985). Since VIP was also a potent relaxant of these muscles it was considered as a possible candidate for the NANC transmitter. However, recent evidence has suggested that this is not the case, since anococcygeus muscles which had been rendered insensitive to VIP (by VIP desensitization, VIP antiserum, or α-chymotrypsin) responded normally to NANC field stimulation (Carvajal *et al.*, 1986). The present results confirm that VIP is not the inhibitory NANC transmitter in the mouse anococcygeus; NANC relaxations were inhibited by NMH and potentiated by M&B 22948, but neither drug had any effect on relaxations to VIP.

ATP has also been detected in anococcygeus muscles by quinacrine fluorescence microscopy (Burnstock *et al.*, 1978; Olson & Alund, 1979) and it is released during trains of NANC field stimulation (Burnstock *et al.*, 1978). However, the sensitivity of anococcygeus muscles from different species to relaxation by ATP is very variable (Gillespie, 1980) and, in some species, good relaxations are only observed in the presence of indomethacin (Burnstock *et al.*, 1978; Gibson & Wedmore, 1981). This variability of response, together with the present observations that relaxations to ATP and NANC stimulation are affected differently by NMH and M&B 22948, makes it unlikely that ATP is the NANC transmitter

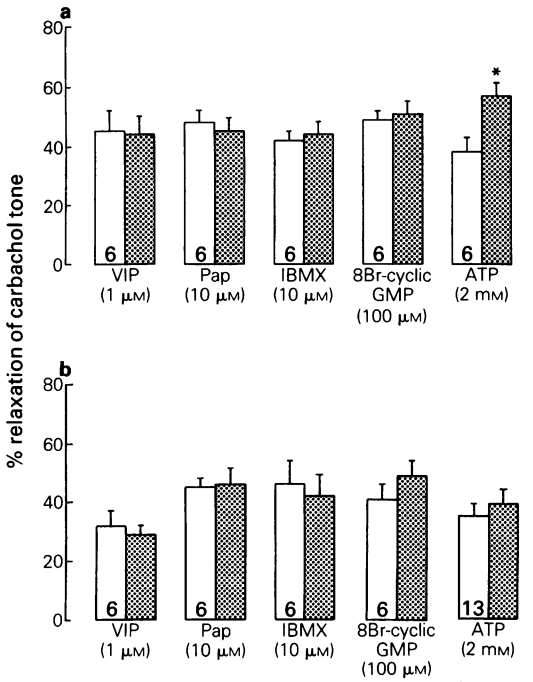

Figure 8 Histograms showing the effect of N-methylhydroxylamine (2 mM, a) and of M&B 22948 (10 μM, b) on relaxations of the mouse anococcygeus to submaximal concentrations of vasoactive intestinal peptide (VIP), papaverine (Pap), 3-isobutyl-1-methylxanthine (IBMX), 8-bromo-cyclic guanosine monophosphate (8Br-cyclic GMP), and adenosine 5'-triphosphate (ATP). The columns represent the % relaxation of carbachol (50 μM)-induced tone (mean with s.e. shown by vertical bars) observed in the absence (open columns) or presence (stippled columns) of either NMH or M&B 22948. The figure within the open column gives the number of muscle preparations studied with each relaxant. * $P < 0.05$: value significantly different from corresponding open column. M&B 22948 had no effect on any of these drugs; NMH had no effect on VIP, Pap, IBMX, or 8Br-cyclic GMP, but potentiated ATP.

in the anococcygeus. At present, we are unable to explain the enhanced responses to ATP in the presence of NMH.

If neither VIP nor ATP is the NANC transmitter, then attention must focus on the inhibitory factor. Although most studies on inhibitory factor have employed the retractor penis, the material can be extracted from, and produces relaxations of, the rat anococcygeus (Gillespie & Martin, 1980; Bowman & Gillespie, 1982). The actions of inhibitory factor can be mimicked by nitrovasodilator drugs, and procedures which block or potentiate NANC relaxations of the retractor penis have the same effect on nitrovasodilator drugs and inhibitory factor (Bowman & Drummond, 1985). It has therefore been proposed that the inhibitory factor (or a closely related substance) is the NANC transmitter of the retractor penis and that it may resemble the nitrovasodilator drugs; indeed, recent evidence has suggested that the inhibitory factor, like nitrovasodilators, may contain a NO moiety which must be liberated to initiate smooth muscle relaxation (Martin *et al.*, 1988; Gillespie & Sheng, 1988). In the present study, relaxations to nitrovasodilator drugs, like those to NANC stimulation, were blocked by NMH and potentiated by M&B 22948; this suggests that the NANC transmitter of the mouse anococcygeus resembles, in its mode of action, the nitrovasodilators and would be consistent with the possibility that the inhibitory factor, or a closely related substance, is the transmitter released by NANC nerves in the anococcygeus (Bowman & Gillespie, 1982). Whatever the transmitter, it seems clear that NANC transmission in the anococcygeus is the same as that in the retractor penis since NANC relaxations of both tissues are blocked by NMH (Bowman & Drummond, 1984; 1985; this study) and haemolysed blood (Bowman & Gillespie, 1982; Gibson & Tucker, 1982; Gibson & Yu, 1983), are potentiated by M&B 22948 (Bowman & Drummond, 1984; 1985; this study), and are insensitive to block by apamin (Bowman & Gillespie, 1982; Gibson & Tucker, 1982).

In the retractor penis, Bowman & Drummond (1984, 1985) have suggested that relaxation results from activation of guanylate cyclase by the NANC transmitter, with subsequent generation of the second messenger substance, cyclic guanosine monophosphate (cyclic GMP). Several observations suggest that this may also be the case in the mouse anococcygeus. First, NANC relaxations were blocked by NMH, which has been shown to prevent activation of guanylate cyclase by various nitrovasodilator-like compounds in nervous tissue (Deguchi *et al.*, 1978); the mechanism by which NMH inhibits nitrovasodilator drugs is not known, but the small molecular changes required to convert a potent relaxant of the anococcygeus (hydroxylamine) to an inhibitor of relaxation (NMH) and then to a compound with little activity (N,N-dimethylhydroxylamine) suggest that the drugs, and the NANC transmitter, interact at a relatively specific recognition site. Secondly, NANC relaxations were potentiated by M&B 22948, which enhances the cellular actions of cyclic GMP by inhibiting cyclic GMP-phosphodiesterase (Waldman & Murad, 1987). Thirdly, 8Br-cyclic GMP, a cell-permeant, stable analogue of cyclic GMP (Miller *et al.*, 1973) produced relaxations, thereby mimicking the effects of NANC stimulation. Further, responses to 8Br-

cyclic GMP were not blocked by NMH, confirming that the site of action of NMH precedes generation of cyclic GMP; as expected, M&B 22948 did not alter relaxations to 8Br-cyclic GMP which is resistant to breakdown by phosphodiesterases (Miller *et al.*, 1973). Since relaxations to papaverine and IBMX were unchanged in the presence of NMH or M&B 22948 their mechanisms of action differ from that of NANC stimulation and the nitrovasodilators; although both papaverine and IBMX are non-specific inhibitors of phosphodiesterase, the involvement of cyclic nucleotides in their smooth muscle relaxing actions remains controversial (Bryson & Rodger, 1987). Clearly, the role of the guanylate cyclase/cyclic GMP system in NANC and drug-induced relaxation of the anococcygeus muscle requires further attention.

The authors thank May & Baker, Dagenham, Essex, UK for the gift of M&B 22948.

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Factors inducing endothelium-dependent relaxation in the guinea-pig basilar artery as estimated from the actions of haemoglobin

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1 Factors inducing dilatation of guinea-pig basilar artery were investigated in intact and endothelium-denuded tissues by measurement of isometric tension and by electrophysiological methods.

2 The amplitudes of contractions induced by 9,11,epithio-11,12-methanothromboxane A_2 (STA_2) and by high K^+ were enhanced by haemoglobin (oxyhaemoglobin, Hb) in a concentration-dependent fashion (above 1 μM). For the high K^+-induced contraction, the initial tonic component was enhanced to a greater extent than the secondary phasic component. Mechanical responses evoked by STA_2 and by high K^+ were greater in endothelium-denuded tissues, but Hb (below 10 μM) had no effect on them.

3 Hb (10 μM) had no effect on the contractile proteins as estimated from the actions of Hb on Ca^{2+}-induced contractions in skinned muscle tissues. Further, Hb had no effect on the release of Ca^{2+} from intracellular stores but it accelerated the Ca^{2+} accumulation into the sarcoplasmic reticulum as judged from the caffeine- or STA_2-induced contraction generated in intact tissues.

4 Acetylcholine (ACh) relaxed tissues that were precontracted by STA_2 but Hb prevented this relaxation, in a concentration-dependent fashion. The ACh-induced relaxation was sustained for over 10 min in the absence of Hb, but following application of Hb, ACh caused only a transient relaxation.

5 STA_2 (up to 100 nM) did not modify the resting membrane potential of smooth muscle cells of the basilar artery. ACh (10 μM) caused transient hyperpolarization which was only slightly inhibited by Hb (10 μM) whether or not STA_2 was present. The hyperpolarization induced by ACh required the presence of endothelial cells.

6 A23187 (0.01–1 μM) relaxed tissues which were precontracted by STA_2, in a concentration-dependent fashion but had no effect on the membrane potential.

7 These results suggest that in guinea-pig basilar artery, ACh induces relaxation of tissues that were precontracted by STA_2 by causing release of both endothelium-derived relaxing (EDRF) and endothelial dependent hyperpolarizing factor (EDHF) (sustained and initial transient relaxation, respectively), but via different mechanisms. Hb inhibits the former and to a lesser extent, the latter. Since A23187 produced relaxation of pre-contracted tissue but caused no detectable change in the membrane potential, this agent may release EDRF but not EDHF.

Introduction

Furchgott & Zawadzki (1980) demonstrated that the vasodilator action of acetylcholine (ACh) on the rabbit aorta was mediated indirectly by release from endothelial cells of a relaxant substance which they later termed endothelium-derived relaxing factor (EDRF) (Cherry *et al.*, 1982). The action of EDRF is associated with a rise in cyclic GMP synthesized in the cytosol of vascular smooth muscle cells (Holzmann, 1982; Rapoport & Murad, 1983; Diamond & Chu, 1983; Furchgott & Jothianandan, 1983; Ignarro *et al.*, 1984) and this stimulation appears to be inhibited both by haemoglobin (Hb) and myoglobin but not by methaemoglobin (Miki *et al.*, 1977; Murad *et al.*, 1978; Mittal *et al.*, 1978). Furthermore, both the endothelium-dependent relaxation and the rise in cyclic GMP induced by ACh in

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bovine coronary and intrapulmonary arteries are blocked by methylene blue (Holzmann, 1982; Ignarro *et al.*, 1984; Martin *et al.*, 1985). Recently Komori & Suzuki (1987a,b) reported that in the rabbit saphenous artery ACh and oxotremorin release EDRF through activation of muscarinic M_2-receptors, and the former but not the latter induces an endothelial-dependent hyperpolarization by releasing a hyperpolarizing substance which we propose to term EDHF (endothelial-dependent hyperpolarizing factor) through activation of muscarinic M_1-receptors. Hb inhibited the relaxation to a greater extent than the hyperpolarization (Chen & Suzuki, 1988). Such endothelium-dependent hyperpolarizations induced by agonists have been reported (Bolton *et al.*, 1984; Bolton & Clapp, 1986; Southerton *et al.*, 1987; Feletou & Vanhoutte, 1988).

Although the action of EDRF on various vascular smooth muscle cells was inhibited by Hb through inhibition of guanylate cyclase, Hb itself or haemolysate produces contraction of basilar arteries in many species (Tanishima, 1980; Toda *et al.*, 1980; Wellum *et al.*, 1982; Fujiwara & Kuriyama, 1984; Fujiwara *et al.*, 1986; Connor & Feniuk, 1987). Thus, the vasoconstriction that occurs after subarachnoid haemorrhage following rupture of cerebral aneurysms may be related to the actions of Hb (Saito *et al.*, 1977; Fisher *et al.*, 1980; Wellum *et al.*, 1982; Kassel *et al.*, 1985; Boullin, 1985).

The present experiments were intended to clarify some of the features of endothelium-dependent relaxation in the guinea-pig basilar artery by use of microelectrode and tension recording techniques in intact and endothelium denuded tissues.

Methods

Guinea-pigs of either sex (250–350 g) were killed by decapitation, taking care to avoid subarachnoid haemorrhage. After craniotomy the basilar artery with brain stem was removed and placed in a small chamber filled with Krebs solution. After dissecting the basilar artery, the arachnoid membrane and connective tissue were carefully removed. Under binocular microscope, circularly cut strips (0.03–0.05 mm in thickness, 0.3–0.5 mm in length and 0.1 mm in width) were prepared for recording tension, and whole muscle tissues (0.2–0.3 mm in diameter and 5 mm in length of vascular tissues without dissection) were prepared for recording membrane activity. To remove the endothelium, the intimal surface was gently rubbed with small knives made from pieces of razor blade (Kanmura *et al.*, 1987). Satisfactory ablation of the endothelium was histologically verified under a light microscope.

Solution

The ionic composition of the Krebs solution was as follows (mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.6, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134.4, and glucose 11.5. The solution was bubbled with 97% O_2 and 3% CO_2, and the pH was adjusted to 7.4. High K^+ solution or Ca^{2+}-free solution was prepared by isoosmotic replacement of NaCl with KCl or of $CaCl_2$ with $MgCl_2$ (with 2 mM EGTA), respectively.

Recording of mechanical activity

The circularly cut strip was set up in a small 1.0 ml chamber, through which the test solution could be changed rapidly by injecting fresh solution from one end and by sucking simultaneously from the other end with the water pump. Both ends of the preparation were fixed between pieces of Scotch double-sided tape via thin silk, and isometric tension was recorded with a strain gauge transducer (U-gauge, Shinko Co.). The temperature of the perfusate was kept at 25°C. All experiments were started after at least 90 min equilibration, under a resting tension of approximately 0.5 mg.

To record the Ca^{2+}-induced contraction in skinned muscle tissues, tissues were exposed to saponin (40 $\mu g\,ml^{-1}$) for 20 min in relaxing solution (mM; K-methanesulphonate 90, piperazine-N-N'-bis-(2-ethanesulphonic acid) (PIPES) 20, Mg-methansulphonate 5.1, ATP 5.2 and EGTA 4: Itoh *et al.* 1982) at 25°C. The tension-pCa relationship was obtained by cumulative application of increasing Ca^{2+} concentrations buffered by EGTA (Itoh *et al.*, 1982) in a stepwise manner. Hb was applied during the Ca^{2+}-induced contraction after tension had reached a steady level.

Recording of membrane activity

The preparation was mounted in an organ bath with a capacity of 1 ml and then superfused with warmed Krebs solution (35°C) at a flow rate of about 2 $ml\,min^{-1}$. The experiments were carried out after 120–150 min of superfusion. Electrical responses of the membrane were recorded via a glass capillary electrode (Higenberg Glass) filled with KCl (3 M). The resistance of the electrode tip ranged between 40–60 MΩ. The microelectrode was inserted from the outer surface of the artery.

Drugs

Oxyhaemoglobin was prepared by reducing commercial bovine Hb containing 75% methaemoglobin (Sigma) as described by Martin *et al.* (1985). Briefly

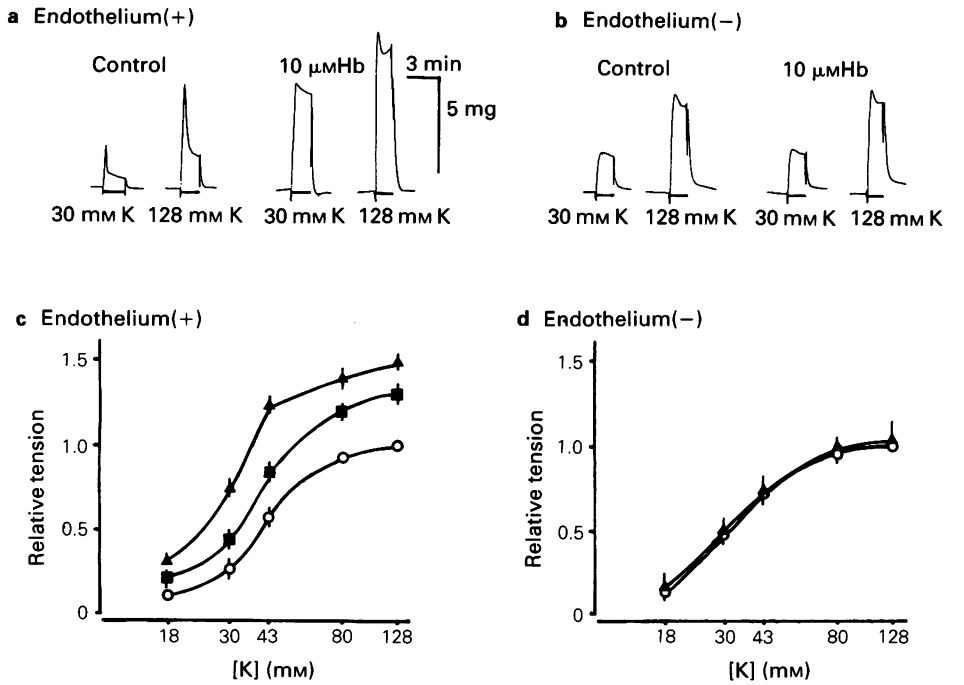

Figure 1 Effects of oxyhaemoglobin (Hb) on contractions evoked by various concentrations of K^+ in the presence (a and c) or absence of endothelial cells (b and d). Effects of Hb (10 μM) on the individual contractions evoked by K^+ (30 mM and 128 mM) in intact tissues (a), or in the endothelium-denuded tissues (b). Grouped data showing effects of Hb (1 and 10 μM) on K^+-induced phasic contraction in intact ($n = 5$) and endothelium-denuded ($n = 4$–6) tissues are shown in (c) and (d). The amplitude of the contraction evoked by K^+ (128 mM) in intact tissues was normalised as a relative value of 1.0: (○) control; (▲) 10 μM Hb; (■) 1 μM Hb.

after adding 10 times sodium dithionite to Hb (1 mM), dialysis by 200 times distilled water was carried out at 4°C. The identification and final concentration of oxyhaemoglobin were determined spectrophotometrically. Hereafter the term Hb is used to mean oxyhaemoglobin. The chemicals used were acetylcholine Cl (Daiichi), saponin (ICN Pharmac. Co.) 9,11-epithio-11,12,-methano-thromboxane A_2 (STA_2; Ono Pharmac. Co.), nifedipine (Bayer Pharmac. Co.), caffeine (Wako Pure Chem.), A23187 (calimycin, free acid; Sigma) and ethyleneglycol-bis (β-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA, Dozin Pharmac.).

Statistics

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

Results

Excitatory effects of haemoglobin on the K^+- and STA_2-induced contractions in intact and in endothelium-denuded tissues

Hb itself did not produce any detectable contraction on muscle strips prepared from the guinea-pig basilar artery. All of the studies described here relate to the effects of Hb on the pharmacological action of other agents. In endothelium-intact tissues, application of K^+ (30 mM) produced a rapid (phasic) contraction followed by a low amplitude (tonic) contraction. The maximum amplitude of contraction that could be induced by K^+ was evoked by a concentration of 128 mM. However, when Hb (10 μM) was applied 10 min before and during application of high K^+ solutions, the amplitudes of both the phasic and tonic contractions were markedly enhanced (Figure 1a). When the amplitude of the phasic contraction evoked by 128 mM K^+ in the absence of Hb was normalized as a relative tension of 1.0, Hb

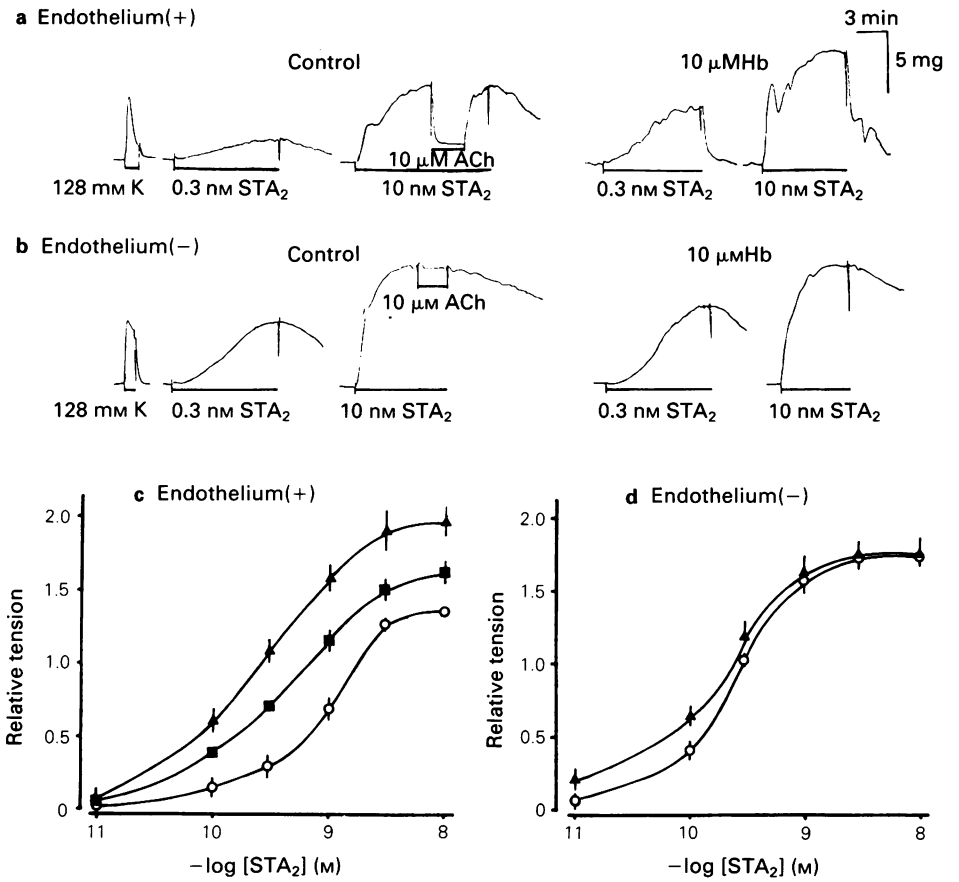

Figure 2 Effects of oxyhaemoglobin (Hb) on the STA_2-induced contraction in intact and endothelium denuded tissues. (a) Examples of contractions evoked by STA_2 (0.3 nM and 10 nM) in the presence or absence of Hb (10 μM) in intact tissues. The contraction evoked by K^+ (128 mM) was taken as the control. (b) The same experimental protocol as in (a), following removal of the endothelium. In (a) and (b), acetylcholine (ACh, 10 μM) was applied during the STA_2-induced contraction to establish whether the endothelium had been removed from the tissue. (c and d) Grouped data showing the effects of Hb on the STA_2-induced contraction before ($n = 3$–8) and after ($n = 4$–6) removal of endothelial cells. The contraction amplitude evoked by K^+ (128 mM) in intact tissues was normalized as 1.0: (○) control; (▲) in the presence of Hb (10 μM) and (■) Hb (1 μM), respectively. Hb was applied 10 min before application of STA_2.

(above 1 μM) consistently enhanced the phasic contraction amplitude at any given concentration of K^+, in a concentration-dependent fashion (Figure 1c).

Following removal of endothelium, the tonic contraction evoked by high K^+ solutions (30 mM and 128 mM) was markedly enhanced (Figure 1b) and reached virtually the same amplitude as the phasic contraction. Any enhancement of the phasic contraction amplitude relative to that observed in intact muscle tissues was minimal. Application of Hb (10 μM) had no effect on the contraction amplitude (Figure 1b). In fact, Hb had no effect on the contractile amplitude in endothelium-denuded tissue at any concentration of K^+ (Figure 1d).

Application of STA_2 (0.3 nM or 10 nM) induced contraction, and application of STA_2 (10 nM) induced a contraction of much higher amplitude than that induced by K^+ (128 mM) and the relaxation of the tissue after removal of STA_2 was very much slower than after removal of K^+ (Figure 2a). Application of Hb (10 μM) enhanced the STA_2-induced contraction (Figure 2a). In endothelium-denuded tissues, the amplitude of the contraction provoked by STA_2 was enhanced compared to that observed in intact tissues, but application of Hb (10 μM) then produced

no further enhancement of the contraction amplitude (Figure 2b). To ascertain whether the endothelium was removed, the effect of ACh (10 μM) was examined (compare Figure 2a and b); the results showed that ACh caused relaxation only in the intact tissue. In intact muscle tissues, Hb (1 and 10 μM) enhanced the contraction evoked by STA_2 in a concentration-dependent fashion (Figure 2c). However, in the endothelium-denuded tissue (Figure 2d), the contraction amplitude was slightly enhanced by Hb only at low concentrations of STA_2 (below 1 nM).

Effects of Hb on the STA_2-induced contraction in Ca^{2+}-free solution or in the presence of Ca antagonist

The effects of Hb on STA_2-induced contractions in intact muscle tissues in Ca^{2+}-free solution or in the presence of nifedipine were also studied (Figure 3). After removal of Ca^{2+} from the Krebs solution containing EGTA (2 mM) for 7 min, STA_2 (10 nM) produced a small amplitude contraction and re-addition of Ca^{2+} (2.6 mM) restored the contraction amplitude to that observed in the presence of Ca^{2+}. When Hb (10 μM) was applied 5 min before application of STA_2 in Ca^{2+}-free solution, the amplitude of the contraction evoked by STA_2 (10 nM) was not affected, but that evoked by STA_2 following re-addition of Ca^{2+} (2.6 mM) was enhanced. When Ca^{2+} was removed again, the tissue relaxed rapidly (Figure 3a). The effects of Hb on the STA_2-induced contraction during treatment with nifedipine are shown in Figure 3b. Since nifedipine (0.3 μM) blocked the K^+-induced contraction in guinea-pig basilar arteries which were endothelium-denuded (Nishiye *et al.* 1988), this same concentration of nifedipine was applied for 10 min before application of STA_2 (10 nM). Nifedipine reduced the contraction amplitude to half of the control, but pre-application of Hb (10 μM) restored the contraction amplitude evoked by STA_2 (10 nM) so that there was only a slight reduction in the amplitude compared with that observed in the absence of nifedipine. Furthermore, ACh (10 μM) inhibited the contraction evoked by STA_2 (10 nM) to 0.6 times the control in the presence of nifedipine (0.3 μM; $n = 3$). The extent of this inhibition was roughly the same as observed in the absence of nifedipine.

Effects of Hb on the caffeine-induced contraction

To investigate the actions of Hb on intracellular Ca^{2+} stores, the effects of Hb on the caffeine-induced contraction were observed in intact tissues in Ca^{2+}-free solution. As shown in Figure 4a, the first application of caffeine (2 mM) produced a transient contraction. A second application of caffeine (2 mM) given immediately after the first contraction had little effect (not shown), but application of caffeine (20 mM) after an interval of 5 min, produced a contraction that was small in comparison to that evoked

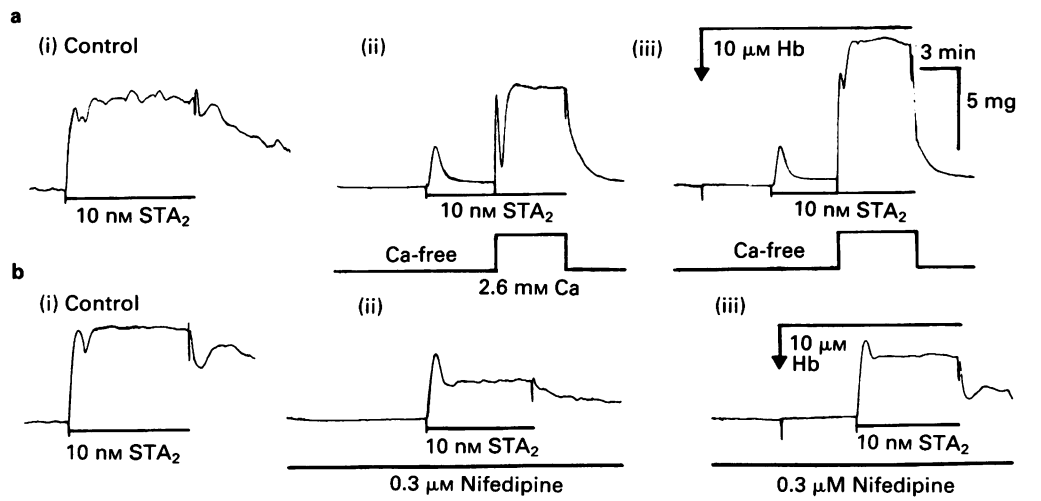

Figure 3 Effects of oxyhaemoglobin (Hb) on the STA_2-induced contraction in intact tissues in Ca^{2+}-free solution containing EGTA (2 mM) or in the presence of nifedipine. (a) (i) Contraction induced by STA_2 (10 nM) was recorded as the control. (ii) Seven min after transition to Ca^{2+}-free solution STA_2 (10 nM) was applied, then Ca^{2+} (2.6 mM) was applied for 5 min. (iii) The same protocol as in (ii) but 5 min before application of STA_2, Hb (10 μM) was added. (b) The response to STA_2 (10 nM; control, (i)), in the presence of nifedipine (0.3 μM), applied 10 min before application of STA_2, (ii), and with the addition of Hb (iii).

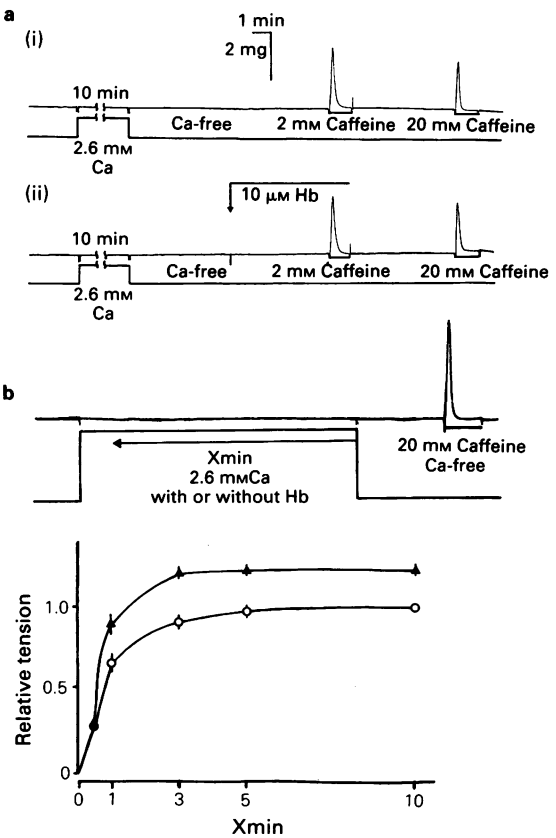

Figure 4 Effects of oxyhaemoglobin (Hb) on the Ca^{2+} release from and Ca^{2+} accumulation into intracellular storage sites as estimated from the amplitude of caffeine-induced contraction in intact tissues. (a) (i) After a period in Ca^{2+}-free solution so as to deplete cellular Ca^{2+} stores, Ca^{2+} (2.6 mM) was applied for 10 min and then the Ca^{2+}-free solution was restored. Ten min later caffeine (2 mM then 20 mM) was applied with an interval of 5 min. (ii) The same protocol as described in (i), but Hb (10 μM) was applied before application of caffeine (indicated by arrow). (b) After depletion of Ca^{2+} stored in smooth muscle cells as in (a), Ca^{2+} (2.6 mM) was applied with or without Hb for various periods (X min), then 3 min after return to Ca^{2+}-free solution, caffeine (20 mM) was applied. The grouped data are all shown in graph below. The contraction amplitude evoked by 10 min application of Ca^{2+} was normalized as a relative tension of 1.0: (○) control and (▲) in the presence of Hb (10 μM).

by the first application of caffeine. When Hb (10 μM) was applied 5 min before and during applications of caffeine (2 mM) in the above experiments, the amplitude of the contractions evoked by 2 or 20 mM remained the same as those observed before application of Hb [Figure 4a, (i) vs (ii)].

Figure 4b shows the effects of Hb on caffeine-induced contraction of intact muscle tissues. The tissue was perfused with Ca^{2+}-free solution and caffeine (20 mM) was repetitively applied to deplete the Ca^{2+} stored in the cells, then Ca^{2+} (2.6 mM) was applied for various periods (up to 10 min) in the presence or absence of Hb (10 μM). After a subsequent period of 3 min in Ca^{2+}-free solution, caffeine (20 mM) was applied so as to allow an estimation of the amount of Ca^{2+} stored in the sarcoplasmic reticulum. Amplitudes of the caffeine-induced contraction increased in a time-dependent fashion and reached a steady level 3 min after application of Ca^{2+}. When Hb (10 μM) was added to the Ca^{2+} containing solution, the contraction amplitude provoked by 1 min after application was consistently enhanced.

Effects of Hb on the Ca^{2+}-induced contraction in skinned muscle tissues

In skinned muscle tissues, the minimum concentration of Ca^{2+} required to produce a contraction was 0.1 μM, and the maximum response was evoked by 10 μM Ca^{2+}. The maximum amplitude of the contraction induced by STA_2 (10 nM) or K^+ (128 nM) was 50–70% of that induced by Ca^{2+} (10 μM). These observations confirm the previous observations made on these tissues (Nishiye *et al.*, 1988). After the contraction had reached a steady amplitude following cumulative accumulation of stepwise increase in Ca^{2+}, Hb (10 μM) had no effect on the contraction evoked by any given concentrations of Ca^{2+} (not shown).

Prevention of the EDRF-induced relaxation by Hb in the guinea-pig basilar artery

To observe the effects of Hb on the ACh-induced relaxation of intact tissues that were contracted by STA_2, ACh (10 μM) was applied for 15 min. As shown in Figure 5a, ACh (10 μM) relaxed the precontracted tissue by STA_2 (0.3 nM or 3 nM) nearly completely and the relaxation was maintained over 15 min with only a slight restoration in the contraction amplitude. When Hb (10 μM) was applied 10 min before and during application of STA_2, ACh transiently relaxed the tissues to a level close to the resting level but the contraction re-developed. Consistently, the amplitude of the contraction and the initial relaxation induced by ACh (10 μM) evoked by STA_2 (3 nM) and STA_2 (0.3 nM) were the same in the presence as in the absence of Hb (10 μM), but the relaxation was gradually attenuated in the presence of Hb. Figure 6 summarises the effects of Hb on the ACh-induced relaxation of tissues that were precontracted by STA_2 against time.

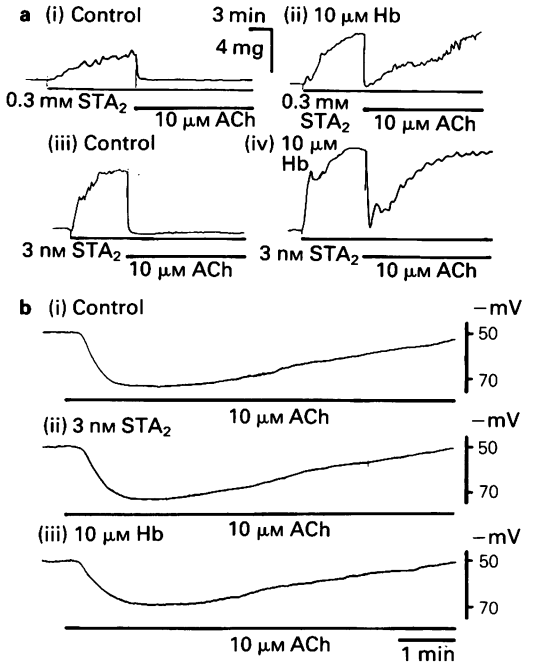

Figure 5 Effects of oxyhaemoglobin (Hb, 10 μM) on the time courses of relaxation of tissues that were precontracted by STA_2 (a) and on hyperpolarization induced by acetylcholine (ACh, 10 μM) in the presence or absence of STA_2 (3 nM) (b). (a) Relaxations were induced by ACh (10 μM) following application of STA_2 (0.3 nM and 3 nM) in the presence (ii) and (iv) or absence of Hb (10 μM) (i) and (iii). Hb was applied 10 min before application of STA_2. (b) Amplitudes and time courses of the hyperpolarization induced by ACh (10 μM) in the presence or absence of Hb. (i) effect of ACh (10 μM); (ii) effect of ACh following application of STA_2 (3 nM); (iii) effect of Hb (10 μM) on the ACh-induced hyperpolarization following applications of STA_2. STA_2 and Hb were applied 10 min before application of ACh.

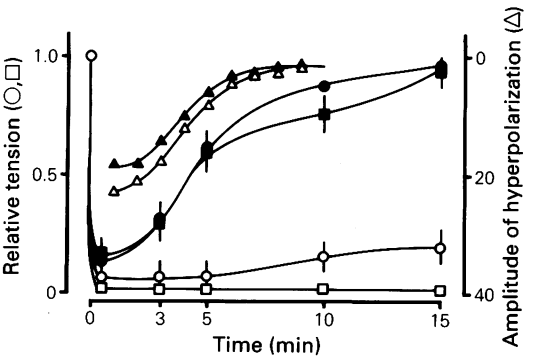

Figure 6 Time courses of the relaxation and hyperpolarization induced by acetylcholine (ACh) in the presence or absence of oxyhaemoglobin (Hb, 10 μM). Left vertical axis indicates the relative tension (the amplitude of the contraction evoked by STA_2 (0.3 or 3 nM) in the presence or absence of Hb were normalized as 1.0, respectively). Right vertical axis indicates the membrane potential (indicated by the absolute value). The STA_2-induced contractions (0.3 nM and 3 nM) in absence of Hb (○, □), in the presence of Hb (●, ■); (△, ▲) membrane potential measured before and after application of Hb. ACh was applied just after 0 min.

Effects of Hb on the ACh-induced changes in membrane potential in smooth muscle cells of the guinea-pig basilar artery

The resting membrane potential of smooth muscle cells of the guinea-pig basilar artery was -50.8 ± 1.2 mV ($n = 30$, 3 preparations) and this value was much the same as that measured in other investigations (guinea-pig: Fujiwara & Kuriyama, 1983; 1984; dog: Fujiwara *et al.*, 1982). The smooth muscle cells were electrically quiescent and spontaneous discharges were not obtained.

Application of ACh (10 μM) caused marked hyperpolarization of the membrane (the peak amplitude was 18 mV, $n = 5$) and this gradually attenuated over several minutes (Figure 5b(i)). When STA_2 (3 nM) was applied, there was no change in membrane potential and subsequent application of ACh (10 μM) hyperpolarized the membrane to the same extent as observed on application of ACh alone (Figure 5b(ii)). Pretreatment with Hb (10 μM) did not modify the membrane potential but did slightly reduce the peak amplitude of the hyperpolarization induced by ACh (10 μM) in the presence of STA_2 (at 3 nM; 15 mV, $n = 5$), without affecting the duration of the hyperpolarization (Figure 5b(iii)). In endothelium-denuded tissue, ACh (10 μM) did not produce hyperpolarization. In Figure 6, the time course of hyperpolarization induced by ACh in the presence or absence of Hb can be compared with the relaxation induced by the same procedures. As can be seen, the time course of the hyperpolarization observed both in the presence and in the absence of Hb coincided well with the time course of the relaxation induced by ACh in the presence of Hb.

A23187 (0.01–1 μM) relaxed the STA_2-contracted tissues in a concentration-dependent fashion (Figure 7a). A23187 (0.01–0.1 μM) did not modify the membrane potential but at a concentration of 1 μM, A23187 caused a slight though statistically insignificant hyperpolarization. The effects of A23187 on the STA_2-induced contraction and membrane potential are summarized in Figure 7b.

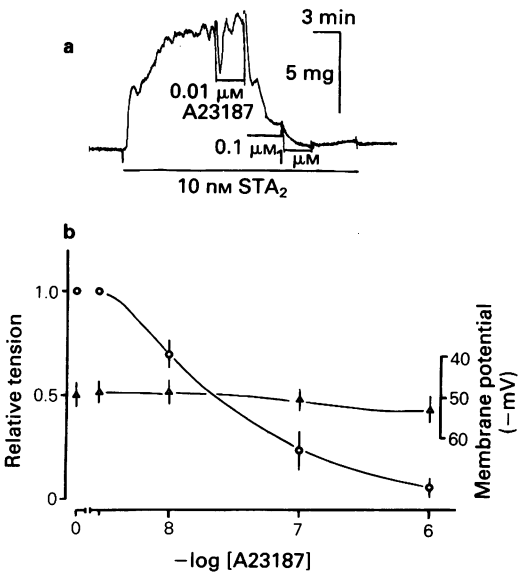

Figure 7 The effects of A23187 on the STA_2-induced contraction (a) and on the membrane potential and mechanical response (b). (a) A23187 was cumulatively applied during the contraction evoked by STA_2 (10 nM). (b) Effects of various concentrations of A23187 on the membrane potential (△, $n = 10$–15; 3 preparations) and mechanical responses (○, $n = 5$). (The STA_2-induced contraction before application of A23187 was normalized as 1.0.)

Discussion

The present study shows that in the guinea-pig intact basilar artery, ACh relaxes preparations that were precontracted by STA_2 and suggest that this is caused by release of EDRF and an endothelial-dependent hyperpolarizing factor (EDHF). It is possible that the latter, like EDRF, is released from the endothelium. As estimated from the actions of Hb and the time course of hyperpolarization, it seems that EDRF produced the long lasting relaxation and the EDHF contributed to an initial transient relaxation.

The thromboxane A_2 (TXA_2) analogue, STA_2, produced a larger contraction amplitude in this tissue in comparison to that induced by ACh, 5-hydroxytryptamine or other agonists (Nishiye *et al.*, 1988). This contrasts with the rabbit coronary artery, in which the maximum contractile amplitude induced by ACh was larger than that evoked by STA_2 (100 nM). Kanmura *et al.* (1987) deduced that the STA_2-induced contraction was due to activation of receptor-operated Ca^{2+} influx and also to intracellular release of Ca^{2+} from the sarcoplasmic reticulum following activation of the TXA_2 receptor. The present results on the nature of contraction evoked in Ca^{2+}-free solution containing EGTA support that conclusion. In the presence of a Ca antagonist (nifedipine), the amplitude of the contraction evoked by STA_2 was reduced but Hb partially restored it. Therefore, the release of EDRF may not be but the Ca^{2+} mobilisation in smooth muscle may be inibited by nifedipine. However, the underlying mechanism of restoration of the nifedipine-induced inhibition by Hb has not been resolved in the present experiments.

In intact tissues, Hb enhanced the amplitude of the contraction produced by high K^+ or by STA_2. The tonic component of the K^+-induced contraction was enhanced to a greater extent by Hb than the phasic, but both components of responses induced by STA_2 were enhanced by Hb. Hb did not modify the release of Ca^{2+} from the intracellular stores induced by application of STA_2 or by caffeine in Ca^{2+}-free solution containing EGTA but Hb did enhance the accumulation of Ca^{2+}, as estimated from the amplitude of the caffeine-induced contraction amplitude. Since Hb had no effect on the Ca^{2+}-induced contraction in skinned muscle tissues, it may be that Hb can inhibit the action of EDRF released spontaneously or by stimulation with an agonist. Thus enhancement of the amplitude of the contraction probably occurs by increasing the amount of free Ca^{2+} in the cytosol rather than by direct actions on the contractile proteins.

It is known that Hb inhibits the relaxation of precontracted tissue caused by release of EDRF, via inhibition of guanylate cyclase in the cytosol (soluble guanylate cyclase) using the same mechanism as the inhibition of relaxation induced by nitro-compounds (Miki *et al.*, 1977; Murad *et al.*, 1978; Mittal *et al.*, 1978; Gruetter *et al.*, 1979; 1981; Itoh *et al.*, 1985). In fact, EDRF-induced relaxation is thought to be related to synthesis of cyclic GMP in the cytosol through a nitric oxide residue of EDRF (Furchgott & Jothianandan, 1983; Palmer *et al.*, 1987). Nitroglycerin and nicorandil apparently reduce the amount of free Ca^{2+} in the cytosol, judging from experiments with the fluorescent indicator quin2 (Sumimoto *et al.*, 1987). Moreover, cyclic GMP is known to accelerate the Ca pump at the sarcolemma (Suematsu *et al.*, 1984; Itoh *et al.*, 1985; Popescu *et al.*, 1985; Sumimoto *et al.*, 1987). Since Hb enhanced the amplitude of the contraction evoked by STA_2 to a greater extent than the contraction induced by high K^+, STA_2 may cause more release of EDRF than high K^+. However, the tonic component of the high K^+-induced contraction was markedly enhanced by Hb, so the possibility that high K^+ itself induces EDRF release cannot be ruled out. These findings are consistent with previously

published data obtained with the dog coronary artery (Robertson, 1985). Furthermore, it is also apparent from the present results that in endothelium-denuded tissue, the amplitude of the tonic contraction was of much the same amplitude as the phasic component. Thus the relatively low amplitude of tonic component in comparison to the phasic component of the K^+-induced contraction in intact tissues could be partly explained by release of EDRF by high K^+.

Hb inhibited the ACh-induced relaxation of tissues that were precontracted by STA_2, such that the ACh-induced relaxation was sustained for over 15 min with only a gradual recovery. However, in the presence of Hb, ACh caused transient relaxation. This indicates that Hb does not completely prevent the endothelium-dependent relaxation.

Komori & Suzuki (1987a,b) found that in the rabbit saphenous artery, stimulation of endothelium by ACh releases EDRF and EDHF. The hyperpolarization induced by ACh was transient and ceased within several minutes. Such hyperpolarization and sustained relaxation also occurred in the pig coronary artery (Beny *et al.*, 1986) and in the rat aorta (Southerton *et al.*, 1987). Furthermore, Komori & Suzuki (1987a) observed that although both ACh and oxotremorine cause release of EDRF and relax the precontracted rabbit saphenous artery, only ACh hyperpolarizes the membrane. Similarly Bolton & Clapp (1986) reported that substance P produces relaxation of guinea-pig mesenteric artery that had been precontracted by noradrenaline without inducing any hyperpolarization of the membrane. Thus, ACh seems to release EDRF and EDHF from the endothelium in both tissues but by independent mechanisms. The present results obtained from the guinea-pig basilar artery suggest that ACh causes relaxation by releasing both EDRF and EDHF; the initial relaxation is mainly due to the hyperpolarization of the membrane as observed in the presence of Hb and the sustained relaxation is mainly due to release of EDRF. It should however be noted that the electrical and mechanical activities of the basilar artery in response to ACh were recorded at different temperatures.

The Ca ionophore, A23187 is also known to be a stimulant of EDRF release (Rapoport & Murad, 1983; Griffith *et al.*, 1984a,b; Cocks *et al.*, 1985) and in fact, A23187 relaxed the basilar artery which had been precontracted by STA_2. However, A23187 did not produce hyperpolarization of the membrane. This is surprising since release of EDRF has been reported to be Ca-dependent (Winquist *et al.*, 1985), moreover, we found that nifedipine did not prevent EDRF-induced relaxation. These observations suggest that the cellular mechanisms responsible for release of EDRF and EDHF differ (Komori & Suzuki, 1987b) and also that unlike EDRF, EDHF release cannot be evoked by the Ca^{2+} ionophore.

Fujiwara & Kuriyama (1983, 1984) reported that in the guinea-pig basilar artery haemolysate caused depolarization of the membrane which was associated with an increase in membrane resistance. Presumably haemolysate reduced the K^+ conductance of the membrane. Since we have now found that Hb does not depolarize the membrane in this tissue, it seems reasonable to postulate that factors in the haemolysate other than Hb may have contributed to the depolarization.

In the basilar artery, multiple substances may be released during activation of endothelial cells, not only EDRF and EDHF but also endothelium-derived contracting factor (De Mey & Vanhoutte, 1983; Nagao & Suzuki, 1987) and also relaxing substances, such as prostacyclin (PGI_2) (Moncada *et al.*, 1977; Baenziger *et al.*, 1977; Eldor *et al.*, 1981; Domae & Kuriyama, 1986). It is unlikely that the effects of ACh on the guinea-pig basilar artery can be accounted for by the release of PGI_2 since the relaxant response to ACh is unchanged by indomethacin and PGI_2 does not hyperpolarize the membrane (unpublished observations). Most of these substances except for endothelium-derived contracting factor tend to produce relaxation of vascular tissues. Cerebral vasospasm, occurring following subarachnoid haemorrhage, may be partly caused by synthesis of thromboxane A_2 or leukotriene D_4 (Tagari *et al.*, 1983; Rosenblum, 1985; Busija *et al.*, 1986) by the platelets, leucocytes and erythrocytes contained within the clot (coagulate). Thus, Hb contained in the clot may act as a stimulant of vascular smooth muscle cells and also as an inhibitor of EDRF-induced relaxation, so causing vasoconstriction. Thus, if endothelial cells were damaged following subarachnoid haemorrhage, vasoconstriction may well be accelerated.

The present results demonstrate that EDRF and EDHF may be released from the endothelium in the guinea-pig basilar artery via different mechanisms and thus may contribute to the maintenance of low resting muscle tone. Cerebral vasospasm provoked following subarachnoid haemorrhage may in part be caused by damage of endothelium and/or by substances released from the clot, such as Hb (Fisher *et al.*, 1980; Ohta *et al.*, 1980).

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Modulation of phospholipase A_2 activity in human fibroblasts

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1 Human embryonic skin fibroblasts (HSF) incubated overnight with either human recombinant interleukin-1α (rIL-1α) or interleukin-1β (rIL-1β) released large amounts of prostaglandin E_2 (PGE_2).

2 rIL-1β, bradykinin (Bk) and arachidonic acid (AA) significantly stimulated PGE_2 release from HSF incubated overnight in the presence of either interleukin.

3 Hydrocortisone inhibited the PGE_2 release induced by rIL-1β and Bk, but not by AA.

4 The steroid inhibitory effect was reversed by actinomycin D as well as by an anti-lipocortin monoclonal antibody.

5 The results suggest that in HSF, rIL-1β is able to stimulate both cyclo-oxygenase and phospholipase A_2 (PLA_2) activity.

6 The stimulation of PLA_2 activity by rIL-1β is inhibited by hydrocortisone, probably via induction of lipocortin-like proteins.

Introduction

Interleukin-1 (IL-1) is a polypeptide with potent amplifying effects on the inflammatory response (Dinarello, 1988; Martin & Resch, 1988). Among these effects the ability of IL-1 to stimulate prostaglandin E_2 (PGE_2) production in several cell types has been well established (Dayer *et al.*, 1981; Whiteley & Needleman, 1984). It has been proposed that the mechanism of this stimulation is an enhanced cyclo-oxygenase activity due to *de novo* protein synthesis induced by IL-1 (Newton & Covington, 1987; O'Neill *et al.*, 1987). The rate limiting step in eicosanoid biosynthesis is thought to be the availability of the arachidonate substrate released by enzymes like phospholipase A_2 (PLA_2, Flower & Blackwell, 1976). The effect of IL-1 on PLA_2 activation seems to be variable: IL-1 stimulated PLA_2 activity in chondrocytes (Chang *et al.*, 1986) and synovial cells (Godfrey *et al.*, 1987), but not in fibroblasts (Bernheim & Dinarello, 1985; Newton & Covington, 1987).

Lipocortins are a family of PLA_2-inhibitory proteins induced by corticosteroids (Di Rosa *et al.*, 1984; Wallner *et al.*, 1986). The inhibition of PLA_2 might explain, at least in part, the anti-inflammatory effect of lipocortins and corticosteroids (Parente *et al.*, 1984; Cirino *et al.*, 1987). The purpose of the present work was to investigate the mechanism of PLA_2 activation and inhibition in human fibroblasts in order to clarify a possible functional antagonism between IL-1 and lipocortin.

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Methods

Cell culture

Human embryonic skin fibroblasts (HSF) were obtained from the Interferon Unit, Sclavo, Italy. Cells were maintained in Dulbecco's modified Eagle's medium with 10% foetal calf serum, 2 mM L-glutamine together with 50 $\mu g\,ml^{-1}$ gentamycin and were passaged by trypsin treatment. Cells were routinely split twice weekly and used between passages 5 and 15.

Prostaglandin E_2 production by human embryonic skin fibroblasts

HSF were seeded at 1×10^4 cells per well of Cluster[96] plates (Costar, U.S.A.) in culture medium with 10% foetal calf serum, and incubated for 72 h at

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37°C in a controlled humidified incubator in 5% CO_2. HSF were then rinsed three times and incubated overnight (16 h) in serum-free culture medium either with or without IL-1. After overnight incubation, supernatants were collected for evaluation of PGE_2 levels. In some experiments, after overnight incubation and collection of supernatant, HSF were rinsed three times and further incubated for fixed times with different drug treatments (see Tables 2 to 5). Supernatants were then removed for assessment of PGE_2 levels. PGE_2 determinations were performed by specific radioimmunoassay with minimal (3.7%) cross-reactivity with PGE_1 (NEN-E.I. DuPont de Nemours and Co., Inc., U.S.A.). Cell viability was always >95% as measured by the trypan blue exclusion test.

Materials

Dulbecco's modified Eagle's medium was purchased from Flow Laboratories, U.S.A.; glutamine from Serva Feinbiochemica GmbH & Co., West Germany; foetal calf serum from Gibco Ltd., U.K. Gentamycin, bradykinin, arachidonic acid sodium salt, hydrocortisone 21-hemisuccinate sodium salt and actinomycin D were obtained from Sigma Chemical, U.S.A. Human recombinant IL-1α (specific activity 1×10^7 units mg^{-1}) was purchased from Genzyme Biochemicals, U.K., human recombinant IL-1β (specific activity 1×10^7 units mg^{-1}) was produced at Sclavo, Italy. Endotoxin contamination was $<20\,pg\,\mu g^{-1}$ as measured by the Limulus amoebocyte lysate chromogenic assay. Anti-lipocortin monoclonal antibody RM23 and control monoclonal RT97 were kindly provided by Professor R.J. Flower, University of Bath (Flower *et al.*, 1984).

Statistics

The statistical analysis was performed using Student's *t* test for unpaired samples. Differences were considered statistically significant when $P < 0.05$.

Results

Effect of recombinant IL-1α and IL-1β on PGE_2 release by HSF

Table 1 shows that both rIL-1α and rIL-1β incubated overnight with HSF induced a 20 fold increase in PGE_2 release compared to cells incubated in medium alone. However, the recombinant interleukins were unable to stimulate PGE_2 release when incubated for 30 min with cells preincubated overnight in medium alone (Table 2) whereas, both

Table 1 Release of prostaglandin E_2 (PGE_2) by human embryonic skin fibroblasts (HSF) incubated overnight in the presence of human recombinant interleukin (rIL-1)

Treatment	*Concentration* ($pg\,ml^{-1}$)	*PGE_2* ($ng\,ml^{-1}$)
Medium	—	2.2 ± 0.5
rIL-1α	250	40.9 ± 4.7***
rIL-1β	250	42.9 ± 5.1***

Results shown are mean ± s.e.mean of 4 different experiments performed in quadruplicate.
Significantly different from medium: ***$P < 0.001$.

Table 2 Release of prostaglandin E_2 (PGE_2) by human embryonic skin fibroblasts (HSF)

Overnight incubation	*30 min subsequent incubation* (PGE_2 $ng\,ml^{-1}$)				
	Medium	*rIL-1α* ($250\,pg\,ml^{-1}$)	*rIL-1β* ($250\,pg\,ml^{-1}$)	*Bk* (1 μM)	*AA* ($1\,\mu g\,ml^{-1}$)
Medium	3.4 ± 0.2	3.0 ± 0.2^{NS}	3.8 ± 0.2^{NS}	10.4 ± 1.2**	24.4 ± 1.5***
rIL-1α ($250\,pg\,ml^{-1}$)	8.8 ± 0.8	11.5 ± 1.5^{NS}	16.9 ± 1.9**	40.9 ± 5.9**	ND
rIL-1β ($250\,pg\,ml^{-1}$)	9.9 ± 0.9	12.5 ± 0.3*	16.6 ± 1.5**	27.3 ± 3.7**	48.3 ± 3.7***

HSF were incubated overnight with medium or recombinant interleukin (IL-1), washed 3 times, then incubated for further 30 min with medium or stimuli (see Methods). Bk, bradykinin; AA, arachidonic acid. Results shown are mean ± s.e.mean of 4 different experiments performed in quadruplicate. Statistical differences vs 30 min incubations in medium: NS, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ND, not done

Table 3 Effect of hydrocortisone (HCT) (20 μM) on prostaglandin E_2 (PGE_2) release by human embryonic skin fibroblasts (HSF)

Overnight incubation		*Subsequent incubations* (PGE_2 ng ml^{-1}) 60 min / 30 min		
		rIL-1β (250 pg ml^{-1})	*Bk* (1 μM)	*AA* (1 μg ml^{-1})
rIL-1β (250 pg ml^{-1})	Medium	18.8 ± 2.4	29.6 ± 4.0	52.4 ± 4.0
	HCT	7.3 ± 0.7**	15.8 ± 0.4*	66.9 ± 4.5^{NS}

HSF were incubated overnight with recombinant interleukin-1β (rIL-1β), washed 3 times, then incubated for further 60 min with medium or hydrocortisone and further 30 min with stimuli (see Methods). Bk, bradykinin; AA, arachidonic acid. Results shown are mean ± s.e.mean of 4 different experiments performed in quadruplicate. Statistical differences vs incubations in medium: NS, not significant; *$P < 0.05$; **$P < 0.01$.

bradykinin (Bk) and arachidonic acid (AA) stimulated a highly significant increase in PGE_2 release over 30 min. Table 2 also shows that rIL-1β (and to a lesser extent rIL-1α) incubated for 30 min significantly stimulated PGE_2 release from cells preincubated overnight with either interleukin. In these cells, Bk and AA exhibited a similar but more potent effect over 30 min.

Effect of hydrocortisone, actinomycin D and monoclonal antibodies on PGE_2 release by HSF

In order to investigate the effect of hydrocortisone, the steroid was added to HSF incubated overnight with rIL-1β. After 60 min cells were stimulated with rIL-1β, Bk or AA and incubated for a further 30 min. Table 3 shows that hydrocortisone treatment was able to inhibit PGE_2 release induced by both rIL-1β and Bk, but not by AA in the 30 min incubation. When hydrocortisone was added together with actinomycin D the inhibitory effect on PGE_2 release stimulated by IL-1 was abolished (Table 4). The hydrocortisone inhibitory effect was also blocked by anti-lipocortin monoclonal antibody RM 23. A control monoclonal RT 97 was ineffective (Table 5). The control release of PGE_2 was not affected by either monoclonal antibody (data not shown).

Discussion

The up-regulation of cyclo-oxygenase activity in fibroblasts plays an important role in the stimulation of PGE_2 release induced by IL-1 (Newton & Covington, 1987; Raz *et al.*, 1988). This is confirmed by our results shown in Table 2. HSF incubated overnight in the presence of rIL-1β released twice as much PGE_2 as cells incubated overnight in medium when stimulated by AA. This effect is probably due to an increased cyclo-oxygenase activity in cells incubated with rIL-1β. However, the rate-limiting step in eicosanoid formation is the availability of AA released by the action of enzymes like phospholipase C (PLC) and PLA_2 (Irvine, 1982). It has been shown that in fibroblasts bradykinin receptors are coupled to both PLC and PLA_2 and that the latter enzyme catalyzes release of arachidonate for prostaglandin biosynthesis (Burch & Axelrod, 1987). Data shown in Table 2 demonstrate that HSF incubated overnight with the interleukins released larger amounts of PGE_2 than cells incubated overnight with medium when stimulated by Bk for 30 min, confirming previous results (O'Neill & Lewis, 1987). It is conceivable that Bk-induced activation of PLA_2 released free arachidonate which in turn has been metabolized to PGE_2 by cyclo-oxygenase. Like Bk, rIL-1β in the 30 min incubation stimulated PGE_2 release from cells incubated overnight in the presence of either interleukin suggesting an increased release of AA (Table 2). rIL-1α was less potent than rIL-1β in releasing PGE_2 in the 30 min incubation from cells incubated overnight with either interleukin. It is noteworthy that differences in potencies of the two interleukins in the stimulation of AA metabolism has been reported in different cell types (Xiao & Levine, 1986). These results suggest that in

Table 4 Effect of hydrocortisone (HCT, 20 μM) and actinomycin D (Act D, 0.8 μM) on prostaglandin E_2 (PGE_2) release by human embryonic skin fibroblasts (HSF)

Overnight incubation	*Subsequent incubations* 60 min	30 min	*PGE_2* (ng ml^{-1})
	Medium		18.8 ± 2.4
rIL-1β	HCT	rIL-1β	7.3 ± 0.7
250 pg ml^{-1}	HCT + Act D	250 pg ml^{-1}	19.5 ± 0.9***
	Act D		15.2 ± 1.6^{NS}

HSF were incubated overnight with recombinant interleukin 1β (rIL-1β), washed 3 times, then incubated for further 60 min with medium or drugs and for further 30 min with rIL-1β (see Methods). Results shown are mean ± s.e.mean of 4 different experiments performed in quadruplicate. Statistical differences: ^{NS} not significant vs incubation in medium; ***$P < 0.001$ vs incubation with HCT alone.

Table 5 Effect of hydrocortisone (HCT, 20 μM) and monoclonal antibodies on prostaglandin E_2 (PGE_2) release by human embryonic skin fibroblasts (HSF)

Overnight incubation	*Subsequent incubations* 60 min	30 min	*PGE_2* (ng ml^{-1})
	Medium		18.8 ± 2.4
	HCT		7.3 ± 0.7
	HCT + RM 23 (1:1000)		17.6 ± 1.0***
rIL-1β 250 pg ml^{-1}	HCT + RM 23 (1:2000)	rIL-1β 250 pg ml^{-1}	13.4 ± 1.0**
	HCT + RT 97 (1:1000)		9.5 ± 0.7^{NS}
	HCT + RT 97 (1:2000)		8.4 ± 1.0^{NS}

HSF were incubated overnight with recombinant interleukin-1β (rIL-1β), washed 3 times, then incubated for further 60 min with medium or HCT or HCT + antibodies (RM23 and RT97) at the dilutions shown, and for further 30 min with rIL-1β (see Methods). Results shown are mean ± s.e.mean of 4 different experiments performed in quadruplicate. Statistical differences vs incubations with HCT alone: ^{NS} not significant; $^{**}P < 0.01$, $^{***}P < 0.001$.

HSF, rIL-1β is able to activate both cyclo-oxygenase and PLA_2 activity. The activation of cyclo-oxygenase requires protein synthesis (Raz *et al.*, 1988) and longer incubation times whilst the activation of PLA_2 is more rapid in onset and appears to be a receptor-mediated mechanism like the Bk effect (Burch & Axelrod, 1987).

The PLA_2 stimulatory effect by rIL-1β is supported also by the experiments with hydrocortisone. Hydrocortisone incubated with HSF for 60 min significantly reduced PGE_2 release induced by rIL-1β and Bk but not by AA, indicating a probable inhibition of the precursor release (Table 3). The inhibitory effect of the steroid was counteracted by actinomycin D, an inhibitor of RNA synthesis (Table 4) and by an anti-lipocortin monoclonal antibody (Table 5). It is likely that hydrocortisone induced in HSF the formation of lipocortin-like proteins which inhibited the activation of PLA_2 induced by both rIL-1β and Bk. Indeed, it has been shown that corticosteroids can induce the synthesis or release of lipocortin in HSF (Errasfa *et al.*, 1985) and that these drugs are ineffective on PLC activation induced by Bk (Burch & Axelrod, 1987).

In conclusion, we have shown that in HSF, rIL-1β can activate PLA_2. This activation is counteracted by corticosteroids via induction of lipocortin-like proteins. It has been reported that IL-1 can stimulate the release of both adrenocorticotropic hormone and glucocorticoids (Besedowski *et al.*, 1986). It is therefore tempting to suggest that interleukin-1 and lipocortin might modulate PLA_2 activity in inflammation according to a regulatory feedback mechanism.

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Effect of a new potent CCK antagonist, lorglumide, on caerulein- and bombesin-induced pancreatic secretion and growth in the rat

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1 The effect of lorglumide, a new potent cholecystokinin (CCK) antagonist, on pancreatic secretion and growth induced by caerulein and bombesin was studied in the rat.

2 Pancreatic exocrine secretion was studied both *in vitro* (isolated and perfused pancreatic segments) and *in vivo* (anaesthetized animals with cannulation of the common bile duct) whereas the trophic effect was investiged after short-term (5 days) administration of the peptides and/or lorglumide.

3 Both caerulein and bombesin stimulated amylase release from *in vitro* pancreatic segments in a concentration-dependent manner. Although the efficacy of both peptides was virtually identical, the potency of caerulein was higher than that of bombesin. Lorglumide displaced the concentration-response curves to caerulein to the right without affecting the maximum response, suggesting a competitive antagonism. The Schild plot analysis of data gave a straight line with a slope not significantly different from unity. The calculated pA_2 for lorglumide was 7.31 ± 0.45. The antagonist, however, was completely ineffective when tested against bombesin-induced amylase release.

4 *In vivo* experiments confirmed results from *in vitro* studies since lorglumide (5 and $10\,mg\,kg^{-1}$) significantly reduced pancreatic exocrine secretion induced by caerulein without affecting the response to bombesin.

5 Administration of either peptide increased the weight of the pancreas, the total pancreatic protein and DNA, trypsin and amylase content. Lorglumide ($10\,mg\,kg^{-1}$), administered together with caerulein, reduced the peptide-induced increase in pancreatic weight, protein and enzyme content. On the contrary, when lorglumide was given together with bombesin, all the parameters that were examined were not altered by concomitant administration of the antagonist.

6 These results have demonstrated the ability of lorglumide to antagonize the effects on the pancreas of a CCK-analogue, caerulein, and its inability to affect bombesin-induced pancreatic secretion and growth, suggesting that lorglumide is a potent and selective antagonist of CCK-receptors in the pancreas.

Introduction

It is now well established that cholecystokinin (CCK) exerts a major role in the physiological regulation of pancreatic enzyme secretion (for review see Chey, 1980). *In vivo* experiments have shown that both endogenous and exogenous CCK increase enzyme output from the pancreas of different animal species, including man (for review see Dockray, 1981). The presence of specific receptors for CCK-like peptides in the mammalian pancreas was confirmed by *in vitro* studies on pancreatic acini or slices from mouse (Williams *et al.*, 1983; Jensen *et al.*, 1986) rats (Jensen *et al.*, 1986; Niederau *et al.*, 1986) and guinea-pigs (Jensen *et al.*, 1986).

Along with its effect on secretion, the ability of CCK alone, or in combination with secretin, to

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produce both hyperplasia and hypertrophy of the exocrine pancreas has been well documented (for review see Solomon, 1981; Fölsch, 1984). It increases pancreatic weight without affecting the endocrine component (i.e. Islets of Langerhans) (Petersen *et al.*, 1978).

Caerulein, a decapeptide first isolated from the skin of Australian frogs (Bertaccini, 1976), has a C-terminal octapeptide sequence that differs only in one amino acid residue from CCK. As a consequence, these two peptides possess the same spectrum of biological actions (Bertaccini, 1976). From an experimental point of view, caerulein presents the advantage of being more resistant to enzymatic cleavage than is CCK (Tokiwa *et al.*, 1975). As expected, caerulein was able to stimulate exocrine pancreatic secretion in several animal species (Bertaccini, 1976) and to promote pancreatic growth after repeated administration in rats (Solomon *et al.*, 1978; Varga *et al.*, 1985; Damge *et al.*, 1988).

Bombesin is a tetradecapeptide from frog skin that has been found to have counterparts of similar immunological characteristics in mammalian gut (Anastasi *et al.*, 1971; Brown & Vale, 1979; Erspamer & Melchiorri, 1980). Bombesin-like immunoreactivity was found to be present throughout the alimentary tract (Walsh *et al.*, 1979; Yanaihara *et al.*, 1980) and pancreas of several animal species including man (Ghatei *et al.*, 1984; Holst *et al.*, 1983). This broad localisation in the gut raises questions about the possible physiological significance of bombesin-like peptides.

The presence of bombesin-like immunoreactivity in both animal and human pancreas suggests that this peptide can affect either endocrine and exocrine pancreatic function. Bombesin was found to increase the release of pancreatic hormones (insulin, glucagon and pancreatic polypeptide) in several animal species, including man (McDonald *et al.*, 1988; Scarpignato, 1988). Exocrine pancreatic function was also affected by bombesin and a stimulatory effect of bombesin on pancreatic enzyme secretion was observed in rats (Namba *et al.*, 1986; Lamers *et al.*, 1987), dogs (Erspamer & Melchiorri, 1980; Konturek *et al.*, 1976), pigs (Lilja *et al.*, 1984) and man (Basso *et al.*, 1976). *In vitro* studies have provided conflicting results. Bombesin, has been found to stimulate amylase release from rat (Iwatsuki & Petersen, 1978; Otsuki *et al.*, 1987), guinea-pig (Jensen *et al.*, 1984), mouse (Iwatsuki & Petersen, 1978) and human (Susini *et al.*, 1986) pancreatic acini, whereas it is ineffective in the same dog preparation (Bommelaer *et al.*, 1981).

The mechanism by which bombesin affects the exocrine pancreas is not well established, as yet. The presence of specific bombesin receptors on pancreatic acini (Jensen *et al.*, 1984) suggests a direct effect of the peptide on the gland. However bombesin releases several gastrointestinal peptides in both animals and man (Ghatei *et al.*, 1982; Knigge *et al.*, 1984), some of which may affect pancreatic growth and secretion. Among these, CCK is the most probable candidate as a mediator of the action of bombesin on rat pancreas.

Peptide receptor antagonists are important tools for characterizing peptide-receptor interaction and evaluating their involvement in physiological processes but until recently few selective CCK antagonists were available. Among these, dibutyryl cyclic GMP (db cyclic GMP) and proglumide were the most extensively studied. These compounds lack potency and selectivity in that they inhibit some gastrin-mediated physiological processes and that their activity *in vitro* is displayed at concentrations in excess of 10^{-4} M (for review see Silverman *et al.*, 1987). The low potency of these agents limits their usefulness as CCK-receptor antagonists *in vivo*, moreover, db cyclic GMP was found to be toxic when administered at effective doses in the intact animal (Grendell *et al.*, 1985).

Among the structural analogues of proglumide, the most potent CCK-receptor antagonists were 4-benzamido-N,N-dialkyl-glutaramic acid derivatives, especially lorglumide (compound CR 1409, Figure 1). This compound was found to be approximately 5600 times more potent than proglumide in inhibiting CCK-induced contractions in guinea-pig gall bladder *in vitro* (Makovec *et al.*, 1985). Other studies have demonstrated that lorglumide is 4000 and 1600 times more potent than proglumide in its ability to inhibit CCK-induced amylase secretion from rat (Niederau *et al.*, 1986) and guinea-pig (Jensen *et al.*, 1986) pancreatic acini, respectively. These results, although providing different potency ratios depending on different experimental conditions, suggest that this proglumide derivative may be useful for *in vivo* studies of the action of CCK.

In the present investigation the activity of lorglumide was evaluated against caerulein-induced pancreatic secretion and growth. The effect of this compound against bombesin was also investigated in

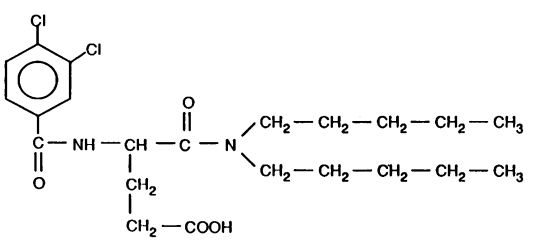

Figure 1 Structural formula of lorglumide (CR 1409).

order to elucidate the involvement of CCK in the actions of bombesin on exocrine pancreas.

Methods

Animals

OFA rats (derived from the Sprague-Dawley strain), weighing 60–80 g or 180–200 g, were used. The animals were housed in light-controlled (12 h light-dark cycle) and air-conditioned (25°C) rooms. Food and drinking water were given *ad libitum*.

In vitro *experiments*

Experiments were performed according to the method of Pearson *et al.* (1984), with minor modifications. Rats (weighing 180–200 g) were killed by a blow to the head and the pancreas was quickly removed and placed in a modified Krebs-Henseleit solution of the following composition (mM): NaCl 103, KCl 4.7, $CaCl_2$ 2.56, $MgCl_2$ 1.13, $NaHCO_3$ 25, NaH_2PO_4 1.15, D-glucose 2.8, sodium pyruvate 4.9, sodium fumarate 2.7, and sodium glutamate 4.9. The pH of the solution was adjusted to 7.4. The solution was maintained at 37°C and gassed with 95% O_2:5% CO_2 mixture.

The pancreas was cut into small segments (3–5 mg) and a total weight of about 150 mg was placed in a tissue flow chamber of 1 ml capacity and superfused at a constant rate of 1.4 ml min^{-1} with the modified Krebs-Henseleit solution. The effluent in the first 30 min was discarded, then fractions were collected at 3 min intervals.

The amylase concentration in the effluent was measured by the method described below. The stimulus-evoked amylase release (i.e. that above basal output) was expressed in terms of the output at the peak of the response in u min^{-1} 100 mg^{-1} tissue. Peptides and lorglumide were added directly to the superfusion solution at known concentrations. When the inhibitory effect of lorglumide against caerulein or bombesin was tested, lorglumide superfusion was started 15 min before the application of the peptides and continued throughout the experiment.

Cumulative concentration-response curves were constructed, since in preliminary experiments no tachyphlaxis was observed. Each concentration was applied for 12 min (i.e. 4 consecutive 3-min collections) because we previously found that the response reaches its peak in the 3rd collected fraction. Two preparations were made from the pancreas of each rat. The first one was used for control (agonist alone) experiments and the second for antagonist studies. This is because two identical concentration-response curves cannot be constructed for the same preparation, due to the partial depletion of enzyme store.

In vivo *experiments*

Two sets of experiments were carried out; the first concerning the effect of compounds on pancreatic secretion, and the second one dealing with the interference of lorglumide on the trophic action of caerulein and bombesin.

Secretory studies Six groups of animals (180–200 g) were used and studied in the same run. They were anaesthetized with urethane (1.2 g kg^{-1}, i.p.), the pylorus and common bile duct at the hepatic hilum were ligated and a polyethylene cannula (PE-50, i.d. 0.58 mm and o.d. 0.96 mm) introduced into the duodenal part of the duct for collecting pure pancreatic juice. Basal pancreatic juice was collected for 60 min. Then each rat of the first three groups received caerulein 1 $\mu g\,kg^{-1}$ intraperitoneally. This dose of the peptide was previously shown to induce a submaximal stimulation of enzyme secretion (Varga *et al.*, 1988b). Animals of group 1 were pretreated intraperitoneally (15 min before caerulein) with saline and those of groups 2 and 3 with lorglumide, 5 and 10 mg kg^{-1}, respectively. The rats of the remaining three groups were given bombesin (10 $\mu g\,kg^{-1}$) intraperitoneally. This dose of the peptide was chosen because several studies (Gardner & Jensen, 1981; Namba *et al.*, 1986; Otsuki *et al.*, 1987) have shown bombesin to be equally effective, but 10 times less potent than caerulein. Animals of group 4 were pretreated with saline whereas those of groups 5 and 6 were given lorglumide (doses as above). After stimulation with either caerulein or bombesin, pancreatic juice was collected for another period of 60 min. Its volume was determined by weighing on an analytical balance to the nearest mg. Protein concentration was measured according to the method of Lowry *et al.* (1951) with bovine albumin used as standard.

Effect of lorglumide on pancreatic trophic response to caerulein or to bombesin Six different groups of animals (60–80 g) were used. They were treated with the following compounds given three times daily, for five days: group 1 with saline, group 2 with lorglumide (10 mg kg^{-1}), group 3 with caerulein (1 $\mu g\,kg^{-1}$), group 4 with caerulein + lorglumide (doses as above), group 5 with bombesin (10 $\mu g\,kg^{-1}$) and group 6 with bombesin + lorglumide (doses as above). Peptides were administered subcutaneously whereas lorglumide was injected intraperitoneally 15 min before caerulein or bombesin. The last dose of the peptide was injected 12 h before the rats were killed after which the entire pancreas was excised.

Biochemical analysis

The pancreas was carefully trimmed of fat, mesentery and lymph nodes, weighed and homogenized in buffer (pH 8.0) of the following composition (mM): Tris-HCl 100, KCl 100 and $CaCl_2$ 20. Protein concentration, trypsin and amylase activity were then measured in the pancreatic tissue. Protein was determined as described above. Amylase activity was measured according to the method of Bernfeld (1955) while trypsin was estimated by a spectrophotometric method (Hummel, 1959) after activation of trypsinogen (Solomon *et al.*, 1978). Tissue DNA content was extracted according to Munro & Fleck (1966) and measured by Burton's method (1956), with calf thymus DNA used as a standard.

Evaluation of data

In *in vitro* studies, all the responses were expressed as a percentage of the maximum effect elicited by caerulein or bombesin. The pD_2 values were obtained from the regression lines calculated by the method of least squares as the percent of the response versus log dose. The degree of antagonism was calculated from the dose-ratio (DR) according to Gaddum *et al.* (1955), comparing the concentration-response curve in the presence of antagonist to that obtained in the absence of antagonist. The dose-ratio was calculated by using agonist concentration giving 50% of the maximal response for each curve. The pA_2 values were determined according to the following equation, derived from Arunlakshana & Schild (1959):

$$pA_2 = -\log [B] + \log (DR - 1)$$

where [B] is the molar concentration of the antagonist. The comparison of the slopes of the regression lines was carried out by using a modified *t* test to determine differences between observed and theoretical slopes (Goldstein, 1964). All these calculations were made according to Tallarida & Murray (1981) by using a computer programme running on an Apple II.

In secretory studies, data were expressed as difference between the observed value after each treatment and basal.

All the values are presented as a mean ± s.e.mean. Comparison among the groups was performed by analysis of variance (ANOVA) and Dunn's multiple range test (Dunn, 1961).

Compounds

Caerulein (synthetic decapetide, m.w. 1352) and bombesin (synthetic tetradecapeptide, m.w. 1620) were a generous gift from Dr Chiara De Paolis (Farmitalia-Carlo Erba Research Labs, Milan, Italy); lorglumide (CR 1409, DL-4-(3,4-dichlorobenzoyl-amino)-5-(di-n-pentylamino)-5-oxo-pentanoic acid was kindly provided by Dr Lucio Rovati (Rotta Research Laboratorium, Monza, Italy). All other chemicals were from Sigma (St Louis, MO, U.S.A.).

Results

In vitro *secretory studies*

Results obtained by using isolated fragments of rat pancreas are summarised in Figures 2–4. In our experimental conditions, basal release of amylase was $1.05 \pm 0.22\,U\,min^{-1}\,100\,mg^{-1}$ of tissue ($n = 30$). This value was comparable to that obtained in rats by Pearson *et al.* (1984), who used the same preparation but a different amylase assay. Caerulein and bombesin, added at increasing concentrations to the superfusion solution, increased enzyme release in a concentration-dependent manner (Figures 2 and 4). The maximum effect was not significantly different for each of the peptides (i.e. $3.61 \pm 0.30\,u\,min^{-1}\,100\,mg^{-1}$ and $3.92 \pm 0.35\,u\,min^{-1}\,100\,mg^{-1}$ for caerulein and bombesin, respectivley). Calculated pD_2 values were 9.97 ± 0.21 for caerulein and 8.29 ± 0.11 for bombesin. Therefore, the potency but not the efficacy of caerulein was higher than that of bombesin.

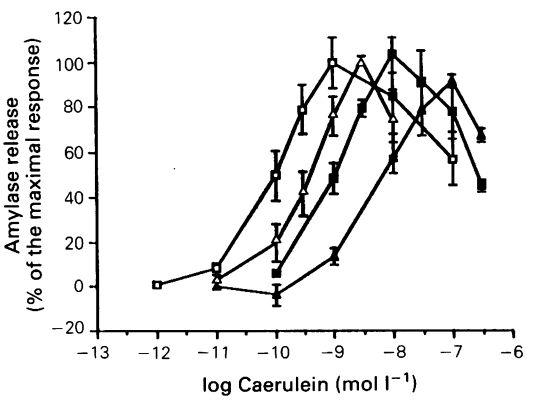

Figure 2 Amylase release from isolated and perfused fragments of rat pancreas. Concentration-response curves to caerulein alone or caerulein plus various concentrations of lorglumide: control (□); lorglumide 3×10^{-7} M (△); 1×10^{-6} M (■); 1×10^{-5} M (▲). Results are expressed as a percentage of the maximum response. They represent the mean of the values obtained from 6 experiments; s.e.mean shown by vertical bars.

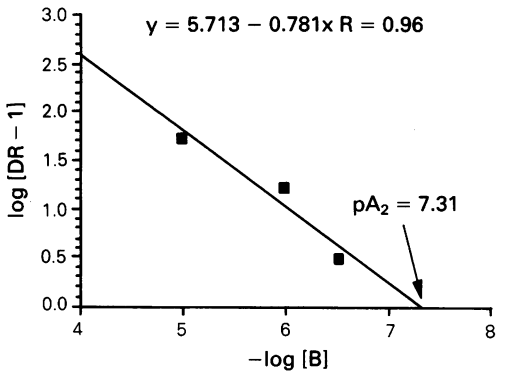

Figure 3 Schild plot for the inhibition of caerulein-stimulated amylase release from rat pancreas by lorglumide. The line represents the calculated least-squares regression line.

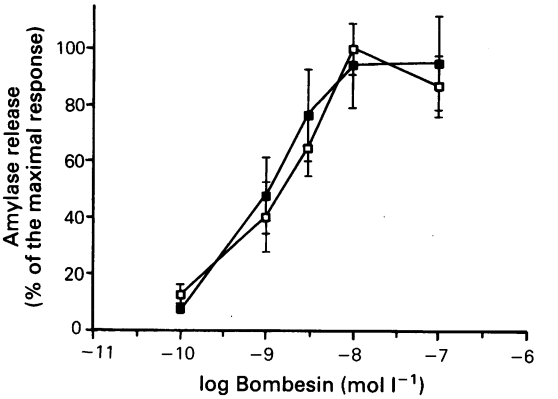

Figure 4 Amylase release from isolated and perfused fragments of rat pancreas. Concentration-response curves to bombesin alone (□) or bombesin plus lorglumide (1×10^{-5} M) (■). Results are expressed as percentage of the maximum response. They represent the mean of the values obtained from 6 experiments; s.e.mean shown by vertical bars.

Lorglumide, added to the bath 15 min before caerulein, displaced the concentration-response curves of the peptide to the right without affecting the maximum response (Figure 2), thus suggesting a typical competitive antagonism. This was confirmed by the Schild plot analysis that yielded a straight line with a slope of 0.78 ± 0.22, not significantly different from unity (Figure 3). The calculated pA_2 value was found to be 7.31 ± 0.45. In contrast, when the antagonist was tested against bombesin-induced amylase release it was completely ineffective (Figure 4). Lorglumide, administered alone at concentrations up to 10^{-4} M, did not affect basal amylase release.

In vivo *secretory studies*

In our experimental conditions the volume of pancreatic juice collected during basal secretion was $26 \pm 3\,\mu l\ 60\,min^{-1}$ and the mean protein output from the pancreas was $1.22 \pm 0.26\,mg\ 60\,min^{-1}$. Caerulein, administered by the intraperitoneal route, caused a five fold increase in the volume of pancreatic juice and concomitantly increased the protein output more than 11 fold (Table 1). Similarly, bombesin, at the dose used in the present experiments, was equally effective. Lorglumide was able to reduce caerulein-induced pancreatic exocrine secretion, although the effect of the lower dose of the compound on the volume of pancreatic juice was not statistically significant (Table 1). Conversely, the pancreatic stimulatory effect of bombesin was not modified by lorglumide administration (Table 1).

In vivo *trophic studies*

Final body weight was similar in each of the six groups of animals studied (Figure 5). As previously

Table 1 Effect of lorglumide (Lor) on caerulein- ($1\,\mu g\,kg^{-1}$) and bombesin- ($10\,\mu g\,kg^{-1}$) induced pancreatic secretion in anaesthetized rats

Treatment	*Juice flow* ($\mu l\ 60\,min^{-1}$)	*Protein output* ($mg\ 60\,mg^{-1}$)
Control	26 ± 3	1.2 ± 0.3
Caerulein	119 ± 10	14.3 ± 1.1
Caerulein + Lor ($5\,mg\,kg^{-1}$)	101 ± 11	10.2 ± 1.2*
Caerulein + Lor ($10\,mg\,kg^{-1}$)	56 ± 9*	5.7 ± 1.3*
Bombesin	119 ± 12	14.4 ± 1.5
Bombesin + Lor ($5\,mg\,kg^{-1}$)	120 ± 10	14.8 ± 1.8
Bombesin + Lor ($10\,mg\,kg^{-1}$)	128 ± 7	15.4 ± 1.1

Values are presented as mean (± s.e.mean) of the values obtained from 7–10 animals.
* $P < 0.05$ versus caerulein value.

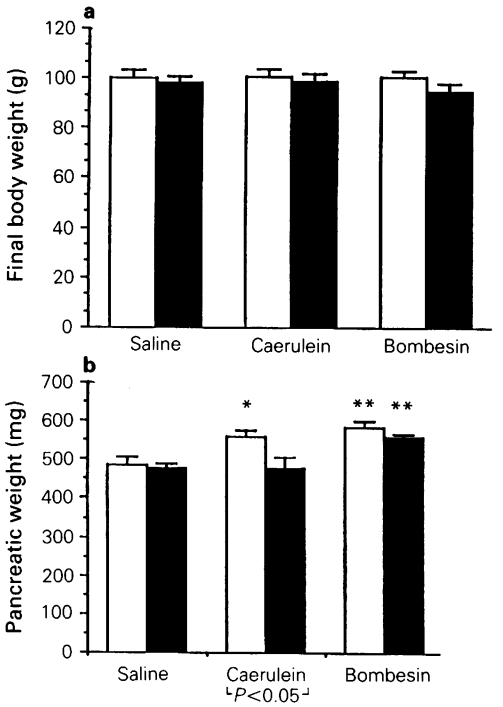

Figure 5 Final body weight (a) and pancreatic weight (b) in rats after short-term treatment (5 days) with saline, caerulein or bombesin with (solid columns) and without (open columns) lorglumide. Each column refers to the mean of the values obtained from 7 animals. Vertical bars are s.e.mean. * $P < 0.05$ versus saline value; ** $P < 0.01$ versus saline value.

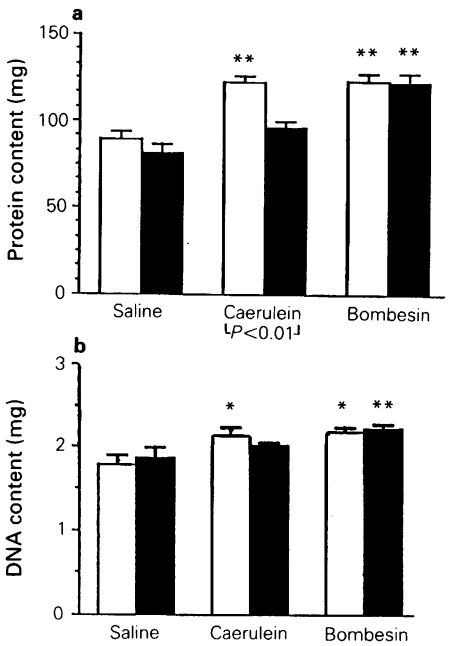

Figure 6 Pancreatic protein content (a) and DNA (b) in rats after short-term treatment (5 days) with saline, caerulein or bombesin with (solid columns) and without (open columns) lorglumide. Each column refers to the mean of the values obtained from 7 animals. Vertical bars are s.e.mean. * $P < 0.05$ versus saline value; ** $P < 0.01$ versus saline value.

described (Varga *et al.*, 1985; 1988a) administration of either caerulein or bombesin increased the weight of the pancreas and the total pancreatic protein and DNA, trypsin and amylase contents (Figures 5–7). Lorglumide, administered alone did not significantly affect pancreatic size and composition; it did, however, significantly reduce the caerulein-induced increase in pancreatic weight, protein and enzyme content (Figures 5–7). On the contrary, when lorglumide was given together with bombesin, all these parameters were not significantly different from those observed after bombesin alone (Figure 5–7).

Discussion

The results of the present investigation confirm the powerful stimulatory effect of both caerulein and bombesin on exocrine pancreatic secretion, already pointed out in different *in vitro* (Bertaccini, 1976; Gardner & Jensen, 1981; Jensen *et al.*, 1984; Otsuki *et al.*, 1987) and *in vivo* (Bertaccini, 1976; Namba *et al.*, 1986; Varga *et al.*, 1988a, b) experimental conditions.

In our *in vitro* system, which was used for the first time to assess the pancreatic secretory action of these peptides, caerulein proved to be of equal efficacy compared to bombesin. Its potency, however, was one order of magnitude higher than that of bombesin. This potency ratio was comparable to that found in rat (Otsuki *et al.*, 1987) and guinea-pig (Gardner & Jensen, 1981) pancreatic acini. The results obtained with lorglumide demonstrate that this compound is a potent and selective antagonist of CCK-induced amylase release. The pA_2 value calculated for lorglumide under our experimental conditions was of the same order of magnitude as that obtained in different *in vitro* preparations, i.e. guinea-pig gallbladder (Makovec *et al.*, 1985) and intestine (Bartho *et al.*, 1987) as well as rat pancreatic acini (Niederau *et al.*, 1986) or dispersed acinar cells (Chen *et al.*, 1988). This suggests that CCK receptors of

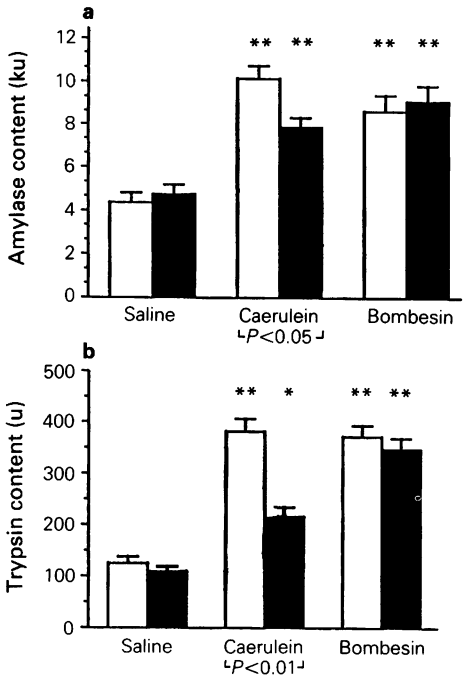

Figure 7 Pancreatic amylase (a) and trypsin content (b) in rats after short-term treatment (5 days) with saline, caerulein or bombesin with (solid columns) and without (open columns) lorglumide. Each column refers to the mean of the values obtained from 7 animals. Vertical bars are s.e.mean. * $P < 0.05$ versus saline value; ** $P < 0.01$ versus saline value.

these organs are not heterogeneous in nature (Bertaccini & Coruzzi, 1981). The antagonism by lorglumide of caerulein-induced amylase release was specific, since this compound was completely ineffective against bombesin, thus showing that the latter peptide acts through different receptors.

These *in vitro* results were confirmed by *in vivo* studies. In these experiments lorglumide, although reducing dose-dependently the caerulein-stimulated output of pancreatic juice and protein, did not affect the pancreatic secretory response to bombesin. This suggests that release of endogenous CCK is not involved in the stimulatory action of bombesin on rat exocrine pancreas. These results are in line with the recent observations of Lamers *et al.* (1987), who showed that immunoneutralisation of CCK by means of a specific antiserum does not affect bombesin-stimulated pancreatic secretion in rats. On the other hand, when CCK was infused in rats to mimic plasma hormone levels obtained after bombesin infusion, pancreatic protein secretion increased by only 10% compared with 50% observed with bombesin (Lamers *et al.*, 1987). All these data, together with the demonstration of specific bombesin receptors in pancreatic acini (Jensen *et al.*, 1984), are consistent with the idea that this amphibian peptide (and most probably its mammalian counterpart gastrin releasing peptide GRP) affects exocrine pancreas through stimulation of specific receptors. It is worth mentioning, however, that the effect of bombesin on dog exocrine pancreas seems to be dependent on the release of endogenous CCK (Erspamer *et al.*, 1974; Konturek *et al.*, 1976) since the peptide is completely ineffective on dog pancreatic acini (Bommelaer *et al.*, 1981). Thus, the involvement of CCK seems to be a species-related phenomenon.

Rothman & Wells (1967) first showed that treatment of rats with CCK caused an increase in pancreatic weight and enzyme content as welll as increased acinar cell size, suggesting acinar cell hypertrophy. Subsequent studies in rats (for review see Solomon, 1981; Fölsch, 1984) have confirmed these observations and, in addition, have shown an increased content and rate of synthesis of DNA, suggesting hyperplasia to be a constant finding after chronic CCK treatment. The ability of caerulein to mimic this biological effect of CCK was first reported by Solomon *et al.* (1978) and was recently confirmed by Varga *et al.*, (1985).

The results presented here confirm once more the ability of caerulein to increase pancreatic growth in rats. The ability of lorglumide to reduce the trophic effect of the amphibian peptide is in line with the recent results of Niederau *et al.* (1987) who demonstrated the ability of CR 1409 to counteract the growth-promoting effect of endogenous and exogenous CCK in the mouse. According to recent preliminary experiments (Damgé *et al.*, 1987), the compound was able to block the trophic action of both CCK and caerulein even when administered orally.

Lorglumide failed to modify the bombesin-induced changes in pancreatic growth and composition, thus suggesting the lack of involvement of CCK in this growth promoting effect of the amphibian peptide. In agreement with our results, Damgé *et al.* (1987) reported the inefficiency of oral lorglumide against bombesin- and GRP-induced pancreatic growth.

In conclusion, these results demonstrate that lorglumide is a potent and selective CCK receptor antagonist. With this compound, direct evidence was provided for the presence of specific CCK receptors in rat exocrine pancreas and of their involvement in both secretory and trophic actions of the hormonal peptide. Similarly, it was demonstrated that the action of bombesin on exocrine pancreas is not mediated by CCK. Lorglumide, therefore, can be considered a useful tool for characterizing CCK-receptor interactions in peripheral organs.

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Electrophysiological and antiarrhythmic effects of UK 52,046-27 during ischaemia and reperfusion in the guinea-pig heart

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1 We studied the antiarrhythmic and electrophysiological effects of UK 52,046-27 (10^{-8} M and 5×10^{-8} M), a highly selective α_1-adrenoceptor antagonist, during global ischaemia (flow reduced to 10% of control for 30 min) and reperfusion, in isolated, buffer-perfused hearts of guinea-pigs.

2 The compound had few electrophysiological effects during normal perfusion, although action potential amplitude and $\dot{V}_{max}$ were reduced with 10^{-8} M (by 9% and 22%) and refractory period was increased with 5×10^{-8} M (by 13%) compared to control hearts.

3 Perfusion with 5×10^{-8} M UK 52,046-27 reduced the incidence of ventricular tachycardia during ischaemia from 67% to 25%, and during reperfusion reduced the incidence of ventricular tachycardia (from 83% to 17%) and ventricular fibrillation (from 67% to 8%).

4 The compound prolonged significantly action potential duration and refractory period during ischaemia and reperfusion. $\dot{V}_{max}$ was reduced to a greater extent during reperfusion in the treated hearts, while greater increases in QRS width and stimulation threshold occurred during ischaemia in the treated group.

5 These results confirm that blockade of the α_1-adrenoceptor subpopulation during myocardial ischaemia and reperfusion decreases the incidence of arrhythmias and alters cellular electrophysiology during ischaemia and reperfusion.

Introduction

Several recent studies (Sheridan *et al.*, 1980; Stewart *et al.*, 1980; Penny *et al.*, 1985) have indicated that α-adrenoceptor blockade has significant antiarrhythmic effects during myocardial ischaemia *in vivo* and *in vitro*. These studies suggested that both selective and non-selective α-adrenoceptor antagonists were effective. Thandroyen *et al.* (1983) suggested that α_2-adrenoceptor blockade may be more effective than α_1, and proposed that the relatively high concentrations of α_1-adrenoceptor blocking agents required to achieve an antiarrhythmic effect would indicate a direct myocardial action rather than one on adrenoceptors. Recent studies in the guinea-pig heart have demonstrated that selective α_1-adrenoceptor stimulation is arrhythmogenic during myocardial ischaemia and reperfusion (Culling *et al.*, 1987) and we considered that selective antagonism of this subpopulation of adrenoceptors during ischaemia and reperfusion should therefore be antiarrhythmic. To assess the efficacy of α_1-adrenoceptor antagonism, we studied the antiarrhythmic and electrophysiological effects of UK 52,046-27, a highly selective α_1-antagonist (M.J. Davey, personal communication) during global ischaemia and reperfusion in isolated, buffer-perfused hearts of guinea-pigs.

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Methods

Hearts were rapidly removed from stunned male guinea-pigs (Dunkin-Hartley, 500–600 g) and mounted for perfusion by the Langendorff technique. Perfusion was performed with oxygenated aqueous Krebs-Henseleit buffer prewarmed to 32°C, containing (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4

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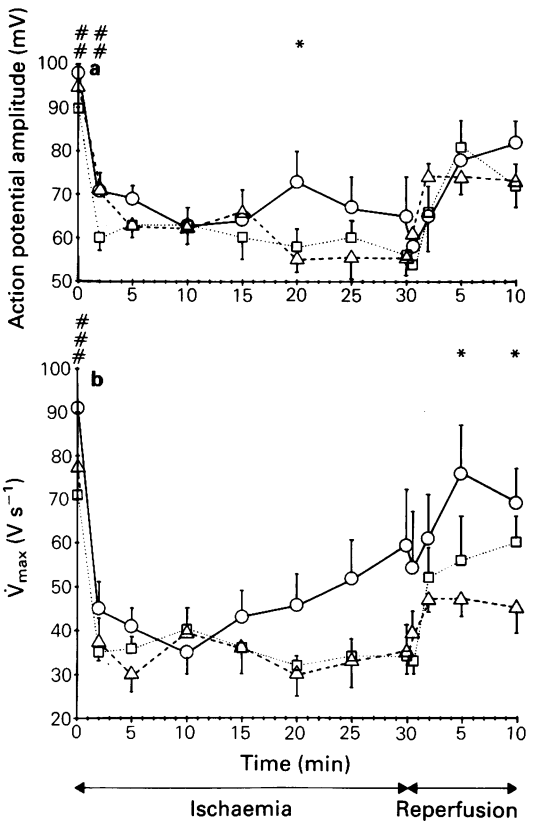

Figure 1. Changes in action potential amplitude (a) and upstroke velocity ($\dot{V}_{max}$) (b) during ischaemia and reperfusion in control hearts (○) and in hearts treated with 10^{-8} M (□) and 5×10^{-8} M (△) UK 52,046-27. The statistical significance levels indicated refer to differences between the control and drug-treated groups: * $P < 0.05$; ** $P < 0.02$ and *** $P < 0.01$, for 5×10^{-8} M; # $P < 0.05$; ## $P < 0.02$ and ### $P < 0.01$, for 10^{-8} M UK 52,046-27.

1.1, $NaHCO_3$ 24, $CaCl_2$ 2.5, glucose 9 and pyruvate 2 (pH 7.4) at a constant flow rate of 5 ml min^{-1} g^{-1}. Hearts were supported horizontally in the organ bath and superfused with aqueous buffer (as above) at 32°C to avoid alterations in temperature associated with myocardial ischaemia. Heart rate was maintained constant to 3.3 Hz (just above the spontaneous sinus rate) by right ventricular pacing at twice the diastolic pacing threshold. Action potentials, refractory periods, electrocardiograms and perfusion pressure were recorded as previously described (Penny & Sheridan, 1983). Action potentials were analysed with an on-line microcomputer, designed to provide real-time analogue reconstruction of digitised data prior to analysis (Sheridan *et al.*, 1983). Transmembrane action potentials were recorded by the floating microelectrode technique from epicardial cells in the apical region of the right ventricle. With this method, the heart is impaled at each time point and, once a stable impalement is achieved, ten action potentials are analysed. This is repeated in each heart, at each time point and means obtained during normal perfusion, global myocardial ischaemia and reperfusion ($n = 11$ impalements per heart.)

Myocardial ischaemia was induced by reducing global coronary flow to 0.5 ml min^{-1} g^{-1} (10% of control) for 30 min, and reperfusion was initiated by restoring flow to control. The time of onset and duration of spontaneously occurring ventricular tachycardia (VT) and ventricular fibrillation (VF) were noted during ischaemia and reperfusion. The incidence of VT and VF, and electrophysiological changes were studied in 12 control hearts, in 15 hearts perfused with buffer containing 10^{-8} M UK 52,046-27, and in 12 hearts which were perfused with buffer containing 5×10^{-8} M UK 52,046-27 during ischaemia and reperfusion.

Electrophysiological measurements are expressed as the mean and standard error of the mean for each group. Comparisons are made between the groups using the non-paired t test or chi-squared analysis, and within groups using the paired t test, with $P < 0.05$ defining the probability value indicating statistical significance.

UK 52,046-27 (4-amino-6,7-dimethoxy-2-(1,2,3,4-tetrahydro-6,7-dimethoxyisoquinol-2-yl)quinoline methanesulphonate) was obtained from (Pfizer Central Research, Sandwich, Kent).

Results

Perfusion with 5×10^{-8} M UK 52,046-27 reduced the incidence of ventricular tachycardia during ischaemia from 67% to 25% ($P < 0.05$), and during reperfusion reduced the incidence of ventricular tachycardia from 83% to 17% and reduced the incidence of ventricular fibrillation from 67% to 8%. During ischaemia, ventricular fibrillation occurred in 25% of control hearts and 17% of treated hearts. Spontaneous onset of arrhythmias occurred during ischaemia at 15.9 ± 1.0 min in the control group and at 20.3 ± 2.3 min in the treated group. Perfusion with 5×10^{-8} M reduced the duration of ventricular tachycardia during ischaemia from 9.9 ± 2.1 min to 2.8 ± 1.1 min. During normal perfusion of the hearts, refractory period was greater in hearts treated with the 5×10^{-8} M UK 52,046-27 (145 ± 3 ms vs. 128 ± 4 ms).

During perfusion with 10^{-8} M UK 52,046-27, ventricular tachycardia occurred during ischaemia in 40% and during reperfusion in 60%. Ventricular

fibrillation occurred in no hearts during ischaemia and in 47% during reperfusion with this concentration. Spontaneous onset of arrhythmias during ischaemia was delayed from 15.9 ± 1.0 min to 21.7 ± 2.0 min with 10^{-8} M UK 52,046-27. During ischaemia, ventricular tachycardia lasted for 9.9 ± 2.1 min in control hearts and 8.3 ± 2.0 min in hearts perfused with 10^{-8} M UK 52,046-27. During normal perfusion of the hearts with this concentration of the compound, action potential amplitude was reduced from 98 ± 2 mV in the control group to 89 ± 2 mV and $\dot{V}_{max}$ was similarly reduced from $91 \pm 5\,V\,s^{-1}$ to $71 \pm 4\,V\,s^{-1}$.

Figure 1 shows that action potential amplitude was similarly and significantly reduced by ischaemia in both control and treated hearts. UK 52,046-27 5×10^{-8} M prevented the spontaneous recovery in action potential amplitude seen in the control hearts at 20 min of ischaemia (73 ± 7 mV vs. 55 ± 3 mV). Ischaemia significantly reduced $\dot{V}_{max}$ to about 50% of control values in both groups (Figure 1). Recovery of $\dot{V}_{max}$ during reperfusion was prevented in the hearts treated with 5×10^{-8} M UK 52,046-27 ($76 \pm 11\,V\,s^{-1}$ in control hearts vs. $47 \pm 4\,V\,s^{-1}$ in the treated hearts at 5 min of reperfusion (Figure 1)).

Figure 2 shows that action potential duration (APD) was significantly reduced in all groups during ischaemia. UK 52,046-27 5×10^{-8} M modified this, prolonging APD at 10 and 15 min of ischaemia and after 30 s, 5 and 10 min of reperfusion. Thus, although the compound had no effect on APD during normal perfusion, it prolonged APD during ischaemia (145 ± 3 ms vs 131 ± 4 ms, $P < 0.01$, at 10 min and 133 ± 4 ms vs 118 ± 5 ms, $P < 0.05$ at 15 min), and increased APD during early and later reperfusion (111 ± 8 ms vs 134 ± 4 ms, $P < 0.02$ at 30 s, and 162 ± 3 ms vs 177 ± 3 ms, $P < 0.01$ at 5 min, Figure 2). A rapid, significant, early increase in refractory period occurred during ischaemia in all groups (24% in control hearts, 41% in 10^{-8} M and 17% in 5×10^{-8} M treated hearts all at 5 min, Figure 2). Refractory periods increased to a similar extent during ischaemia in all groups, and at 10 min were longer in the drug-treated groups before returning towards control values. UK 52,046-27, at the higher concentration increased refractory periods throughout reperfusion (162 ± 4 ms vs 120 ± 9 ms, at 2 min, Figure 2).

Conduction time increased similarly and significantly in all groups during ischaemia (compared to normal perfusion), Figure 3. QRS width followed a similar pattern (Figure 3), increasing significantly during ischaemia by 36%, 41% and 57% in control, 10^{-8} M treated and 5×10^{-8} M treated hearts respectively at 10 min but with greater values in the group perfused with the higher concentration compared to control hearts during early ischaemia (42 ± 4 ms vs 32 ± 2 ms after 5 min). Stimulation threshold was also significantly increased by ischaemia (Figure 3). This effect was also more pronounced in the hearts treated with 5×10^{-8} M UK 52,046-27 at 15 and 20 min of ischaemia (5.94 ± 0.61 V vs 3.90 ± 0.63 V at 15 min), and during early and later reperfusion when stimulation threshold was elevated in these hearts (5.40 ± 0.42 V vs 2.90 ± 0.95 V at 2 min and 5.07 ± 0.52 V vs 3.00 ± 0.75 V at 10 min).

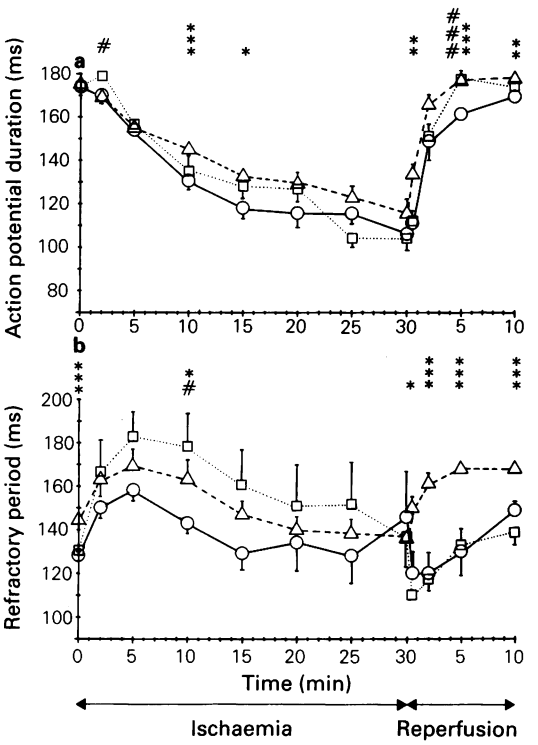

Figure 2 Changes in action potential duration (APD) (a) and refractory period (RP) (b) during ischaemia and reperfusion in control hearts (○) and hearts treated with 10^{-8} M (□) and 5×10^{-8} M (△) UK 52,046-27. Note that the higher concentration of the drug prolonged APD and RP during ischaemia and reperfusion. The statistical significance levels indicated refer to differences between the control and drug-treated groups: $^{*}P < 0.05$, $^{**}P < 0.02$ and $^{***}P < 0.01$ for 5×10^{-8} M; $^{\#}P < 0.05$; $^{\#\#}P < 0.02$ and $^{\#\#\#}P < 0.01$, for 10^{-8} M UK 52,046-27.

Discussion

In recent years, evidence has accumulated which suggests that α-adrenoceptors play an important role in

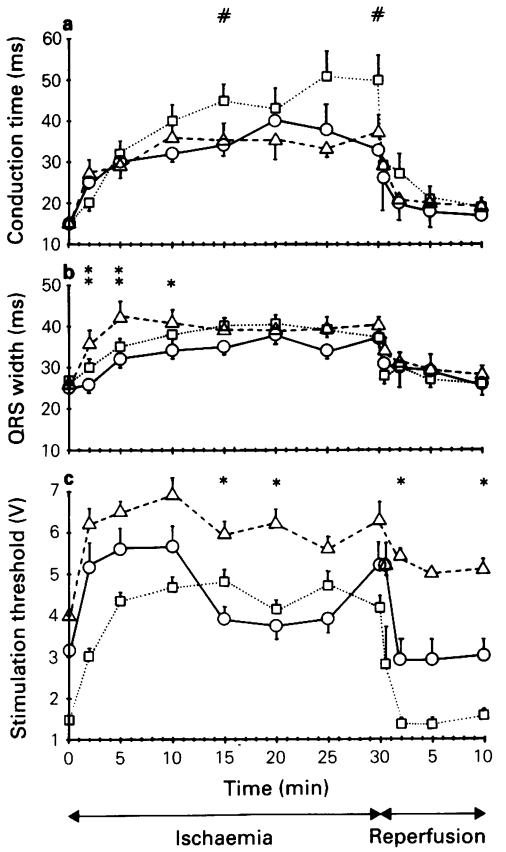

Figure 3 Changes in conduction time (a), QRS width (b) and stimulation threshold (c) during ischaemia and reperfusion in control hearts (○) and hearts treated with 10^{-8} M (□) and 5×10^{-8} M (△) UK 52,046-27. The higher concentration of the drug prolonged QRS width and increased stimulation threshold during ischaemia. The statistical significance levels indicated refer to differences between the control and drug-treated groups: $^{*}P < 0.05$; $^{**}P < 0.02$ and $^{***}P < 0.01$ for 5×10^{-8} M; $^{\#}P < 0.05$; $^{\#\#}P < 0.02$ and $^{\#\#\#}P < 0.01$ for 10^{-8} M UK 52,046-27.

mediating the arrhythmogenic effects associated with myocardial ischaemia and reperfusion (Sheridan *et al.*, 1980; Stewart *et al.*, 1980; Thandroyen *et al.*, 1983; Penny *et al.*, 1985). α-Adrenoceptor antagonists produce haemodynamic effects, although these can be reversed without attenuating the antiarrhythmic action (Sheridan *et al.*, 1980), suggesting that their antiarrhythmic effect is independent of such peripheral actions. Cellular electrophysiological studies support this (Penny *et al.*, 1985; Culling *et al.*, 1984), and although direct myocardial electrophysiological effects can be demonstrated for some α-adrenoceptor antagonists in high concentrations (Rosen *et al.*, 1971) which might explain their antiarrhythmic activity, these effects are not apparent at lower concentrations which are potently antiarrhythmic (Penny & Sheridan, 1982; Penny *et al.*, 1985). Further evidence supporting a role for α-adrenoceptors in mediating the arrhythmogenic effects of myocardial ischaemia comes from studies in which α_1-adrenoceptor stimulation has been shown to be arrhythmogenic, but without effect during normal perfusion (Culling *et al.*, 1987).

Thandroyen *et al.* (1983) suggested that antiarrhythmic effects of α_2-adrenoceptor blockade may be more effective than α_1. The present results indicate that selective blockade of the α_1 subpopulation of adrenoceptors during myocardial ischaemia and reperfusion is effective in decreasing the incidence of arrhythmias and further demonstrates that a relatively low concentration (5×10^{-8} M) is required for antiarrhythmic activity. Possible changes in the numbers of α-adrenoceptors during myocardial ischaemia which may complicate the interpretation of data from such studies have been suggested (Corr *et al.*, 1981), but not always demonstrated by other workers (Broadley *et al.*, 1985; Crome *et al.*, 1985). The high affinity of UK 52,046-27 for myocardial α_1-adrenoceptors and its selectivity as an α_1-adrenoceptor antagonist ($IC_{50} = 6$ pM for α_1-adrenoceptors vs 25 nM for α_2-adrenoceptors vs 8.1 μM for β-adrenoceptors vs >10 μM for 5-HT_2-receptors from radioligand binding studies; $pA_2 = 8.91$ in rabbit papillary muscles; M.J. Davey, personal communication) may help to explain its efficacy in low concentrations compared to other non-specific antagonists. The results obtained also demonstrate that this antiarrhythmic action is mediated by altering cellular electrophysiology during ischaemia and reperfusion with relatively little effect on normally perfused myocardium. The prolongation in APD, and prevention of spontaneous recovery of action potential amplitude and upstroke velocity during ischaemia and reperfusion are likely to be important in the prevention of arrhythmias, but would not have been predictable from a study of the compound's action during normal perfusion at a concentration which has antiarrhythmic effects during myocardial ischaemia and reperfusion (5×10^{-8} M). The electrophysiological effects of UK 52,046-27 during ischaemia and reperfusion are broadly similar to those seen with other α-adrenoceptor antagonists (Penny *et al.*, 1985). In previous studies we have shown that these effects are similar to those produced by depletion of myocardial catecholamines and that additional electrophysiological effects are not seen in the absence of endogenous myocardial catecholamines (Culling *et al.*, 1984). Such findings indicate that adrenergic mecha-

nisms, rather than a direct action on sarcolemmal properties, mediate their effects.

Although some early anecdotal reports suggest that α-adrenoceptor blockade may be anti-arrhythmic in man (Gould *et al.*, 1969) there are as yet no convincing studies available. The electrophysiological effects of UK 52,046-27 demonstrated here, and its claimed cardioselectivity (M.J. Davey, personal communication) if substantiated, suggest that it may be a suitable agent with which to test this important hypothesis in man.

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Evidence for co-transmitter role of neuropeptide Y in the pig spleen

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1 The possible involvement of neuropeptide Y (NPY) in relation to noradrenaline (NA) and adenosine triphosphate (ATP) mechanisms in the sympathetic nervous control of the vascular tone and capsule contraction in the blood perfused pig spleen was investigated *in vivo*.

2 Local injections or infusions of NA, NPY and α-, β-methylene ATP (mATP) caused vasoconstriction (perfusion pressure increase) and capsule contraction (increased venous blood flow). ATP only evoked vasodilatation. NPY was about 50 fold more potent than NA as a vasoconstrictor, and the NPY response was more long-lasting. Reserpine treatment did not change the effects of NPY.

3 Electrical stimulation of the splenic nerves in control animals caused a frequency-dependent, guanethidine-sensitive output of both NPY-like immunoreactivity (-LI) and NA, suggesting co-release. The output of NPY-LI relative to NA was enhanced at high frequency stimulation. Furthermore, α-adrenoceptor blockade by phentolamine enhanced both the output of NPY-LI and NA while inhibition of the neuronal uptake of NA with desipramine reduced the low frequency stimulation-evoked overflow of NPY-LI. Preganglionic denervation did not change the output of NPY-LI or NA.

4 Reserpine treatment reduced both the splenic content of NA and NPY-LI. Preganglionic denervation inhibited the reserpine-induced depletion of the NPY content but not of NA in terminal areas. The stimulation-evoked NPY overflow was markedly enhanced, especially at low-frequency stimulation after resperpine, and the plasma levels of NPY-LI in the venous effluent were then in the nmolar range (i.e. where exogenous NPY induced vasoconstriction). The perfusion-pressure increase upon stimulation in reserpine-treated, preganglionically-denervated animals was highly correlated ($r = 0.91$) to the NPY overflow. The functional 0.5 Hz responses were reduced after reserpine, while at higher frequencies the functional effects were of similar magnitude to controls but longer-lasting.

5 Tyramine induced a release of NA but not of NPY-LI. Furthermore, the increase in perfusion pressure induced by tyramine was absent after reserpine.

6 After tachyphylaxis to the vasoconstrictor effects of mATP, the nerve stimulation-evoked, functional response as well as the NA and NPY-LI overflow were unchanged. After reserpine treatment, both the perfusion-pressure increase and NPY-LI overflow to nerve stimulation were reduced after mATP tachyphylaxis.

7 In conclusion, release of NPY rather than ATP may explain the long-lasting, non-adrenergic, splenic functional responses in reserpinized animals upon sympathetic stimulation. However, NA is most likely the main splenic transmitter when low-frequency stimulation is used under control conditions.

Neuropeptide Y (NPY), a peptide with 36 amino acid residues which was originally isolated from porcine brain (Tatemoto, 1982), co-exists with noradrenaline (NA) in postganglionic, sympathetic nerves in a variety of peripheral organs (Lundberg *et al.*, 1982) including the pig spleen (Lundberg *et al.*, 1988). Increasing evidence suggests that NPY is involved in sympathetic neurotransmission. NPY is

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co-released with NA upon electrical stimulation of sympathetic nerves (Lundberg *et al.*, 1984a; 1986b,c) and during reflex activation under physiological conditions in man, such as physical exercise (Lundberg *et al.*, 1985b; Pernow *et al.*, 1986a). NPY binds to specific, high-affinity sites with receptor characteristics in membranes from the rat brain (Saria *et al.*, 1984) and the pig spleen (Lundberg *et al.*, 1988). NPY exerts prejunctional inhibitory actions on the nerve stimulation-evoked [^{3}H]-NA release from sympathetic nerves (Lundberg & Stjärne, 1984; Pernow *et al.*, 1986b) and induces vasoconstriction in a variety of vascular beds (Lundberg & Tatemoto, 1982; Edvinsson *et al.*, 1983; Lundberg *et al.*, 1984a,b) including the pig spleen (Lundberg *et al.*, 1986c; 1988).

Increasing evidence suggests that part of the vasoconstrictor response to sympathetic nerve stimulation in several organs *in vivo* (see Lundberg & Hökfelt, 1986) and isolated blood vessels *in vitro* (Burnstock & Kennedy, 1986) is resistant to pretreatment with α- and β-adrenoceptor-blocking agents. However, it has been difficult to exclude the possibility that high concentrations of locally released NA activate a separate class of adrenoceptors (see Neild & Hirst, 1984). Furthermore, very little of the sympathetic vasoconstrictor response to nerve stimulation in the spleen remains following blockade of NA mechanisms with α-adrenoceptor antagonists (Cubeddu *et al.*, 1974) or NA depletion due to reserpine treatment (Dixon *et al.*, 1979). Reserpine treatment in the guinea-pig and cat is associated with a concomitant depletion of NPY-LI in the heart and spleen through enhanced neuronal release (Lundberg *et al.*, 1984b; 1985c,d; 1986a,b). In contrast, adenosine triphosphate (ATP) mechanisms are uninfluenced by reserpine (Kirkpatrick & Burnstock, 1987).

In the present study the effects of adrenoceptor blocking agents and reserpine pretreatment on the sympathetic control of the pig spleen in relation to NA, NPY and ATP mechanisms have been investigated *in vivo*.

Methods

Fifty-two pigs (b.w. 25–35 kg) were premedicated with ketamine (25 mg kg^{-1}, i.m.) and atropine (0.05 mg kg^{-1}, i.m.). Anaesthesia was induced with sodium pentobarbitone (20 mg kg^{-1}, i.v.) and maintained by continuous infusion of this agent (5 mg kg^{-1} h^{-1}). The pigs were intubated and ventilated artificially and their body temperature was maintained at 37°C. Skeletal muscle paralysis was then induced with pancuronium (0.2 mg kg^{-1}, i.v.) and maintained with repeated doses every hour. After heparinization (1000 iu kg^{-1}), one femoral artery was cannulated for recording of systemic arterial blood pressure, using a pressure transducer connected to a Grass polygraph. The femoral vein on this side was used for continuous infusions of Ringer solution or Macrodex to compensate for blood losses (see below). The contralateral femoral artery and vein were also cannulated to allow blood perfusion of the spleen. The spleen was dissected free from the mesentery and all accessory vessels (at caudal and cranial portions) were ligated. After ligation of the main splenic vessels close to the coeliac artery, the spleen was excised and placed in saline at +37°C. The main splenic artery was rapidly cannulated and constantly perfused with blood from the femoral artery at a rate of 25–35 ml min^{-1}, using a roller pump. The venous outflow from the spleen was directed back into the femoral vein. Perfusion pressure in the system on the arterial side (reflecting vascular resistance changes) was monitored continuously via an inlet close to the spleen, by use of a pressure transducer. The perfusion rate was adjusted to obtain a perfusion pressure of 75–80 mmHg. The blood flow in the splenic venous effluent (which is related to capsule contractions) was recorded in parallel with an electromagnetic flowmeter (Nycotron, Norway).

After perfusion of the spleen had been established (within 5 min of removal from the animal), the postganglionic nerves along the main splenic vessels were carefully dissected free and placed on platinum electrodes. A resting period of 30–45 min followed before any stimulations or infusions were performed. Electrical, nerve stimulations were delivered using a Grass S88 stimulator at 10 V, 5 ms with varying frequencies or patterns, giving a total of 240 shocks. Continuous stimulations at 0.5 Hz were performed for 8 min, at 2 Hz for 2 min and at 10 Hz for 24 s. In addition, intermittent stimulation with bursts at 20 Hz for 1 s every 10 s for 2 min (20b) were also used. Stimulation was carried out at 20 min intervals and, in an initial series of experiments, the reproducibility of the model was shown by repeating each type of stimulation twice in a random fashion ($n = 4$). After these four types of stimulation, a second series of 20 b stimulations was also applied after administration of different blocking agents. In controls, a further 5 stimulations at 20 b were carried out, and in reserpinized animals 3 or 4 stimulations (see Results).

Blood samples (5 ml during and 3 ml before and after stimulation) were collected in ice-chilled tubes containing EDTA (final concentration 10^{-2} M), from the venous outflow just before and after $\frac{2}{3}$ of the respective stimulation periods, as well as 30 s, 2, 5, 10 and 15 min after the stimulation. Arterial blood samples were obtained before and 5 and 15 min after

the stimulations. Samples were kept in an ice bath during the experiments and then centrifuged at 2000 r.p.m. at +4°C for 15 min. Plasma samples (0.5 ml for each type of analysis) were collected and frozen at −70°C for later determination of NPY-LI and endogenous catecholamines. Endogenous plasma NA and adrenaline (Ad) were determined by high performance liquid chromatography (h.p.l.c.) with electrochemical detection (Hallman *et al.*, 1978). The content of NA was also quantified in biopsies from the spleen, kidney, right atrium of the heart and the quadriceps skeletal muscle obtained at the end of the experiments. Plasma NPY-LI was determined after acid ethanol extraction, by radioimmunoassay (RIA) with the NPY antiserum N1 (for details, see Theodorsson-Norheim *et al.*, 1985). Bolton-Hunter labelled [^{125}I]-porcine NPY was used as a tracer. The content of NPY-LI in biopsies obtained at the end of the experiments from the spleen, kidney, the right atrium of the heart and from the quadriceps skeletal muscle was also determined after extraction with acetic acid at 95°C (see Theodorsson-Norheim *et al.*, 1985). The output of NA from the spleen was corrected for local uptake by using corresponding uptake values for plasma Ad. The total output of NA and NPY-LI during and for 15 min immediately after nerve stimulation was calculated by multiplying the veno-arterial concentration differences by the plasma flow.

Local intra-arterial infusions or injections of drugs were made via an inlet close to the spleen. Substances were delivered via infusion at a rate of 200 μl min^{-1}, by means of a micro-infusion pump. Since the amount infused and the arterial plasma flow were known, the doses of drugs have been given as estimated plasma concentrations. Bolus injections were made with 0.3 ml of the tested agent followed by 0.3 ml saline.

Reserpine pretreatment (1 mg kg^{-1}, i.v.) was performed with a single dose in an ear vein following premedication with ketamine (25 mg kg^{-1}, i.m.). Five different groups of reserpinized pigs were used. The first group ($n = 6$) was maintained under anaesthesia after reserpinization for 16 h before the start of experiments. The second group was given reserpine 24 h before the experiments and allowed to regain consciousness ($n = 5$). The third and fourth groups were subjected to sectioning of the left splanchnic nerve simultaneously with ($n = 8$) or one week before the reserpine injections 24 h before the experiments began ($n = 6$). These nerves have been shown in the cat to contribute with preganglionic fibres to about 80% of the noradrenergic sympathetic ganglionic cells in the coeliac ganglion innervating the spleen (Brown *et al.*, 1961). The denervation procedure was performed after the same premedication, and under the same sodium-pentobarbitone anaesthesia and skeletal muscle paralysis, as described above. A retroperitoneal approach was used via a left subcostal incision under sterile conditions. About 1 cm of the left splanchnic nerve was removed and the wound closed by sutures. Atropine (0.05 mg kg^{-1}, i.v.) and neostigmine (0.1 mg kg^{-1}, i.v.) were then given, and the tracheal tube was removed after spontaneous breathing had resumed. The fifth group of pigs was preganglionically denervated at the same time as reserpine was given, 72 h before the experiments. A separate control group of animals was preganglionically denervated without being reserpine treated 24 h before the experiments. The postoperative period was free from complications in all pigs. In the reserpine-treated and preganglionically denervated animals, the same series of stimulations were performed as in the control animals before and after local infusion of drugs. Injections of tyramine, NPY, NA, ATP and α-, β-methylene ATP (mATP) were made in four additional animals.

Drugs

Reserpine (Serpasil), phentolamine methanesulphonate (Regitin), guanethidine sulphate (Ismelin) and desipramine hydrochloride were obtained from Ciba Geigy, Mölndal, Sweden. The following were also used: propranolol (Inderal, ICI, England); porcine neuropeptide Y (CRB, Cambridge, England); Bolton-Hunter labelled [^{125}I]-NPY (Amersham, England); [^{3}H]-noradrenaline (NEN, USA); atropine sulphate (Atropin); sodium pentobarbitone (Membumal) and neostigmine (ACO, Sweden); ketamine hydrochloride (Ketalar, Parke Davies, USA); pancuronium bromide (Pavulon, Organon, The Netherlands); tyramine hydrochloride, ATP (sodium salt and α-, β-methylene ATP lithium salt, Sigma, USA).

Statistics

Data are presented as means ± s.e.mean. Statistical significance was calculated using Kruskal-Wallis analysis of variance with multiple comparisons (for further details, see Theodorsson-Norheim, 1986) or Student's t test.

Results

Effects of nerve stimulations in control animals

Under basal conditions plasma NA and Ad levels were about 50–60% higher in the arterial input than in the venous effluent suggesting uptake in the spleen (Table 1). No corresponding gradient for the plasma

Table 1 Mean arterial blood pressure (BP, mmHg) and plasma levels of noradrenaline (NA, nM), adrenaline (Ad, nM) and NPY-LI (pM) in aorta (a) and splenic vein (v) from anaesthetized control pigs, 1 h after pretreatment with desipramine (DMI; 0.5 mg kg^{-1}, i.v.), 16 h after reserpine (1 mg kg^{-1}, i.v.) in pigs kept under anaesthesia and 24 h as well as 72 h after reserpine treatment of conscious animals; in one reserpine 24 h group and in the 72 h group the left splanchnic nerve was sectioned at the time of the reserpine injection

	BP (mmHg)	*NA* (nM)		*Ad* (nM)		*NPY-LI* (pM)	
		a	v	a	v	a	v
Control	106 ± 2	2.1 ± 0.3	1.3 ± 0.2	0.4 ± 0.1	0.2 ± 0.04	30 ± 4	36 ± 4
DMI	110 ± 3	1.3 ± 0.1	1.4 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	26 ± 3	27 ± 4
Reserpine 16 h + anaesthesia	75 ± 4***	3.0 ± 0.5	1.6 ± 0.5	1.6 ± 0.2**	1.2 ± 0.2**	459 ± 26***	465 ± 25***
Reserpine 24 h	93 ± 4**	3.4 ± 0.5	1.2 ± 0.3	3.6 ± 1.2**	1.8 ± 0.6**	167 ± 48**	190 ± 46**
Reserpine 24 h + pregangl. denerv.	88 ± 3**	3.8 ± 0.4	1.7 ± 0.3	3.2 ± 0.9**	1.5 ± 0.4**	152 ± 37**	181 ± 38**
Reserpine 72 h + pregangl. denerv.	97 ± 3	2.6 ± 0.3	1.6 ± 0.2	1.50 ± 0.2**	1.1 ± 0.2**	29 ± 4	32 ± 5

Data represent means ± s.e.mean (n = 6–15). **P < 0.01, ***P < 0.001 compared to control, using Kruskal Wallis analysis of variance.

NPY-LI was observed. Pretreatment with desipramine (DMI) abolished the splenic NA and Ad gradients (Table 1). Electrical stimulation of the splenic nerves increased perfusion pressure (Figure 1), blood flow in the splenic venous effluent (not shown) and the output of NA and NPY-LI (Figure 2). The perfusion-pressure increase and output of NA and NPY-LI were frequency-dependent (Figure 2A) and reproducible. The blood-flow increase, however, diminished upon repeated stimulations due to the depletion of the stored blood cells. Therefore, this parameter for capsule contractions has only been quantified for the initial series of stimulations. However, the blood flow information was used when calculating the total output of NA and NPY-LI.

Intermittent stimulation with 20 b caused a significantly larger perfusion-pressure increase (about 40%; P < 0.01) and enhancement (3 fold) of the output of NPY-LI relative to NA (1.6 fold), as compared to a continuous stimulation at 2 Hz, giving the same total number of impulses (Figure 2A). After DMI, the functional responses were enhanced following 20 b stimulations (by 30 and 40% regarding perfusion pressure and blood flow, respectively), in parallel with an increased NA overflow (by 60%). At 0.5 Hz the output of NPY was reduced significantly

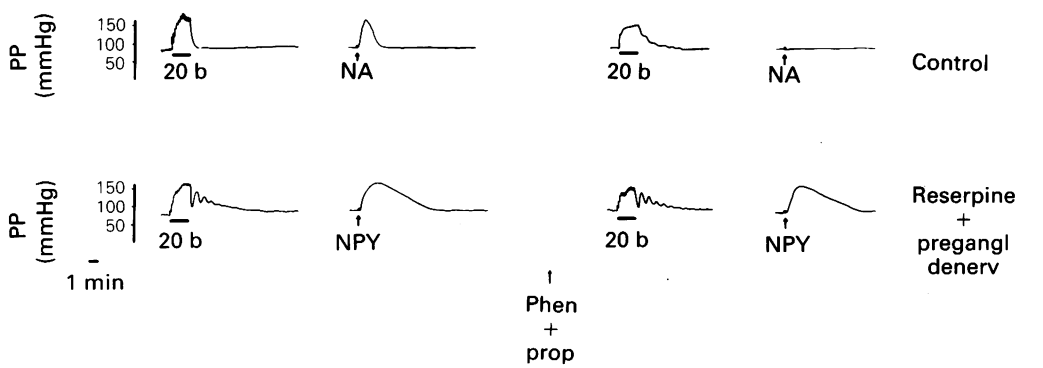

Figure 1 The effects of intermittent nerve stimulations with bursts at 20 Hz for 1 s every 10 s for 2 min (20b) or local bolus injections of noradrenaline (NA, 650 nmol) or neuropeptide Y (NPY, 2 nmol) on perfusion pressure (PP, mmHg) in the blood-perfused pig spleen *in vivo*. The effects under control conditions (top panel) are compared with the responses 24 h after reserpine (1 mg kg^{-1}, i.v.) treatment in animals where the spleen was denervated preganglionically one week prior to reserpine injections (lower panel). Local intra-arterial infusion of phentolamine (Phen) and propranolol (prop) (estimated local plasma concentrations of 5 × 10^{-5} and 10^{-6} M, respectively) was performed during the repeated stimulations or injections. Bar indicates time scale for 1 min.

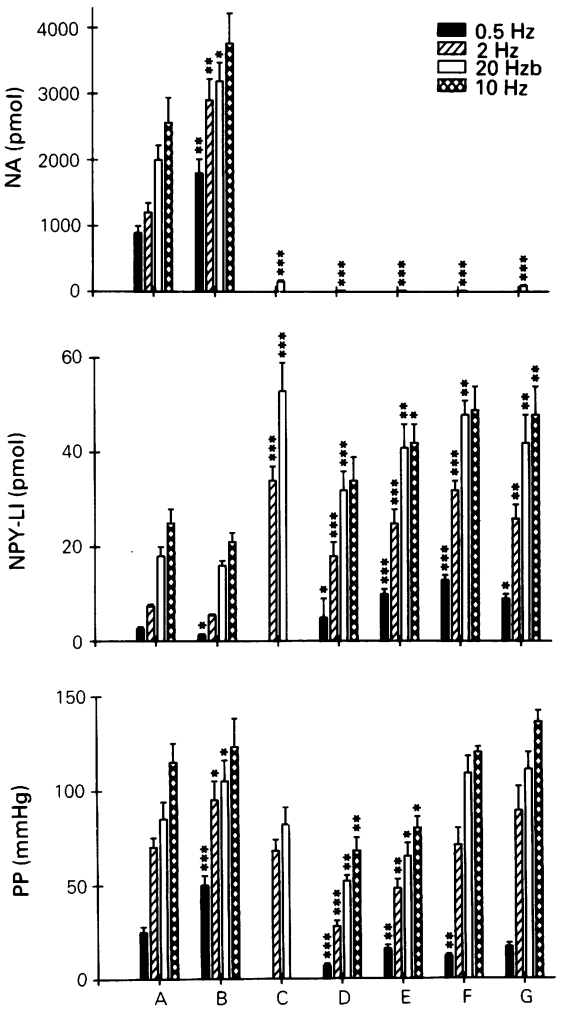

Figure 2 Perfusion-pressure increase (PP, mmHg) and the integrated output of neuropeptide Y-like immunoreactivity (NPY-LI) and noradrenaline (NA) (pmol) from the blood-perfused pig spleen *in vivo* during and the first 15 min after electrical stimulation of the splenic nerves with 0.5 Hz for 8 min, 2 Hz for 2 min and 10 Hz for 24 s, giving a total of 240 shocks. The effects of intermittent stimulation with bursts at 20 Hz for 1 s every 10 s for 2 min (20 b) are also illustrated. The responses are given as means (with s.e.mean shown by vertical bars) in (A) control animals ($n = 5$), (B) after pretreatment with desipramine ($n = 5$), (C) after resperpine treatment of pigs kept under anaesthesia for 16 h ($n = 7$), and 24 h (D, E, F) or 72 h (G) following resperine treatment of conscious pigs in intact (D) ($n = 5$) or preganglionically denervated preparations (1 week) (E), 24 h (F), or 72 h (G) ($n = 6$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to responses in control animals, using Kruskal-Wallis analysis of variance with multiple comparisons.

(by 45%) compared to the response in controls (Figure 2B). Local pretreatment with phentolamine (estimated plasma concentration 5×10^{-5} M) caused a parallel enhancement of the output of both NPY-LI (from 20 ± 4 to 37 ± 4 pmol) and NA (from 1200 ± 150 to 2400 ± 450 pmol) upon 20 b stimulations.

Preganglionic denervation 24 h before the experiments did not change the functional responses or the overflow of NA and NPY-LI upon nerve stimulation ($n = 4$; not shown).

Effects of reserpine treatment

Sixteen and 24 h after reserpine treatment, the pigs had a lowered systemic arterial blood pressure (Table 1). Furthermore, plasma Ad and NPY-LI were markedly elevated (Table 1). Seventy-two hours after reserpine, blood pressure, plasma NA and NPY-LI were close to normal, while plasma Ad was still elevated (Table 1). After reserpine treatment of pigs kept either under anaesthesia or normal conscious conditions, the splenic NA content (Table 2) and the nerve stimulation-evoked output of NA from the spleen were markedly reduced (>90%) (Figure 2 C–G). In the pigs kept under anaesthesia after reserpine, the depletion of the NA content in the spleen (93%) and the reduction in the nerve stimulation-evoked output of NA (87%) were slightly less than in the animals which had been conscious for 24 h after the reserpine injection, where both parameters were reduced by 98–99% (Figure 2 C, D; Table 2). Preganglionic denervation for one week did not prevent the reserpine-induced depletion of NA in the spleen (Table 2). Three days after the injection of reserpine, about 10% of the initial NA content had recovered in the spleen (Table 2).

A marked depletion (>98%) of NA content 24 h after reserpine treatment also occurred in the right atrium of the heart (control: 15.4 ± 1.9 nmol g^{-1}, $n = 11$). Furthermore, the content of NA in the kidney (control: 1.8 ± 0.1 nmol g^{-1}, $n = 10$) and skeletal muscle (control: 0.12 ± 0.02 nmol g^{-1}, $n = 6$) were both reduced by 98% 24 h after the reserpinization of conscious pigs. In the adrenal gland, however, the content of NA was only reduced by 37% (from 5002 ± 489 nmol g^{-1} to 3165 ± 294 nmol g^{-1}; $n = 6$) and Ad levels by 77% (from 4323 ± 755 nmol g^{-1} to 983 ± 134 nmol g^{-1}; $n = 6$) 24 h after reserpine.

Reserpine treatment was also associated with a depletion of NPY-LI in the spleen, as revealed in biopsies obtained after the end of the stimulation experiments (Table 2). Furthermore, the contents of NPY-LI in the right atrium of the heart (control: 24.0 ± 3.0 pmol g^{-1}, $n = 8$), kidney (control: 1.8 ± 0.09 pmol g^{-1}, $n = 5$) and skeletal muscle

Table 2 The levels of neuropeptide Y-like immunoreactivity (NPY-LI, pmol g^{-1}) and noradrenaline (NA) (nmol g^{-1}) in the pig spleen from (1) controls, (2) animals where the spleen was preganglionically denervated 24 h earlier, (3) reserpine (1 mg kg^{-1}, i.v.) pretreated pigs kept under anaesthesia for 16 h, (4) reserpine-pretreated pigs that were conscious for 24 h after the bolus injection, conscious reserpine-pretreated pigs where the spleen also was preganglionically denervated 1 week earlier (5) or simultaneously with the reserpine injection (6) and (7) conscious reserpine-treated pigs where the injection and preganglionic denervation were carried out 72 h before the experiment

	NPY-LI	%	*NA*	%
1 Control	19.1 ± 1.4 (12)	100	11.6 ± 1.0 (12)	100
2 Pregangl. denerv. 24 h	15.5 ± 0.8 (4)	81	10.7 ± 1.3 (4)	92
3 Reserpine 16 h + anaesthesia	11.7 ± 0.8 (6)	61**	0.8 ± 0.1 (7)	7***
4 Reserpine 24 h	5.3 ± 0.6 (5)	28***	0.1 ± 0.05 (5)	1***
5 Reserpine 24 h + pregangl. denerv. 1 week	10.1 ± 0.5 (6)	53***	0.1 ± 0.03 (6)	1***
6 Reserpine 24 h + pregangl. denerv. 24 h	15.5 ± 1.2 (8)	81	0.2 ± 0.04 (4)	2***
7 Reserpine 72 h + pregangl. denerv. 72 h	12.8 ± 1.5 (4)	67*	1.3 ± 0.3 (4)	11***

Data are given as means ± s.e.mean (n = 4–12). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ denote significant changes from control, using Kruskal-Wallis analysis of variance with multiple comparisons.

(control: 0.12 ± 0.02 pmol g^{-1}, $n = 4$) were reduced by 83, 78 and 42%, respectively 24 h after reserpine treatment of conscious animals. The adrenal in control pigs contained low levels of NPY-LI (0.3 ± 0.01 pmol g^{-1}) and after reserpine a slight reduction to 0.1 ± 0.05 pmol g^{-1} was observed ($n = 4$). When preganglionic denervation was combined with the reserpine treatment, a partial prevention of the NPY depletion in the spleen was observed with one week of denervation and close to a normal content after 24 h denervation (Table 2).

The perfusion-pressure increase in the spleen upon electrical nerve stimulation, when using high stimulation frequencies (20 b and 10 Hz) was well preserved in reserpine-treated animals (Figures 1, 2). Furthermore, the increase in blood flow in the splenic venous effluent was approximately 60% of the response to 20 b and 10 Hz in controls (not shown). However, the perfusion pressure response both to 0.5 and 2 Hz stimulations were reduced (by about 50%) in the non-denervated and 1 week-denervated groups, which had been conscious after the reserpine injection (Figure 2 D, F). The blood-flow increase upon 0.5 and 2 Hz stimulations was 40 and 50%, respectively, as compared to controls in the reserpine-treated animals where the spleen had been preganglionically denervated one week earlier. In the pigs that were preganglionically denervated simultaneously with reserpine injection, a major decrease in the perfusion-pressure response was observed only at 0.5 Hz (Figure 2 F). The perfusion-pressure responses in the animals, which had been preganglionically denervated and conscious after the reserpine injection were long-lasting compared to the effects under control conditions (Figure 1). Repeated stimulation in reserpinized animals caused a progressively diminishing functional response (not shown) (Table 3).

The output of NPY-LI upon nerve stimulation was markedly enhanced after reserpine treatment (Figure 2). Thus, after reserpine treatment performed simultaneously with or one week after a preganglionic denervation, the output of NPY-LI upon stimulation increased about 4, 3, 2 and 1.5 fold at 0.5, 2, 20 b and 10 Hz, respectively. There was a good correlation between the remaining perfusion-pressure responses and the overflow of NPY-LI (expressed as the total output ($r = 0.91$, $P < 0.001$) or as a maximal, veno-arterial concentration gradient ($r = 0.92$, $P < 0.001$) in the reserpinized animals (Figure 3a,b). The maximal increase in the plasma levels of NPY-LI in the splenic venous effluent upon 10 Hz stimulations in reserpinized animals was 1250 pmol l^{-1}. The output of NPY-LI, however, declined in the reserpinized animals upon repeated 20 b stimulation (not shown). Since a maximal output of around 60 pmol NPY-LI was recovered in the splenic venous effluent upon a 20 b stimulation for 2 min in reserpinized pigs, at least 3% of the total splenic NPY content (which was 1795 ± 132 pmol in control spleens; $n = 8$, corresponding to 8 μg of NPY) was released. Therefore, a considerable portion of the initial splenic NPY content is likely to have been depleted during the series of stimulations in the reserpinized animals before tissue samples were obtained (Table 2).

Pretreatment with propranolol did not influence the perfusion-pressure increase or the output of NPY-LI evoked by nerve stimulation in control or reserpinized pigs (Table 3). The perfusion-pressure increase to 20 b in control animals was prolonged and reduced by 35% after combined α- and β-adrenoceptor blockade, with phentolamine and propranolol (Figure 1), while the output of NPY-LI was enhanced as compared to controls (Table 3). In the conscious reserpine-treated animals, the

Table 3 The effects of local treatment with propranolol (10^{-6} M), phentolamine (5×10^{-5} M) plus propranolol (10^{-6} M) or guanethidine (3 mg kg^{-1}, i.v.) on nerve stimulation (20 b)-evoked perfusion-pressure increase and the output of NPY-LI from pig spleen

	Control		*Reserpine Anaesthesia*		*Reserpine Concious + 24th pregangl. denerv.*		*Reserpine Concious + 72 h pregangl. denerv.*	
	Perfusion pressure increase	*Output NPY-LI*	*Perfusion pressure increase*	*Output NPY-LI*	*Perfusion pressure increase*	*Output NPY-LI*	*Perfusion pressure increase*	*Output NPY-LI*
Basal	100 ± 6	100 ± 15	100 ± 11	100 ± 11	100 ± 11	100 ± 12	100 ± 0	100 ± 15
Repeat	95 ± 8	107 ± 17	—	—	92 ± 2	92 ± 2	90 ± 2	85 ± 9
Propranolol	99 ± 4	89 ± 10	91 ± 7	108 ± 12	—	—	—	—
Phentolamine + propranolol	65 ± 4**	170 ± 27**	77 ± 3*	75 ± 15*	77 ± 6*	82 ± 4*	75 ± 10*	78 ± 6*
Guanethidine	3 ± 1***	4 ± 2***	—	—	3 ± 1***	2 ± 1***	—	—

Data are shown from controls, reserpinized animals that were simultaneously preganglionically denervated 24 or 72 h before the experiments. Values (means ± s.e.mean) have been expressed in percentage of the initial 20 b stimulation in each experiment ($n = 5$–6). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to the basal stimulation response in each group, using Kruskal-Wallis analysis of variance with multiple comparisons.

Figure 3 Correlation between perfusion-pressure increase (PP, mmHg), and (a) the recovered integrated output of neuropeptide Y-like immunoreactivity (NPY-LI) (pmol/stimulation) or (b) the maximal veno-arterial (v-a) plasma-concentration gradient of NPY-LI in the splenic venous effluent upon nerve stimulations with different frequencies of the blood-perfused spleen of reserpinized pigs (1 mg kg^{-1} given i.v. 24 h prior to the experiments in both control and preganglionically denervated animals).

perfusion-pressure responses (Figure 1) and the output of NPY-LI were also somewhat reduced after combined α- and β-adrenoceptor blockade (Table 3). Phentolamine and propranolol treatment *per se* increased the perfusion pressure, especially in the conscious reserpinized pigs (at 24 h, by 25 ± 4 mmHg and at 72 h, by 30 ± 5 mmHg). In the reserpinized animals kept under anaesthesia, pretreatment with phentolamine and propranolol prolonged and reduced the functional response in parallel with a slight reduction in the NPY output (Table 3). Guanethidine pretreatment virtually abolished the release of NPY-LI and the functional responses in control and reserpinized pigs (Table 3).

Effects of locally administered agents

Bolus injections of tyramine caused a long-lasting increase in the perfusion pressure in control animals. Simultaneously, there was an increased veno-arterial gradient of NA over the spleen, suggesting release

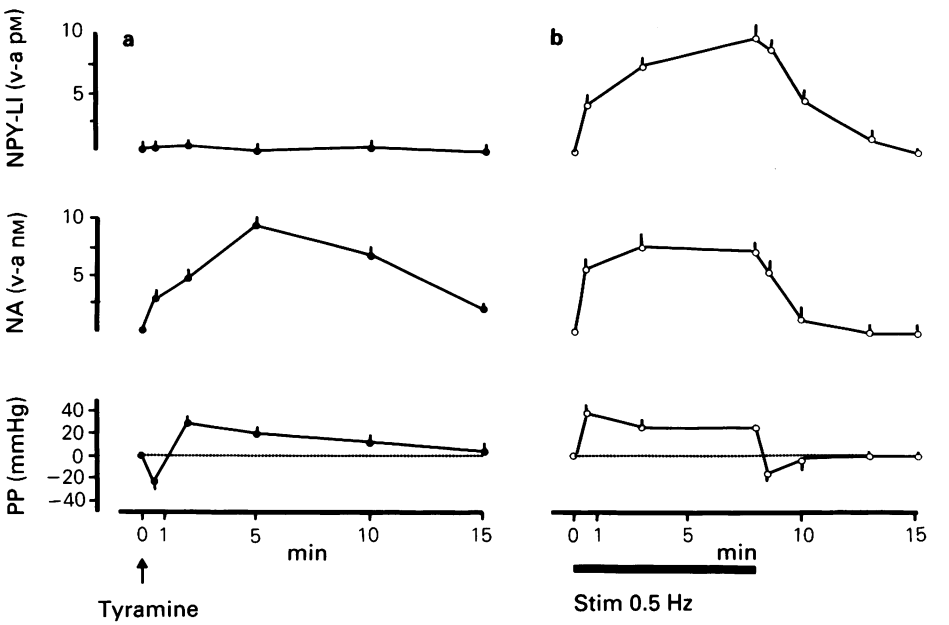

Figure 4 Effects upon local bolus injection of tyramine 4 μmol (arrow) or nerve stimulation with 0.5 Hz for 8 min on perfusion pressure (PP, mmHg) as well as veno-arterial (v-a) gradients of noradreneline (NA, nM) and neuropeptide Y-like immunoreactivity (NPY-LI, pM) from pig spleen. Values are given as means with s.e.mean shown by vertical lines, $n = 4$.

largely similar to the effect of the stimulation with 0.5 Hz, while no change in the NPY output occurred (Figure 4). In the reserpinized animals, no functional effects or NA release were evoked by tyramine (not shown).

NPY and NA were infused locally or given as bolus injections into control or reserpinized spleens which were not subjected to release experiments. NPY was about 50 fold more potent on a molar basis than NA in increasing the perfusion pressure with a threshold effect at 1000 pM. A very good recovery of the locally infused NPY was obtained in the splenic venous effluent as revealed by RIA. Thus, the levels of NPY-LI detected were 0.9 ± 0.1; 8.8 ± 1.2 and 85 ± 13 nM ($n = 4$) at the infusions, giving estimated local plasma concentrations of 1, 10 and 100 nM, respectively. The perfusion-pressure responses to bolus injections of NPY (Figure 1) or infusions of NPY were more long-lasting than those of NA and similar in control and reserpinized animals. Thus, the perfusion-pressure increases were 9 ± 1 mmHg vs. 11 ± 2 at 10^{-9} M NPY and 48 ± 5 vs. 52 ± 6 mmHg at 10^{-8} M NPY ($n = 4$) in controls and 24 h after reserpine combined with preganglionic denervation, respectively. Furthermore, both NA and NPY infusions were associated with an increased splenic venous blood flow, suggesting capsule contraction of a comparable magnitude both in control and reserpinized animals (not shown).

The perfusion-pressure effects upon the infusion of 5×10^{-6} M NA (98 ± 5 mmHg) were abolished after pretreatment with phentolamine (5×10^{-5} M) and propranolol (10^{-6} M), while the response to NPY was not influenced by these adrenoceptor antagonists (not shown).

Local infusions of ATP (10^{-7} to 10^{-3} M) caused a dose-dependent reduction in the perfusion pressure (maximally by 70% at 10^{-3} M). Furthermore, the bolus injections of ATP (4 nmol to 4 μmol) also reduced the perfusion pressure (80% maximal reduction at 4 μmol ATP). The ATP analogue mATP caused a dose-dependent increase in the perfusion pressure and venous blood flow with a similar potency to NA. The mATP effect was not influenced by reserpine or the adrenoceptor antagonists. Upon continuous infusion of mATP (10^{-5} M), the initial marked perfusion-pressure increase was followed by a decline with time (Figure 5). Then, tachyphylaxis to the perfusion-pressure effects of mATP developed (Fig. 5). The maximal functional response to a 20 b stimulation as well as the output of NPY-LI and NA were not significantly changed upon mATP desensitization (Figures 5, 6). However, the perfusion-pressure response to nerve stimulation was reduced

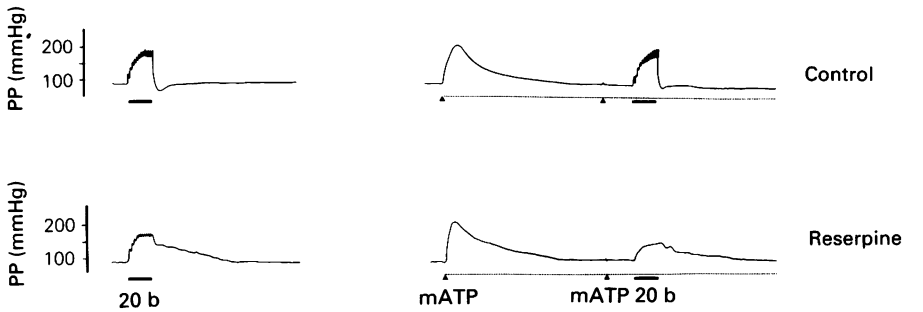

Figure 5 Effects of the infusion of mATP (10^{-5} M) on basal perfusion pressure (PP, mmHg) and the response to nerve stimulation (20 b for 2 min) in a control pig (top panel) and in an animal that was pretreated with reserpine combined with preganglionic denervation 24 h earlier (lower panel). A new mATP infusion (10^{-5} M) was started at the second right arrow head.

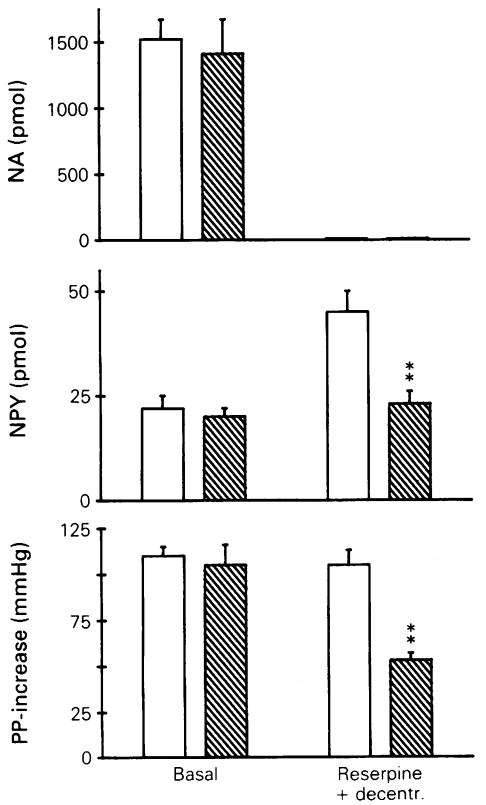

Figure 6 Summary of the effects of mATP, desensitization on the stimulation-evoked perfusion-pressure increase (PP, mmHg) as well as the integrated output of neuropeptide Y-like immunoreactivity (NPY-LI, pmol) and noradrenaline (NA, pmol) from the spleen of control and reserpinized pigs (24 h combined with preganglionic decentralization). Open columns, control; hatched columns, mATP. Data are given as means with s.e.mean shown as vertical bars ($n = 4$). $^{**}P < 0.01$, comparing the responses before and after mATP tachyphylaxis.

by about 50% after mATP tachyphylaxis in reserpinized pigs (Figures 5, 6). The output of NPY-LI was then reduced in parallel (Figure 6).

Discussion

The present data show that in spite of pharmacological blockade of NA mechanisms by adrenoceptor antagonists and an almost total NA depletion after reserpine pretreatment, large functional responses (perfusion-pressure and blood-flow increases) remain in the pig spleen upon sympathetic nerve stimulation *in vivo*. In contrast, the functional effects of tyramine were absent in reserpine-treated animals. Thus, in the present *in vivo* model, no evidence was obtained that the tyramine response was non-adrenergic or associated with the release of NPY-LI, which is in apparent contrast to the *in vitro* data from the rat vas deferens reported by Cheng & Shen, 1987. It should be emphasized that adrenergic transmission has been reported to occur with small amounts of NA in reserpinized animals (see Andén & Henning, 1966; Antonaccio & Smith, 1974). However, the largely parallel reduction of the functional effects as well as the NPY overflow after phentolamine and propranolol pretreatment in the reserpinized animals indicate that the 10% of the initial content of NA remaining is not sufficient to contribute as a main splenic transmitter. Furthermore, the enhancement of the stimulation-evoked NPY overflow after reserpine was similar regardless of whether the NA content was 10 or 1% of control.

The prolongation of the nerve-stimulation responses by phentolamine and propranolol as well as the perfusion-pressure increase by these agents *per se* in reserpinized animals may be related to the inhibition of a vasodilator tone due to the high Ad levels in arterial plasma. Thus, the reserpine dose used, only partially depleted the adrenal catecholamines. Propranolol treatment in control animals did not

influence the basal or stimulation-evoked perfusion-pressure increase, suggesting that the Ad levels were then too low to influence the vascular tone and that neuronally released NA does not activate the vascular β-adrenoceptors to any major extent. The prolongation of the perfusion-pressure responses after adrenoceptor blockade in control animals (see also Lundberg & Tatemoto, 1982) and after reserpine treatment may also be related to the enhanced overflow of NPY-LI. In addition, metabolic production of vasodilator factors, such as adenosine, via adrenergic mechanisms (see Fredholm, 1976) are likely to contribute to the relatively rapid decline in the perfusion-pressure response observed after the stimulation under control conditions.

The absence of detectable NA release and the 99% depletion of NA content in the tissue 24 h after reserpine treatment of conscious animals suggest that activation of classical or novel adrenoceptors (see Nield & Hirst, 1984) is unlikely to account for the observed remaining splenic functional effects under these conditions. Since bolus injections of NPY mimicked these long-lasting functional responses and NPY is released upon splenic nerve stimulation, as indicated by increased overflow (see also Lundberg *et al.*, 1984a; 1986c) this peptide is a possible candidate for the mediation of these apparently 'non-adrenergic' effects. Furthermore, the plasma levels of NPY-LI in the splenic venous effluent upon nerve stimulation at 20 b and 10 Hz after reserpine treatment were in the same range (nM) as the arterial plasma levels during the infusions of exogeneous NPY which are associated with vasoconstriction (see also Lundberg *et al.*, 1986c; Rudehill *et al.*, 1987). In addition, it is likely that the local concentrations of NPY-LI close to the nerve terminals after release are considerably higher than the spill-over recovered in the venous effluent. A close relationship between NPY and the functional responses in reserpinized pigs was further supported by the excellent correlation ($r = 0.91$) between the remaining perfusion-pressure responses upon splenic nerve stimulation and the recovered output of NPY-LI in the splenic venous effluent. When using a low continuous stimulation frequency (0.5 Hz), most of the functional effects under control conditions are, however, likely to be related to NA, considering the relatively modest functional responses present in spite of a large increase in the corresponding output of NPY-LI in the reserpinized animals.

The release of NPY-LI upon low-frequency stimulation was slightly reduced when neuronal uptake was inhibited by DMI and enhanced after the administration of the α-adrenoceptor antagonist phentolamine. Furthermore, the NPY output was to an even greater extent enhanced when NA was depleted after reserpine pretreatment. This suggests that NA exerts an inhibitory, prejunctional feedback influence on the release of NPY-LI via α-receptors, especially at low frequency stimulation (see also Lundberg *et al.*, 1984a; 1986c). The release of NA was also enhanced after local phentolamine treatment in accordance with the view that the NA release from the spleen is subjected to autoregulation by prejunctional α-adrenoceptors (see Cubeddu *et al.*, 1974).

Guanethidine pretreatment abolished the release of NPY-LI and perfusion-pressure increase upon splenic nerve stimulation in both control and reserpinized pigs. This observation is in accordance with the view that the NPY release originated from adrenergic nerve terminals capable of taking up guanethidine (see Boura & Green, 1965).

It has recently been shown that pretreatment with high doses of reserpine also depletes NPY-LI from certain sympathetic nerve terminals via a mechanism likely to involve neurogenic activation and release of the peptide in an excess of resupply by axonal transport (Lundberg *et al.*, 1985c,d; 1986a). The content of NPY-LI in the pig spleen was in the present study also reduced after reserpine pretreatment, as determined after the stimulations. Part of this reduction, however, may be attributed to the stimulation-evoked release and depletion of terminal stores during the experiments, considering slow replacement of NPY by axonal transport.

The enhanced, stimulation-evoked NPY output from the pig spleen after reserpine treatment is in agreement with the increased release (see Dixon *et al.*, 1979) and depletion of the splenic contents (Arnaiz *et al.*, 1978) of dopamine-β-hydroxylase, another component of storage granules. This suggests that the increased impulse activity, which has been reported to be present in the sympathetic nerves of reserpine-treated animals (see Heusler, 1974), as well as the present observations of an enhanced release of NPY-LI per nerve impulse, created a situation in the non-denervated animals where resupply of the peptide was inadequate. Part of the increased release of NPY-LI after reserpine may be related to a loss of the inhibitory prejunctional feedback by NA, although other actions of reserpine may also contribute (see Cubeddu & Weiner, 1975).

Increasing evidence based on observations from *in vitro* experiments on isolated blood vessels (Kugelgen & Starke, 1985; Kennedy *et al.*, 1986; Burnstock & Warland, 1987; Muramatsu, 1987) and vas deferens (Fedan *et al.*, 1981; Sneddon *et al.*, 1982; Stjärne & Åstrand, 1985), suggests that ATP is a co-transmitter with NA in the electrical and/or contractile responses of smooth muscle cells evoked by sympathetic nerve stimulation. It seems clear, however, that the proposed ATP-mediated com-

ponent of the smooth muscle response of isolated blood vessels is a rapid short-lasting contraction where the ATP contribution relative to NA is inversely related to stimulation frequency (i.e. relatively largest at low stimulation frequencies) (Kennedy *et al.*, 1986; Burnstock & Warland, 1987). In contrast, the remaining nerve stimulation-evoked responses in the pig spleen after reserpine were slowly developing, long-lasting and were most pronounced at high frequencies. The depolarization-evoked ATP release is considered to be unaffected by reserpine treatment (Katsuragi & Su, 1981; Kirkpatrick & Burnstock, 1987; see Burnstock & Kennedy, 1986), and the non-adrenergic, presumably ATP-mediated, vasoconstrictor effects are also uninfluenced by reserpine (Muramatsu, 1987). This is not in line with the decline of the functional responses upon repeated stimulation in the spleen of reserpinized animals. It has, in fact, not been possible to provide conclusive evidence that nerve stimulation is associated with release of ATP from the perfused spleen (Stjärne *et al.*, 1970) or a depletion of the splenic storage-vesicle content of ATP (Blaschke & Uvnäs, 1979). In addition, the reserpine-induced depletion of NA in the rat heart was not accompanied by any corresponding change in the vesicular content of ATP (Potter & Axelrod, 1963).

In the pig-spleen model, infusion of the metabolically stable ATP analogue mATP, which acts on P_2-purinoceptors (see Burnstock & Kennedy, 1986), caused a perfusion-pressure increase *in vivo*, suggesting vasoconstriction (see Delbro *et al.*, 1985; Burnstock & Kennedy, 1986) both in control, in reserpinized animals and after adrenoceptor blockade. However, ATP only induced vasodilatation even in very high doses, which may be related to P_2-purinoceptor activation on endothelial cells (see Furchgott, 1981) and/or rapid degradation to adenosine, which is a potent vasodilator agent acting on P_1-purinoceptors on the vascular smooth muscle cells (see Burnstock & Kennedy, 1986).

The functional perfusion-pressure increase in the pig spleen to stimulation under control conditions was not influenced by mATP tachyphylaxis in spite of short bursts of impulses being used that would have favoured the putative ATP mechanisms (see Burnstock & Kennedy, 1986). In accordance with earlier reports, the nerve stimulation-evoked release of NA (Kugelgen & Starke, 1985; Stjärne & Åstrand, 1985) and NPY-LI (present results) was not reduced upon mATP tachyphylaxis in control animals. When tachyphylaxis had developed to the perfusion-pressure response of mATP after reserpine treatment, the maximal perfusion-pressure effects upon splenic nerve stimulation were reduced. Then, the output of NPY-LI was inhibited in parallel. This suggests that mATP under these conditions caused an inhibitory effect on NPY release, via the activation of prejunctional P_2-purinoceptors (Fujioka & Cheung, 1987) or alternatively by some other pharmacological effect, such as the formation of adenosine or prostaglandins (Needleman *et al.*, 1974; Frew & Baer, 1979) due to prolonged vasoconstriction. Furthermore, stable ATP analogues have been reported to depress the stimulation-evoked acetylcholine release from the intestine (Wiklund *et al.*, 1985). The inhibitory effect of mATP on sympathetic transmission in the pig spleen seemed to be absent under control conditions, possibly due to a dominance of the powerful α-adrenergic feed-back regulation. Earlier findings using the pithed rat model *in vivo* have also shown that stimulation of the sympathetic outflow under control conditions results in a vasopressor response, which is not influenced by mATP tachyphylaxis (Flavahan *et al.*, 1985). In contrast, the vasopressor effect to nerve stimulation after reserpine is reduced by mATP in agreement with the present data from the pig spleen. The influence of mATP on NPY release in reserpinized animals has then to be taken into account.

In conclusion, reserpine treatment combined with preganglionic denervation creates a stiuation where the high-frequency, stimulation-evoked, long-lasting, functional responses are likely to depend on the release of a non-adrenergic mediator, such as NPY rather than ATP. However, the possible importance of NPY compared to the classical transmitter NA (Peart, 1949) in the sympathetic control of the pig spleen during physiological conditions remains to be established by use of specific antagonists.

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Prostanoid-induced contraction of human bronchial smooth muscle is mediated by TP-receptors

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1 A range of naturally-occurring prostaglandins sulprostone, 16,16-dimethyl prostaglandin E_2 (DME_2) and the thromboxane A_2 (TXA_2)-mimetic, 11α,9α-epoxymethano prostaglandin H_2 (U-46619) have been tested for contractile agonist activity on human isolated bronchial smooth muscle.

2 Prostaglandin D_2 (PGD_2), $PGF_{2\alpha}$, 9α,11β-PGF_2 (11β-PGF_2) and U-46619 all caused concentration-related contractions. U46619 was at least 300 fold more potent than the other prostanoids with a mean EC_{50} of 12 nM. Sulprostone caused contraction only at the highest concentration tested (30 μM). PGE_2 and PGI_2 caused relaxations at low concentrations, and only caused contractile responses at high concentrations ($\geqslant$ 10 μM). In contrast, DME_2 caused small contractions at low concentrations but relaxation at the highest concentration tested (30 μM).

3 The rank order of contractile agonist potency was: U-46619 $\gg$ 11β-PGF_2 $\simeq$ $PGF_{2\alpha}$ > PGD_2 > PGE_2 > PGI_2 $\simeq$ sulprostone $\simeq$ DME_2.

4 The TP-receptor blocking drug, AH23848 (1 μM) antagonized the contractile effects of U-46619, PGD_2, $PGF_{2\alpha}$ and 11β-PGF_2, but had no effect against contractions to carbachol. In a single experiment, a pA_2 of 8.3 (slope = 1.2) was obtained for AH23848 against U-46619.

5 In most preparations, administration of AH23848 (1 μM) to human bronchus resulted in small, transient contractile responses.

6 The results obtained with both the agonists and the antagonist, AH23848 are therefore consistent with prostanoid-induced contractions of human bronchial smooth muscle being mediated by TP-receptors.

Introduction

Since Mathé and his colleagues (Mathe' *et al.*, 1973) demonstrated that asthmatics may be up to 8,000 times more sensitive than non-asthmatics to the bronchoconstrictor effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), there has been an interest in the possible role of prostanoids as causative factors in bronchial asthma. It is now well known that human isolated bronchial smooth muscle can contract in response to prostanoids (Strandberg & Hedqvist, 1977; Karim *et al.*, 1980; Black *et al.*, 1986; Seibert *et al.*, 1987), and that not only $PGF_{2\alpha}$, but also PGD_2 and the principle metabolite of PGD_2, 9α,11β-PGF_2(11β-PGF_2), all cause bronchoconstriction in man (Robinson *et al.*, 1987). A preliminary investigation into the nature of the receptors mediating this effect has recently been reported by McKenniff *et al.* (1988), who speculated that contraction of human isolated bronchus may be mediated by a novel prostanoid receptor.

In the present study, therefore, we have evaluated the constrictor effects of a range of prostanoids on human isolated bronchial smooth muscle. We have also investigated the interaction of some of these agonists with the TP-receptor blocking drug, AH23848 (Brittain *et al.*, 1985). From the results of these studies, some conclusions have been drawn as to the nature of the receptors mediating prostanoid-induced bronchoconstriction in man.

Methods

Tissue preparation

Samples of human bronchus were obtained from patients undergoing surgical resection of the lung. Bronchial tissue was dissected clear of lung par-

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enchyma and vascular tissue, and stored overnight in modified Krebs solution at 20°C. The composition of the Krebs solution was as described by Apperley *et al.* (1976). Bronchus of lumen diameter 3–4 mm was cut into rings of 3–4 mm width, which were then opened to form strips. These strips were mounted in organ baths and bathed in Krebs solution at 37°C, containing indomethacin (2.8 μM) and bubbled with 5% CO_2 in O_2. A resting tension of 1 g was applied to each preparation.

Experimental design and evaluation of agonist and antagonist potency

In all agonist studies, control cumulative concentration-effect curves to the standard, the thromboxane A_2 mimetic U-46619 (Coleman *et al.*, 1981), were repeated at approximately 90 min intervals until constant responses were obtained, after which a similar curve was constructed to another agonist. Agonist potency is expressed as equipotent molar concentration (EPC, U-46619 = 1), defined as:

$$\frac{EC_{50} \text{ test agonist}}{EC_{50} \text{ U-46619}}$$

In antagonist studies, agonist cumulative concentration-effect curves were repeated at approximately 90 min intervals until constant responses were obtained, and then a single concentration of AH23848 (1 μM) was added to the bathing solution before a further agonist concentration-effect curve was constructed. An antagonist contact time of between 30 and 45 min was used. Antagonist potency is expressed as concentration-ratio (CR), defined as:

$$\frac{\text{agonist } EC_{50} \text{ in presence of antagonist}}{\text{agonist } EC_{50} \text{ in absence of antagonist}}$$

In a single experiment, a pA_2 value for AH23848 against U-46619 was determined by the method of Arunlakshana & Schild (1959).

Drugs used

The following drugs were used: [1α(Z),2β,5α]-(±)-7-[5-[[(1,1'-biphenyl)-4-yl]methoxy]-2-(4-morpholinyl)-3-oxocyclopentyl]-4-heptenoic acid, calcium salt (AH23848, Glaxo Group Research); carbamylcholine chloride (carbachol, BDH); indomethacin (Sigma); PGD_2 (Glaxo Group Research); PGE_2 (Upjohn); $PGF_{2\alpha}$ tromethamine (Upjohn); PGI_2 Na salt (Glaxo Group Research); 11β-PGF_2 (Glaxo Group Research); 11α,9α-epoxymethano PGH_2 (U-46619, Glaxo Group Research); sulprostone (Glaxo Group Research) and 16,16-dimethyl PGE_2 (DME_2, Glaxo Group Research). Carbachol was dissolved in 0.9% w/v NaCl solution (saline), indomethacin, PGD_2, PGE_2, 11β-PGF_2, DME_2 and U-46619 were all dissolved in 1.0% $NaHCO_3$ in saline, sulprostone was dissolved in 3% EtOH/0.01% Tween 80 in saline, AH23848 was dissolved in 6% EtOH/0.01% Tween 80 in saline. All dilutions were made in saline. PGI_2 was dissolved in Tris/HCl buffer pH 9.0 on the day of the experiment, and dilutions made in Tris/HCl buffer pH 8.0 immediately before use.

Results

Agonist studies

PGD_2 (0.1–30 μM), $PGF_{2\alpha}$ (0.1–30 μM), 11β-PGF_2 (0.1–30 μM) and U-46619 (0.001–0.3 μM) all caused concentration-related contractions of human bronchial strips, while sulprostone caused a contraction at the highest concentration tested (30 μM) only. In contrast, low concentrations of PGE_2 (0.01–1.0 μM) and PGI_2 (0.1–10 μM) caused concentration-related relaxation, higher concentrations (PGE_2, 10–30 μM and PGI_2, 30 μM) causing contractions. In a single experiment, DME_2 caused small contractions at 1.0–10 μM and relaxation at 30 μM. U-46619 was the most potent agonist, with a mean EC_{50} of 12 nM (95% confidence limits = 7–17, $n = 7$), determined from those experiments in which a maximum response was clearly established. Mean concentration-effect curves to the agonists tested are shown in Figure 1 and the contractile agonist potencies of the prostanoids, relative to U-46619, are summarised in Table 1.

Due to their relatively low potency, full contractile concentration-effect curves were only obtained in a proportion of experiments with 11β-PGF_2 ($n = 3/4$), $PGF_{2\alpha}$ ($n = 2/4$) and PGD_2 ($n = 2/4$), in the concentration-range tested. Thus the maximum

Table 1 Mean equipotent molar concentration (EPC) values (U-46619 = 1) for prostanoids in contracting human isolated bronchial smooth muscle

Agonist	*EPC*	(95% CL)	n
U-46619	1	–	21
11β-PGF_2	319	(47–2176)	4
$PGF_{2\alpha}$	383	(177–830)	4
PGD_2	628	(58–6804)	4
PGE_2	1260	(540–2930)	4
PGI_2	>2000	–	2
Sulprostone	9400–15000[a]	–	2
DME_2	>1000	–	1

[a] Range of EPC values.

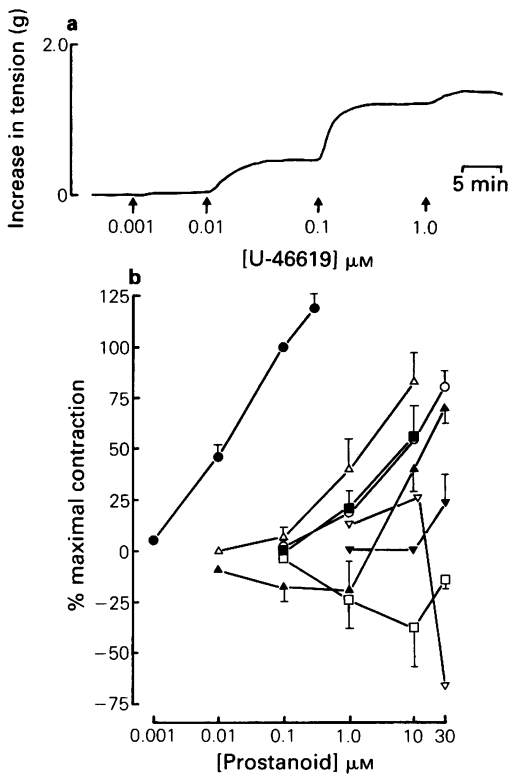

Figure 1 (a) Typical concentration-effect curve to U-46619 on human bronchus. (b) Mean concentration-effect curves to U-46619 (●), 11β-PGF_2 (△), PGD_2 (■), $PGF_{2\alpha}$ (○), PGE_2 (▲), PGI_2 (□), sulprostone (▼) and DME_2 (▽) on human bronchus. All responses were calculated as a percentage of the response obtained to U-46619 (0.1 μM) in the final control curve. Each point is the mean of 4 experiments except for U-46619 ($n = 21$), PGI_2 and sulprostone ($n = 2$) and DME_2 ($n = 1$). Vertical bars represent s.e.mean except for PGI_2 and sulprostone, where they represent range.

obtainable responses to $PGF_{2\alpha}$ and PGD_2 were consistently (7–25%) less than the corresponding maximum responses obtained to U-46619. In two of those experiments in which 11β-PGF_2 produced full concentration-effect curves, response maxima were identical to those to U-46619, whereas in the other experiment, the maximum was only 58% of the U-46619 maximum.

Antagonist studies

AH23848 (1 μM) was tested for its ability to inhibit contractile responses to U-46619, PGD_2, $PGF_{2\alpha}$, 11β-PGF_2 and carbachol. In some experiments (15/20), addition of AH23848 (1 μM) caused modest, transient contractile responses. The mean amplitude of these contractions was 53 (±13) mg as compared with 791 (±181) mg obtained with U-46619 (0.1 μM). In all cases, the AH23848-induced contraction had disappeared within 3–8 min, despite the continued presence of the drug. AH23848 caused parallel rightward shifts of curves to U-46619, with a mean concentration-ratio (CR) (95% confidence limits) of 313 (109–897, $n = 8$), consistent with a pA_2 value of 8.5 as determined by the Gaddum equation (Gaddum, 1957). In a single experiment, AH23848 at concentrations of 0.03, 0.3 and 3.0 μM caused parallel, rightward shifts of concentration-effect curves to U-46619 resulting in CRs of 11, 150 and 2600 respectively. From these data a pA_2 value of 8.3 (slope = 1.2) was calculated. In all experiments in the presence of AH23848, contractions to $PGF_{2\alpha}$ ($n = 2$), PGD_2 ($n = 3$) and 11β-PGF_2 ($n = 3$) were small or absent even at the highest concentration tested, such that accurate CR values could not be determined. In the presence of AH23848, $PGF_{2\alpha}$ at concentrations of 10–100 μM actually caused small relaxations. In contrast, curves to carbachol were unaffected by AH23848 (1 μM), giving a mean CR of 1.5 (0.4–6.3, $n = 4$). Mean concentration-effect curves to U-46619, $PGF_{2\alpha}$, 11β-PGF_2 and carbachol in the absence and presence of AH23848 (1 μM) are illustrated in Figure 2.

Discussion

In the present study, we have confirmed the findings of Seibert *et al.* (1987) who showed that 11β-PGF_2 contracts human isolated bronchial smooth muscle, and that its potency is similar to that observed with $PGF_{2\alpha}$ and PGD_2. We have also demonstrated that the rank order of prostanoid contractile agonist potency on human bronchial smooth muscle is similar to that previously reported in preparations containing TP-receptors, such as guinea-pig lung and rat aorta (Kennedy *et al.*, 1982), with U-46619 being at least two orders of magnitude more potent than PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2. In support of this, we have shown that AH23848 specifically inhibits the contractile agonist actions of U-46619, PGD_2, $PGF_{2\alpha}$ and 11β-PGF_2, suggesting not only that human bronchial smooth muscle contains TP-receptors, but that all of these prostanoids exert their contractile activities through this receptor. Furthermore, the similarity in the pA_2 value determined for AH23848 against U-46619 on human bronchus with those previously obtained on blood platelets and vascular smooth muscle from a range of species (Brittain *et al.*, 1985), suggests that the TP-receptors in all of these tissues are of the same type. The weak contractile actions of PGE_2, DME_2, sulprostone

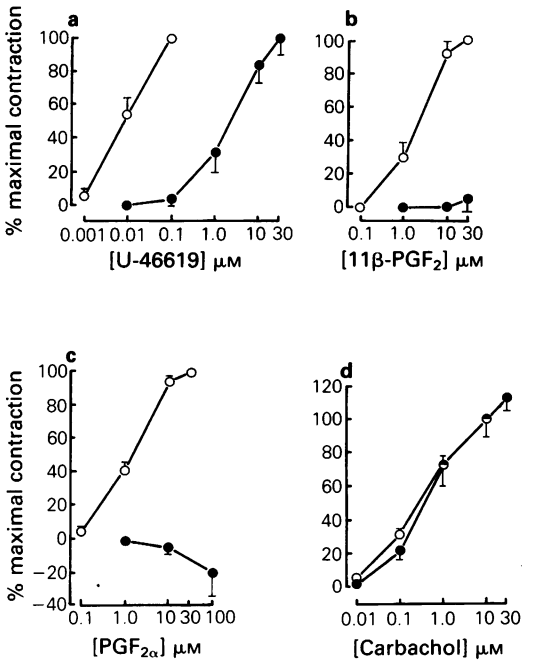

Figure 2 Mean concentration-effect curve to (a) U-46619, (b) 11β-PGF_2, (c) $PGF_{2\alpha}$, and (d) carbachol in the absence (○) and presence (●) of AH23848 (1 μM) on human bronchus. The ordinate scale shows % contraction, 100% being defined as the response in the absence of AH23848 to (a) U-46619 (0.1 μM), (b) 11β-PGF_2 (30 μM), (c) $PGF_{2\alpha}$ (30 μM) and (d) carbachol (10 μM). All responses are expressed as a percentage of this 100% response. Each point is the mean of 6–7 (a), 3 (b), 2 (c), 4 (d) experiments. Vertical bars represent s.e.mean (U-46619 and carbachol) or range (11β-PGF_2 and $PGF_{2\alpha}$).

and PGI_2 are presumably also mediated by TP-receptors, but to date AH23848 has not been tested against any of these prostanoids. Transient contractile agonist actions of AH23848, like those observed in the present study, have previously been reported in other TP-receptor containing tissues, such as human pulmonary vascular preparations and dog saphenous vein (Brittain *et al.*, 1985). It is believed that this activity results from a partial agonist action of AH23848 at TP-receptors (Humphrey *et al.*, 1986).

The results in this study differ from those obtained in a limited study by McKenniff and her colleagues (McKenniff *et al.*, 1988). They found DME_2 to be a potent contractile agonist which was not antagonized by either the TP-receptor blocking drugs, AH23848 and EP092, or the EP_1-receptor blocking drug, AH6809. These results are consistent with the presence of contractile EP-receptors which are insensitive to AH6809 (i.e. EP_3-receptors, Coleman *et al.*, 1987a). However, we have found that DME_2 is only a very weak contractile agonist on human bronchus. Furthermore, the potent EP_1- and EP_3-receptor selective agonist, sulprostone (Coleman *et al.*, 1987a), is also a very weak contractile agonist, being approximately 1000 times less potent than U-46619. We have therefore been unable to confirm McKenniff's observations with DME_2 and have found no evidence for the presence of either EP_1- or EP_3-receptors mediating the contractile effects of prostanoids in this tissue. Our results with the TP-receptor blocking drug AH23848 are also in disagreement with those obtained by McKenniff. In our experiments, AH23848 is a potent TP-receptor blocking drug, whereas McKenniff found AH23848 to be at least an order of magnitude weaker. A possible explanation for the differences in the results obtained with AH23848 and DME_2 in the two studies may be related to the part of the bronchial tree from which the tissue was taken. In our studies, we have used bronchus of lumen diameter 3–4 mm, whereas McKenniff used bronchus of much larger diameter, between 6–10 mm, presumably from higher up the bronchial tree. Whether receptor differences exist in the prostanoid receptor populations at different levels of the bronchial tree or whether there is some other reason for the differences in the results from the two studies remains to be determined.

In addition to TP-receptors mediating contraction, human bronchial smooth muscle also contains prostanoid receptors mediating relaxation since PGE_2, PGI_2 and DME_2 all caused relaxant responses. Indeed, in the presence of TP-receptor blockade with AH23848, even $PGF_{2\alpha}$ sometimes caused relaxation. An action of PGD_2 and $PGF_{2\alpha}$ at relaxant receptors in human bronchus, coupled with their relatively low potency at TP-receptors may explain the apparent inability of these prostanoids to elicit maximal contractile responses equal to those to U-46619. The identity of such relaxant receptors cannot be determined from the present study. However, other evidence is consistent with these receptors being similar to those mediating relaxation of tracheal smooth muscle from guinea-pig and cat (Gardiner, 1986), which we have previously classified as EP_2-receptors (Coleman *et al.*, 1987b).

In conclusion, the present results suggest that if, as we believe, prostanoids play a role in asthmatic bronchoconstriction, this effect will be mediated by TP-receptors. If so, such bronchoconstriction will be inhibited by TP-receptor blocking drugs like AH23848, irrespective of the particular type of prostanoid concerned. AH23848 and other such drugs should not only serve as tools to determine the extent to which prostanoids contribute to the pathogenesis of bronchial asthma, but may also represent valuable drugs in the treatment of this disease.

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Capsaicin and nicotine-sensitive afferent neurones and nasal secretion in healthy human volunteers and in patients with vasomotor rhinitis

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1 Applications of capsaicin, nicotine and methacholine were made locally onto the nasal mucosa in human controls and patients suffering from hyperreactive nasal disorders. Perception of sensation was registered as a sympton score and secretion quantified. The sensory reaction (irritation – pain) to capsaicin was similar in the three groups studied, i.e. controls, a group of patients with the diagnosis of vasomotor rhinitis and a group of patients with increased nasal secretion as the main symptom of the hyperreactive disorder. Nicotine induced only a mild itching sensation in the three groups. However, capsaicin and nicotine challenge caused a significantly larger secretory response in the last group than in the unselected vasomotor rhinitis group and in the control group.

2 Pretreatment with muscarinic receptor antagonists almost completely abolished the secretory response to both capsaicin and nicotine, and blocked methacholine-induced secretion. Furthermore, pretreatment with a combination of local anaesthetic and vasoconstrictor agent abolished the capsaicin-induced irritation, as well as the capsaicin- and nicotine-induced secretion on both the ipsilateral and the contralateral side. Therefore, no clearcut contribution seems to be exerted by locally released peptides from sensory neurones as direct trigger substances for the secretory response to capsaicin.

3 In conclusion, the nasal secretory response, in man, to both capsaicin and nicotine, seems to be mediated via cholinergic parasympathetic reflexes. In patients with hyperreactive non-allergic disorders of the nasal mucosa with rhinorrhea as the main complaint, the enhanced secretion may be due to a hyperreactive efferent cholinergic mechanism rather than hypersensitive irritant receptors on capsaicin- and nicotine-sensitive sensory neurones. Challenge with irritant agents seems a useful test for the evaluation of both afferent and efferent reflexogenic responses in hyperreactive disorders of the nasal mucosa.

Introduction

The pathophysiology of non-allergic hyperreactive disorders of the nose, i.e. vasomotor rhinitis, is unclear. Increasing evidence from experimental studies suggests that unmyelinated, capsaicin-sensitive sensory C-fibres play an important role in a variety of protective reflexes, such as vascular and secretory reactions, in the nasal mucosa upon inhalation of irritants (Lundblad *et al.*, 1985). The capsaicin-sensitive nerves in the nose have been shown to contain tachykinins such as substance P (SP) (Hökfelt *et al.*, 1977; Lundblad *et al.*, 1983; Uddman *et al.*, 1983) and calcitonin gene-related peptide (CGRP) (Uddman *et al.*, 1985). These SP- and CGRP-containing nerves are distributed in a

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dense network under and within the lining epithelium of the nasal mucosa as well as around blood vessels (Lundblad *et al.*, 1983, Uddman *et al.*, 1985.

Capsaicin, the pungent agent in hot pepper and paprica, which selectively activates chemosensitive C-fibre afferents, induces a local and central release of SP, CGRP and other neuropeptides from these neurones (Gamse *et al.*, 1979; Saria *et al.*, 1986; Hua *et al.*, 1986). In a preliminary study, we demonstrated that local application of capsaicin to the human nasal mucosa induces sneezing and secretion (Lundblad *et al.*, 1987a,b). The mechanism underlying this effect in man is unclear, even though SP is known to be a powerful secretagogue in some experimental animals (Pettersson *et al.*, 1985). Nicotine on the other hand, excites sensory nerves in the nasal mucosa which are probably different from those activated by capsaicin (Lundblad *et al.*, 1985).

The parasympathetic nervous system innervates the glands of the nasal mucosa (Cauna *et al.*, 1972). Muscarinic receptor stimulation by methacholine induces a profuse watery secretion upon local challenge (Borum 1979). The preganglionic parasympathetic fibres to the nasal mucosa run together with the sympathetic fibres in the Vidian nerve to the sphenopalatine ganglion. It has been shown that Vidian neurectomy has an inhibitory effect on the rhinorrhea observed in patients with nasal allergy and intramuscular injection of atropine blocks the hypersecretion (Konno & Togawa, 1979a).

Histamine and methacholine challenge have been suggested as useful tests for evaluation of nasal hyperreactivity (Borum *et al.*, 1983). Histamine stimulates sensory receptors and produces reflex secretion from the nasal glands but in addition has a direct effect on the nasal glands, inducing secretion and transudation (Konno & Togawa, 1979b).

Methacholine on the other hand, acts directly on the glandular cells. Therefore, methacholine challenge evaluates only the final step of the efferent part of the reflex arc. To characterize nasal hyperreactivity, a substance which selectively activates the reflex arc by stimulating afferent sensory receptors in the nasal mucosa would be preferable.

The aim of the present study was to quantify and characterize the subjective sensation and secretory effect induced by two sensory irritants, capsaicin and nicotine. The secretory effects of the substances were compared to the effect of methacholine. The agents were applied locally to the human nasal mucosa in three different groups tested: normal subjects, patients with unspecific vasomotor rhinitis and patients with increased nasal discharge as the main complaint. Furthermore, anticholinoceptor drugs and local anaesthetics were given as pretreatment before capsaicin, nicotine and methacholine application to investigate the nature of the reflex mechanism.

Methods

Normal subjects were selected from medical students (mean age: 32 years, male/female: 50/50%) at the Karolinska Hospital. They had no history of nasal disease or nasal allergy, smoking or ongoing drug treatment. The patients with vasomotor rhinitis came from the outpatient department at the ENT-clinic (mean age: 39 years, male/female: 37/63%). Vasomotor rhinitis was diagnosed by the following clinical symptoms: sneezing and rhinorrhea and/or nasal congestion. There was no history of atopy or allergic disease. We chose to study an unselected group of patients with the diagnosis of vasomotor rhinitis (the group is referred to in the text as VMR) and compare it to another group of patients with vasomotor rhinitis with sneezing and rhinorrhea as dominating symptoms (VMR-d).

To evaluate the subjective sensation caused by the application of capsaicin to the nasal mucosa a symptom score was used (scale graded from 0–10, where 1 represented slight irritation, 5 burning sensation and 10 severe pain). The subject was asked to mark a subjective score on the scale after each application. The number of sneezing discharges within 2 min of application of the substances was also registered.

The exocrine secretion from the nasal mucosa was absorbed by a rectangular piece of absorbant paper, 5 × 50 mm (Knowles *et al.*, 1981; Pettersson *et al.*, 1985) preweighed in a test tube. The piece of paper was placed against the septal wall in the nasal cavity to be tested. In addition, in some experiments a piece of paper was also inserted into the contralateral nostril. After 2 min the paper was removed and again weighed. Drugs were applied in a volume of 50 μl to the anterior part of the inferior turbinate with a micropipette. The following substances, in stock solution, were used for application: capsaicin (initially dissolved in 70% ethanol and further diluted in saline; Sigma), nicotine bitartrate (diluted in saline) and methacholine bromide (diluted in saline). Atropine, 0.5 mg (ACO), was given i.m. in one group and in four of the VMR-d patients combined with ipratropium bromide 0.25 mg ml^{-1}, 200 μl (Atrovent, Boehringer-Ingelheim). The ipratropium was sprayed into the nasal cavity 30 min before the application of capsaicin.

Cotton strips were soaked in lignocaine chloride 4% (Xylocaine, Astra); naphazoline chloride

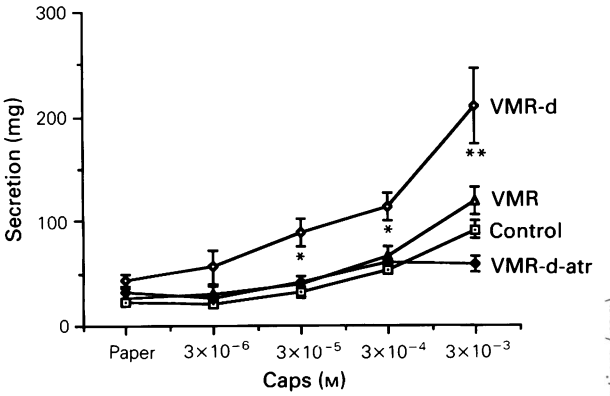

Figure 1 Secretory response upon the introduction of filter paper into the nasal cavity and subsequently upon local applications of capsaicin to the inferior turbinate of the human nasal mucosa in dilutions of 3.3×10^{-6} M – 3.3×10^{-3} M in a volume of 50 μl. Control group (□, $n = 17$), VMR-group (Δ, $n = 10$), VMR-d-group (◇, $n = 8$) and VMR-d pretreated with i.m. atropine and local ipratropium bromide (VMR-d-atr) (◆, $n = 4$). Responses are means with s.e.mean shown by vertical bars. $*P < 0.05$, $**P < 0.01$ compared to control. Student's t test.

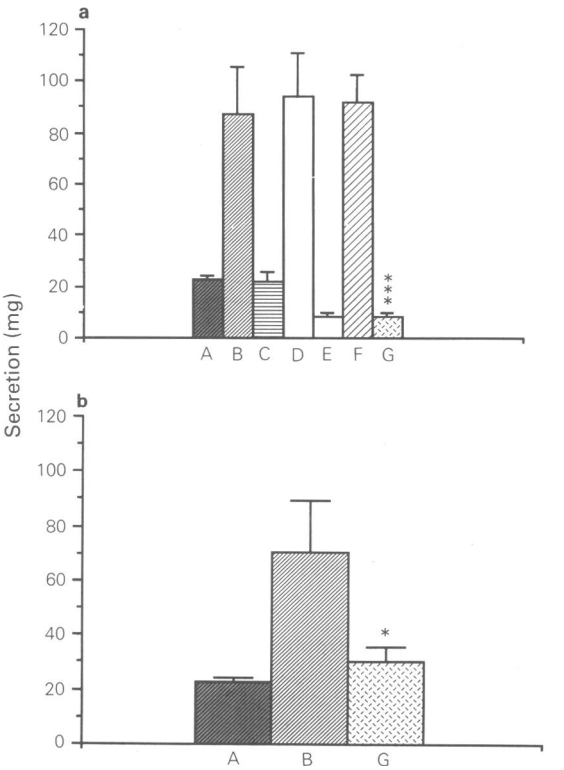

Figure 2 Secretory response upon local application of capsaicin, 3.3×10^{-3} M, in a volume of 50 μl to the inferior turbinate on the ipsilateral side (a) and on the contralateral side (b), before and after pretreatment on the stimulated side with lignocaine chloride 4% naphazoline chloride 0.02 mg ml^{-1} or lignocaine chloride 3.4% mixed with naphazoline chloride 0.02 mg ml^{-1}. (A) Control; (B) capsaicin 3.3×10^{-3} M; (C) lignocaine; (D) capsaicin and lignocaine; (E) naphazoline; (F) capsaicin and naphazoline; (G) capsaicin, naphazoline and lignocaine. The responses are means with s.e.mean shown by vertical bars. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Quade analysis. $n = 5$.

0.02 mg ml^{-1} (Naphazoline, Leo); naphazoline chloride 0.02 mg ml^{-1} mixed with lignocaine chloride 3.4% or ipratropium bromide 0.25 mg ml^{-1}. The strips were packed into the nasal cavity and left there for 15 min. Capsaicin 3.3×10^{-3} M, nicotine 6.5×10^{-3} M or methacholine 5×10^{-4} M, in a volume of 50 μl, was then applied locally to the inferior turbinate in the pretreated nostril.

The study was approved by the ethical committee of the Medical Faculty at the Karolinska Hospital. Informed consent was obtained from all the subjects included in the study.

Statistical and numerical analysis: the mean and standard error of the mean (s.e.mean) are used to describe central tendency and variation throughout the study. Student's t test and Quade analysis (Theodorsson-Norheim, 1987) were used to test the statistical significance. P values of <0.05 were taken as significant.

Results

Upon local nasal application of capsaicin all subjects responded with a concentration-dependent increase in nasal sensations, as revealed by the symptom score, and nasal secretion. Sneezing discharge was discarded as a parameter since it was quite clear that subjects could suppress the reaction. In a low dose the subjects mainly perceived irritation, while upon higher concentrations increasingly severe pain was reported. The subjective sensation plotted on a symptom score scale (not shown) showed no statistical differences between the groups. The secretory effect of capsaicin, on the other hand, was significantly greater in the VMR-d group than in the other groups in all but the lowest concentration (see Figure 1). There was also a tendency, although not statistically significant, for the VMR group to secrete more than the controls.

Atropine i.m. had no effect on nasal secretion induced by capsaicin application ($n = 10$, not

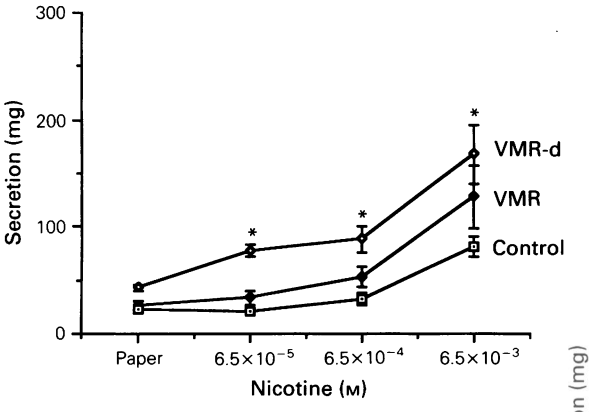

Figure 3 Secretory response upon local applicaion of nicotine to the inferior turbinate in dilutions of 6.5×10^{-5} M – 6.5×10^{-3} M in a volume of 50 μl. Control group ($n = 10$), VMR-group ($n = 10$) and VMR-d-group ($n = 4$). The responses are means with s.e.mean shown by vertical bars. $^{*}P < 0.05$; $^{**} < 0.01$. Student's t test, compared to controls.

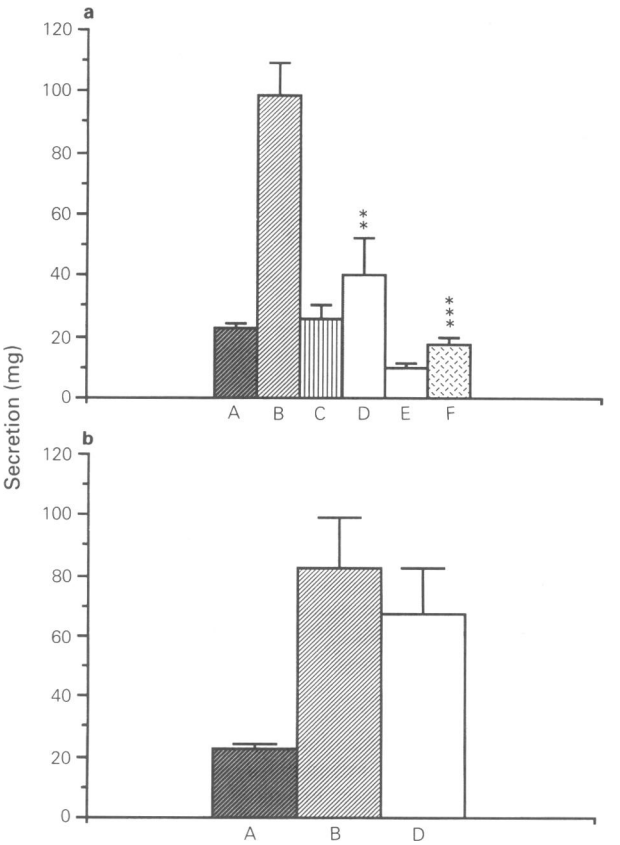

Figure 4 Secretory response upon local application of nicotine 6.5×10^{-3} M in a volume of 50 μl to the inferior turbinate on the ipsilateral side (a) and on the contralateral side (b), before and after pretreatment on the stimulated side with ipratropium bromide 0.25 mg ml^{-1} or lignocaine chloride 3.4% mixed with naphazoline chloride 0.02 mg ml.$^{-1}$ The responses are means with s.e.mean shown by vertical bars. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Quade analysis. $n = 5$. (A) Control; (B) nicotine 6.5×10^{-3} M; (C) ipratropium; (D) nicotine and ipratopium; (E) naphazoline; (F) nicotine, naphazoline and lignocaine.

shown). However, atropine i.m. in combination with ipratropium, sprayed locally into the nasal cavity, caused a marked reduction of the secretory response in the VMR-d group (Figure 1).

When capsaicin (3.3×10^{-3} M) was applied to the inferior turbinate, a matched secretion was also evoked from the contralateral side which was not significantly lower than that on the stimulated side (see Figures 2a and b). Pretreatment with 4% lidocaine did not have any effect on basal secretion (see Figure 2a). Capsaicin (3.3×10^{-3} M), when applied in the lidocaine pretreated nostril, induced a secretory effect which was not significantly lower than the control response (see Figure 2a). Furthermore, the individuals reported a symptom score of the sensation which was similar to the control situation (not shown). Naphazoline, a vasoconstrictor agent, caused a significant reduction *per se* (about 60%) in the basal secretion level (see Figure 2a). However, when capsaicin (3.3×10^{-3} M) was applied to the inferior turbinate of the naphazoline pretreated side, the secretory effect was not inhibited (see Figure 2a). To improve the action of local anaesthetics, lignocaine was combined with naphazoline as pretreatment. Upon subsequent capsaicin (3.3×10^{-3} M) application, the subjects perceived no sensation of irritation and, furthermore, the secretory effect was abolished (the ipsilateral secretion level measured was lower than the basal level obtained when inserting the filter paper in the unstimulated nostril) (see Figures 2a and b).

Nasal application of nicotine produced a concentration-dependent secretion in all the groups studied (see Figure 3). There was a significantly greater secretory response in the VMR-d group compared to the normal subjects (controls). Nicotine induced only a mild itching sensation, which did not seem to be dose-dependent (not shown). Nicotine (6.5×10^{-3} M) caused a secretory response on the contralateral side that was similar to that on the stimulated side (see Figures 4a and b). Unilateral pretreatment with ipratropium caused a significant reduction of the secretion on the nicotine stimulated

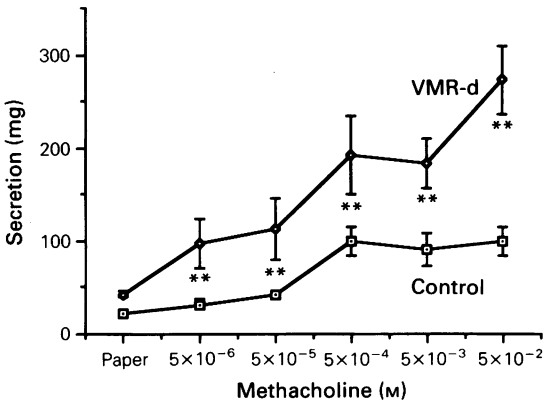

Figure 5 Secretory response upon local application of methacholine to the inferior turbinate in dilutions of 5×10^{-6} M – 5×10^{-2} M in a volume of 50 µl. Control group ($n = 10$), VMR-d-group ($n = 8$). The responses are expressed as means with s.e.mean shown by vertical bars. $*P < 0.05$, $**P < 0.01$, $***P$0.001. Student's t text.

side, but not on the contralateral side (see Figures 4a and b). After pretreatment with a combination of lignocaine and naphazoline the secretory effect of nicotine was abolished (see Figure 4a).

Nasal application of methacholine induced a concentration-dependent secretion (see Figure 5). The methacholine response was significantly higher

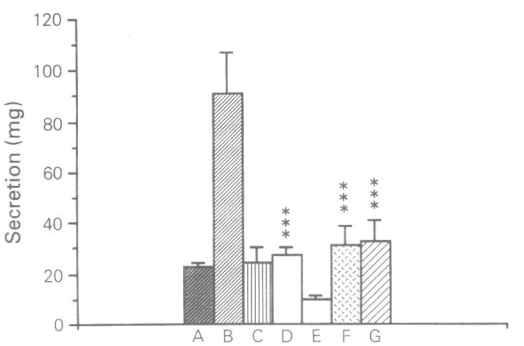

Figure 6 Secretory response upon local application of methacholine, 5×10^{-4} M, to the inferior turbinate before and after pretreatment with ipratropium bromide 0.25 mg ml^{-1}, naphazoline chloride 0.02 mg/ml^{-1} or lignocaine chloride 3.4% mixed with naphazoline chloride 0.02 mg ml^{-1}. (A) Control, (B) methacholine; (C) ipratropium; (D) methacholine and ipratropium; (E) naphazoline; (F) methacholine, naphazoline and lignocaine; (G) methacholine and naphazoline. The responses are means with s.e.mean shown by vertical bars. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Quade analysis. $n = 5$.

in the VMR-d group than in the controls (see Figure 5). Methacholine did not produce any subjective sensation. After pretreatment with ipratropium, the secretory response to methacholine 5×10^{-5} M was abolished (see Figure 6). Pretreatment with lignocaine combined with naphazoline caused a significant reduction of the secretory response to methacholine 5×10^{-4} M (see Figure 6). However, pretreatment with naphazoline alone also caused a marked reduction in the secretory response to methacholine (Figure 6).

Discussion

In the present study, capsaicin locally applied to the human nasal mucosa elicited a concentration-dependent sensation of irritation and pain as well as a secretory response. The capsaicin-induced secretion was considerably larger in the vasomotor rhinitis patients with enhanced secretion as the main complaint. When using methacholine challenge to reveal secretory hyperreactivity (Borum, 1979), there has been some question whether nasal absorption of the applied substance was more prominent in the hyperreactive group so explaining the observed increased response. The present study indicates that this is not the case with capsaicin. Thus, there was no significant difference in the subjective symptom scores in the groups upon capsaicin application.

Various methods have been described for measuring nasal secretion to a challenge. Konno & Togawa (1979a) used suction, Secher *et al.* (1982) preferred weighing handkerchiefs after nose blowing and Johansson & Deuschl (1976), Linder *et al.* (1983) and Naclerio *et al.* (1983) all describe different techniques for nasal lavage with or without a tracer substance. The technique used in our study for the collection of nasal secretion has been described earlier by Knowles and co-workers (1981) and by Pettersson *et al.* (1985). This technique obviously has its limitations, since the method will only permit the collection of secretion from a selected area of the nasal mucosa. However, an equal proportion was likely to have been obtained in each experiment since in all subjects the filter paper and substances were applied to the same anatomical site. However, it seems that the method underestimates a high secretory response.

The mechanism underlying the secretory effect of capsaicin and nicotine may be complex. It has been shown that SP- and CGRP-IR nerves are present in the human nasal mucosa around some exocrine elements, mainly ducts (Stjärne *et al.*, unpublished observations). SP is known to be a potent secretagogue, at least in the nasal mucosa of the rat and

dog (Rackham *et al.*, 1981; Pettersson *et al.*, 1985). Hypothetically, the SP-induced secretion may be secondary to mast cell degranulation (Hägermark *et al.*, 1978) and subsequent histamine release (Konno & Togawa, 1979b). From experimental studies (Lundblad & Lundberg, 1984), it can be deduced that the mechanism underlying the secretory response to capsaicin may be: (1) a local axon reflex with the subsequent release of mediators from sensory nerves acting on the exocrine elements in the nasal mucosa, (2) a parasympathetic reflex relayed via axon collaterals of sensory nerves in the sphenopalatine ganglion, or (3) a centrally-mediated parasympathetic reflex (see Figure 7).

The secretory effect of capsaicin could be totally blocked, both on the side of application and on the contralateral side, using a combined pretreatment with local anaesthetic and a vasoconstrictor agent. This indicates that the major portion of the secretory effect of capsaicin is reflex mediated. The failure of lignocaine alone to block the secretion is probably due to the fact that the nasal mucosa is highly vascularized, which prevents the relatively more hydrophilic lignocaine molecule penetrating as deeply into the tissue as capsaicin, which is known to be very lipophilic. This is supported by the fact that subjects upon capsaicin application after lignocaine pretreatment indicated a symptom sensation score that was not significantly lower than in the control situation. In subjects pretreated with lignocaine/naphazoline, where the secretion was almost abolished, no sensation was perceived upon the capsaicin application. To verify that the blocking effect of lignocaine/naphazoline on the secretory response, was due to a nerve blockade and not a secondary effect due to the vasoconstriction induced by naphazoline, pretreatment with naphazoline alone was performed. The subsequent capsaicin application induced a nasal secretion which was not significantly lower than in the control situation with stimulation by capsaicin in the untreated nostril. This suggests that the blood flow decrease induced by naphazoline, which reduced the secretory response seen on methacholine application, was counteracted upon capsaicin application by an associated vasodilatation induced by local release of neuropeptides, most likely of both sensory (SP, CGRP) and parasympathetic (vasoactive intestinal polypeptide, VIP) origin (Lundblad *et al.*, 1983).

The reflex seems to be centrally-mediated since the ipsilateral application of capsaicin induces a contralateral secretory response. The finding that locally applied ipratropium together with systemic pretreatment with atropine almost abolished the secretory response to capsaicin strongly suggests a parasympathetic cholinergic involvement in the reflex arc, acting on muscarinic receptors as the final step

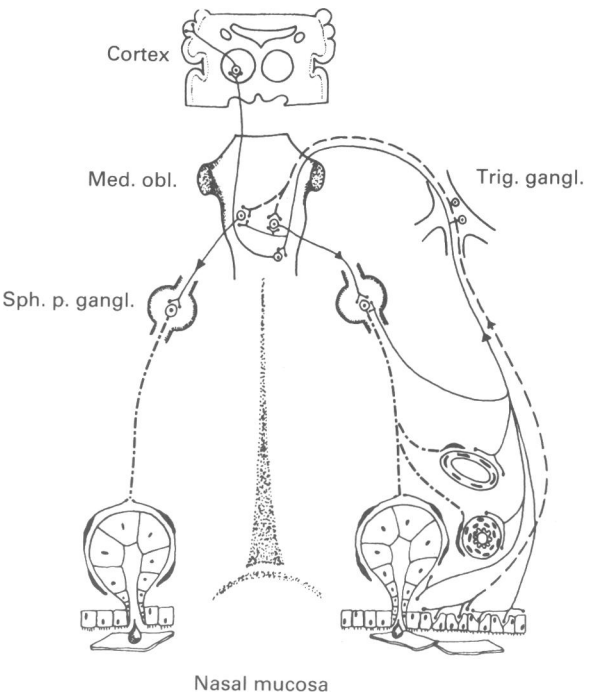

Figure 7 Schematic drawing of the vascular and secretory innervation of the human nasal mucosa. At least two different populations of chemo-sensitive sensory neurones seem to be present. One is the capsaicin-sensitive C-fibre afferents (———), containing substance P and calcitonin gene-related peptide, with axonal branches to the arterioles and venous sinosoids, to the ductal portion of the exocrine glands and to the epithelium which they penetrate. The other population is the nicotine-sensitive afferents (- - - - -). Both types of sensory nerves terminate in the medulla oblongata, (Med. obl.,) where they are relayed to the cortex and to the preganglionic parasympathetic neurones that project to the sphenopalatine ganglion cells (Sph. p. gangl) in the pterygo-palatine fossa; here they synapse with postganglionic cholinergic neurones (–.–.–), also containing vasoactive intestinal peptide, in the final step of the reflex arc to exocrine secretion.

(Davies *et al.*, 1982). Similar results have been obtained with capsaicin-induced salivation in man (Duner-Engström *et al.*, 1986). However, a slight nasal secretory response was still present at high doses in the present study, indicating either incomplete blockade of muscarinic receptors or an effect on the exocrine glands mediated by locally released neuropeptides.

Nicotine is likely to activate a population of sensory nerves in the nasal mucosa other than the

capsaicin-sensitive C-fibre afferents, at least as regards sneezing discharges in the guinea-pig (Lundblad *et al.*, 1985). A similar situation seems to be present in man, since completely different sensations were perceived upon nicotine and capsaicin applications. Nicotine, however, also induced nasal secretion, which indicates that at least two populations of sensory nerves in the human nasal mucosa are linked to parasympathetic reflexes. This was also verified by the finding that pretreatment with lignocaine/naphazoline blocked the nasal secretory response upon nicotine application and that local pretreatment with ipratropium significantly reduced the nasal secretion upon nicotine application on the stimulated side, whereas it had no effect on the secretory response on the unpretreated contralateral side. Concerning the irritant effect of cigarette smoke on the nasal mucosa, however, vapour phase components other than nicotine seem to be the main irritants, at least in the guinea-pig (Lundblad & Lundberg, 1984).

According to Mygind (1978), the vasomotor rhinitis patients may be divided into two 'main symptom' groups suffering from nasal congestion ('blockers') or hyperrhinorrhea ('runners'). It is possible that these two groups represent conditions with different pathophysiological mechanisms. Assuming that the same population of sensory nerve fibres is involved in sensation and in the reflex arc evoked secretion, our finding that there was no significant differences between the groups regarding the symptom score for irritation and pain upon capsaicin application, together with the fact that the VMR-d group produced a significantly higher secretory response than the controls on capsaicin, nicotine and methacholine application, would indicate that the hyperreactivity is most likely not directly related to the sensory nerve endings. Thus, the hypersecretory response may be related to a change at the cholinoceptor site on the glandular cell and/or glandular hypertrophy since the capsaicin-induced secretion, but not sensation, was almost abolished by ipratropium pretreatment. In patients with nasal allergy (Ishibe *et al.*, 1983), it has been demonstrated that there is an increase in the number of muscarinic receptors and a decrease in the number of α_1- and β-adrenoceptors. Whether this occurs in non-allergic disorders of the nasal mucosa is not known.

It is known from earlier studies that tachykinins induce a slow e.p.s.p. from ganglion cells and this could result in a facilitation of ganglionic transmission (Otsuka & Konishi, 1983). However, the symptom score for the subjective sensations in the VMR-d group was not significantly higher than in the other groups, which indicates that sensory transmission to the CNS was not altered in the diseased group. On the other hand, a constant exposure to irritants might, via increased sensory activity and parasympathetic tone, lead to a hypertrophy of the glandular tissue. However, it is important to point out that activation of receptors on the sensory neurones in the nasal mucosa is of primary importance in the protective responses induced by irritants in the environment. In this respect, the parasympathetic secretory reflex is one in an arsenal of protective responses that can be activated via the sensory irritant receptors (Lundblad & Lundberg, 1984). However, it remains to be established whether capsaicin desensitization and damage of the afferent part of the reflex arc can reduce the nasal secretory hyperreactivity to environmental stimuli in man, considering that the nicotine- sensitive sensory nerves are likely to be left intact.

During the completion of our study, Gepetti and co-workers (1988) have published results which in some aspects are contradictory to our findings. These authors proposed that capsaicin induces secretion in the human nasal mucosa primarily via local release of transmitter(s) from peripheral terminals of primary sensory neurones, but reported that local application of SP and CGRP did not reveal any secretory response. Our data with local anaesthetics and muscarinic receptor antagonists suggest that local release of tachykinins and CGRP may only play a minor direct role in the secretory response to local capsaicin application upon the human nasal mucosa *in vivo*. Furthermore, the finding of Gepetti *et al.* (1988) that ipratropium had no effect on capsaicin-induced secretion is also in apparent contradiction to the present data, but may be due to an incomplete blockade of muscarinic receptors.

In conclusion, our findings suggest that the secretory effect of capsaicin and nicotine in the human nasal mucosa is mediated via a central parasympathetic reflex arc with a final muscarinic receptor mechanism. Capsaicin and nicotine seem to activate different populations of sensory neurones. According to our data, no clearcut contribution is exerted by locally released tachykinins and CGRP as direct trigger substances for the secretory response to capsaicin. We propose that challenge with irritant agents is the appropriate test when evaluating both the afferent and the efferent pathways of reflexes in hyperreactive disorders of the nasal mucosa. The cause of enhanced secretion in patients with hyperreactive non-allergic disorders in the nasal mucosa is less likely to depend on hypersensitive irritant receptors on capsaicin-sensitive sensory neurones, providing that the same population of afferents mediate both the irritation sensation and the reflex cholinergic secretory response.

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Estimation of affinities and efficacies for κ-receptor agonists in guinea-pig ileum

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1 Ethylketocyclazocine (EKC) and U50,488H have been employed widely as κ-receptor agonists in the study of opioid receptor systems. However, the quantification of their agonism in terms of affinities and relative efficacies has not been investigated.

2 In this study, operational model-fitting was used to analyse the effects of irreversible receptor alkylation by β-chlornaltrexamine (β-CNA) on the κ-receptor mediated effects of EKC and U50,488H in the isolated, coaxially stimulated ileum of the guinea-pig.

3 EKC produced monophasic inhibitory concentration-effect curves which were readily amenable to analysis. In contrast U50,488H produced biphasic curves characterized by a higher potency phase of agonism that was susceptible to antagonism by 16-methylcyprenorphine (RX8008M) and a lower potency phase that was apparently non-opioid in nature.

4 Analysis of the κ-receptor-mediated effects of both agonists indicated that EKC has fifteen fold higher affinity than U50,488H and that the two agonists possess similar intrinsic efficacies.

Introduction

Opioid receptor classification studies in isolated tissues have for the most part relied on competitive antagonist affinity estimates. The estimation of opioid agonist affinities and relative efficacies has received little attention despite the contribution such information makes to receptor classification. A few investigators have reported functional estimates of μ-agonist affinities (Porreca & Burks, 1983; Williams & North, 1984; Dougall & Leff, 1987) but no attempts similarly to estimate κ-agonist affinities are evident in the literature to these authors' knowledge.

With the objective of providing more quantitative information than currently available on two established κ-receptor agonists we have analysed the action of ethylketocyclazocine (EKC) and U50,488H in the guinea-pig ileum using the irreversible receptor antagonist β-chlornaltrexamine (β-CNA).

The results of this study are discussed with regard to their implications, for the classification of opioid receptors. A preliminary account of this work was presented to the British Pharmacological Society (Leff & Dougall, 1988b).

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Methods

Guinea-pig isolated coaxially-stimulated ileum

Male albino Dunkin-Hartley guinea-pigs (250–400 g) were killed by cervical dislocation. The terminal ileum was removed and the 10 cm section closest to the ileocaecal junction discarded. Approximately 2 cm portions of ileum were cleared of adherent tissue and the contents gently flushed out before being transferred to 20 ml organ baths containing modified Krebs solution of the following composition (mM): NaCl 118.41, $NaHCO_3$ 25.00, KCl 4.75, KH_2PO_4 1.19, $MgSO_4$ 1.19, glucose 11.10, $CaCl_2$ 2.50. This was maintained at 37°C and continually gassed with 95% O_2 and 5% CO_2. The upper end of the tissue was attached by cotton thread to a Grass FTO3C force-displacement transducer, the bottom end being tied to the tissue holder. Contractions of the tissue were elicited by coaxial supramaximal

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stimulation (0.5 ms duration, 0.1 Hz, 20 V). Changes in isometric force were recorded on Gould BS-272 pen recorders.

Experimental protocols

General At the beginning of each experiment, a force of 1.0 g was applied to each tissue. This was followed by a 30 min stabilization period before electrical simulation was started. At the end of the stabilization period the tension was reinstated. Reproducible responses to supramaximal electrical stimulation were established after about 45 min.

Agonist concentration-effect, E/[A], curves were constructed by cumulative additions of either EKC or U50,488H at 0.5 $\log_{10}$ unit increments. Only a single curve was generated in each tissue. Responses were recorded as percentage inhibitions of the stimulated twitch.

Competitive antagonist studies Tissues from the same animal were incubated with 1 of 4 concentrations (10^{-7} M, 3×10^{-7} M, 10^{-6} M or 3×10^{-6} M) of 16-methylcyprenorphine (RX8008M) (Smith, 1987) or vehicle for 40 min before construction of U50,488H E/[A] curves. This procedure was repeated until 5 replicates were obtained.

Irreversible receptor inactivation Tissues from the same animal were either treated as controls or were incubated with β-CNA (10 nM or 30 nM) for 30 min after which excess alkylating agent was removed by 20 changes of the organ bath Krebs solution over a period of 60 min. EKC or U50,488H curves were then constructed as outlined above. This procedure was repeated until at least 4 replicates were obtained.

Analysis of data

E/[A] curve data obtained in competitive antagonist studies was fitted to a logistic function of the form:

$$E = \frac{\alpha[A]^m}{[A_{50}]^m + [A]^m} \quad (1)$$

in which E and [A] are the pharmacological effect and the concentration of the agonist, respectively; α, $[A_{50}]$ and m are the asymptote, location and slope parameters, respectively. Location parameters were actually estimated as logarithms. This fitting procedure also performed one-way analyses of variance comparing computed slope and asymptote parameters between and within treatment groups. If the treatments did not significantly ($P < 0.05$) modify these parameter estimates, then computed $\log_{10}$ $[A_{50}]$ values were fitted to the following linear form of the Schild equation (Trist & Leff, 1985):

$$\log_{10}[A_{50}] = \log_{10}[A_{50}{}^c] + \log_{10}(1 + [B]^n/K_B) \quad (2)$$

where $[A_{50}{}^c]$ is the estimated control $[A_{50}]$, [B] is the concentration of antagonist, K_B is its equilibrium dissociation constant and n is equivalent to the Schild plot slope parameter (unity for simple competition). If n was not significantly different from unity, it was constrained to this value in order to estimate pK_B ($-\log_{10} K_B$).

Operational model fitting

E/[A] curve data obtained in experiments with β-CNA treatment were fitted to the operational model of agonism (Black & Leff, 1983; Black *et al.*, 1985) in order to estimate agonist dissociation constants and efficacies:

$$E = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n}$$

in which τ defines the efficacy of an agonist in a system; E_m is the maximum possible effect; K_A is the agonist dissociation constant and n is the slope parameter for the transducer relation linking occupancy to effect.

Drugs

Ethylketocyclazocine methane sulphonate (Sterling-Winthrop); U50,488H (*trans*-(±)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide methane sulphonate (Upjohn); β-CNA dihydrochloride and 16-methylcyprenorphine hydrochloride (both prepared by Dr S. Wilkinson, Medicinal Chemistry Department, Wellcome Research Laboratories).

16-Methylcyprenorphine was dissolved in distilled water with the addition of a few drops of 1 M HCl. All other drugs were dissolved and diluted in distilled water.

Results

Interaction between U50,488H and RX8008M

Average concentration-effect data for the interaction between U50,488H and RX8008M are shown in Figure 1a. Both in the absence and presence of the antagonist U50,488H E/[A] curves were clearly biphasic. RX8008M produced displacements of the higher potency phase of U50,488H's agonism which according to analyses of variance did not signifi-

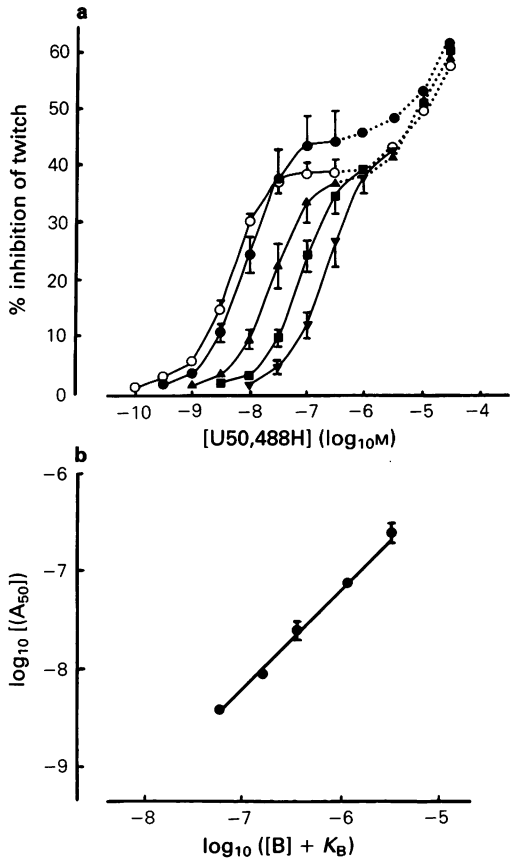

Figure 1 (a) Antagonist effects of RX8008M on U50,488H E/[A] curves. The data are the averages of 5 replicate E/[A] curves with vertical lines indicating s.e. The concentrations of the antagonist were zero (0); 10^{-7} M (●); 3×10^{-7} M (▲); 10^{-6} M (■); 3×10^{-6} M (▼). The dotted lines illustrate the presence of a RX8008M-resistant phase of agonism. (b) The $[A_{50}]$ data in Clark Plot form. The adherence of the data with the unit slope line drawn through them indicates consistency with simple competition. The pK_B value, estimated by fitting equation (2) to the $[A_{50}]$ values was 7.26 ± 0.06 (s.e., 23 d.f.).

cantly deviate from parallelism (Figure 1a). Analysis of the computed $[A_{50}]$ values by Equation (2) indicated that the interaction between RX8008M and U50,488H conformed to simple competition. Figure 1b shows the $[A_{50}]$ data in Clark Plot form. The Schild slope parameter (n) was 1.11 ± 0.06 (s.e.mean, 22 d.f.) and the resulting pK_B estimate with n constrained to unity was 7.26 ± 0.06 (s.e.mean, 23 d.f.). This is consistent with U50,488H activating κ-receptors in this concentration-range since we have previously demonstrated that RX8008M competitively antagonizes EKC in this tissue yielding a pK_B of 7.06 (Leff & Dougall, 1988a). Figure 1a also illustrates the resistance of the lower potency phase (dotted lines) of U50,488H's agonism to antagonism by RX8008M. This agonism was also resistant to naloxone antagonism at a concentration of 300 nM.

Irreversible antagonism of ethylketocyclazocine effects by β-chlornaltrexamine

The average data shown in Figure 2a illustrate the effects of β-CNA (10 nM or 30 nM each for 30 min) on EKC E/[A] curves. The lines drawn through the data are the results of operational model-fitting. The parameter values corresponding to these lines were as follows: $E_m = 63.32\%$; $n = 1.05$; $pK_A = 8.33$; τ_1 (control) = 9.76; τ_2 (10 nM β-CNA) = 0.97; τ_3 (30 nM β-CNA) = 0.45.

Irreversible antagonism of U50,488H effects by β-chlornaltrexamine

The average data shown in Figure 2b illustrate the effects of β-CNA (10 nM for 30 min) on U50,488H E/[A] curves. The solid lines drawn through the data are the results of operational model-fitting. The parameter values corresponding to these lines were as follows: $E_m = 43.07\%$; $n = 1.20$; $pK_A = 7.16$; τ_1 (control) = 12.10; τ_2 (10 nM β-CNA) = 1.15. The dotted lines merely indicate the presence of the resistant phase of agonism (see above) and the data points connected by these lines were not used in the model-fitting analysis.

It is evident from Figure 2 that the E_m values for the two sets of data were different. In fact, the two sets of experiments corresponding to the two panels were conducted at different times. However when EKC and U50,488H were applied to portions of ileum from the same guinea-pig, maximum responses observed were not significantly different (data not shown, $n = 3$) implying that the observed differences in E_m were the result of animal variation.

Discussion

The quantitative classification of opioid receptors has for the most part relied upon estimates of affinity for competitive antagonists. Few attempts have been made to complement these with estimates of affinity and efficacy for agonists. When using agonists, most investigators have been content to quote potency orders despite the fact that these offer unreliable information for receptor classification. Agonist potency depends on both affinity and efficacy (Stephenson, 1956; Black & Leff, 1983) and it is possible for agonists to demonstrate the same potency

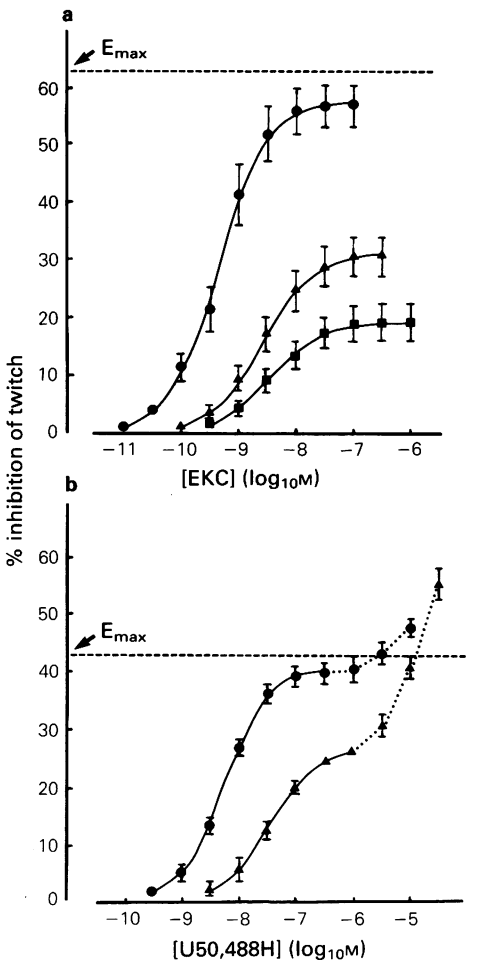

Figure 2 Effect of β-chlornaltrexamine (β-CNA) treatment on ethylketocyclazocine (EKC) and U50,488 E/[A] curves: (a) shows EKC E/[A] curves obtained in the absence of (●) and following 30 min exposure to 10 nM (▲) or 30 nM (■) β-CNA. (b) Shows U50,488H E/[A] curves in the absence of (●) and following 30 min exposure to 10 nM β-CNA (▲). Each point is the mean of 4–7 replicates. Vertical lines show s.e. The solid lines drawn through the data are the results of operational model-fitting. The dotted lines in (b) illustrate the presence of a non-opioid phase of agonism.

orders in two tissues containing different receptors. Also, changes in agonist potency order can occur between different tissues containing the same receptors (Kenakin, 1984). Therefore, in order for results with agonists to contribute reliably to receptor classification, attempts should be made to estimate their affinities and efficacies. The absence of such information on κ-agonists instigated the present study.

Here, we have attempted to quantify the action of two commonly used κ-agonists, EKC and U50,488H. Initially, the agonist effects of the two compounds were confirmed as being κ-receptor mediated using the competitive antagonist RX8008M. This antagonist is reported to have similar affinities for μ- and δ-receptors and less, though still substantial affinity for κ-receptors (Smith, 1987); in this study, it demonstrated competitive dynamics against U50,488H. We have previously reported similar behaviour when EKC is employed as the agonist (Leff & Dougall, 1988a) and in both cases the pK_B values were consistent with the antagonist's reported affinity for κ-receptors (Smith, 1987). Attempts were made to support these results by use of a putative κ-receptor selective antagonist nor-binaltorphimine (Portoghese *et al.*, 1987; Birch *et al.*, 1987). However, although it blocked the responses to both agonists in the concentration-range expected on the basis of its reported κ-receptor affinity, it did not demonstrate simple competition and pK_B estimates could not be made (data not shown).

Assuming that EKC and U50,488H were κ-receptor agonists in this tissue they were subjected to analysis using the operational model. According to this analysis these agonists possess similar efficacies (10 and 12 respectively in this tissue), but different affinities, EKC having some fifteen fold higher affinity than U50,488H. It is important to emphasise that the difference in the curve maxima between the two agonists was due to E_m differences, reflecting a tissue difference rather than intrinsic efficacy differences between the agonists.

Unlike EKC, U50,488H clearly demonstrated a biphasic E/[A] curve in the presence and absence of β-CNA treatment. It is known that the guinea-pig ileum possesses both μ- and κ-receptors and, in principle, the lower affinity phase of agonism could have been due to activation of μ-receptors.

Sheehan *et al.* (1986) have suggested that U50,488H is able to activate μ-receptors in this tissue. Those authors used β-CNA in the presence of the selective μ-receptor agonist [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (DAGOL) to produce a preponderantly μ-receptor-enriched tissue. Under those conditions U50,488H produced agonist effects in a concentration range 150 fold higher than under control conditions. These effects were attributed to μ-receptor activation.

However, the present study indicates that the lower potency phase of U50,488H's agonism was not mediated by opioid receptors, due to its resistance to both RX8008M and naloxone. Although the study of Sheehan *et al.* (1986) did not reveal a clearly-defined biphasic curve for U50,488H it is notable that the resistant phase described here occupies the same

concentration-range as the apparently μ-receptor phase of agonism described in their study. As those authors did not apply naloxone or any other antagonist in their study it would seem reasonable to adopt the present explanation. However, although the lower potency phase of U50,488H's agonism was not μ-receptor mediated, the present study did not rule out μ-receptor properties of EKC and U50,488H. Until any such properties associated with these compounds are adequately quantified their classification will remain incomplete and interpretation of *in vivo* data limited.

In conclusion, the present analysis represents the first attempt to quantify the action of κ-opioid receptor agonists in terms of their affinities and efficacies. Similar attempts to provide such information would do much to further the classification of opioid receptors.

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Augmentation by external Mg ions of β-adrenoceptor-mediated actions in the longitudinal muscle of rat uterus

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1 The longitudinal muscle isolated from the uterus of oestrogen-treated rats was not spontaneously active in Locke solution, and electrical stimulation evoked phasic contraction. Isoprenaline (3×10^{-11}–10^{-8} M) and dibutyryl cyclic AMP (db cyclic AMP, 0.1–0.8 mM) depressed the phasic contraction; the depression was enhanced in the presence of 0.6 mM Mg.

2 The contracture generated by 40 mM K was partially relaxed by isoprenaline (10^{-11}–10^{-8} M) and db cyclic AMP (0.1–0.8 mM). Mg (0.6 mM) enhanced the isoprenaline-induced relaxation, but not that induced by db cyclic AMP.

3 The membrane potential of the muscle was −61 mV, and electrical stimulation induced an action potential which consisted of spike and plateau components. Application of isoprenaline and db cyclic AMP mainly reduced the duration of the plateau potential. The effect was potentiated by 0.6 mM Mg.

4 The membrane was hyperpolarized, accompanied by a decrease in membrane resistance, when 10^{-8} M isoprenaline or 0.8 mM db cyclic AMP was applied. The effects of isoprenaline were prominently augmented in the presence of 1.2 mM Mg, while those of db cyclic AMP were slightly potentiated.

5 Forskolin (0.1 μM) or papaverine (10 μM) inhibited the phasic contraction and the K-contracture. The effect on the phasic contraction was potentiated by 0.6 mM Mg, while that on the K-contracture was not affected.

6 Forskolin shortened the action potential at 0.3 μM, and hyperpolarized the membrane with a decrease in membrane resistance at 3.0 μM. The membrane effects were augmented by 0.6 and 1.2 mM Mg, respectively.

7 It was hypothesized that external Mg ions could affect at least two processes involved in actions at β-adrenoceptors on rat myometrium; receptor-agonist interaction and cyclic AMP-mediated inhibition of membrane excitability.

Introduction

Mg ions are an essential constituent of serum, but less attention has been paid to their physiological significance than to other cations (e.g., Na, K and Ca ions). In vascular smooth muscle, some processes in the plasma membrane are thought to be modulated by external Mg ions: the excitability of the muscle membrane can be depressed by the stabilizing action of Mg ions (Bohr, 1964); Mg inhibits the entrance of Ca ions from the extracellular space following membrane excitation (Viveros & Somjen, 1968). Hormonal effects on smooth muscle are also influenced by external Mg ions: the effects of vasopressin and oxytocin on vascular, intestinal and myometrial smooth muscle are potentiated by Mg ions (Somlyo *et al.*, 1966; Woo & Somlyo, 1967; Schild, 1969). These actions of Mg are thought to be exerted at the receptor level. It was found that inhibitory effects of isoprenaline (β-adrenoceptor agonist) on uterine circular muscle of the oestrogen-treated rat were potentiated by adding Mg ions to the external solution, but the underlying mechanism remained obscure (Osa & Ogasawara, 1979).

The purpose of the present work was to clarify the process influenced by external Mg ions on the β-adrenoceptor action in rat myometrium. Experiments were based on the following scheme for the

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mechanism of β-action which is generally accepted (see Bülbring & Tomita, 1987, for a recent review): the activation of β-adrenoceptors (binding of agonist to receptor) stimulates adenylate cyclase, and adenosine 3′:5′-cyclic monophosphate (cyclic AMP) production is increased; intracellular cyclic AMP activates protein kinase which, in turn, phosphorylates functional proteins on the plasma membrane and cell organelles. As a result, the concentration of intracellular free Ca ions is decreased, affecting the electrical and mechanical activities of smooth muscle.

We have shown in the present paper that the presence of Mg ions in the external solution augments the effects of isoprenaline, forskolin and dibutyryl cyclic AMP on the electrical activity of the plasma membrane in the uterine longitudinal muscle. On the other hand, the effect on K-contracture is potentiated by Mg only in the case of isoprenaline. It is suggested that there are at least two processes in the β-adrenoceptor action affected by extracellular Mg ions.

Methods

Wistar-strain female rats (200–300 g) were ovariectomized. After more than 10 days the rats were given 50 μg oestradiol-17β benzoate dissolved in sesame oil subcutaneously, and killed 3 days later. The uteri were excised and cut open along the mesometrium. A strip of longitudinal muscle was dissected 1 mm in width and 5 mm in length. The muscle strip was tied at both ends with silk threads and suspended vertically in a 2 ml chamber; one end was fastened at the bottom of the chamber and the other end was attached to a force transducer. Bathing solution (36°C) continuously flowed over the strip at a rate of 8 ml min^{-1}. The muscle strip was stimulated by current pulses when necessary. For measurement of electrical activity, the muscle strip was mounted in a partition chamber (1 ml in volume) as described by Abe & Tomita (1968), and superfused at a rate of 5 ml min^{-1}. Electrical activity of the muscle cell was recorded intracellularly with glass microelectrodes filled with 3 M KCl (30–50 MΩ in resistance). For simultaneous records of electrical and mechanical activity, one end of the muscle strip was tied with a thread and connected to a force transducer. Signals were amplified and recorded by an ink-writing pen recorder. Membrane responses were in some cases stored on a data recorder and reproduced at appropriate sweep speed and magnitude.

The Locke solution had the following composition (mM): NaCl 130.6, KCl 5.9, $NaHCO_3$ 8.0, $CaCl_2$ 2.5 and glucose 11.5, aerated with 95% O_2 plus 5% CO_2 (pH 7.3). The preparations were incubated in the solution at 36°C for 1–2 h before the experiment began. High-K (40 mM) solution was made by replacing NaCl with equimolar KCl. The solution containing Mg ions was prepared by adding an appropriate quantity of 1 M $MgCl_2$ to the Locke or high-K solution. Drugs used were isoprenaline hydrochloride (Nikken), dibutyryl cyclic AMP monosodium (db cyclic AMP, Sigma), papaverine hydrochloride (Nakarai), and forskolin (Sigma). Forskolin was dissolved in 95% ethanol, and was stable for at least 3 months (Seamon *et al.*, 1981). It was diluted in perfusing solution to give the desired concentration. The final concentration of ethanol in the solution was less than 0.03%.

Statistical analysis for the significance of differences between means was by Student's t test.

Results

Isoprenaline and dibutyryl cyclic AMP

The longitudinal muscle strips excised from oestrogen-treated rat uterus were not normally spontaneously active after 1–2 h incubation in Locke solution, and field current stimulation evoked phasic contraction. Isoprenaline (10^{-10} M) inhibited the phasic contraction in amplitude and duration (Figure 1a(i)). After washing out isoprenaline, the effects of 10^{-10} M isoprenaline were again examined in the presence of 0.6 mM Mg. Phasic contraction was more strongly inhibited by isoprenaline in the presence of Mg (Figure 1a(ii)). Dibutyryl cyclic AMP (0.2 mM) also inhibited the phasic contraction (Figure 1b(i)), but the onset of the drug action appeared 3–5 min after the application of db cyclic AMP. The delay is probably due to the time required for db cyclic AMP to penetrate into the muscle cell. The inhibitory effect of db cyclic AMP was again augmented in solutions containing 0.6 mM Mg ions (Figure 1b(ii)).

The membrane and contractile effects of isoprenaline and db cyclic AMP were examined in depolarized muscle (Figure 2). Phasic contractions corresponded well with evoked action potentials in solutions containing 0.6 mM Mg (Figure 2a). When the muscle was exposed to high-K solution, the membrane was depolarized to -25 mV and a contracture was produced. The effects of isoprenaline (3×10^{-9} M) and db cyclic AMP (0.4 mM) were examined in the high K solution; neither drug affected the membrane potential, but the contracture was partly relaxed (Figure 2b,c). Therefore, effects of isoprenaline and db cyclic AMP via change in membrane potential can be ignored in the depolarized muscle.

Effects of isoprenaline and db cyclic AMP were

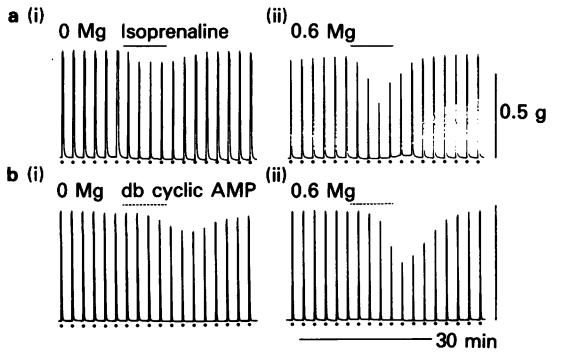

Figure 1 Effects of isoprenaline (a) and dibutyryl cyclic AMP (db cyclic AMP) (b) on the phasic contractions of uterine longitudinal muscle in the absence (i) and presence (ii) of Mg ions. Isoprenaline (10^{-10} M) and db cyclic AMP (0.2 mM) were applied during the periods indicated by solid and broken lines, respectively. Mg (0.6 mM) was added to the bathing solution 1–2 h before the 2nd application of the drugs. Dots indicate electrical stimulation (1 s in duration, supramaximal intensity). Records (a) and (b) were obtained from different preparations.

investigated in the depolarized preparation in the absence and presence of Mg ions (Figure 3). The contractures induced by 40 mM K were partly relaxed by 10^{-10} M isoprenaline and 0.2 mM db cyclic AMP under Mg-free conditions (Figure 3a). In the presence of 0.6 mM Mg, the relaxation of K-

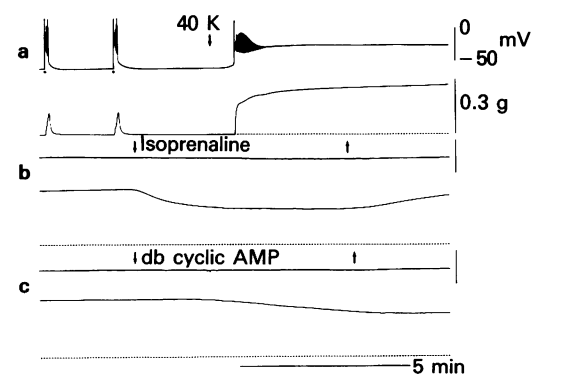

Figure 2 Simultaneous records of electrical (upper trace) and mechanical (lower trace) activities, and effects of isoprenaline and dibutyryl cyclic AMP (db cyclic AMP) in high-K solution. The bathing solution contained 0.6 mM Mg. External K concentration was raised to 40 mM at the arrow (a). Isoprenaline (3×10^{-9} M) and db cyclic AMP (0.4 mM) were applied between the arrows (b and c, respectively). Broken lines show the initial level of resting tension. Dots indicate electrical stimulation (500 ms, supramaximal intensity). All records are from the same preparation.

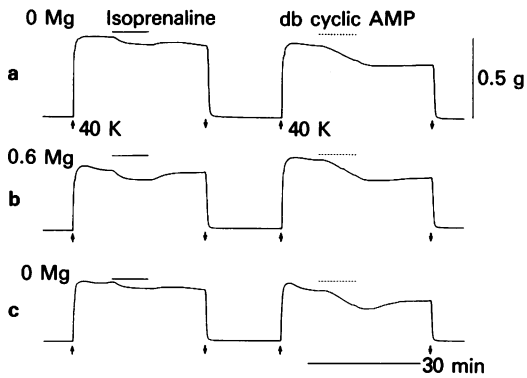

Figure 3 Effects of isoprenaline (left) and dibutyryl cyclic AMP (db cyclic AMP) (right) on the K-contracture in the absence (a,c) and presence (b) of Mg ions. External K concentration was raised to 40 mM at the upward arrow, and returned to 5.9 mM at the downward one. Isoprenaline (10^{-10} M) and db cyclic AMP (0.2 mM) were applied during the periods indicated by solid and broken lines, respectively. Mg (0.6 mM) was added to the bathing solution 70 min before the second application of isoprenaline (b), and removed 25 min before the third application (c). All records are from the same preparation.

contracture by isoprenaline increased but that by db cyclic AMP was unchanged (Figure 3b).

The inhibitory effects of 10^{-10} M isoprenaline and 0.2 mM db cyclic AMP on the mechanical activities are summarized in Table 1. It was proved that the effect of isoprenaline and db cyclic AMP on the phasic contraction and the effect of isoprenaline on the K-contracture were potentiated by Mg, while the effect of db cyclic AMP on the K-contracture was not affected by Mg.

The effects of isoprenaline and db cyclic AMP on mechanical activity were evaluated at various concentrations both in the absence and presence of 0.6 mM Mg (Figure 4). In the case of phasic contractions (Figure 4a(i)(ii)), the reduction of the amplitude caused by either drug was larger at each concentration in the Mg-containing solution than in the Mg-free solution, except at relatively low concentrations in one preparation (isoprenaline, triangle). The depression of K-contracture by isoprenaline was also potentiated at each concentration by Mg, and the dose-response curves tended to be shifted to the left in the presence of Mg (Figure 4b(i)). In contrast, the dose-response curves for db cyclic AMP were little influenced by Mg (Figure 4b(ii)).

Intracellular recording of membrane activity was carried out, and the effects of isoprenaline and db cyclic AMP were examined in the absence and presence of external Mg ions. The resting membrane potential (mean ± s.d.) was −61.8 ± 3.2 mV ($n = 37$,

Table 1 Contractile effects of isoprenaline and dibutyryl cyclic AMP (db cyclic AMP) in the absence and presence of Mg

	Phasic contraction (% of control)			*K-contracture* (% of control)		
	n	0 mM Mg	0.6 mM Mg	n	0 mM Mg	0.6 mM Mg
Isoprenaline 10^{-10} M	5	80.9 ± 11.7	58.6 ± 20.5*	6	83.5 ± 5.4	76.8 ± 5.9*
db Cyclic AMP 0.2 mM	5	65.5 ± 16.8	40.4 ± 19.4*	4	80.1 ± 6.5	81.8 ± 8.9

Values are means ± s.d. *n*, number of preparations. Effects of drugs were first examined in the absence of Mg, and then in the presence of 0.6 mM Mg after 1–2 h exposure to the Mg-containing solution.
The minimum amplitude of contraction during the exposure to drugs (12 min) is expressed as a percentage of the amplitude of contraction immediately before the drug application.
* Significantly different from the value at 0 mM Mg ($P < 0.05$).

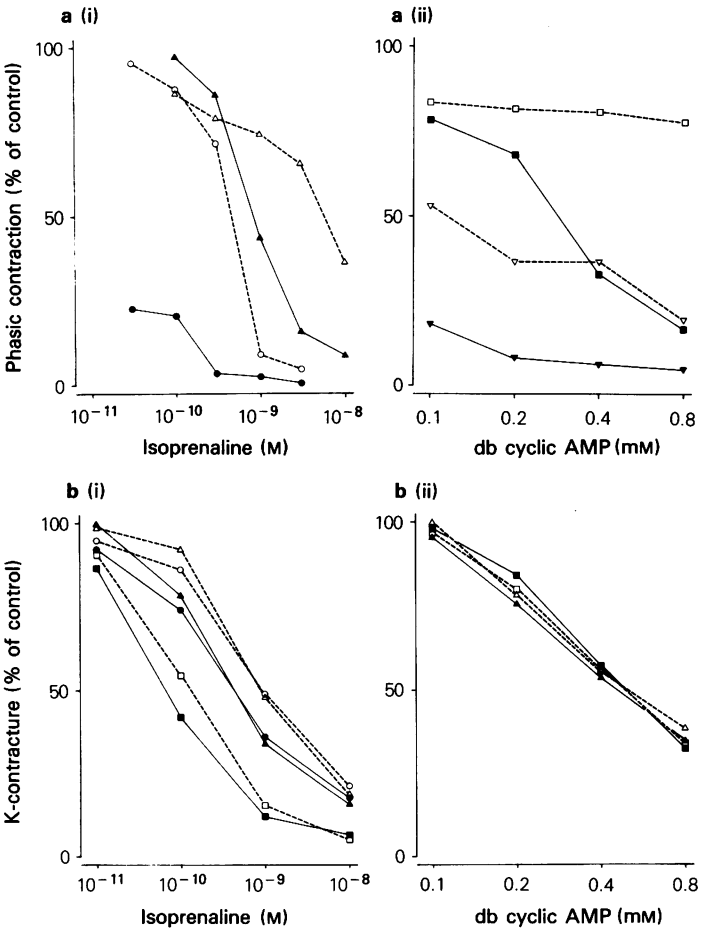

Figure 4 Dose-response relationships for the effects of isoprenaline (i) and db cyclic AMP (ii), on the phasic contraction (a) and the K-contracture (b) in the absence (open symbol, broken line) and presence (filled symbol, solid line) of 0.6 mM Mg. In each graph, the same type of symbol represents the same preparation. The drugs were applied cumulatively. In the case of K-contracture, external K concentration was raised to 40 mM 10 min before the application of drugs. The experiments in the presence of Mg were carried out after the preparations were incubated with the Mg-containing solution for 1–1.5 h. Ordinates: the minimum amplitude of contraction during the exposure to drugs is expressed as percentage of the amplitude of contraction immediately before the drug application. Abscissae: concentrations of drugs.

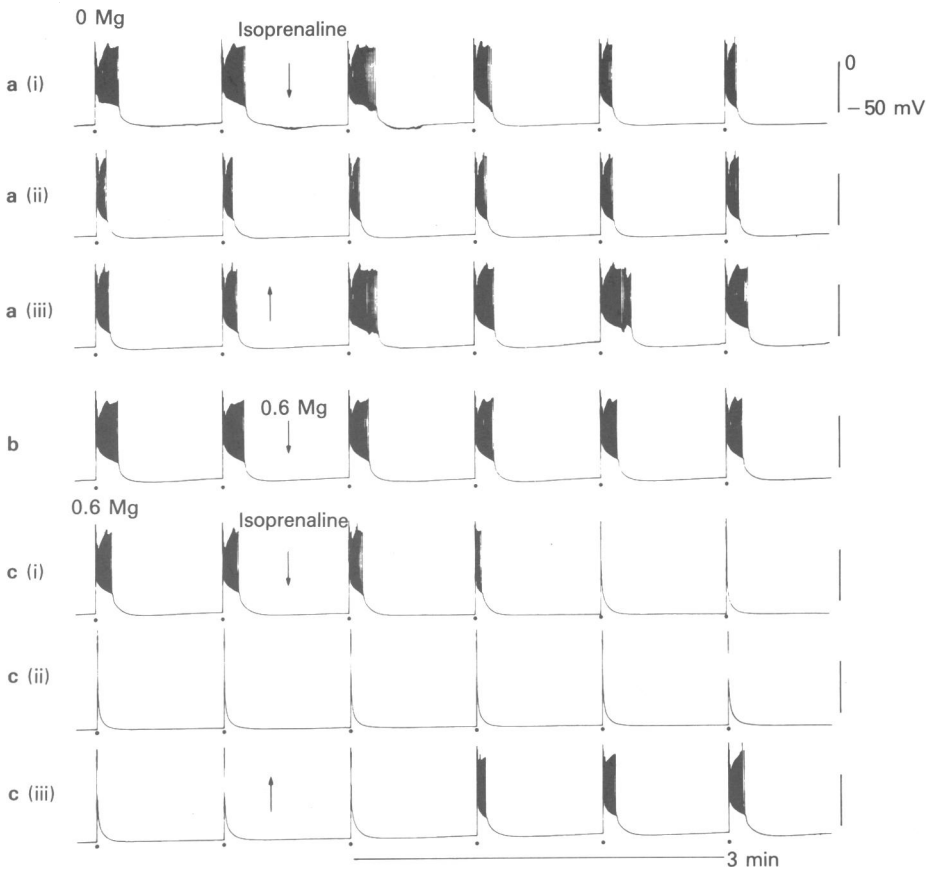

Figure 5 Effects of isoprenaline on the electrical activity in the absence (a) and presence (c) of Mg ions. Isoprenaline (3×10^{-9} M) was applied at the downward arrow and washed out at the upward one. In record (b), Mg (0.6 mM) was added to the bathing solution. Record (c(i)) was taken 30 min after the Mg application. Dots indicate electrical stimulation (500 ms, supramaximal intensity). All records were obtained from the same cell.

no Mg), -62.5 ± 3.8 mV ($n = 22$, 0.6 mM Mg) and -61.6 ± 3.5 mV ($n = 20$, 1.2 mM Mg).

Electrical stimulation evoked an action potential which consisted of an initial spike and a plateau potential under Mg-free conditions; repetitive spike discharge was observed on the plateau (Figure 5a). The main effect of 3×10^{-9} M isoprenaline was to shorten the duration of the action potential. In the presence of 0.6 mM Mg (Figure 5b), the duration of the action potential discharge was slightly reduced, and 3×10^{-9} M isoprenaline progressively shortened the action potential, until finally only a few spikes were generated by current stimulation (Figure 5c).

Figure 6 shows the effects of db cyclic AMP on the membrane activity in the absence and presence of Mg. The action potential did not change in its configuration during the first 4–5 min after the application of 0.4 mM db cyclic AMP under Mg-free conditions (Figure 6a(i)), and thereafter decreased mainly in duration (Figure 6a(ii)). The lag time corresponds with the delay observed in the mechanical response (Figure 1b). In the presence of 0.6 mM Mg, the action potential was greatly shortened by 0.4 mM db cyclic AMP and finally became a single spike (Figure 6b(i)(ii)).

The effects of isoprenaline on the membrane potential and membrane resistance were compared in the absence and presence of external Mg ions (Figure 7). In the cell illustrated, application of 10^{-8} M isoprenaline hyperpolarized the membrane by 8 mV (Figure 7a(i)), and reduced the amplitude of the electrotonic potential by 7.2% (Figure 7a(iii) vs 7a(ii)) in Mg-free solution. Membrane potential and electrotonic potential were little altered by the application of 1.2 mM Mg (physiological Mg concentration), but isoprenaline (10^{-8} M) hyperpolar-

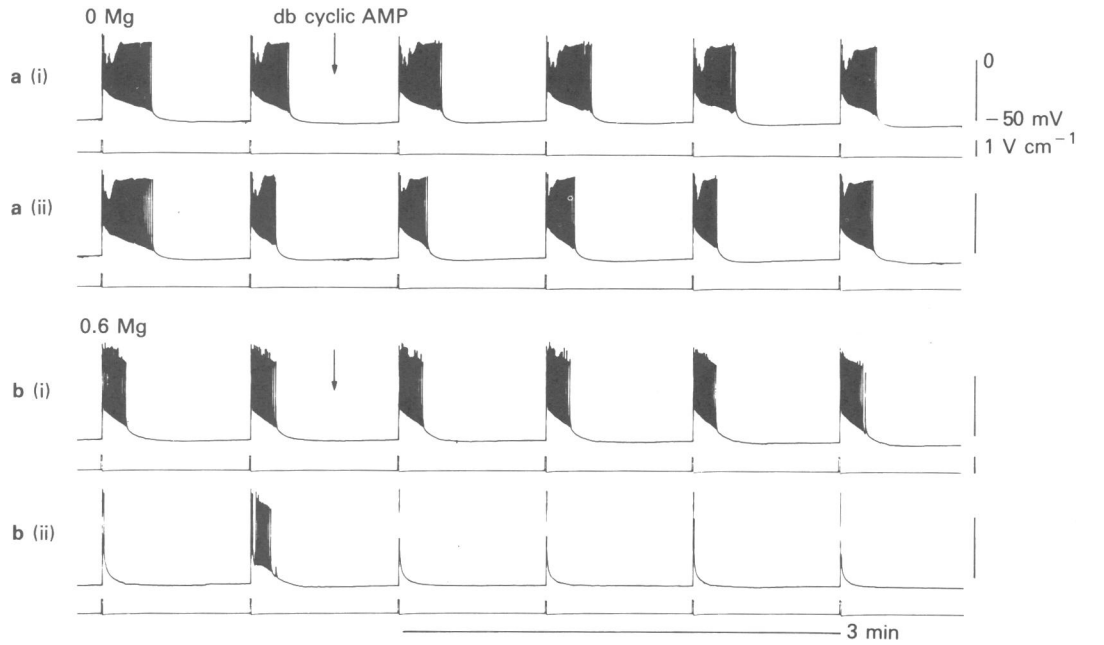

Figure 6 Effects of dibutyryl cyclic AMP (db cyclic AMP) on the electrical activity in the absence (a) and presence (b) of Mg ions. db Cyclic AMP (0.4 mM) was applied at the downward arrow. Mg (0.6 mM) was added to the bathing solution 1.5 h before the 2nd application of db cyclic AMP. Upper and lower traces show electrical activity and stimulating current monitor, respectively. Outward current pulses (500 ms, supramaximal intensity) were applied at intervals of 60 s. Records (a) and (b) were obtained from the same preparation, but from different cells.

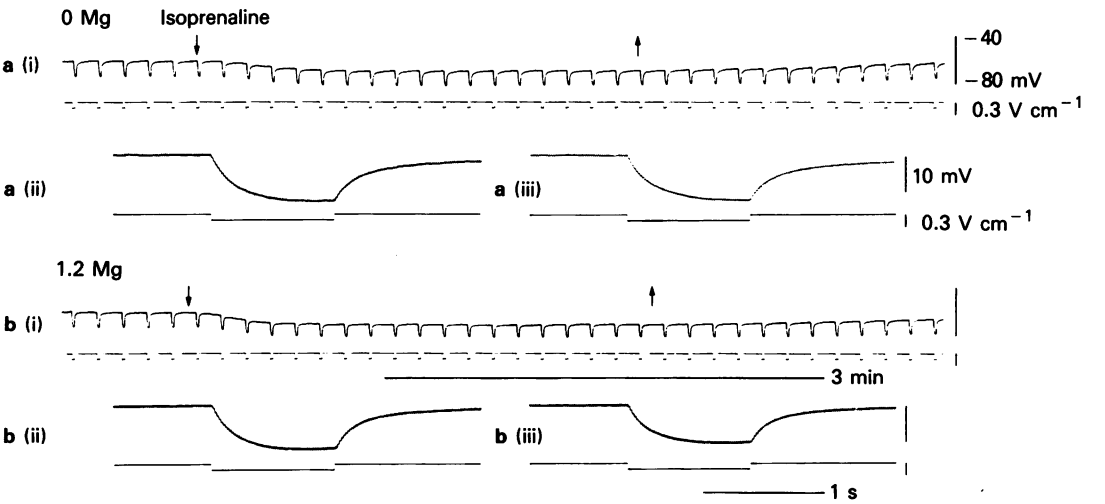

Figure 7 Effects of isoprenaline on the membrane potential and membrane resistance in the absence (a) and presence (b) of Mg ions. Isoprenaline (10^{-8} M) was applied between the arrows (a(i), b(i)). Mg (1.2 mM) was added to the bathing solution 55 min before the second application of isoprenaline. Upper and lower traces show membrane potential and stimulating current monitor, respectively. Inward current pulses (1 s) at constant intensity were applied at intervals of 10 s. Magnified electrotonic potentials with a faster sweep mode are reproduced under (a(i)) and (b(i)); (a(ii)), (b(ii)) and (a(iii)), (b(iii)) are the electrotonic potential 10–20 s before and 140–160 s after the application of isoprenaline, respectively. All records were obtained from the same cell.

ized the membrane by 12 mV (Figure 7b(i)), and reduced the amplitude of the electrotonic potential by 16.0% (Figure 7b(iii) vs 7b(ii)). In other cells, membrane hyperpolarization by 10^{-8} M isoprenaline was as follows (mean ± s.d.): no Mg, 8.5 ± 2.9 mV ($n = 6$); 1.2 mM Mg, 12.3 ± 2.3 mV ($n = 6$); the means are significantly different ($P < 0.025$). Therefore membrane hyperpolarization produced by isoprenaline was potentiated by Mg ions.

In similar experiments with db cyclic AMP, the membrane hyperpolarized gradually and the electrotonic potential decreased slightly, when 0.8 mM db cyclic AMP was applied (not illustrated). The hyperpolarization by 0.8 mM db cyclic AMP was: no Mg, 3.2 ± 1.6 mV ($n = 5$); 1.2 mM Mg, 5.4 ± 1.7 mV ($n = 5$); the means are significantly different ($P < 0.05$). The effect of db cyclic AMP on the membrane potential seems to be less influenced by Mg ions than the effect of isoprenaline.

Forskolin and papaverine

Forskolin increases intracellular cyclic AMP concentration in uterine smooth muscle by directly stimulating adenylate cyclase activity (Marshall & Fain, 1984). Figure 8 shows the influence of external Mg ions on the mechanical effects of forskolin. Forskolin (0.1 μM) had only a slight inhibitory effect on the phasic contraction in Mg-free solution (Figure 8a(i)), but the effect was much stronger in the presence of 0.6 mM Mg (Figure 8a(ii)). Spontaneous contractions took place when Mg was removed in this preparation (Figure 8a(iii)). The spontaneous activity was abolished by forskolin, and electrical stimulation evoked phasic contractions of large amplitude. On the other hand, the forskolin (0.1 μM)-induced inhibition of K-contracture was nearly the same in the absence (Figure 8b(i)) and presence (Figure 8b(ii)) of Mg.

Papaverine inhibits phosphodiesterase activity in rat myometrium (Uruno *et al.*, 1974), and cyclic AMP accumulates in the muscle cell. Similar results to those for forskolin were obtained with papaverine for the influence of Mg on the mechanical responses (not illustrated).

Table 2 is a summary of the mechanical effects of forskolin and papaverine in the absence and presence of Mg ions. For both drugs, inhibition of the phasic contraction was significantly augmented by Mg, while inhibition of the K-contracture was not significantly influenced by Mg.

External Mg ions also augmented inhibition of the action potential by forskolin (Figure 9). In Mg-free solution, application of 0.3 μM forskolin shortened the action potential (Figure 9a). Moreover, it depressed the amplitude of the plateau potential, which perhaps resulted in an increase in amplitude of the spikes superimposed on the plateau. These effects were greater in solutions containing 0.6 mM Mg, and finally the action potential was generated only during the period when the current pulse was applied (Figure 9b).

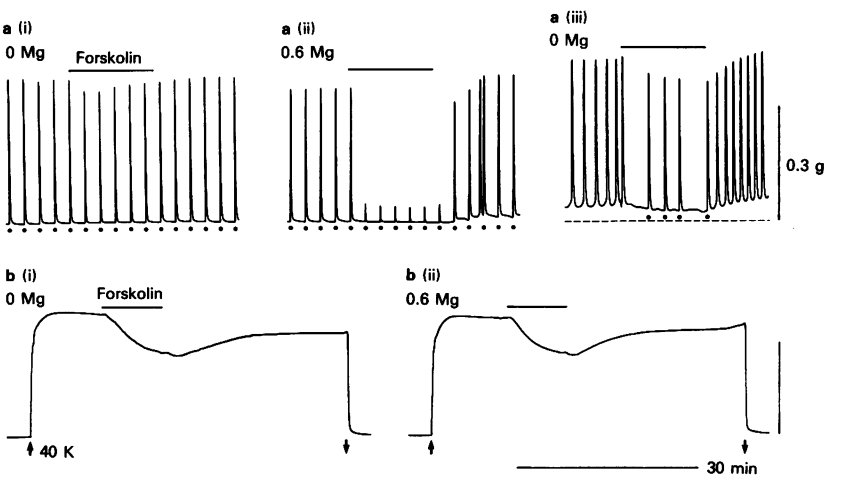

Figure 8 Effects of forskolin on the phasic contraction (a) and K-contracture (b) in the absence and presence of Mg ions. In the records (b), external K concentration was raised to 40 mM at the upward arrow, and returned to normal at the downward one. Forskolin (0.1 μM) was applied during the periods indicated by solid lines. Mg (0.6 mM) was added to the bathing solution 2 h before the second application of forskolin (a(ii), b(ii)), and removed 1 h before the third application (a(iii)). The broken line (a(iii)) shows the initial level of resting tension. Dots indicate electrical stimulation (1 s, supramaximal intensity). Records (a) and (b) were obtained from different preparations.

Table 2 Contractile effects of forskolin and papaverine in the absence and presence of Mg

	Phasic contraction (% of control)			*K-contracture* (% of control)	
	n	0 mM Mg	0.6 mM Mg	n	0 mM Mg	0.6 mM Mg
Forskolin 0.1 μM	7	82.0 ± 22.3	34.6 ± 25.9*	4	70.1 ± 7.1	72.4 ± 7.3
Papaverine 10 μM	6	72.7 ± 18.4	39.3 ± 22.1*	4	52.9 ± 6.5	51.0 ± 8.5

Values are means ± s.d. *n*, number of preparations. Effects of drugs were first examined in the absence of Mg, and then in the presence of 0.6 mM Mg after 1–2 h exposure to the Mg-containing solution.
The minimum amplitude of contraction during the exposure to drugs (12 min) is expressed as a percentage of the amplitude of contraction immediately before the drug application.
* Significantly different from the value at 0 mM Mg ($P < 0.025$).

The effects of a higher concentration (3 μM) of forskolin on the membrane potential and membrane resistance were investigated in the absence and presence of 1.2 mM Mg. Figure 10 is a representative record. The membrane was hyperpolarized by 3 mV (Figure 10a(i),(ii)) and the amplitude of the electrotonic potential decreased by 6.5% (Figure 10a(iv) vs 10a(iii)), when forskolin was applied under Mg-free conditions. In Mg-containing solution, forskolin hyperpolarized the membrane by 10 mV (Figure 10b(i),(ii)) and reduced the amplitude of the electrotonic potential by 13.4% (Figure 10b(iv) vs 10b(iii)).

Discussion

The present results indicate that the effects of isoprenaline, forskolin and db cyclic AMP on the membrane responses (inhibition of action potential, hyperpolarization and reduction of electrotonic potential) were potentiated in the presence of external Mg ions whereas only the inhibition of the K-contracture caused by isoprenaline was markedly potentiated by Mg. In addition, external Mg potentiated the inhibition of the phasic contraction by isoprenaline, forskolin and db cyclic AMP, which

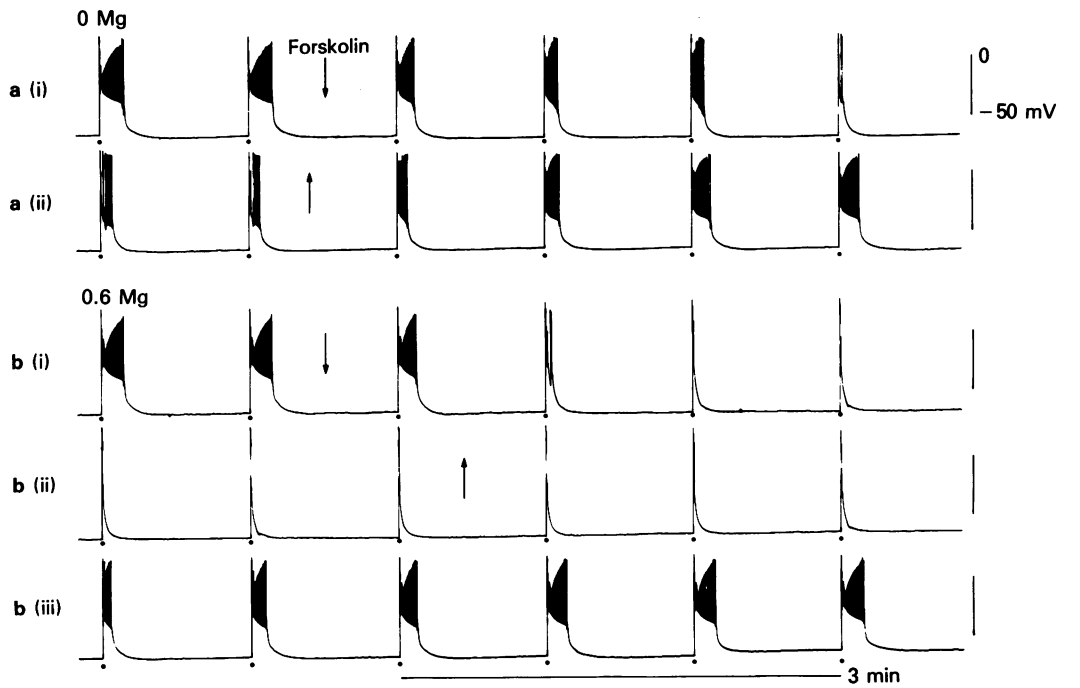

Figure 9 Effects of forskolin on the electrical activity in the absence (a) and presence (b) of Mg ions. Forskolin (0.3 μM) was applied at the downward arrow and washed out at the upward one. Mg (0.6 mM) was added to the bathing solution 1 h before the 2nd application of forskolin. Dots indicate electrical stimulation (500 ms, supramaximal intensity). All records are from the same preparation.

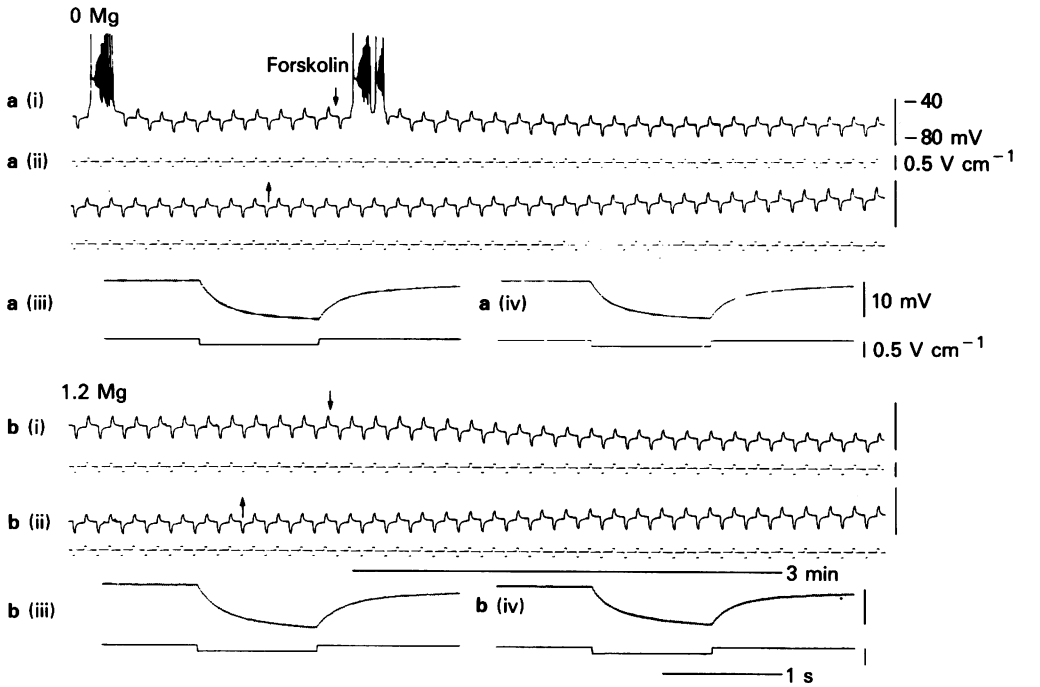

Figure 10 Effects of forskolin on the membrane potential and membrane resistance in the absence (a) and presence (b) of Mg ions. Forskolin (3 μM) was applied at the downward arrow, and removed at the upward one. Mg (1.2 mM) was added to the bathing solution 80 min before the 2nd application of forskolin. Upper and lower traces show membrane potential and stimulating current monitor, respectively. Inward and outward current pulses (1 s) at constant intensity were applied alternately at intervals of 5 s. Magnified electrotonic potentials with a faster sweep mode are reproduced below (a(ii)) and (b(ii)); (a(iii)), (b(iii)) and (a(iv)), (b(iv)) are the electrotonic potential 10–20 s before and 205–225 s after the application of forskolin, respectively. All records are from the same preparation.

occurs as a result of the action potential. The fact that external Mg ions enhanced the effect of db cyclic AMP indicates that the process depending on external Mg ions exists at a relatively 'late' phase of the β-adrenoceptor response. On the other hand, involvement of Mg in the process prior to cyclic AMP-mediated action ('early' phase) is also possible, which seems to be revealed by the results on K-contracture.

It has been postulated in rat myometrium that membrane hyperpolarization in response to β-adrenoceptor activation is due to an increase in K conductance (Kroeger & Marshall, 1973), which is supported by a decrease in membrane resistance in the present work (Figures 7, 10). In the β-action, cyclic AMP may phosphorylate the K channels either directly or via cyclic AMP-dependent protein kinase as reported in *Helix* neurone (Ewald *et al.*, 1985). Mg is considered to augment the activation of K channels by cyclic AMP through the process. In vascular smooth muscle, Somlyo *et al.* (1972) found that no significant membrane hyperpolarization was observed on the application of 1 mM db cyclic AMP with 4 mM theophylline after 90 min exposure to Mg-free solution, and further application of Mg, still in the presence of db cyclic AMP and theophylline, hyperpolarized the membrane by about 10 mV. They suggested that the hyperpolarization was caused by the activation of Mg-ATPase mediating cation extrusion rather than through opening of K channels.

Action potential duration in uterine longitudinal muscle of oestrogen-treated rats was shortened by lowering external K and Ca concentration, and was prolonged by the application of TEA and tetracaine (Maruta & Osa, 1987). It was concluded that an increase in K conductance was responsible for the repolarization of the action potential. Since the shortening of the action potential by isoprenaline, forskolin and db cyclic AMP was potentiated in the presence of Mg, it is speculated that cyclic AMP facilitates the activation of delayed outward K current, and the action is potentiated by Mg ions. It is not yet clear whether the K channel concerned

with the repolarization of action potential is identical with the K channel responsible for the membrane hyperpolarization.

In depolarized smooth muscle, it is thought that Ca influx through voltage-dependent Ca channels produces the contracture directly or via Ca-induced Ca release (Bolton, 1979). Since the K-contracture was partially relaxed without any change in membrane potential by isoprenaline or db cyclic AMP (Figure 2), the relaxation would be ascribed to intracellular events. It is known that cyclic AMP (or cyclic AMP-dependent protein kinase), as a result of β-adrenoceptor activation, accelerates the uptake of cytoplasmic free Ca ions into storage sites, and directly interferes with the contractile machinery (Bülbring & Tomita, 1987). Hence, the fact that relaxation by forskolin, db cyclic AMP and papaverine of the K-contracture was not altered by external Mg may indicate that cyclic AMP production by adenylate cyclase and the cyclic AMP-mediated effects as described above are not sensitive to 'extracellular' Mg. On the other hand, enhancement by Mg of the isoprenaline-induced relaxation of K-contracture would be ascribed to an increase in affinity of the β-adrenoceptor for isoprenaline, because the concentration-response curves of isoprenaline shifted to the left in the presence of Mg ions (Figure 4b(i)). Augmentation of receptor-agonist interaction by external Mg ions has also been reported in other systems (oxytocin and vasopressin, Somlyo *et al.*, 1966; Woo & Somlyo, 1967; Schild, 1969: adenosine, Murphy & Snyder, 1983: endothelium-derived relaxant factor, Altura & Altura, 1987).

The site of action of Mg for the interaction of isoprenaline and the β-receptor probably exists on the outer surface of the plasma membrane. On the other hand, it is difficult in the cyclic AMP-mediated membrane response to determine whether the site of action of Mg is inside or outside the membrane. According to the data obtained by atomic absorption spectrophotometry and nuclear magnetic resonance spectroscopy, the intracellular content of Mg ions was little influenced when external Mg concentration was altered (uterus, Moawad & Daniel, 1971; arterial wall, Palatý, 1971; taenia coli, Nakayama *et al.*, 1987). It is therefore probable that the site where Mg ions participate in the cyclic AMP-mediated membrane response is located on the outer surface of membrane. However, it also seems possible that local Mg existing on and near the inner surface of the membrane is labile and this Mg is responsible for the cyclic AMP-mediated membrane response. This idea is reasonable since cyclic AMP acts on the plasma membrane from the inside. The cyclic AMP-dependent protein kinase purified from bovine brain is reported to have an absolute requirement for a divalent metal such as Mg, Mn and Co when stimulated by cyclic AMP (Miyamoto *et al.*, 1969). In Mg-free external solution, K channels may be hard to activate by the cyclic AMP-dependent protein kinase which exists in the peripheral region of the cytoplasm.

In conclusion, it is proposed that extracellular Mg potentiates the β-adrenoceptor-mediated action in rat myometrium at least by two processes: one is the acceleration of receptor-agonist interaction at the outside of the membrane; the other is presumably the potentiation of cyclic AMP-mediated activation of the K-channel exerted on the inside of a membrane. In view of the effects on K-contractures, a cyclic AMP-mediated Ca uptake by intracellular organelles affected by the extracellular Mg can be excluded.

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A comparison of vasodilator activity of agents activating cyclic nucleotides with those inhibiting their metabolism in rabbit isolated ear artery

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1 The effects of forskolin, a direct activator of adenylate cyclase and sodium nitroprusside, a direct activator of guanylate cyclase, were studied on rabbit isolated ear arteries preconstricted with 80 mM potassium.

2 Bolus injection of these two compounds resulted in vasodilatation. They had similar potencies in this tissue but forskolin had a significantly longer duration of action than sodium nitroprusside.

3 In the same tissue, perfusion with isobutylmethylxanthine (IBMX), a non-selective phosphodiesterase (PDE) inhibitor, or zaprinast, selective for the PDE primarily responsible for the metabolism of guanosine 3':5'-cyclic monophosphate (cyclic GMP), resulted in vasodilatation. However, SK&F 94120 selective for cyclic AMP-PDE (PDE III), primarily responsible for the metabolism of adenosine 3':5'-cyclic monophosphate (cyclic AMP), resulted in vasodilatation only at very high concentrations. The rank order of potency for the compounds was IBMX > zaprinast > SK&F 94120.

4 The effects of these three PDE inhibitors were studied on the vasoconstriction produced by perivascular sympathetic nerve stimulation in the absence of raised potassium. IBMX and zaprinast, caused a reduction in the response at 50 Hz stimulation frequency and a shift in the frequency-response curve to the right. SK&F 94120 did not displace the frequency-response curve but did reduce the response at 50 Hz. The same order of potency for the inhibition of the vasoconstrictor responses to perivascular sympathetic nerve stimulation was found as for vasodilatation i.e. IBMX > zaprinast > SK&F 94120.

5 These results indicate that in the same tissue direct activation of adenylate and guanylate cyclase results in vasodilatation. Non-specific PDE and cyclic GMP-PDE inhibition also resulted in vasodilatation and inhibition of vasoconstrictor responses to sympathetic nerve stimulation. However a selective cyclic AMP-PDE (PDE III) inhibitor did not result in vasodilatation, except at very high concentrations, or inhibit sympathetic vasoconstrictor responses except to reduce the response at 50 Hz stimulation. These findings provide further support for the ability of PDE inhibitors to be tissue selective.

Introduction

Changes in intracellular cyclic nucleotide concentrations are known to mediate the effects of many drugs and hormones on vascular smooth muscle. The relaxation of smooth muscle by β-adrenoceptor agonists is associated with a rise in intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) concentration (Andersson, 1973; Poch & Holzmann, 1980). On the other hand, the relaxation in response to nitrates is brought about by a rise in intracellular guanosine 3':5'-cyclic monophosphate (cyclic GMP) concentration (Itoh *et al.*, 1985). Thus both cyclic nucleotides are implicated in the relaxation of vascular smooth muscle and studies on the time course of these events have revealed that the rise in intracellular cyclic nucleotide concentrations precedes the mechanical events (Kukovetz *et al.*, 1979).

Compounds which inhibit phosphodiesterase (PDE), responsible for the metabolism of cyclic nucleotides, also result in vasodilatation (Kramer & Wells, 1979). Recently, different PDE isoenzymes have been identified and characterized. Selective inhibitors for these isoenzymes have been identified (Weishaar *et al.*, 1986; Reeves *et al.*, 1987) and measurement of their effects on vascular smooth muscle

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may yield further information for the role of cyclic nucleotides in mediating smooth muscle relaxation.

We have investigated the vasodilator effects of PDE inhibitors with differing isoenzyme selectivity; (a) isobutylmethylxanthine (IBMX) which is non-selective, (b) zaprinast which primarily inhibits the metabolism of cyclic GMP and (c) SK&F 94120 (5-(4-acetamidophenyl)pyrazin-2[1H]-one) which inhibits the metabolism of cyclic AMP (Reeves *et al.*, 1987), and compared their effects with forskolin and sodium nitroprusside which activate adenylate and guanylate cyclase respectively (Seamon & Daly, 1981; Itoh *et al.*, 1985). The tissue used was the rabbit isolated ear artery perfused at constant flow.

Methods

New Zealand white rabbits of either sex (2–4 kg) were pretreated with heparin (Pularin, Evans Medical) 1,250 iu kg^{-1} i.v. immediately prior to anaesthesia with a mixture of sodium pentobarbitone (Sagatal, May & Baker) 17 mg kg^{-1} and sodium-5-ethyl-5-1-methyl-propyl-2-thiobarbiturate (Inactin, Byk Gulden) 45 mg kg^{-1} i.v. and killed by exsanguination. The central ear arteries were cannulated and a 3–4 cm length dissected free from the ear and placed in a silicone envelope to prevent drying out.

The arteries were perfused at 2 ml min^{-1} (Watson-Marlow 502s pump) at 32°C. The vascular perfusion pressure, recorded by a pressure transducer (Bell & Howell type 4-422), was measured from a side-arm cannula close to the perfusion cannula. The output was amplified (Lectromed 3552 conditioning amplifier) and recorded (Lectromed Multitrace 4 type 5041). Changes in perfusion pressure were used as an index of changes in the resistance of the artery; a fall in perfusion pressure indicated vasodilatation, a rise, vasoconstriction. The perfusion pump produced a pulsatile pressure, this was not electrically meaned since the time course of the R-C circuits would have biased the measurement of the duration of action. All responses were measured from the minimum of the pulse pressure and are the peak responses. For the comparison of the potencies of the drugs, doses required to produce a given dilatation or constriction were calculated by straight line interpolation from bracketing doses. The data were analysed by Student's paired t test to compare the effects of bolus injection of forskolin and sodium nitroprusside, and the effects of the different PDE inhibitors on perivascular sympathetic nerve stimulation. Student's t test was used for the comparison of the effects of perfusion with different PDE inhibitors. The data were analysed by use of a statistical package (RSE, BBN Software Products) on a VAX 785 computer (Digital Equipment Corporation). Differences were considered to be significant when $P < 0.05$.

Preconstricted arteries

The arteries were preconstricted by perfusion with 80 mM KCl in modified Krebs-Henseleit solution of the following composition (mM): NaCl 43.9, KCl 78.8, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, KH_2PO_4 1.2, glucose 5.0, Na pyruvate 2.0, bubbled with 95% O_2 and 5% CO_2 to give a pH of 7.3–7.4. Bolus injection of compounds into the perfusate were made by injecting 10 or 30 μl volume with a Hamilton syringe through a short length of thick walled rubber tubing just proximal to the artery. Injection of 30 μl of the vehicles used to dissolve forskolin and sodium nitroprusside had no effect on vascular tone. For continuous infusion of compounds, the appropriate molar concentrations were dissolved in the Krebs-Henseleit solution which perfused the arteries.

Perivascular sympathetic nerve stimulation

The arteries were prepared as described above, and perfused with Krebs-Henseleit solution of composition (mM): NaCl 118.0, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, KH_2PO_4 1.2, glucose 5.0, Na pyruvate 2.0, bubbled with 95% O_2 and 5% CO_2 to give a pH of 7.3–7.4. Stimulation of the perivascular sympathetic nerves was achieved by placing the proximal end of the artery over a bipolar silver stimulating electrode using stimulus parameters of 1 to 50 Hz, 5 ms duration, and 20 V which was supramaximal, from a Grass S11 stimulator (Grass Instruments, Quincy, Mass., U.S.A.). The control frequency-response curve was obtained, followed by perfusion with Krebs-Henseleit solution containing the inhibitor for 20 min before obtaining the second frequency-response curve, this was then followed by a period of 1 h of perfusion with Krebs-Henseleit solution to washout the inhibitor, after which the third curve was obtained. Three successive frequency-response curves, without compound present, were shown to be highly reproducible over the time taken to complete the protocol.

Drugs

The following compounds were used and prepared as follows: forskolin (Calbiochem, Cambridge, U.K.) dissolved in absolute ethanol to make a 10^{-2} M stock solution. Sodium nitroprusside (Sigma Chemical Co., St. Louis, MO., U.S.A.) dissolved in distilled water to make a 10^{-2} M stock solution. All stock solutions were diluted with Krebs-Henseleit solution. SK&F 94120 (Smith Kline and French Labs Ltd, Welwyn Garden City), zaprinast (May & Baker, Dagenham)

and IBMX (Aldrich Chemical Co. Gillingham) were dissolved in 0.5 ml 1 M NaOH diluted with one litre of Krebs-Henseleit solution to make a 10^{-4} M solution (pH 7.3–7.4).

Results

Vasodilator effects of forskolin and sodium nitroprusside in potassium constricted arteries

In eight arteries injection of either compound produced a dose-dependent dilatation of the preconstricted arteries. Threshold doses were 3×10^{-10} mol, for forskolin and nitroprusside. The doses required to produce a 25 mmHg fall in perfusion pressure were not significantly different, 8.7 ± 0.4, 12.9 ± 1.0 10^{-10} mol for forskolin and nitroprusside respectively. Solubility of forskolin limited the highest concentration of forskolin that could be injected to 3×10^{-8} mol which resulted in a dilatation of 89.7 ± 8.5 mmHg, sodium nitroprusside at this same dose resulted in a dilatation of 90.1 ± 19.1 mmHg. For sodium nitroprusside this was also the highest dose that could be given which allowed recovery of the perfusion pressure to the control values. These results are illustrated in Figure 1.

In these eight arteries the duration of responses to forskolin was much greater than that to nitroprusside. For forskolin, 1×10^{-9} mol, the time to 50% recovery ($t_{50\%}$) of the peak dilatation, 6.1 ± 1.7 min, was significantly greater than that for nitroprusside, 2.9 ± 0.4 min, at 1×10^{-9} mol (Figure 2).

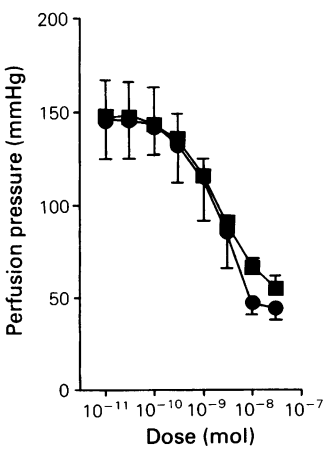

Figure 1 Vasodilatation in rabbit isolated ear arteries preconstricted with K^+ (80 mM) following bolus injection of forskolin (●) or sodium nitroprusside (■) each tested on the same 8 arteries. Points are mean with s.e.mean shown by vertical bars.

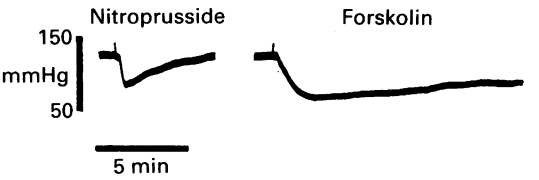

Figure 2 Rabbit isolated ear arteries: an example of the vasodilator responses to bolus injection of sodium nitroprusside 10^{-9} mol, and forskolin 10^{-9} mol.

Vasodilator effects of continuous infusion of different phosphodiesterase isoenzyme inhibitors

Each inhibitor was tested on a separate group of eight arteries. Perfusion with the non-specific PDE inhibitor IBMX and the PDE I selective inhibitor zaprinast resulted in concentration-dependent vasodilatation. The concentrations required to produce a 50 mmHg fall in perfusion pressure of the arteries were $<10^{-6}$ M for IBMX and $2.18 \pm 0.01 \times 10^{-6}$ M for zaprinast. Perfusion with the selective PDE III inhibitor SK&F 94120 resulted in a minimal dilatation, statistically significant only at 3×10^{-4} M. These data are shown in Figure 3.

Effects of different phosphodiesterase inhibitors on the vasoconstrictor responses to sympathetic perivascular nerve stimulation

Supramaximal electrical stimulation resulted in a frequency-dependent increase in perfusion pressure

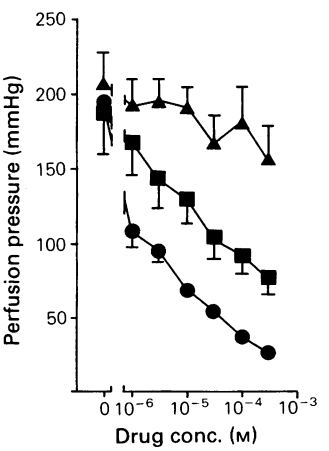

Figure 3 Vasodilatation in rabbit isolated ear arteries preconstricted with K^+ (80 mM)-containing Krebs-Henseleit solution following addition of isobutylmethylxanthine (●), zaprinast (■), or SK&F 94120 (▲). Points are mean with s.e.mean shown by vertical bars; 8 separate arteries for perfusion with each drug.

of the arteries over the range 1 to 50 Hz. At 100 Hz responses did not exceed those at 50 Hz and usually were reduced. In eight arteries three repeated frequency-response curves were reproducible; the mean frequencies required to produce a 50 mmHg rise in perfusion pressure (EF_{50}) were: 5.8 ± 1.1 Hz for curve 1 control; 4.4 ± 0.8 Hz for curve 2, carried out 20 min after curve 1 and equivalent to the curve in the presence of drug; 4.7 ± 0.7 Hz for curve 3, carried out 1 h after curve 2 and equivalent to the curve performed after 1 h washout of the drug. These EF_{50} values were not significantly different. There was no significant difference in the responses at 50 Hz for the three curves: they were 143 ± 30, 155 ± 31, 123 ± 20 mmHg, curves 1 to 3 respectively. These data are shown in Figure 4a, for clarity only curves 1 and 2 are shown.

In eight arteries the inclusion of IBMX, 1×10^{-4} M, in the perfusion fluid reduced vasoconstrictor responses to sympathetic nerve stimulation. The EF_{50} significantly increased from 4.1 ± 0.5 to 27.3 ± 7.1 Hz and the response at 50 Hz was significantly reduced from 168 ± 23 to 67 ± 19 mmHg (Figure 4b). Following washout for 1 h the EF_{50} returned towards control values, 11.7 ± 6.5 Hz, as did the response at 50 Hz, 128 ± 27 mmHg; neither response was significantly different from control curve values.

Zaprinast, 1×10^{-6} M in eight arteries, significantly increased the EF_{50} from 3.7 ± 0.5 to 8.3 ± 1.7 Hz, and decreased the response at 50 Hz from 191 ± 17 to 114 ± 18 mmHg. Following washout for 1 h the EF_{50} only partially returned to control values, 7.1 ± 1.1 Hz, as did the response at 50 Hz, 139 ± 29 mmHg; these responses were significantly different from control curve values; Figure 4c (only two curves shown, the control and that in the presence of the drug).

In contrast SK&F 94120, 1×10^{-4} M in eight arteries, did not significantly alter the EF_{50} value, 5.4 ± 0.5 to 9.3 ± 2.1 Hz, but the response at 50 Hz was significantly reduced, 182 ± 31 to 146 ± 29 mmHg. Following washout for 1 h the EF_{50} was not significantly different from the control value 8.1 ± 1.3 Hz; however, the maximum response was slightly reduced further to 130 ± 27 mmHg, still significantly different from control (Figure 4d, only 2 curves, control and in the presence of drug shown).

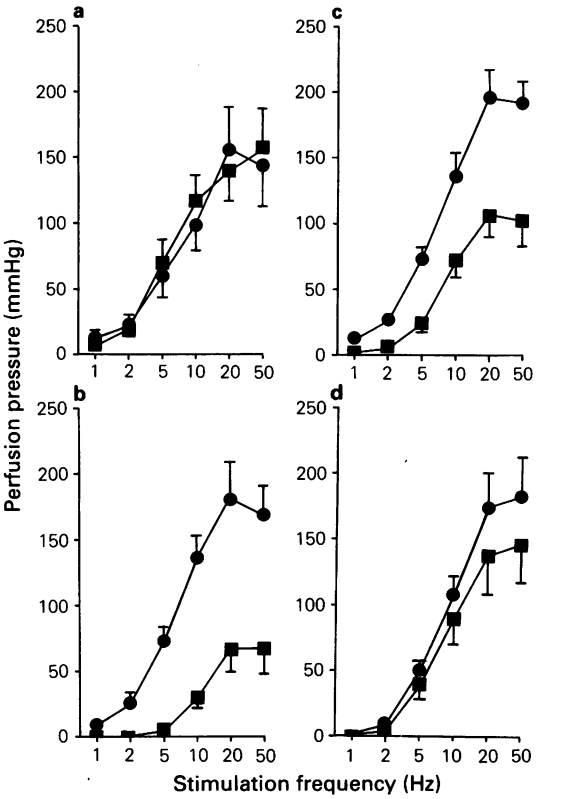

Figure 4 Vasoconstriction during sympathetic nerve stimulation in rabbit ear arteries. Frequency-response curves are shown before (●) and during (■) perfusion of the arteries with (a) Krebs-Henseleit solution without drug, (b) Krebs-Henseleit solution containing isobutylmethylxanthine, 10^{-4} M, (c) Krebs-Henseleit solution containing zaprinast, 1×10^{-4} M or (d) Krebs-Henseleit solution containing SK&F 94120, 1×10^{-4} M. All points are mean with s.e.mean shown by vertical bars, for clarity, two of three curves performed in the same 8 arteries are shown. The third curve was performed after 1 h washout of the drug (not shown). A different set of arteries was used to test each drug and to test the reproducibility of the 3 curves in the absence of drug.

Discussion

These experiments have demonstrated similar vasodilator potencies for compounds which, in the same tissue, activate guanylate or adenylate cyclase directly i.e. sodium nitroprusside and forskolin respectively. However, there was a significant difference in the duration of action of the compounds. In the same tissue, PDE inhibitors with differing isoenzyme selectivity have been shown to result in vasodilatation in potassium precontracted tissues and also to inhibit the responses to perivascular sympathetic nerve stimulation, in each case with a similar order of potency, IBMX > zaprinast > SK&F 94120.

The weak vasodilator activity of SK&F 94120 was unexpected, since Gristwood *et al.* (1986) showed that it was a vasodilator *in vivo*. In that paper, SK&F 94120 (0.1 mg kg^{-1} min^{-1}) resulted in a significant decrease in total peripheral vascular resist-

ance, resulting from reductions in vascular resistance of the heart, kidneys, duodenum, and stomach. No *in vitro* vasodilator data were described in the paper, yet SK&F 94120 was shown to have inotropic effects *in vitro* in guinea-pigs, cats, marmosets, and dogs.

Penetration of SK&F 94120 into vascular cells is needed to achieve inhibition of the tissue enzyme. Its IC_{50} for the low K_m cyclic AMP-PDE (PDE III) is 11 μM, with no inhibition of cyclic GMP-PDE (PDE I), PDE II or the high K_m cyclic AMP-PDE (PDE IV) (Leigh *et al.*, 1987). SK&F 94120 has been shown to act as a positive inotrope over the same concentration range as it inhibits PDE III (Gristwood *et al.*, 1986) suggesting that myocyte penetration is no barrier to activity on the tissue enzyme. Thus the weak vasodilator activity in the rabbit ear artery may imply little or no turnover of cyclic AMP which is metabolised by PDE III.

IBMX is non-selective with IC_{50} values of 11.5, 4.2, 13.0 μM for PDE I, III and IV respectively, and zaprinast is selective for PDE I, IC_{50} values 10, 400, 300 μM for PDE I, III and IV (Leigh *et al.*, 1987). The greater potency of IBMX relative to zaprinast may reflect its ability to inhibit a wider range of PDE enzymes or, if PDE I is the primary basis for activity, reflect greater cell penetration.

Previous investigations of PDE inhibitors have shown that PDE inhibition results in relaxation of preconstricted vascular smooth muscle (Ljung *et al.*, 1975; Kramer & Wells, 1979; Kukovetz *et al.*, 1981; Schoeffter *et al.*, 1987). However, a correlation between the relaxant effects of the PDE inhibitors and increases in intracellular cyclic nucleotide concentrations has not been established. Vegesna & Diamond (1986) used a series of xanthine derivatives and papaverine with phosphodiesterase inhibitory activity in a rabbit aortic ring preparation. They showed dissociation between vasodilator activity and phosphodiesterase inhibition i.e. some compounds were more potent in causing relaxation than would be expected from their low potency for the inhibition of the hydrolysis of cyclic nucleotides, whilst others were more potent for the inhibition of cyclic nucleotide metabolism, than for relaxation of smooth muscle. They also reported that some agents which were of equivalent potency for the relaxation of smooth muscle, caused differential increases in cyclic nucleotide concentrations. Fujioka (1984), using basilar arteries of dog and guinea-pig, also failed to find a causal relationship between cyclic AMP and the effects of papaverine, which was thought to act by inhibition of voltage-dependent influx of calcium and inhibition of receptor activated release of intracellularly stored calcium.

The use of more selective inhibitors of the different PDE isoenzymes by Schoeffter *et al.* (1987) in rat thoracic aorta, demonstrated a correlation between intracellular cyclic AMP concentrations and relaxation. However they concluded that an increase in intracellular cyclic GMP by inhibition of the cyclic GMP-PDE with zaprinast did not by itself lead to relaxation of the tissue. They reported a 3.3 fold increase in cyclic GMP with zaprinast, 50 μM, yet they measured a 10, 20, 60% relaxation at 5, 50, 500 μM. Our findings are at odds with those of Schoeffter *et al.* (1987), because we have shown a greater potency of action for zaprinast for the relaxation of vascular smooth muscle with 50% reduction in perfusion pressure at 55 μM compared to 500 μM needed in their study.

The arteries used in our investigation were perfused with pulsatile pressure at constant flow. Recent studies have shown that this may cause the release of EDRF (Holtz *et al.*, 1984; Rubanyi *et al.*, 1986) which is known to increase cyclic GMP concentrations and so result in vasodilatation (Holzmann, 1982; Rapoport & Murad, 1983). Griffith, (1985) showed that responses to EDRF could be potentiated by zaprinast thus confirming the role of cyclic GMP in mediating the response of EDRF. Griffith *et al.* (1987) provided evidence for a possible physiological role for the endogenous release of EDRF. They showed that EDRF may control the perfusion of an arterial vascular bed, the rabbit ear artery, during high rates of perfusion by maintaining a constant pressure gradient across each vessel, particularly in the first order branching vessels from the main artery. The endothelium is intact in our preparations, despite being perfused with high potassium Krebs-Henseleit solution (Griffith *et al.*, 1984), since in four arteries perfused under identical conditions and for the same time required to perform the dose-response curves to perfusion with PDE inhibitors, carbachol, 1×10^{-8} mol injected as a bolus, resulted in a peak dilatation of 31 ± 6 from 97 ± 16 mmHg (unpublished observations). The effectiveness of IBMX and zaprinast in causing vasodilatation in our preparations, compared to the findings of Schoeffter *et al.* (1987), may therefore be due to an increased basal turnover of cyclic GMP caused by EDRF release, since the actions of PDE inhibitors must be in part dependent upon the turnover rate of cyclic nucleotides. The lack of effect of SK&F 94120 in our preparations could therefore be accounted for by a corresponding lack or very slow rate of turnover of cyclic AMP, since it would appear from the equipotency of forskolin and sodium nitroprusside that both cyclic nucleotides result in vasodilatation.

Tissue selectivity of cyclic AMP-PDEs may however account for the lack of effect of SK&F 94120. Schoeffter *et al.* (1987) used rolipram and Ro 20-1724 as cyclic AMP-PDE inhibitors whereas we have used SK&F 94120. Reeves *et al.* (1987) showed that SK&F 94120 is selective in its inhibition of

PDE III which is a subtype of the cyclic AMP-PDE and that rolipram and Ro 20-1724 are inhibitors of PDE IV, a clearly distinct subtype of the cyclic AMP-PDE with a high K_m for cyclic AMP. The activities of these cyclic AMP-PDE subtypes has previously been shown to have differential functional significance in another tissue (Gristwood & Owen, 1986) where PDE III inhibition by SK&F 94120 causes an increase in cyclic AMP of the heart associated with an increase in the contractility, whereas rolipram also caused an increase in the cyclic AMP but did not affect the contractility of the heart. With this precedent in heart muscle, the apparently different effects of cyclic AMP-PDE inhibition on vascular smooth muscle could be accounted for by the inhibition of these different subtypes of cyclic AMP-PDE.

There is a further significant difference between the two studies, that of the precontracting agent used. Thus the differences in the vasodilator effects of cyclic AMP-PDE inhibition could be the result of using potassium to preconstrict the arteries in our experiments, and the use of 5-hydroxytryptamine by Schoeffter *et al.* (1987). Work to study possible differences in potency of PDE inhibitors caused by differing precontracting agents is currently under way. Preliminary results obtained by Morrison & Pollock (1988) indicate that sodium nitroprusside relaxations of rat aortic rings were significantly smaller with potassium as a precontracting agent than with noradrenaline. Therefore reduced vasodilator potencies of drugs tested against potassium precontraction may explain the minimal effect of the cyclic AMP-PDE inhibitor SK&F 94120 in our preparations.

We have shown for the first time that PDE inhibition can attenuate the vasoconstrictor responses to perivascular sympathetic nerve stimulation. The frequency-response relationship was shifted to the right and the maxima of the responses were reduced by IBMX and zaprinast. However SK&F 94120 only weakly inhibited the response at 50 Hz. Thus the same order of potency was found for the inhibition of neural vasoconstrictor responses, as for dilatation of the tissue when preconstricted with potassium. It would therefore seem that there is some common underlying mechanistic reason for the differences in potency of cyclic nucleotide PDE inhibitors for the relaxation of vascular smooth muscle, perhaps either basal turnover rates of cyclic nucleotides or tissue selectivity of the inhibitors.

In order to resolve these differences in potency of the PDE inhibitors and the differences in duration of action of forskolin, and sodium nitroprusside the correlation of time course and concentrations of intracellular cyclic nucleotides and resulting vasodilator effects should be investigated. These studies are in progress.

It remains for the functional significance of this inhibition to be determined *in vivo*. It could be expected that non-specific PDE inhibition and inhibition of cyclic GMP-PDE would result in vasodilatation. *In vivo* there might be an elevated turnover of cyclic AMP compared to that *in vitro* which may account for the activity of SK&F 94120 as a vasodilator *in vivo* (Gristwood *et al.*, 1986).

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Variable, voltage-dependent, blocking effects of nitrendipine, verapamil, diltiazem, cinnarizine and cadmium on adrenomedullary secretion

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1 Catecholamine release from cat adrenal glands perfused at a high rate (4 ml min^{-1}) at 37°C with modified Krebs solutions lacking Ca and containing 1.2 mM K (hyperpolarizing solution) or 118 mM K (depolarizing solution) was triggered by 10-s pulses of Ca (0.5 mM) in the presence of 118 mM K. Hyperpolarized glands released 1280 ± 135 ng per pulse and depolarized glands 831 ± 98 ng per pulse ($n = 29$).

2 While the dihydropyridine Ca channel blocker nitrendipine inhibited secretion in hyperpolarized glands with an IC_{50} of 214 nM, in depolarizing conditions the drug was much more potent ($IC_{50} = 0.99$ nM). In contrast, the inorganic Ca channel blocker cadmium inhibited secretion with the same potency both in hyperpolarized or depolarized glands.

3 Cinnarizine, diltiazem and verapamil exhibited intermediate degrees of voltage-dependence in blocking secretion. The IC_{50} ratios between hyperpolarized and depolarized glands were 215, 36, 19, 8 and 0.76 respectively for nitrendipine, cinnarizine, diltiazem, verapamil and cadmium. Because the experimental design (strong depolarization in the absence of Ca) favours the highest opening probability of Ca channels, it seems that these drugs bind preferentially to their receptors when these channels are in their open state.

4 Variable voltage-dependent effects of the five Ca channel blockers on adrenomedullary catecholamine release suggests different sites and mechanisms of action on, or near L-type Ca channels in chromaffin cells. In addition, these findings might help to explain why these drugs exhibit tissue selectivity and why they act differently in normal polarized as compared to ischaemic depolarized cells.

Introduction

Since both the early activation and inactivation of Ca uptake into K-depolarized chromaffin cells proceeds in a few seconds, and closely parallels the activation and inactivation of catecholamine release (Artalejo *et al.*, 1986) it seems plausible that voltage-dependent Ca channels located on the plasma membrane constitute an early step in the modulation of cytosolic processes leading to secretion (Artalejo *et al.*, 1988a). These channels were first analysed electrophysiologically by Neher's group (Fenwick *et al.*, 1982) and are likely to be of the L-type (Nowicky *et al.*, 1985) since both Ca uptake and secretion are very sensitive to dihydropyridine (DHP) Ca channel activators such as Bay K 8644, CGP28392 or (+)-Sandoz 202-791 (García *et al.*, 1984; Montiel *et al.*, 1984; Ladona *et al.*, 1987; Fonteriz *et al.*, 1987) and blockers such as (+)-PN200-110, nitrendipine, nifedipine, niludipine or nimodipine (Ceña *et al.*, 1983; Montiel *et al.*, 1984; Ladona *et al.*, 1987; Gandia *et al.*, 1987).

Voltage-dependence of DHPs, phenylalkylamines and benzothiazepines is now fairly well established, chiefly on the basis of electrophysiological data from Ca current kinetic analysis (Lee & Tsien, 1983; Sanguinetti & Kass, 1984; Bean, 1984; Uehara & Hume, 1985; Gurney *et al.*, 1985; Sanguinetti *et al.*, 1986; Rane *et al.*, 1987); functional data to define the importance of such effects are therefore clearly needed. These data may show: (a) the relevance of the voltage-dependence of the major categories of Ca channel blockers; (b) the mechanism of action of the Ca channel blockers and the fundamentals of their

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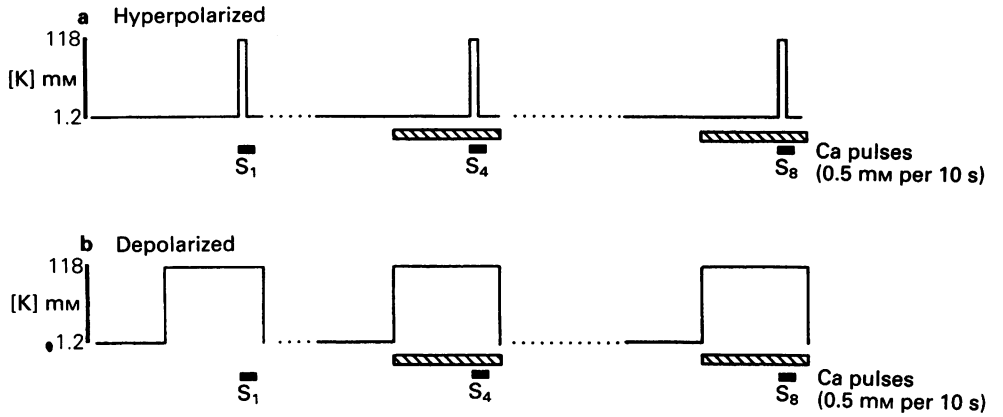

Figure 1 Scheme representing the protocol used to study the voltage-dependent effects of Ca channels blocking drugs on catecholamine release from hyperpolarized (a) or depolarized (b) perfused cat adrenal glands. Hatched bar indicates blocking drug. See Methods for further detail.

tissue selectivity according to variable membrane resting potential of several excitable cells in physiological or ischaemic (depolarized) conditions; (c) whether the electrophysiological findings have a functional correlate as far as voltage-dependence is concerned; and (d) by means other than radioligand binding studies in isolated membranes, whether different degrees of voltage-dependence in blocking a physiological function suggest different binding sites on the Ca channel complex where the four categories of drugs (nitrendipine, cinnarizine, diltiazem and verapamil) might exert their blocking effects through different mechanisms. In addition, in view of the fact that these drugs are widely used to treat cardiovascular diseases and their therapeutic indications are being extended to many other diseases, knowledge of whether they affect other non-cardiovascular physiological functions (for instance, catecholamine release) at pharmacologically relevant concentrations has clinical implications.

In the light of these observations, it seemed appropriate to perform the experiments presented here demonstrating that the various categories of Ca channel blockers exhibit different degrees of voltage-dependence in inhibiting catecholamine release from perfused cat adrenal glands.

Methods

Cat adrenal glands were isolated and prepared for retrograde perfusion at a rate of 4 ml min^{-1}, at 37°C with Krebs-HEPES solution pH 7.4, continuously bubbled with pure O_2 and having the following composition (mM): NaCl 144, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.5, HEPES 10 and glucose 11. Glands were initially perfused with this solution for 60 min to allow their equilibration with the experimental conditions.

Experimental design

After 1 h, two glands from the same cat were stimulated to secrete catecholamines with a four-step pattern of perfusing solutions (see protocols in Figure 1): (1st) both glands were perfused for 20 min with 1.2 K/0 Ca (a Krebs-HEPES solution containing 1.2 mM K and no Ca); (2nd) one gland was perfused for an additional 10 min with this solution (hyperpolarized gland) and the contralateral (depolarized gland) was perfused with 118 K/0 Ca (a Krebs-HEPES solution in which 118 mM NaCl was substituted by 118 mM KCl, and lacking Ca); (3rd) 10 s with 118 K/0.5 Ca (secretion test pulse) to allow a brief Ca entry and secretion in both glands; and (4th) 50 s back to 1.2 K/0 Ca solution (hyperpolarized) or 118 K/0 Ca solution (depolarized) to collect the secreted catecholamines in 10 s periods. These stimulation cycles were repeated 8 times in each pair of glands at 30 min intervals.

Control glands were exposed to vehicle only (ethanol). Drugs were tested by adding increasing concentrations (10^{-10}–10^{-5} M) during steps 2, 3 and 4 of each stimulation cycle; they were absent during the first step (20 min in 1.2 K/0 Ca). These protocols are similar to those used when measuring Ca current inactivation by conditioned prepulses (Fenwick *et al.*, 1982): we tried to promote the inhibition of catecholamine release by various Ca channel blockers by pre-exposing the adrenals to them and high K in the absence of Ca (118 K/0 Ca), before initiating release by addition of external Ca (0.5 mM for 10 s), and comparing this release with secretion obtained in hyperpolarized glands.

Catecholamine assay and quantitation of results

Catecholamines present in each 10-s collection sample (600 μl of medium acidified with perchloric acid to 0.05 N) during the last 10 s of the 2nd stimulation step (basal secretion) and steps 3 and 4, were measured fluorometrically according to Shellenberger & Gordon (1971). Data were expressed as ng of total catecholamine release per Ca test pulse and are means $\pm$ their s.e. To calculate the concentrations of drugs blocking the secretory process by 50%, the sigmoid inhibition curves were converted into straight lines by plotting values in the ordinates as log $(y/100 - y)$; the intercepts with the abscissae ($y = 0$) gave the IC_{50} s.

Results

Profile of secretory responses evoked by Ca test pulses

Figure 2 shows the time-course of secretion evoked by 10-s Ca test pulses in hyperpolarized (a) and depolarized (b) adrenal glands. Secretion increased immediately to reach a peak, and then quickly declined to basal resting levels within the 50-s period following the Ca test pulse. The initial secretory Ca tests gave figures of 1280 $\pm$ 135 and 831 $\pm$ 98 ng per pulse ($n = 29$) for hyperpolarized and depolarized adrenals respectively.

To test the effects of increasing concentrations of drugs on secretion in the same gland to which Ca test pulses were sequentially applied, it was of utmost importance that those pulses evoked similar secretory responses throughout the experiment. In Figure 2c, catecholamine releases were normalized as % of the individual secretion obtained in S_3 for each experiment. We realised in earlier experiments that secretory responses were fairly stable from the third Ca pulse onwards; therefore, the amounts of catecholamine released in each experiment in S_3 were taken as 100% and from S_4 to S_8 was expressed as % of S_3.

Effects of nitrendipine on catecholamine release evoked by Ca pulses in hyperpolarized and depolarized adrenal glands

Figure 3a shows that nitrendipine caused a concentration-dependent blockade of Ca-evoked catecholamine release in both, hyperpolarized and depolarized adrenal glands. However, the drug could not fully inhibit secretion even at the high concentrations of 1 μM if given to glands preperfused with 1.2 K/0 Ca solution. In contrast, 10 nM nitrendipine caused 80% blockade in depolarized glands. The

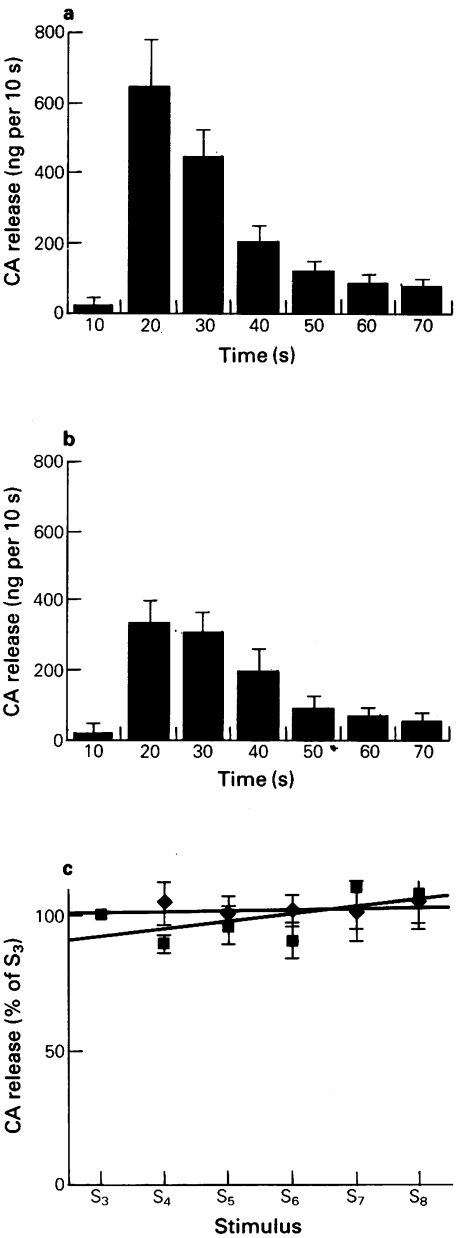

Figure 2 Time-course of catecholamine release evoked by reintroducing Ca (0.5 mM for 10 s) into glands preperfused with hyperpolarizing (1.2 K/0 Ca) (a) or depolarizing (118 K/0 Ca) (b) solutions. Data are taken from the third test pulse and represent means of 29 paired experiments; s.e. shown by vertical bars. In (c) secretory responses obtained in 5 subsequent Ca pulses (S_4 to S_8) are expressed as a % of those obtained in S_3; data are means $\pm$ s.e. of 12 paired experiments: (■) hyperpolarized; (◆) depolarized.

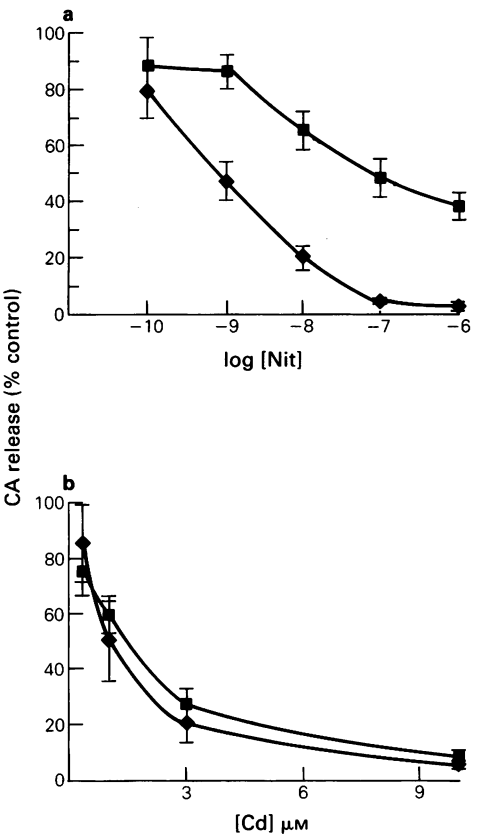

Figure 3 Concentration-dependent blockade by nitrendipine (a) and Cd (b) of catecholamine release evoked by 10-s Ca pulses given at 30 min intervals to glands pre-perfused with 1.2 K/0 Ca (hyperpolarizing) or 118 K/0 Ca (depolarizing) solutions. Increasing concentrations of nitrendipine (a) or Cd (b) were given 10 min before and during stimulations with 118 K/0.5 Ca (Ca pulses). See Methods for further detail of the experimental protocol. Secretion data in ng were normalized to 100% (S_3) and are expressed as % of catecholamine release obtained in S_3 (ordinates); they are expressed as means of 10 paired experiments, s.e. shown by vertical bars: (■) hyperpolarized; (◆) depolarized.

IC_{50} in hyperpolarization was 214 nM, as opposed to depolarization which was 0.99 nM.

Cadmium effects on secretion

Various inorganic Ca channel blockers inhibit K-evoked catecholamine release in perfused cat adrenal glands; one of the most potent is Cd (Gandia *et al.*, 1987). Therefore, we compared its effects with those of nitrendipine, and determined whether it also exhibited voltage-dependence. In Figure 3b, it can be seen that Cd ions inhibited Ca-evoked secretion equally in both hyperpolarized and depolarized adrenal glands; at 10 μM, secretion was fully inhibited in both types of glands. If anything, the IC_{50} in depolarized glands was slightly greater (1510 nM versus 1150 nM) than in hyperpolarized glands, a result opposite to that obtained with nitrendipine.

Effects of cinnarizine, diltiazem and verapamil on catecholamine release from hyperpolarized and depolarized glands

Ca channel blocking drugs belonging to chemical classes different from DHPs, such as cinnarizine, diltiazem and verapamil, also inhibited Ca-evoked secretion in a concentration-dependent manner; however, they behaved quite differently. For instance, little blockade was observed with verapamil and diltiazem at 1 μM in hyperpolarized glands, but cinnarizine caused over 60% inhibition of secretion. Depolarization enhanced the blocking effects of the three chemicals, though it was more pronounced with cinnarizine, followed by diltiazem and last by verapamil.

It is worth comparing the IC_{50} ratios required to block secretion from hyperpolarized or depolarized glands of the five compounds tested (Table 1). This ratio was highest for the DHP (215), followed by piperazine (36), the benzothiazepine (19) and lastly the benzylalkylamine (8); Cd showed no voltage-dependency.

Discussion

The experiments presented here show that inhibition by various Ca channel blockers of adrenomedullary

Table 1 Variable voltage-dependent effects of five Ca channel blockers on adrenomedullary secretion

Drug	n	IC_{50} 1.2 K	IC_{50} 118 K	$\frac{IC_{50}\,1.2\,K}{IC_{50}\,118\,K}$
Nitrendipine	10	214	0.99	215
Cinnarizine	8	189	5.23	36
Diltiazem	11	2040	106	19
Verapamil	7	1770	217	8
Cadmium	8	1150	1510	0.76

Catecholamine release from perfused cat adrenal glands was evoked by a test pulse of 118 mM K, 0.5 mM Ca for 10 s, after pretreatment with hyperpolarizing (1.2 K/0 Ca) or depolarizing (118 K/0 Ca) solutions for 10 min. IC_{50} is the drug concentration that inhibits by 50% the initial control release. Values are expressed in nM; n = number of paired experiments.

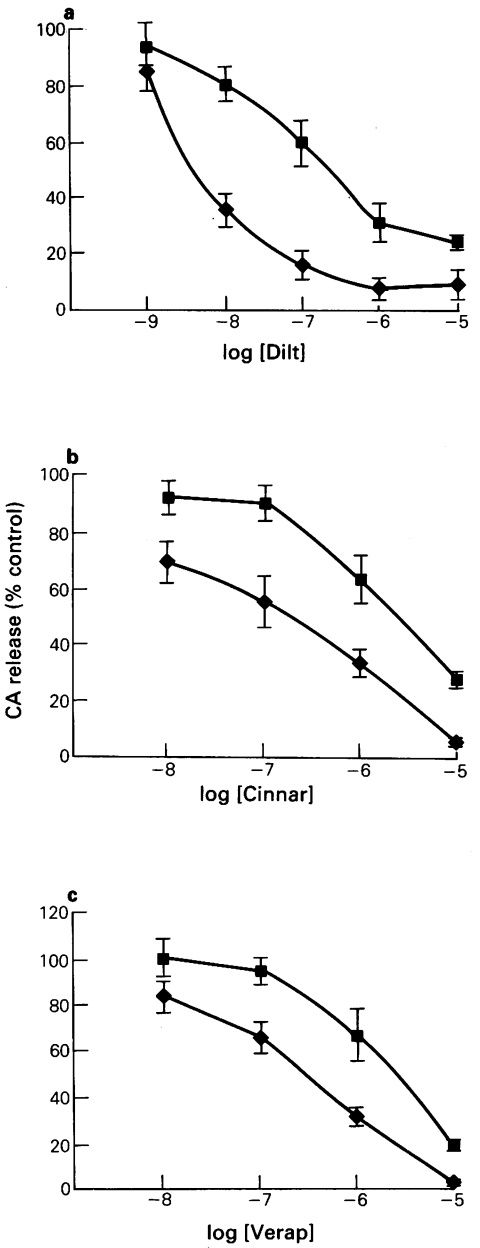

Figure 4 Effects of diltiazem (Dilt) (a), cinnarizine (Cinnar) (b) and verapamil (Verap) (c) on catecholamine release from cat adrenal glands evoked by test pulses of 118 mM K/0.5 mM Ca for 10 s, after pre-perfusion with hyperpolarizing (1.2 K/0 Ca) (■) or depolarizing (118 K/0 Ca) (◆) solutions for 10 min. Data are normalized to percentages of secretion obtained in S_3 in the absence of drug (100%), and represent means of 6–8 paired experiments; vertical bars show s.e.

catecholamine release evoked by brief pulses of Ca is strongly dependent on whether the adrenal glands were exposed to the drugs under hyperpolarizing or depolarizing conditions. Our present studies are based on experimental designs similar to those used by electrophysiologists when measuring Ca current inactivation by conditioned prepulses (Fenwick *et al.*, 1982). There are several new features compared to our previous experiments in which we could not discriminate the voltage-dependent effects of these drugs (Ceña *et al.*, 1983; García *et al.*, 1984; Ladona *et al.*, 1987; Gandia *et al.*, 1987). First, tissues were exposed to drugs under hyperpolarizing (1.2 K/0 Ca) or depolarizing (118 K/0 Ca) conditions with the intention of manipulating the affinities of the drugs for their receptors as a function of the membrane potential. It is now established that the effects of various Ca channel blockers on the heart (Sanguinetti & Kass, 1984; Bean, 1984; Uehara & Hume, 1985; Gurney *et al.*, 1985; Sanguinetti *et al.*, 1986; Rane *et al.*, 1987) and the intact adrenal medulla (Artalejo *et al.*, 1988b) are dependent on membrane potential. Second, secretion was triggered by a brief Ca test pulse applied to adrenals previously perfused with Ca-free solutions containing low (1.2 mM) or high (118 mM) K. Under these conditions, drugs will bind to their receptors with variable affinities, depending on the membrane potential. Thirdly, the duration of the Ca pulses was very short (10 s) in order to decrease the opportunity for drugs to change their equilibrium receptor binding during the pre-test 10-min perfusion period. This is important because the onset of blockade of cardiac Ca currents by DHP is completed in about 10 s (Sanguinetti & Kass, 1984). It is, therefore, clear that in our earlier experiments (Gandia *et al.*, 1987) we were really measuring effects of drugs mainly in depolarized cells since the test secretory pulse lasted over 60 s and the preperfusion period with drugs was performed only under resting (5.9 mM K) conditions. With those earlier protocols we could not determine the possible voltage-dependence of the blocking effects of various drugs. However, in the present studies, we observed that secretory responses in hyperpolarized glands were very resistant to blockade by the four drugs tested, while those obtained in depolarized glands were highly sensitive to the drugs.

The question arises as to whether the observed secretory patterns might be correlated with events taking place in membrane Ca channels; the observation that Cd, a potent inorganic blocker of L-type Ca channels (Nowicky *et al.*, 1985) inhibited both secretory responses, supports this view. Further evidence favouring the correlation between both events comes from the observation that nitrendipine binds with much higher affinity to cat adrenomedullary tissues, and blocks much more efficaciously ^{45}Ca

uptake into and catecholamine release from such tissues, in pre-depolarized than in pre-hyperpolarized conditions (Artalejo *et al.*, 1988b). It is therefore plausible to explain the present results on the basis of the voltage-dependence of nitrendipine, cinnarizine, diltiazem and verapamil in binding to their receptors on L-type Ca channels.

Voltage-dependent Ca channels are dynamic molecular entities that undergo constant transitions between resting, open or inactivated states (Hess *et al.*, 1984). Since sustained depolarization in the absence of Ca (for instance, 10 min in 118 K/0 Ca) causes little inactivation of chromaffin cell Ca channels (Sala *et al.*, 1986; Artalejo *et al.*, 1987), it seems that nitrendipine, cinnarizine, diltiazem and verapamil preferentially bind to their receptors when Ca channels are in their open state. This conclusion is consistent with the interpretation of the mechanism of action of DHPs based on electrical recordings of Ca currents, showing that the drugs display an increased affinity for the L-type channel as the holding potential of the cell is increased (Uehara & Hume, 1985; Cohen & McCarthy, 1987); however, it differs from other reports suggesting that nitrendipine binds tightly to the inactivated state of the channel (Bean, 1984; Sanguinetti & Kass, 1984).

Radioligand binding studies have generated a model for three separate, but allosterically linked binding sites for 1,4-DHPs (nitrendipine), phenylalkylamines (verapamil) and benzothiazepines (diltiazem) (Triggle & Janis, 1987). However, this model has only limited and controversial functional support in non-secretory tissues (DePover *et al.*, 1983; Spedding, 1983; Yousif & Triggle, 1985). Here, we have shown marked differences in potencies for blockade of secretion in hyperpolarized or depolarized glands; voltage-dependent blocking effects are prominent for DHPs, followed by cinnarizine and diltiazem, small for verapamil and absent for the inorganic Ca channel blocker Cd. Though these findings suggest different sites and mechanisms of action of the five agents, they might also be explained by different polarity of the molecules that limit their access to specific receptors sites in depolarizing or hyperpolarizing conditions through the inner or outer part of the channel.

Whatever the mechanism, different degrees of voltage-dependence might help to explain why verapamil and diltiazem are much more cardioactive compounds than DHPs, or why cinnarizine may have some selectivity for certain vascular beds, as well as their different effects on healthy polarized, or depolarized ischaemic tissues. Our data suggest that at clinically relevant concentrations, DHPs might interfere with adrenomedullary secretion; this could constitute an additional contributory mechanism to the well known vasodilator effects of the drugs that make them very useful in treating hypertensive patients.

We thank Prof. F. Hoffmeister, Bayer A.G., FRG for the kind gift of nitrendipine; Knoll Laboratories, FRG for the gift of verapamil, and Doctor Esteve Laboratories, Spain, for the gift of diltiazem and cinnarizine. We also wish to thank Mrs M.C. Molinos for typing the manuscript. This work was supported by grants from Fundación Ramón Areces, CYCYT (No. PB86-0119) and FISS (Spain).

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Tolerance to cromakalim in the rat uterus *in vivo*

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1 Cromakalim (0.1 and 1 mg kg^{-1}) produced inhibition of uterine contractions and falls in mean blood pressure in ovariectomized, non-pregnant rats, the durations of which were dose-dependent. Frequency of contractions was inhibited selectively compared to amplitude.

2 The durations of the uterine effect of cromakalim (1 mg kg^{-1}), when given at 12 h intervals, were less after the second, third and fourth doses compared to the first dose in non-pregnant rats. In ovariectomized rats treated from day 18 of pregnancy, using the same experimental design, no uterine relaxant effects were seen to the third and fourth doses. The timing of delivery and foetal viability were unaffected by cromakalim.

3 The sensitivity of the uterus to cromakalim was tested using small doses before and after three cromakalim doses (1 mg kg^{-1}) given at 8 h intervals in non-pregnant rats. This regime resulted in a 25 fold decrease in the sensitivity of the uterus to cromakalim.

4 Cromakalim can produce long-lasting inhibition of uterine contractions in the rat after bolus i.v. dose but it exhibits little selectivity relative to its vasodepressor action and there appears to be tolerance to the uterine relaxant action of cromakalim *in vivo*.

Introduction

Cromakalim (BRL34915) is a potent relaxant of isolated vascular, gastrointestinal and tracheal smooth muscles (Allen *et al.*, 1986; Hamilton *et al.*, 1986; Weir & Weston, 1986a,b). It is suggested that these effects are achieved by the opening of plasma membrane K^+-channels as relaxation is associated with hyperpolarization to the potassium equilibrium potential, with increased $^{86}Rb^+$ and $^{42}K^+$ efflux and cromakalim is antagonized by K^+-channel blockers. Recently cromakalim has been shown to open K^+-channels in isolated vascular smooth muscle cells when studied by the patch clamp technique (Kusano *et al.*, 1987; Trieschmann *et al.*, 1988).

Cromakalim is also a relaxant of isolated uterus from term pregnant rats (Hollingsworth *et al.*, 1987; Small *et al.*, 1988). The potency and relaxant profile of cromakalim is similar in the uterus as in other isolated tissues and cromakalim is antagonized by several K^+-channel blockers (Hollingsworth *et al.*, 1988). However, cromakalim produced only a small hyperpolarization and no increase in $^{86}Rb^+$ (Hollingsworth *et al.*, 1987) or $^{42}K^+$ (Hollingsworth *et al.*, 1988) efflux in uterus. It was suggested that the relaxant action in the uterus may be exerted by a selective action on K^+-channels in pacemaker cells (Hollingsworth *et al.*, 1987), a proposal supported by other workers (Quast, 1987).

The vasodilator and bronchodilator actions of cromakalim extend to the *in vivo* situation in both animals and in man and the drug is under clinical investigation as an antihypertensive and anti-asthmatic agent (Buckingham *et al.*, 1986; Buckle *et al.*, 1987; Fox *et al.*, 1987; Hamilton, 1988). The aim of this work was, therefore, to determine if the uterine relaxant action *in vitro* also extended to the *in vivo* situation. It was intended to ascertain the pharmacological profile of cromakalim in the rat with regard to the nature of its uterine effect, the selectivity compared to cardiovascular actions and whether the effects on the uterus were maintained with repeated treatment such that the drug could prevent preterm labour. Such a study would enable the profile of cromakalim to be compared with other uterine relaxants such as agonists at β-adrenoceptors (e.g. salbutamol) and calcium entry blockers (e.g. nifedipine). Salbutamol is a potent uterine relaxant but exhibits pronounced tachycardia and some vasodepression at uterine inhibitory doses (Abel & Hollingsworth, 1985). In addition there is pronounced tolerance to salbutamol in the uterus (Abel & Hollingsworth, 1986a) which probably explains its inability to prevent labour in a rat model of preterm

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labour (Abel & Hollingsworth, 1986b). Nifedipine is a uterine relaxant which produces much less tachycardia but slightly greater vasodepression at uterine relaxant doses than does salbutamol (Abel & Hollingsworth, 1985). The uterine inhibitory actions of nifedipine are maintained and the drug is able to prevent preterm labour in the rat (Abel & Hollingsworth, 1986a,b; Downing & Hollingsworth, 1988; Hollingsworth & Downing, 1988). The pharmacological profile of cromakalim has, therefore, been examined in both non-pregnant and pregnant animals.

Methods

Non-pregnant (200–250 g) and day 18 pregnant (280–370 g) Sprague-Dawley rats were purchased from Charles River Ltd. Day of mating was designated day 1 of pregnancy.

Surgical techniques

These techniques were essentially as described previously (Downing *et al.*, 1987; 1988; Downing & Hollingsworth, 1988). Non-pregnant rats were anaesthetized with tribromoethanol (240 mg kg^{-1}, i.p.), bilaterally ovariectomized and the right jugular vein cannulated. In addition, either an intrauterine microballoon was inserted or the right carotid artery was cannulated. Animals were allowed 24 h for recovery. Pregnant rats were anaesthetized with tribromoethanol, bilaterally ovariectomized, an intrauterine microballoon inserted and the right jugular vein cannulated. The number of foetuses *in utero* was determined. Morphine sulphate (5 mg salt kg^{-1} i.p.) was given postoperatively to all rats. Pregnant rats were injected s.c. with oestradiol benzoate (0.5 $\mu g\,kg^{-1}$ s.c. in arachis oil) twice daily starting at the time of surgery. The onset of delivery was defined as the delivery of the first pup. The number of foetuses remaining *in utero* at autopsy was determined and individual foetuses and placentae were weighed. Cromakalim or solvent was given by i.v. injection. Details of experimental designs are given under Results.

Measurements

Intrauterine pressure, blood pressure and heart rate were measured in conscious unrestrained rats and recorded on a polygraph (Grass Instruments, Quincy, MA, U.S.A.) as described previously (Downing *et al.*, 1987; 1988; Downing & Hollingsworth, 1988). Uterine contractions were quantified by integrating the area under the pressure curve above basal resting pressure using a 7P10 integrator. In some experiments frequency of contractions was also measured where a contraction was defined as a rise and fall in pressure of >5 mmHg (1 mmHg = 133 Pa). Amplitude was defined as the maximum contraction within the recording interval. The changes in integral of uterine contractions after cromakalim were expressed as a % of the respective values before drug administration in some experiments. The time interval over which uterine contractions were measured was 15 or 30 min or 1 h dependent upon the experimental design (see Results). If a significant change occurred in a biological parameter in vehicle-treated rats the mean value for a time interval was subtracted from the change observed in individual cromakalim-treated animals at the same time interval.

Statistics

Means of integral and frequency of uterine contractions, blood pressure and heart rate were compared between treatment groups using analysis of variance followed by 2-tailed Student's *t*-test (Sokal & Rohlf, 1969) or paired Student's *t*-test (Armitage, 1971).

Drugs

Cromakalim (BRL34915;(±)-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-benzo-[b]pyrano-3-ol; Beecham) was dissolved in 70% v/v ethanol : distilled water as a 1 or 5 mg ml^{-1} solution. Vehicle refers to 70% v/v ethanol in distilled water, 15% ethanol in saline (tolerance study) or saline. Oestradiol benzoate was obtained from Sigma Chemical Co., tribromoethanol from Fluka Chemicals and morphine sulphate from Evans.

Results

Effects on the uterus and cardiovascular system – single dose

Preliminary experiments in tribromoethanol-anaesthetized animals showed that a bolus injection of cromakalim produced a long-lasting inhibition of uterine contractions; up to 130 min after cromakalim (0.1 mg kg^{-1}) and at least 240 min with cromakalim (1 mg kg^{-1}) (data not shown).

In another series of experiments in conscious animals, uterine contractions or blood pressure and heart rate were measured at 15 min intervals for 30 min before and for 240 min after a single bolus i.v. injection of cromakalim (0.1 or 1 mg kg^{-1}) or vehicle (1 ml kg^{-1}). The vehicle produced an inhibition of uterine contractions, fall in blood pressure and heart rate lasting less than 5 min and thereafter values were not significantly different from pre-vehicle

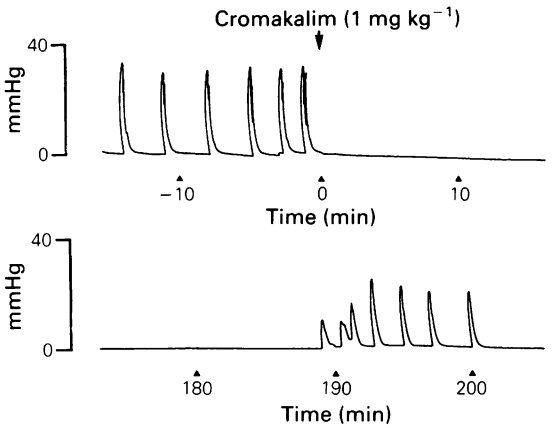

Figure 1 The effect of a bolus i.v. injection of cromakalim (1 mg kg^{-1}) on uterine contractions in the conscious non-pregnant rat.

control values. Cromakalim produced an immediate and complete inhibition of uterine contractions (Figure 1). The duration of the inhibition was dose-dependent as contractions started to reappear

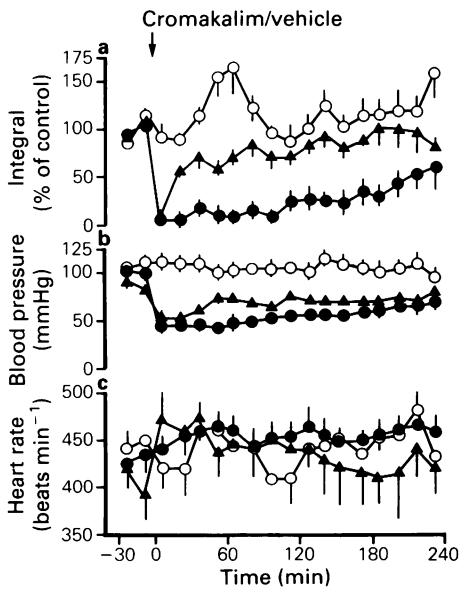

Figure 2 (a) Integral of uterine contractions, (b) blood pressure and (c) heart rate for 30 min before and 240 min after a bolus i.v. injection of either cromakalim (0.1 mg kg^{-1}, ▲; 1 mg kg^{-1}, ●) or vehicle (1 ml kg^{-1}, ○) in non-pregnant rats. Cardiovascular (0.1 mg kg^{-1}, $n = 5$; 1 mg kg^{-1}, $n = 8$; vehicle, $n = 6$) and uterine effects (0.1 mg kg^{-1}, $n = 6$; 1 mg kg^{-1}, $n = 5$; vehicle, $n = 6$) were measured in different animals. Values are means and vertical lines show s.e.mean.

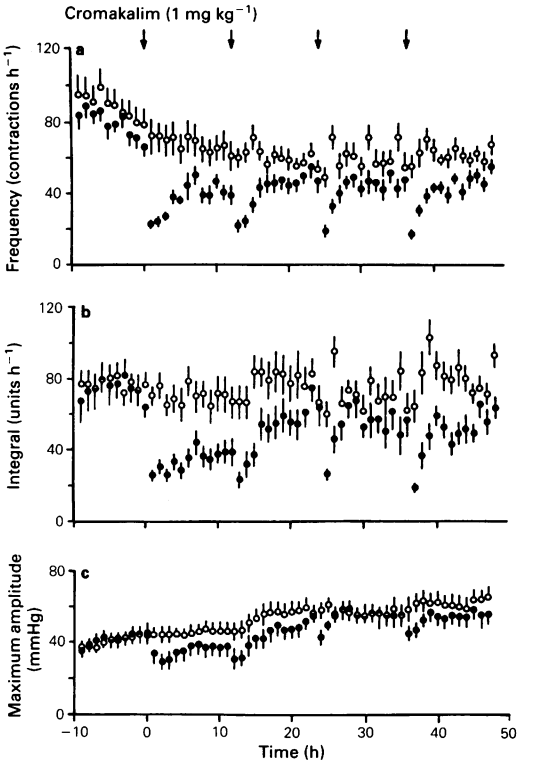

Figure 3 (a) Frequency, (b) integral and (c) maximum amplitude of uterine contractions for 10 h before and 48 h after the first of four bolus i.v. injections of either cromakalim (1 mg kg^{-1}, $n = 10$, ●) or vehicle (1 ml kg^{-1}, $n = 10$, ○) given at 0, 12, 24 and 36 h (shown as ↓) in non-pregnant rats. Data are means and vertical lines show s.e.mean.

approximately 15 min after cromakalim (0.1 mg kg^{-1}) and 90 min after cromakalim (1 mg kg^{-1}) (Figures 1 and 2). Uterine contractions tended to reappear in bursts with quiescent periods between; the individual contractions were of similar amplitude to those in the control situation. Uterine contractions were still significantly reduced ($2P < 0.05$) 215 min after cromakalim (1 mg kg^{-1}). In other animals, cromakalim (0.1 and 1 mg kg^{-1}) produced a fall in mean blood pressure, the duration of which was dose-dependent and with a similar time course to the effect on the uterus (Figure 2). There was no significant change in heart rate after cromakalim.

Effects on the uterus – repeated doses

The aim of this series of experiments was to determine if the effects of cromakalim on the uterus were reproducible with successive doses. Also, in pregnant

rats, whether repeated doses of cromakalim could prevent preterm delivery. Uterine contractions were recorded at 1 h intervals for either 10 h (non-pregnant) or 1 h (pregnant) before and for 48 h after the first of four bolus i.v. injections of either cromakalim (1 mg kg^{-1}) or vehicle (1 ml kg^{-1}) given at 0, 12, 24 and 36 h.

The integral of uterine contractions remained constant in control non-pregnant animals throughout the 58 h recording period (Figure 3). Frequency of contractions declined slightly during the 10 h pre-injection period in controls but thereafter did not change. Amplitude of uterine contractions increased slightly with time in controls. Each dose of cromakalim (1 mg kg^{-1}) produced a peak inhibition of integral and frequency of uterine contractions of 70–80% with only a slight inhibition of the amplitude of contractions. Although the immediate peak effect of cromakalim did not change with repeated dose, it was clear that the durations of the effect of the second, third and fourth doses were less than that of the first dose. It was observed that during recovery from the effect of cromakalim the $\log_{10}$ of the mean % inhibition of the integral of contractions decreased linearly with time. The nature of this relationship enabled a half-time ($t_{1/2}$) for recovery from the effect of cromakalim to be calculated. The $t_{1/2}$ values for the second (2.2 h), third (4.9 h) and fourth (2.7 h) doses were less than that for the first dose (12 h).

This lack of reproducibility of effect with repeated injection was even more evident in pregnant rats (Figure 4). In control animals, frequency of contractions declined during the initial 10 h of recording and then remained constant. The integral of contractions showed a slight diurnal rhythm in control rats, while amplitude of contractions remained constant. The first dose of cromakalim (1 mg kg^{-1}) produced a nearly complete inhibition of integral and frequency of uterine contractions, although amplitude was unaffected. The second dose of cromakalim produced a smaller peak and shorter duration of inhibition of frequency and integral. The subsequent two doses had no significant effect. The time of onset of delivery was unaffected by cromakalim (43.4 ± 2.2 h after first injection in controls, mean ± s.e.mean, $n = 6$; 43.3 ± 2.1 in cromakalim-treated animals, $n = 5$) where delivery had taken place. Only one rat in each group did not deliver any pups. In the majority of dams some foetuses were still *in utero* and alive at the termination of the experiment on day 20. There was no difference in the proportion of foetuses *in utero* in test and control rats (55.0 ± 11.5%, $n = 5$ dams – controls; 60.8 ± 21.1%, $n = 4$ – cromakalim: $2P > 0.05$). Mean foetal weights (2.45 ± 0.25 g, $n = 7$ pups – controls; 2.52 ± 0.26 g, $n = 6$ – cromakalim) and mean placental weights

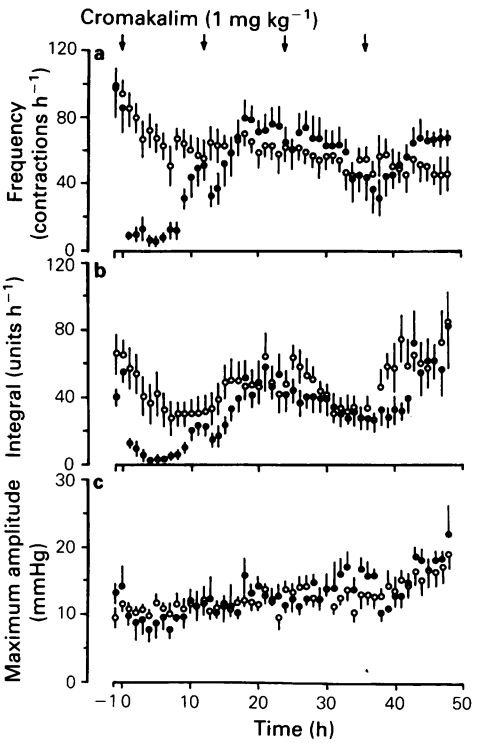

Figure 4 (a) Frequency, (b) integral and (c) maximum amplitude of uterine contractions for 1 h before and 48 h after the first of four bolus i.v. injections of either cromakalim (1 mg kg^{-1}, $n = 6$, ●) or vehicle (1 ml kg^{-1}, $n = 6$, ○) given at 0, 12, 24 and 36 h (shown as ↓) in rats starting on day 18 of pregnancy. Data are means and vertical lines show s.e.mean.

(0.47 ± 0.03 g, $n = 7$ – controls; 0.50 ± 0.05 g, $n = 6$ – cromakalim) did not differ significantly between cromakalim- and vehicle-treated animals.

Tolerance to uterine effects

In the light of the above observations, it was decided to assess more fully the possible tolerance to the uterine effects of cromakalim. Non-pregnant rats were given a single small bolus i.v. dose of cromakalim (0.01, 0.05, 0.1 or 0.5 mg kg^{-1}) to test sensitivity. The integral of uterine contractions was measured for 0.5 h after the sensitivity test dose and expressed as a % of the integral measured for 0.5 h immediately before the bolus dose administration. Four hours later the first of three bolus i.v. doses of cromakalim (1 mg kg^{-1}) or vehicle (1 ml kg^{-1} control animals) were given 8 h apart. The same sensitivity test dose was repeated 4 h after the last dose of cromakalim (1 mg kg^{-1}) or vehicle. At least 60 min of continuous

uterine contractions had been recorded from all animals before post-treatment cromakalim sensitivity was tested. It was not possible to administer more than one sensitivity test dose of cromakalim to each animal due to the prolonged duration of action of the drug and the rapidity with which tolerance to its effect occurred. Therefore, pre- and post-tolerance ID_{50} values were derived from mean data.

Control animals showed a 7.5 fold increase in uterine sensitivity to cromakalim from day 1 to day 2 of the experiment ($2P < 0.001$, paired t-test; Figure 5). The ID_{50} values for the first and second sensitivity tests in controls were approximately 0.083 and 0.011 mg kg^{-1}, respectively. In contrast, cromakalim-treated animals showed a 3.5 fold decrease in sensitivity to the uterine relaxant effects of the drug ($2P < 0.02$). The ID_{50} values for the first and second sensitivity tests in these animals were approximately 0.083 and 0.29 mg kg^{-1}, respectively. Comparison of uterine sensitivity to cromakalim after repeated drug/vehicle treatment showed a 25 fold difference in sensitivity to cromakalim between drug- and vehicle-treated animals. This was also evident in the difference in the magnitude of the inhibitory effects of the test doses of cromakalim after repeated cromakalim or vehicle treatment ($2P < 0.05$–0.01 mg kg^{-1} test dose; $2P < 0.001 - 0.05$ mg kg^{-1}; $2P < 0.001 - 0.1$ mg kg^{-1}; $2P < 0.05 - 0.5$ mg kg^{-1}).

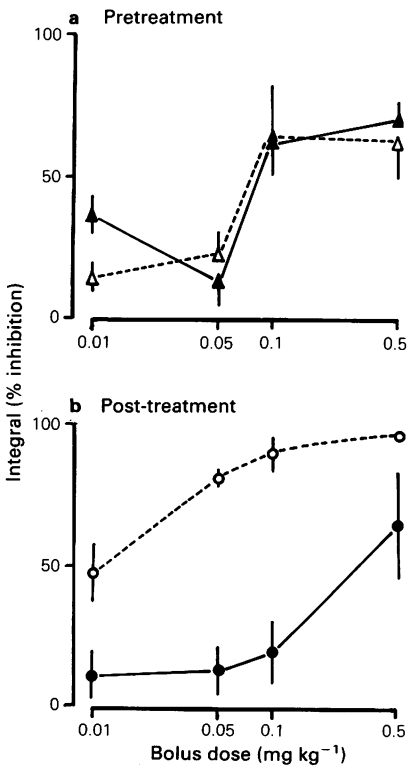

Figure 5 The % inhibition of integral of uterine contractions after bolus i.v. dose, for four sensitivity test doses of cromakalim 4 h before (a) and 4 h after (b) treatment with cromakalim, 3 × 1 mg kg^{-1}, 8 h apart ($n = 5$ rats per bolus dose; ▲ pretreatment, ● post-treatment) or 3 × 1 ml kg^{-1} vehicle 8 h apart ($n = 5$ rats per bolus dose; △ pretreatment, ○ post-treatment). Data are means and vertical lines show s.e.mean.

Discussion

This is the first time that cromakalim, or any well established K^+-channel opener, has been shown to possess uterine relaxant properties *in vivo*. This effect is seen both in non-pregnant and pregnant rats. Previously, cromakalim was shown to be a potent uterine relaxant *in vitro* (Hollingsworth *et al.*, 1987; 1988; Small *et al.*, 1988), although in these studies only tissue from term pregnant rats was used.

Like other uterine smooth muscle relaxants, cromakalim can produce a complete and immediate inhibition of spontaneous contractions. However, the pharmacological profile of cromakalim in the uterus differs in some respects from other uterine relaxants. It was clearly evident that cromakalim produced a selective inhibition of frequency with little inhibition of amplitude of contractions. This is in accordance with similar observations in isolated uterus (Hollingsworth *et al.*, 1987) and portal vein (Hamilton *et al.*, 1986; Quast, 1987; Cook *et al.*, 1988). By contrast, calcium entry blockers, such as nifedipine, produced a selective inhibition of amplitude of uterine contractions relative to frequency (Hollingsworth & Downing, 1988). These findings would support suggestions (Hollingsworth *et al.*, 1987; Quast, 1987; Cook *et al.*, 1988) that the relaxant action of cromakalim is exerted by a selective action on pacemaker cells, while calcium entry blockers selectively affect the cellular mechanism of contraction. It was evident that the effect of cromakalim in the uterus was reversible and that during the decline of its effect contractions returned in an episodic manner. The reason for this episodic nature is unclear but may relate to an action on pacemaker cells, which do not have a fixed position in the uterus (Anderson, 1978). It can be appreciated that if a pacemaker cell can initiate a spike potential this may well be conducted throughout the uterus. The effect of cromakalim on the uterus was prolonged which probably reflects a large pharmacokinetic $t_{1/2}$ of the drug.

Vasodepression was seen with cromakalim in accordance with previous findings (Buckingham *et al.*, 1986; Cook *et al.*, 1988). The cardiovascular and

uterine effects occurred at the same doses. Salbutamol, an agonist at β-adrenoceptors, and nifedipine, a calcium entry blocker, also produced vasodepression and the former a marked tachycardia in rats at uterine inhibitory doses (Abel & Hollingsworth, 1985; Downing & Hollingsworth, 1988; Hollingsworth & Downing, 1988). It is somewhat difficult to compare the uterine:cardiovascular selectivities of drugs with very different durations or profiles of action, but it would appear that cromakalim is no more, and may be less, selective than nifedipine or salbutamol for the uterus. Further studies using other experimental designs and hormonal states are needed to clarify the selectivity of cromakalim.

The most unexpected finding of this study was that the effect of cromakalim on the uterus declined with repeated dosage. As far as we are aware this is the first account of tolerance *in vivo* to cromakalim or any proposed K^+-channel opener. This feature was present in non-pregnant rats and very evident in pregnant animals, such that in the latter there was only a small uterine inhibitory response to the second dose and none to subsequent doses. There are at least three possible explanations. First, if cromakalim is mainly eliminated by hepatic metabolism, there could be enhanced clearance with repeated doses resulting in a reduced $t_{1/2}$. However, in the pregnant rats there was a complete absence of effect within 24 h of starting treatment which is difficult to equate with a change in clearance. Also there are no published data describing a decline in sensitivity of cardiovascular effects to prolonged treatment with cromakalim, which would have been expected if a significant change in metabolism of the drug had occurred. A second explanation is that a metabolite is an antagonist of cromakalim. Although this explanation is compatible with the data from the sensitivity test experiment, there is no published evidence that any of the metabolites of cromakalim have significant pharmacological activity. A third explanation could be that tolerance occurred to cromakalim in the uterus, that is a decrease in sensitivity at its site of action. This is the simplest reason for the observations in the sensitivity test experiment. Quast (1987), Coldwell & Howlett (1986) and Cook *et al.* (1988) have shown that the increase in $^{86}Rb^+$ efflux from isolated portal veins induced by cromakalim is not sustained during prolonged contact with the drug at high concentrations. Also, Nakao *et al.* (1988) have shown that the hyperpolarizing action of high concentrations of cromakalim in the guinea-pig isolated mesenteric artery was not maintained in its continued presence. Whether these *in vivo* and *in vitro* phenomena are related requires further investigation. Clearly further studies, in which plasma concentrations of cromakalim are measured simultaneously with the effect of cromakalim, will help to resolve the question as to whether kinetic or dynamic reasons underlie tolerance.

The tolerance to cromakalim probably explains the inability of the drug to prevent preterm labour although cromakalim initially produced complete abolition of uterine contractions. Tolerance to salbutamol in the uterus and a similar failure to prevent preterm labour has been seen previously (Abel & Hollingsworth, 1986b). If pharmacodynamic tolerance is a phenomenon with cromakalim, it will be interesting to determine whether this is occurring at its binding site, at the K^+-channel or at some second messenger site. This assumes that the uterine relaxant action *in vivo* is due to K^+-channel opening. Tolerance will also be a useful analytical tool to ascertain whether other purported K^+-channel openers share a common mechanism of action with cromakalim.

We would like to express our thanks to Dr T. Hamilton (Beechams) for cromakalim and to Action Research for the Crippled Child, Beechams Pharmaceuticals and the Medical Research Council for financial support.

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Effects of tetrodotoxin on relaxation of pig duodenal circular muscle induced by C-terminal fragments of cholecystokinin

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1 The mechanisms by which cholecystokinin (CCK)-C-terminal peptides relax the isolated duodenal circular muscles of pigs were compared with mechanisms involving high potassium and electrical stimulation.

2 The relaxation (30%) induced by CCK-8 0.263 nM was completely and non-competitively blocked by tetrodotoxin (TTX) 0.313 μM, and was enhanced 2–2.5 times by mesaconitine 0.158 μM, tetraethylammonium (TEA) 0.597 mM and ouabain 0.136 μM.

3 The TTX sensitivity was greater for CCK-8 and CCK-6, than for CCK-5 and CCK-4.

4 High potassium (11.8–23.6 mM)-induced, or electrically stimulated (0.3–30 Hz) relaxation showed similar characteristics except that they were not antagonized by the CCK antagonist P3-I (168 μM).

5 Apamin (0.385–3.85 μM) enhanced relaxation induced by 10.6 mM K^+, but did not affect relaxation induced by CCK-8.

6 These results indicate that: (1) CCK-8, K^+, and transmural electrical stimulation relax duodenal circular muscles by releasing an endogenous inhibitory substance; (2) the latter two do not mediate the release of CCK; and (3) the mechanisms for relaxation by CCK-4 are different from those of CCK-8.

Introduction

Cholecystokinin (CCK) relaxes the lower oesophageal sphincters (Sturdevant & Kun, 1974), the sphincter of Oddi (Sandblom *et al.*, 1935), and the duodenal circular muscles of pigs (Kimura *et al.*, 1983). Two distinct receptors for CCK have been reported: an inhibitory one present in non-cholinergic and non-adrenergic neurones, and an excitatory one in circular muscles (Behar & Biancani, 1977; 1980). The electrophysiological basis of the relaxation is clearly not suppression of Ca^{2+} spikes but hyperpolarization (Kimura *et al.*, 1983).

On the other hand, we demonstrated that duodenal circular muscles were relaxed by high K^+ (Kimura *et al.*, 1984). The relaxation was correlated with hyperpolarization of the muscle membranes and during the K^+-induced relaxation, electrically evoked Ca^{2+} spikes were not suppressed, nor were the Ca^{2+} spikes produced during the CCK-4-induced relaxation. Tetrodotoxin blocked both high K^+-induced relaxation as well as hyperpolarization.

In the present study, we have investigated the mechanisms of relaxation produced by CCK, high K^+ and electrical stimulation-induced relaxation. We have defined further the differences in relaxation mechanism between CCK-8 and CCK-4.

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Method

Pigs of either sex weighing about 90–110 kg were stunned by electrical shock and bled in the slaughter house.

The duodenal segments located about 3–5 cm from the bile duct ampulla were isolated. After the mucous membranes surrounding the segments were cut away, the circular muscles were separated from the longitudinal muscles and from serous membranes, and were then dissected into 2 × 10 mm strips. The circular strips thus obtained were suspended under 0.5 g loading in a 5 ml organ bath filled with modified Krebs solution (mM: NaCl 122, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 15.5 and glucose 11.5). The bath solution was maintained at 37°C, and bubbled with 95% O_2 and 5% CO_2.

For transmural stimulation, circular muscles were placed through the loops of a pair of electrodes (platinum wire) 5.5 mm apart. Every 20 s rectangular

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pulses (5 ms duration) were delivered at 0.3, 3 and 30 Hz. High K^+ solutions (10.6–47.2 mM) were prepared by replacing NaCl with equivalent amounts of KCl. Low Na^+ solutions (107–27.7 mM) were prepared by replacing NaCl with osmotically equivalent amounts of sucrose. For 'Ca^{2+}-free solution' $CaCl_2$ was omitted from and EGTA (2 mM) added to the bathing solution. Isotonic tension was recorded on a polygraph (W1-180, Nihon Kohden) with an isotonic transducer (TD-112S, Nihon Kohden) coupled to a biophysical amplifier (RB-2, Nihon Kohden). Tension changes from the normal tone were expressed as a percentage of the value obtained with the Ca^{2+}-free solution.

The drugs used were tetrodotoxin (TTX, Sankyo), mesaconitine (a gift from Prof. H. Hikino, Dept. of Pharmacognosy, Tohoku University), tetraethylammonium (TEA, Nakarai), apamin (Sigma), ouabain (Merck), CCK-8 (Peptide Institute), CCK-6 and CCK-5 (gifts from Dr G. Tsukamoto, Kanebo Chemical Institute), AOC(tert-amyloxycarbonyl)-CCK-4 (a gift from Dr Y. Ishii, Nihon Kayaku), proglumide-peptide CCK antagonists, P3-I (L-isomer) and P2-II (D) (D-4-carbobenzoxyamido-*N*,*N*-dipropylglutaramic acid, Phe-NH_2) Kanebo Chemical Institute) (Kimura *et al.*, 1987), vasoactive intestinal peptide (VIP, Peninsula Laboratories, Inc.), papaverine HCl (Nakarai), and (±)-isoprenaline HCl (Kaken). TTX, TEA, apamin, ouabain, papaverine, (±)-isoprenaline, and VIP were dissolved in distilled water. Mesaconitine was dissolved in 0.1 N HCl solution, and diluted with distilled water. CCK-8 and P3-I (L-4-benzamido-*N*-*N*-dipropylglutaramic acid, Asp-Phe-NH_2) were dissolved in 0.1 N Na_2CO_3 solution, and diluted with distilled water. P2-II (D) was dissolved in DMF, and diluted with distilled water. A 0.1 ml volume of the 50 fold concentrated solution was added to the 5 ml bath solution.

Results

Effects of tetrodotoxin on relaxations induced by cholecystokinin C-terminal peptides

The effects of TTX on the relaxation induced by CCK-C-terminal peptides were compared with those produced by high K^+ or electrical (transmural) stimulation. All three kinds of relaxation are accompanied by hyperpolarization (Kimura *et al.*, 1983; 1984), suggesting the involvement of some inhibitory transmitters. The effect of CCK-8 was blocked non-competitively by 0.313 μM TTX, and completely abolished by 3.13 μM TTX as shown in Figure 1a. Typical data are shown in Figure 4a. TTX alone in the concentrations shown, had no direct effect on resting tension. The sensitivity to blockade by TTX

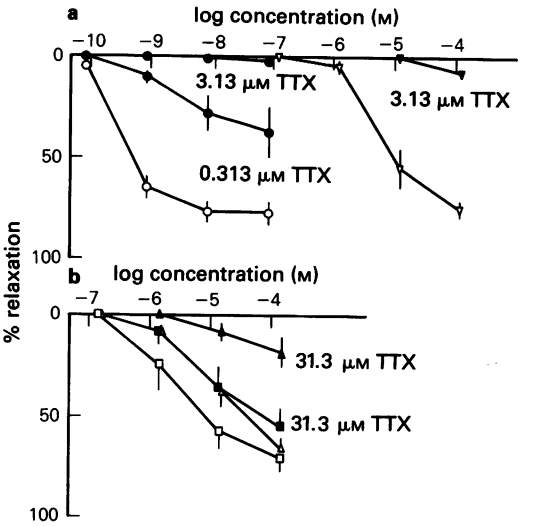

Figure 1 (a and b) Log cumulative concentration-relaxation curves to CCK-8 (○, ●) and CCK-6 (▽, ▼) (a), and CCK-5 (△, ▲) and CCK-4 (□, ■) (b) in the presence (closed symbols) or absence (open symbols) of 0.313–31.3 μM tetrodotoxin (TTX). The relaxation is expressed as a percentage of the maximal reduction in tone on changeover to Ca^{2+}-free Krebs solution containing 2 mM EGTA. Each point represents the mean (n = 3–4) and vertical lines show s.e.mean.

of the response to CCK-6 was similar to that for CCK-8. In contrast, responses to CCK-5 and CCK-4 were affected to a smaller extent, even by high concentrations of TTX (Figure 1b). Thus, the effectiveness of TTX in suppressing the relaxant effect of various CCK-C-terminal peptides is as follows: CCK-8 ⩾ CCK-6 > CCK-5 > CCK-4.

Effects of tetrodotoxin on relaxation to high K^+

The effect of TTX on the responses to high K^+ concentrations was investigated as shown in Figure 2a. Na^+ in the nutrient solution was replaced with K^+ to give 11.8, 13.0, 14.2, 17.7, 23.6, 47.2 and 143.4 mM K^+ solutions. The onset of relaxation was 20–30 s after application of a high K^+ solution. The maximal relaxation, which occurred in 17.7 mM K^+, was estimated to be 82% of that obtained upon changeover to Ca^{2+}-free solution containing 2 mM EGTA. K^+ concentrations higher than 23.6 mM caused a weak relaxation followed by a transient contraction. The contraction was measured from the level of tone in Ca^{2+}-free solution. The low concentration of TTX (0.313 μM) completely suppressed the high K^+-induced relaxation (Figure 2a); a typical effect is shown in Figure 4b.

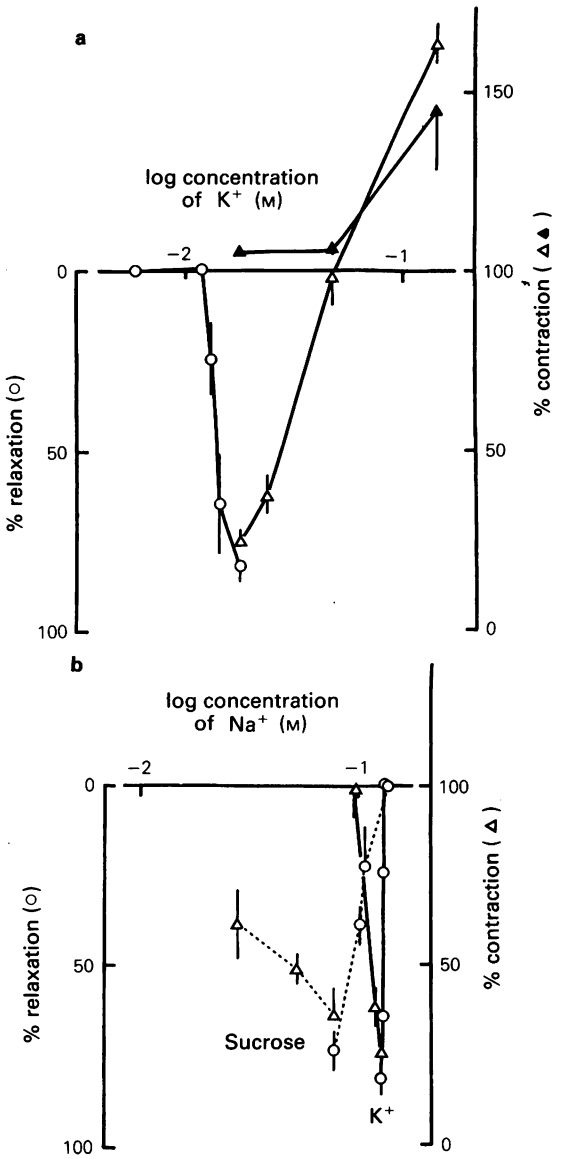

Figure 2 (a) Log concentration-response curves for the relaxation (○) and contraction (△, ▲) induced by increasing concentration of K^+ in the presence (closed symbols) or absence (open symbols) of 0.313 μM tetrodotoxin (TTX). The relaxations are as in Figure 1. The contractions after relaxation were measured from the level of minimum tone due to deprivation of calcium. Each point represents the mean (n = 4–11) and the vertical lines show s.e.mean. (b) Log Na^+ concentration-response curves for relaxations (○) and contractions (△). NaCl was replaced with equivalent amounts of KCl (solid lines) and with sucrose (broken lines). The relaxations and contractions are represented as in (a). Each point represents the mean (n = 4–11), and the vertical lines show s.e.mean.

The patterns of responses to high K^+ were subtly different from those to low Na^+ without changing the K^+ concentration (sucrose substitution) as shown in Figure 2b. The onset of relaxation after applying low Na^+ was comparatively slow (60–90 s); the maximal relaxation was estimated to be 75%. Relaxations to low Na^+ were observed over a comparatively wide range (76.5–107 mM) of concentrations. These results indicate that the effects of high K^+ were not mainly due to the decrease in Na^+ concentration.

Effect of tetrodotoxin on relaxation to transmural stimulation

Circular muscles were stimulated transmurally with trains of pulses (3–3.5 V, 5 ms) with increasing frequency (0.3, 3, and 30 Hz every 20 s). The relaxation response at all frequencies was reduced in a concentration-dependent manner by TTX (0.313 and 3.13 μM) as shown in Figure 3. The higher concentrations of TTX almost completely blocked the relaxation. The sensitivity to TTX of the relaxation in response to field stimulation is thus similar to that observed for CCK-8- or high K^+-induced relaxation.

Comparison of effects of mesaconitine, tetraethylammonium, apamin, ouabain, and CCK antagonists on CCK-8- and high K^+-induced relaxation

Mesaconitine, a Na^+-channel activator (Catterall *et al.*, 1981), TEA, apamin and ouabain were used to investigate the possibility that activation of ion channels and the Na/K pump are involved in CCK-8(0.263 nM)- and high K^+-induced relaxation. Mesaconitine 0.158 μM, TEA 0.597 mM and ouabain

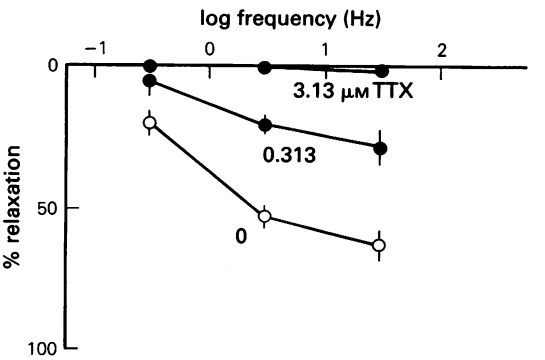

Figure 3 Log stimulation frequency-relaxation curves in the presence (●) or absence (○) of 0.313 or 3.13 μM tetrodotoxin (TTX) (15 min pretreatment). The circular muscle was stimulated with trains of pulses (3–3.5 V, 5 ms) with increasing frequency (0.3, 3 and 30 Hz every 20 s). The extent of relaxation is represented as in Figure 1. Each point represents the mean (n = 3) and the vertical lines show s.e.mean.

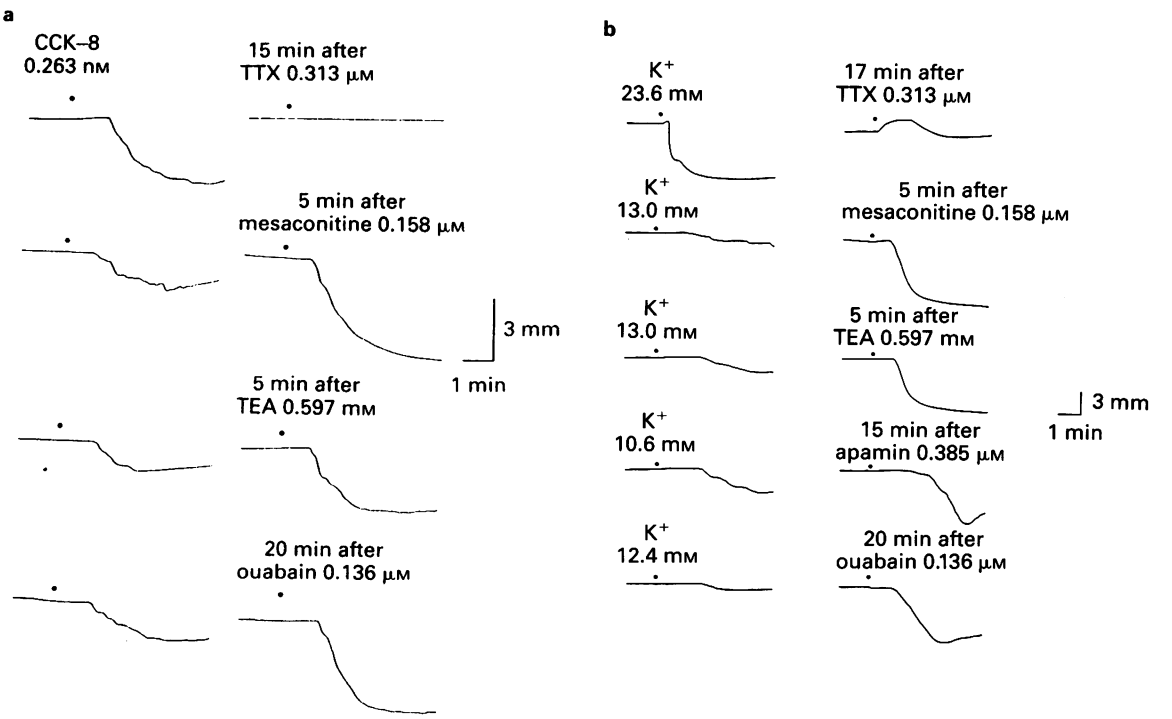

Figure 4 (a) Typical records of the responses induced by 0.263 nM CCK-8 (given at ●) with (right hand column) and without (left hand column) 0.313 μM tetrodotoxin (TTX), 0.158 μM mesaconitine, 0.597 mM tetraethylammonium (TEA), and 0.136 μM ouabain. Similar results were obtained consistently in 4–5 separate experiments. (b) Typical records showing the responses induced by 10.6–23.6 mM K^+ (given at ●) with (right hand column) and without (left hand column) 0.313 μM TTX, 0.158 μM mesaconitine, 0.597 mM TEA, 0.385 μM apamin, and 0.136 μM ouabain. These effects occurred consistently in 2–4 separate experiments.

0.136 μM markedly enhanced the relaxation produced by CCK-8 0.263 nM (Figure 4a), while apamin, even at a high concentration (3.85 μM), had no effect.

The weak relaxation induced by high K^+, up to 13.0 mM, was greatly enhanced by the above treatments with mesaconitine, TEA, or ouabain but also by apamin (0.385 μM) (Figure 4b). The relaxation due to high K^+ (up to 23.6 mM) was hardly affected by P3-I (168 μM), a competitive CCK antagonist (Kimura *et al.*, 1987).

The response to transmural electrical field stimulation was enhanced by TEA to a small but significant extent and by mesaconitine (data not shown), but was little affected by ouabain, P3-I or P2-II (D) (19.6 μM).

Papaverine (0.266 μM–0.266 mM) and (±)-isoprenaline (4.04 nM–4.04 μM) relaxed the muscles in a concentration-dependent manner (data not shown); the relaxations induced by 2.66 μM papaverine or by 40.4 nM isoprenaline were unaffected by the above concentrations of TTX, mesaconitine, or TEA (data not shown).

The vasoactive intestinal peptide concentration-relaxation curve compared with that of CCK-8

The concentration-response curve to CCK-8 was compared with that for VIP, a putative candidate as an inhibitory neurotransmitter released by transmural electrical stimulation (Biancani *et al.*, 1984). The potency of VIP was approximately 2 orders of magnitude lower than that for CCK-8 (Figure 5) and the relative intrinsic activity of VIP was 0.93 which is greater than that of CCK-8 (0.78). The VIP-induced relaxation was not suppressed by 3.13 μM tetrodotoxin (data not shown).

Discussion

CCK receptors have been classified as being of 'peripheral' or 'central' type. Peripheral CCK receptors are further classified as neurogenic (duodenum,

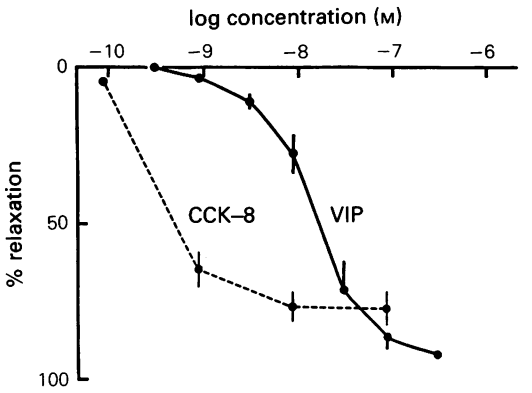

Figure 5 Log concentration-relaxation curve of cumulatively administered (every 5 min) vasoactive intestinal peptide (VIP). The relaxation is represented as in Figure 1a. Each point represents the mean ($n = 4$) and the vertical lines indicate s.e.mean. The curve for CCK-8 is replotted from Figure 1.

sphincter of Oddi, and gallbladder), exocrine (amylase secretion), and endocrine (insulin release). CCK-8 has a higher affinity than CCK-4 for the peripheral receptor. Behar & Biancani (1977, 1980) reported that CCK relaxes the lower oesophageal and choledochal sphincter of the cat by releasing an inhibitory transmitter, since a direct contractile effect can be unmasked by exposure to TTX. TTX is known to abolish almost all neural activity by blocking Na^+ channels. It also blocks neurotransmitter release (Kao, 1972). In the present study relaxation of duodenal circular muscles induced by CCK was inhibited completely by TTX under conditions where no contraction occurred. In contrast, the CCK-4-induced relaxation was suppressed only in part even by a high concentration of TTX. Relaxations in response to CCK-6, which has a lower affinity for CCK receptors and relatively lower intrinsic activity than CCK-8, were, like those with CCK-8, highly sensitive to TTX. Responses to CCK-5 showed a pattern intermediate between that for CCK-6 and CCK-4. In a previous paper (Kimura *et al.*, 1987), we showed different patterns of antagonism with the CCK antagonists, P2-II (D) and P3-I against CCK-8- and CCK-4-induced relaxation. The present results indicate that CCK-8 is completely TTX-sensitive, but that CCK-4 is able to invoke another mechanism for relaxation that is TTX-insensitive, probably by acting directly on muscle cells.

It is expected from the Goldman equation that high K^+ solutions would produce depolarization of the membrane and would produce contraction of the smooth muscle. In circular muscles of pig duodenum, however, it hyperpolarized the membrane and relaxed the muscle (Kimura *et al.*, 1984) as is reported for arterial muscles (Reiner, 1978), anococcygeus muscle (Ishii & Shimo, 1980) and taenia coli (Ishii & Shimo, 1979). The relaxation was concentration-dependent in a very narrow concentration range of K^+. Reducing the Na^+ concentration to less than 20 mM depolarized the circular muscles of the guinea-pig stomach and contracture developed (Sakamoto & Tomita, 1982). However, low Na^+ solutions prepared by replacing NaCl with an osmotically equivalent amount of sucrose relaxed the muscles over a comparatively wide range of concentrations. This is somewhat different from the relaxing pattern of high K^+. In a previous paper (Kimura *et al.*, 1984) we reported that both K^+-induced or electrically stimulated relaxation was inhibited by TTX. Mesaconitine, which increases Na permeability (Catterall *et al.*, 1981), potentiated the three kinds of relaxation by CCK-8, high K^+ or electrical stimulation.

TEA also potentiated the three kinds of relaxation. TEA prolongs the action potential in nerves by preventing the delayed increase in K^+ permeability (Armstrong & Binstock, 1965), and it increases the liberation of a transmitter from the nerve as a result of an increased entry of Ca^{2+} (Gillespie & Tilmisany, 1976). Apamin was reported to inhibit the relaxation induced by noradrenaline and adenosine triphosphate in guinea-pig taenia coli (Weir & Weston, 1986). In the present study, apamin also potentiated the K^+-induced relaxation, while it did not affect that produced by CCK-8. These results indicate that the mechanisms for relaxation by K^+ and CCK-8 are somewhat different and that the activation of the Ca^{2+}-activated K^+-channel is not involved in CCK-8-induced relaxation. Ouabain, which potentiates the release of transmitters from nerves (Paton *et al.*, 1971; Bonaccorsi *et al.*, 1977), potentiated both relaxation induced by CCK-8 and K^+ (12.4–13.0 mM). Ouabain, mesaconitine and TEA had little effect on transmural and electrically stimulated relaxation; the reason for this is unknown.

In previous papers (Kimura *et al.*, 1983; 1984), we reported that the three kinds of treatment hyperpolarized the membrane. Two possible mechanisms are considered to explain the hyperpolarization: activation of the K^+-channel and the Na^+-K^+-pump. CCK-8- and high K^+-induced relaxations, however, were potentiated both by TEA or by ouabain. These results could not be explained in terms of direct action on the smooth muscles. The properties of the three kinds of relaxation are summarized in Table 1. It is postulated that all three agents producing relaxation involve the release of some inhibitory substances. The main difference among the three relaxants clearly concerns both CCK antagonist-

Table 1 The common properties of CCK-8, K^+ and electrically stimulated relaxation in pig duodenal circular muscle

	CCK Antagonist	*Na channel*		*K channel*		*Na^+-K^+ pump*	*Resting membrane potential*
		TTX	*Mesaconitine*	*TEA*	*Apamin*	*Ouabain*	
CCK-8	Inhibits	Inhibits	Potentiates	Potentiates	No effect	Potentiates	(Hyperpolarizes)[a]
K^+	No effect	Inhibits	Potentiates	Potentiates	Potentiates	Potentiates	Hyperpolarizes
Electrical	No effect	Inhibits	Potentiates	Potentiates	—	No effect	Hyperpolarizes

CCK-8 (0.263 nM), K^+ (10.6–23.6 mM), electrical stimulation (3–3.5 V, 5 ms, 0.3–30 Hz for 20 s), CCK antagonist P3-I (168 μM) and P2-II(D) (19.6 μM), TTX (0.313 μM), mesaconitine (0.158 μM), TEA (0.597 mM), apamin (0.385–3.85 μM), and ouabain (0.136 μM).

[a] The effect of CCK-4 (Kimura *et al.*, 1983).

and Ca^{2+}-dependent K^+-channel antagonist-actions, as shown in Table 1.

CCK-8 relaxed the duodenal circular muscles by mechanisms clearly different from those for papaverine or isoprenaline, both of which were TTX-insensitive. VIP, which was proposed by Biancani *et al.* (1984) as a possible neurotransmitter candidate released by transmural stimulation in the lower oesophageal sphincter, relaxed the duodenal circular muscles and this effect was resistant to TTX.

In conclusion, CCK-8, K^+, and transmural electrical field stimulation relaxed duodenal circular muscles by releasing an endogenous inhibitory substance. The latter two mechanisms did not involve the release of CCK. The mechanisms for relaxation by CCK-4 are different from those of CCK-8.

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Vasoactive intestinal polypeptide and non-adrenergic, non-cholinergic inhibition in lower oesophageal sphincter of opossum

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1 Field stimulation or vasoactive intestinal polypeptide (VIP) relaxed lower oesophageal sphincter (LOS) from North American opossum. Pretreatment with carbachol in Cl-ion-containing or Cl-ion-free Krebs solution or with 10^{-3} M 9-aminoacridine abolished or markedly reduced relaxation due to VIP applied exogenously but not that elicited by field stimulation of non-adrenergic, non-cholinergic nerves.

2 Inhibitory junction potentials (7.5 ± 1.2 mV, $n = 5$) could be recorded in LOS strips with the sucrose gap technique. They lacked significant after-depolarizations but were accompanied by decreased membrane resistance ($61 \pm 6\%$, $n = 3$). In these strips, VIP (10^{-6} M) produced small hyperpolarizations (2.1 ± 1.1 mV, $n = 5$) sometimes followed by membrane potential oscillations but no change in conductance.

3 Removal of external chloride depolarized the membranes (7.6 ± 1.7 mV) but did not prevent the hyperpolarization to VIP or the occurrence of inhibitory junction potentials. Restoration of external chloride repolarized the cells. It appears that an appreciable chloride conductance may be present in sphincter muscle cells and this may cause them to be more depolarized than non-sphincter muscle.

4 We conclude that it is very unlikely that VIP is the inhibitory NANC neurotransmitter since it does not mimic the inhibitory junction potential.

Introduction

Previous studies of opossum lower oesophageal sphincter (LOS) *in vivo* revealed the existence of relaxation which could be activated by stimulation of vagal preganglionic nerves, by field stimulation of post-ganglionic non-adrenergic, non-cholinergic nerves (NANC), by swallows or by proximal balloon distention (Rattan & Goyal, 1974; Sarna *et al.*, 1977; Daniel *et al.*, 1979; Goyal & Rattan, 1978). Further studies suggested that the mediator might be vasoactive intestinal polypeptide (VIP) since VIP given intra-arterially relaxed the LOS by a direct action and VIP-antiserum reduced the relaxation to vagal stimulation but not that to isoprenaline (Goyal *et al.*, 1980; Rattan *et al*, 1982).

However, studies of the circular muscle just proximal to the LOS revealed that VIP could not be the mediator of NANC-inhibition since its electrophysiological actions did not mimic those of the mediator. In particular, the mediators released by field stimulation caused an inhibitory junction potential (i.j.p.) by increasing the K^+-conductance of the smooth muscle cell membrane (Daniel *et al.*, 1983; Jury *et al.*, 1985). In contrast, VIP caused slight or no membrane hyperpolarization accompanied by a decreased membrane conductance, followed by membrane repolarization and membrane oscillations, which triggered action potentials on the depolarizing phase (Daniel *et al.*, 1983; 1987). Further, a material similar to VIP (in its electrophysiological effects) was shown to be released by dopamine acting via dopamine (D_1-like) receptors on nerve endings (Daniel *et al.*, 1987). This release was tetrodotoxin-insensitive but sensitive to scorpion venom and solutions containing low Ca and high Mg. Both the effects of dopamine and VIP were abolished by removal of external chloride (Daniel *et al.*, 1987). Dopamine-induced hyperpolarization, caused by the release of an endogenous VIP-like

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material, showed a reversal potential near the chloride-equilibrium potential. Thus, it was not possible that VIP was the transmitter released by NANC inhibitory nerves acting directly on circular muscle of the body of the oesophagus (BCM) since the actions of exogenous VIP and endogenous VIP-like material were similar to each other but differed from the actions of the mediator.

The purpose of the present study was to determine whether VIP could be the mediator of the NANC-relaxation in the LOS itself. Since LOS preparations, which have fewer gap junctions than proximal muscle (Daniel & Posey-Daniel, 1984), often fail to show nerve-mediated responses in the sucrose gap, we also studied VIP- and electrically-evoked relaxations of the LOS in an *in vitro* muscle chamber. We examined whether removal of external chloride or application of 9-aminoacridine (9-AA), a putative potassium-conductance blocking agent (Volle, 1971; Volle & Henderson, 1972) affected relaxations to these two stimuli differently. In addition, experiments were carried out with the double sucrose gap technique to characterize the response of the muscle to VIP and to the nerve mediator.

Methods

North American opossums (*Didelphis marsupialis*) were obtained (Singleton, FL, U.S.A.), handled as previously described (Daniel *et al.*, 1983) and killed by intraperitoneal pentobarbitone ($100\,mg\,kg^{-1}$). The oesophagus and proximal stomach were removed and dissected as previously described (Daniel *et al.*, 1983). After removal of the mucosa, the LOS was identified by its thickened structure and locus and divided into 2 mm wide strips. Strips from the centre of this thickened muscle were included in the results presented. These were mounted through concentric platinum electrodes 1 cm apart in 4 ml muscle baths containing a modified Krebs solution, warmed to 37°C and bubbled with 95% O_2 and 5% CO_2. This solution contained (mM): NaCl 115, KCl 4.5, $NaHCO_3$ 21.9, NaH_2PO_4 1.16, $CaCl_2$ 2.5, $MgSO_4$ 1.16 and glucose 11. Cl-free solution was made by substituting NaCl and KCl with isethionate salts and Calevulinate for $CaCl_2$. The pH of all solutions used was 7.4 at 37°C.

The tissue was tied at one end to the electrode holder and at the other to a strain gauge transducer (Grass FT03C). Tension was recorded on a Beckman R611-8 channel oscillograph. 9-Aminoacridine was obtained from Fluka. Since chloride-free solutions first increased but subsequently abolished the basal active tension of LOS strips, carbachol (10^{-6} M) was added after chloride removal to restore tone. We have shown previously (Daniel *et al.*, 1979) that this does not impair relaxation to field stimulation. Field stimulation was carried out for 10 s at 40 V, 0.5 ms, 5 Hz, parameters shown previously (Daniel *et al.*, 1979) to cause maximal and complete relaxation. We also studied inhibitory junction potentials (i.j.p.) in response to field stimulation (30 Hz, 0.3 ms duration, with supramaximal field strength $20\,V\,mm^{-1}$ for 300 ms trains) by the double sucrose gap technique as previously described (Jury *et al.*, 1985; Daniel *et al.*, 1987). These were compared to the effects of VIP (10^{-6} M). Also, changes in conductance during the i.j.p. and the responses to VIP were compared by applying brief pulses of hyperpolarizing currents. The effects of chloride-free solutions were also studied. Data were expressed as means ± standard error of mean (s.e. mean) and statistical significance was determined using Student's *t* test for paired or unpaired values as appropriate ($P < 0.05$ was taken to be significant).

Results

After the strips of LOS were placed in the muscle bath under 10 mN tension, they rapidly acquired additional tone of about 10 to 15 mN. After 1 h equilibration, field stimulation (FS) caused maximal relaxation, with an amplitude of 13.6 ± 1.2 mN ($n = 49$). This represented the active tension and left a residual passive tension of 5 to 10 mN. Subsequently, VIP in a concentration (10^{-6} M) found in preliminary studies to be maximal in effect, was tested in the same strips. It caused a gradual relaxation equal to 107 ± 11% ($n = 49$) of the rapid relaxation produced by FS in the same tissues. After washout of VIP, the preparations regained active tension. Then Cl-free solution followed by carbachol was used and effects of FS and VIP retested. Substitution of isethionate for chloride resulted in loss of active tension; carbachol-induced contraction was, therefore, induced to allow relaxation to be observed. Figure 1 shows typical results of experiments which are summarized in Table 1. Cl-free medium abolished or nearly abolished the relaxation to VIP (compare traces (i) and (v) in Figure 1a) but had little effect on relaxation to FS (compare traces (i) and (iii) in Figure 1b).

Further analysis showed that the VIP-induced relaxation was already slowed and impaired in magnitude (compare traces (i) and (iii) in Figure 1a) or abolished by the use of carbachol in normal medium to cause contraction. Table 1 shows that on average, VIP-induced relaxation was nearly complete within 2 min in the absence of carbachol but attained a smaller and slower relaxation only after 4 min when carbachol was present. A stability control for

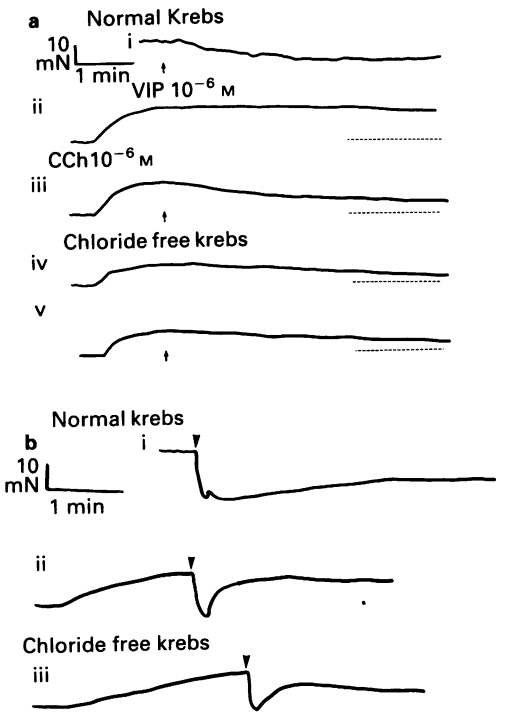

Figure 1 Comparison of relaxant responses to field stimulation (FS) and VIP in carbachol (CCh) and chloride-free media. (a) All tracings from the same strip: (i) control responses to VIP 10^{-6} M; (ii) and (iii) show the response of the tissue to carbachol (10^{-6} M) without or with the addition of VIP (10^{-6} M) at the arrow. The dashed lines show the starting tension in each case. Note that there appears to be a definite but smaller relaxation to VIP. This was not always found. (iv) and (v) show responses to carbachol (10^{-6} M) in chloride-free bathing solution. There was no increase in the decay of tension with time when VIP was added at the arrow. (b) (i) Control relaxation response to FS (10 s, 5 Hz, 0.5 ms, 40 V); (ii) relaxation to FS in CCh 10^{-6} M; (iii) relaxation to FS in CCh added to chloride-free bathing solution. The degree of relaxation is similar in all cases but recovery of tension was faster after CCh.

carbachol-induced tension in the same strips showed that there was usually a significant spontaneous decay of tension induced by carbachol after 4 min (not evident in the second trace in the figure). Removal of chloride from the medium sometimes (as in trace (iv) in Figure 1a) increased the rate of decay of the carbachol-induced tension but did not affect the magnitude of the relaxation which had occurred 4 min after addition of VIP. Thus, the difference between the normal relaxation with time and the VIP-induced relaxation was markedly less in this medium. However, field stimulated relaxations persisted in normal Krebs medium or chloride-free medium with carbachol as a nearly constant percentage of the total active tension (Table 1). Figure 1b shows an example in which FS relaxed the LOS after carbachol in either normal or chloride-free medium. The recovery of active tension after the stimulus had stopped was faster in the presence of this agonist in this and most strips.

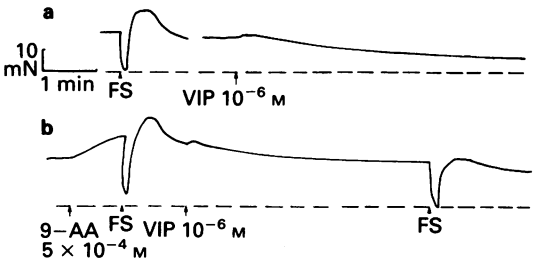

Figure 2 Comparison of the effects of 9-aminoacridine (9-AA) on the relaxant responses to field stimulation (FS) and VIP. (a) Control response to FS, (10 s, 5 Hz, 0.5 ms, 40 V), and VIP (10^{-6} M); (b) responses to FS and VIP evoked in the presence of 9-AA 5×10^{-4} M. Note that FS evoked a more complete relaxation without an 'off-response' above basal tension in the presence of VIP. However, VIP failed to relax the preparation; the decay in the tension after VIP is similar to that in strips treated with 9-AA but not VIP.

The effects of a putative selective potassium channel blocker 9-AA were also tested in the organ bath studies. As illustrated in Figure 2, 9-AA increased the tone of the preparations, did not reduce the relaxation induced by field stimulation, but abolished the VIP-induced relaxation. In 11 LOS strips, which relaxed 14.0 ± 1.2 mN in control tests and 124 ± 20% of those values to 10^{-6} M VIP, 9-AA (10^{-3} M) reduced the VIP-induced relaxation to 19 ± 8% but not FS-relaxation, which remained at 149 ± 29% of control. After 10 to 15 min in 9-AA, all tone gradually disappeared; then, of course, FS had no further relaxant effect. However, in many cases spontaneous phasic contractions occurred and these were transiently abolished after field stimulation (Figure 3), suggesting that inhibitory mediator

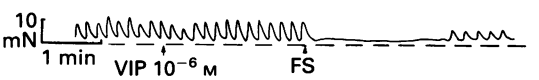

Figure 3 Effects of field stimulation and VIP on contractions induced by 9-aminoacridine (9-AA). In tissue treated with 9-AA (10^{-3} M) for 40 min, persistent active tension disappeared but spontaneous contractions developed in the presence of the putative K-channel blocker. These were not affected by VIP but were inhibited by FS.

Table 1 Effects of carbachol (CCh) and chloride-free medium on relaxation responses to field stimulation and VIP (10^{-6} M)

Normal Krebs			*ΔVIP**			*ΔNo VIP†*	*No FS*
Tension	*ΔFS†*	*Tension*	2 min	4 min	*Tension*	2 min	4 min
11.4 ± 1.9 (n = 8)	−7.8 ± 1.9 (−68%)	10.8 ± 2.2	−5.3 ± 1.4*** (−49%)	−6.4 ± 1.7*** (−59%)	12.3 ± 4.5	−0.5 ± 2.9 (4%)	−0.2 ± 2.5 (2%)
CCh			2 min	4 min		2 min	4 min
27.5 ± 3.6 (n = 8)	−18.3 ± 1.9* (−67%)	23.0 ± 2.5	−1.9 ± 0.6*,** (−8%)	−4.4 ± 1.4** (−19%)	25.6 ± 1.9	−0.1 ± 0.1 (0.4%)	−1.7 ± 0.8 (7%)
Chloride free + CCh			2 min	4 min		2 min	4 min
13.3 ± 2.5 (n = 8)	−8.9 ± 1.9 (−67%)	15.3 ± 2.8	−1.4 ± 0.6*,** (−9%)	−5.0 ± 1.8** (−32%)	14.4 ± 2.8	−0.3 ± 0.3 (2%)	−2.7 ± 1.1 (19%)

Values in mN tension mean ± s.e.mean; values in parantheses; % change in initial tension.
† Δ refers in all cases to the net change in tension; this was measured at the maximum change after field stimulation (FS) and at the times indicated in other cases.
* Significantly different by paired t test ($P < 0.05$) from values in normal Krebs without carbachol.
** Significantly different by paired t test ($P < 0.05$) from magnitude of field-stimulated relaxation.
*** Significantly different from the tensions observed with time in the absence of VIP (right-hand columns); measured at the same time interval after carbachol as when VIP added.

was still active and being released. Effects of 9-AA were not fully reversible on washing out this substance.

Inhibitory junction potentials (i.j.ps) with a peak amplitude of about 8 mV were obtained in 5 strips of LOS (Table 2). These differed from the i.j.ps recorded from body circular muscle (Daniel *et al.*, 1983; 1987; Jury *et al.*, 1985) in being smaller (cf: Table 2 and see Daniel *et al.*, 1983; 1987; Jury *et al.*, 1985) and lacking detectable after-depolarization (Figures 4 and 5). They were accompanied by a significant reduction in the magnitude of electrotonic potentials evoked by hyperpolarizing constant current pulses (Table 2 and Figure 4), indicating that there was a conductance increase. In the same strips, VIP (10^{-6} M) caused a small (about 2 mV) hyperpolarization that was not accompanied by an increase in the membrane conductance (Table 2 and Figure 5). This hyperpolarization induced by VIP did not alter the value of peak negativity of hyperpolarization associated with the i.j.p. (Figure 5), although it did reduce the amplitude of the i.j.p. to 58 ± 12% of control (n = 3). The rate of repolarization of the i.j.p. was, however, usually slowed (not evident at the

Table 2 Electrophysiological effects of field stimulation and VIP (10^{-6} M)

	i.j.p. amplitude (mV)	*Electrotonic potential during i.j.p.* (% control)	*Hyperpolarizing effect of VIP* (mV)	*Electrotonic potential in VIP* (% control)	*i.j.p. amplitude in VIP* (% control)
Normal	8.9 ± 0.8 (5)		2.2 ± 1.1 (5)*	105 ± 4 (5)	74 ± 11 (5)
Krebs	[8.2 ± 0.9] (3)	[61 ± 6 (3)]*	[3.7 ± 1.6] (3) NS	[107 ± 4] (3) NS	[58 ± 12] (3)
Cl-free	7.3 ± 2.0 (3)	—	2.7 ± 1.2 (3) NS*	97 ± 3 (3) NS	89 ± 20 (3)
▲mV Depol.					
7.6 ± 1.7 (3)*					

Mean ± s.e.mean (number of preparations in parentheses).
* Significantly different ($P < 0.05$) from 0 mV or 100%. Value in bottom line (▲) shows membrane potential change due to removal of external Cl ion.
NS not significantly different ($P > 0.05$) by paired comparison; same three strips compared in normal Krebs (values in [ ]) and in Cl-free Krebs.

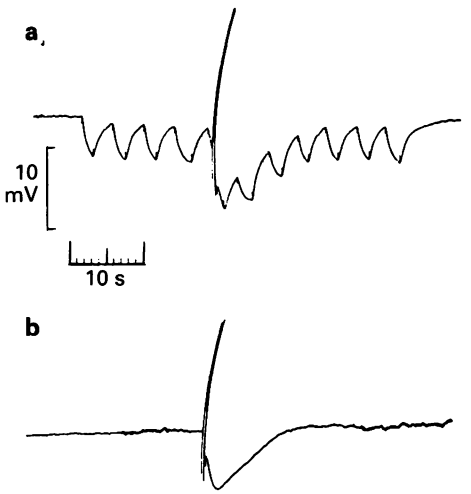

Figure 4 Conductance change associated with inhibitory junction potential (i.j.p.) (sucrose-gap recording). High speed trace of an i.j.p. of opossum oesophagus LOS in response to field stimulation (a) (300 ms, 30 Hz, 0.5 ms, 30 V); initial upward and downward deflections are stimulus artefacts. In (b) repetitive brief (2 s) hyperpolarizing constant current pulses were applied, during which an i.j.p. was elicited. Note that the size of the electrotonic potentials were reduced during the i.j.p. but recovered to control size as the membrane potential recovered. A conditioning hyperpolarization of the same magnitude as the i.j.p. did not affect these brief electrotonic potentials.

chart speed of Figure 5). During prolonged superfusion with VIP the tissue repolarized in its presence, and there were on several occasions persistent membrane oscillations (not shown). Removal of external chloride caused a persistent depolarization of the LOS (about 8 mV). This was sometimes accompanied by a gradual reduction (Figure 5) in the amplitude of the i.j.p. When the i.j.p. was reduced in amplitude (as in Figure 5), the rate of repolarization of the i.j.p. was increased. There was no significant change in the magnitude of the responses to VIP (Table 2 and Figure 5) in chloride-free superfusion medium. Likewise, the magnitude of the i.j.p. in the presence of VIP was not significantly different from the control value in chloride-free medium.

Discussion

These results show that it is possible to abolish or reduce markedly the VIP-induced relaxation or inhibitory response of the LOS by 9-AA or by carbachol in a chloride-free medium without similarly affecting the relaxation response to field stimulation. This implies that, when VIP is ineffective as a relaxant agent, the mediator of NANC relaxation is still effective. Thus, it is unlikely that VIP is that mediator. It is of interest that the induction of tension by carbachol impairs relaxation to VIP. This impairment was so extensive that removal of chloride, which abolished the effects of VIP on body circular muscle (Daniel *et al.*, 1983; 1987), had no further statistically clear effect. A recent report (Thorpy *et al.*, 1986) shows that VIP-induced relaxation of the LOS is accompanied by an increase in cyclic AMP concentrations, whereas that to field stimulation is accompanied by an increase in cyclic GMP; in several tissues carbachol acting at muscarinic receptors causes a decrease in cyclic AMP concentrations and this may explain its ability to oppose the relaxant actions of VIP but not those of field stimulation (Nathanson *et al.*, 1978; Brown & Goldstein, 1986).

Since VIP-induced relaxation could not be tested in the chloride-free medium unless tone was restored by carbachol, we could not determine whether removal of chloride *per se* selectively inhibited responses to VIP as it did in body circular muscle (BCM) (Daniel *et al.*, 1983; 1987). The observed changes in the time-course of the electrotonic potential consequent upon removal of chloride ions are, however, compatible with the passive membrane properties of smooth muscle cells (Jury *et al.*, 1985). In contrast, removal of external chloride had effects on the LOS different from those induced in BCM; there was a persistent depolarization and transient contraction (not shown) in the LOS rather than a hyperpolarization as observed in BCM. In the LOS the membrane repolarized back to the original membrane potential after re-exposure to a chloride-containing superfusion fluid.

The depolarization of the LOS when chloride was removed may imply that there is an appreciable resting chloride conductance, so that the loss of internal chloride into the chloride-free medium causes depolarization and an increase in the time constant of the electrotonic potential, as observed. This depolarization, however, should be transient, disappearing when internal chloride is lost; however, it persisted for at least 30 min in the absence of chloride. This is so far unexplained. An appreciable resting Cl-conductance, absent in the BCM, could lead to lower resting potentials in the LOS if the chloride equilibrium potential is of the order of 30 mV inside negative, as it appears to be in some smooth muscles (see references in Daniel *et al.*, 1987). This could explain the resting active tension in LOS, absent in BCM.

Previous studies in BCM (Daniel *et al.*, 1983; 1987) suggested that VIP caused a decrease in chloride conductance. Thus, chloride replacement with an impermeant anion prevented all responses to

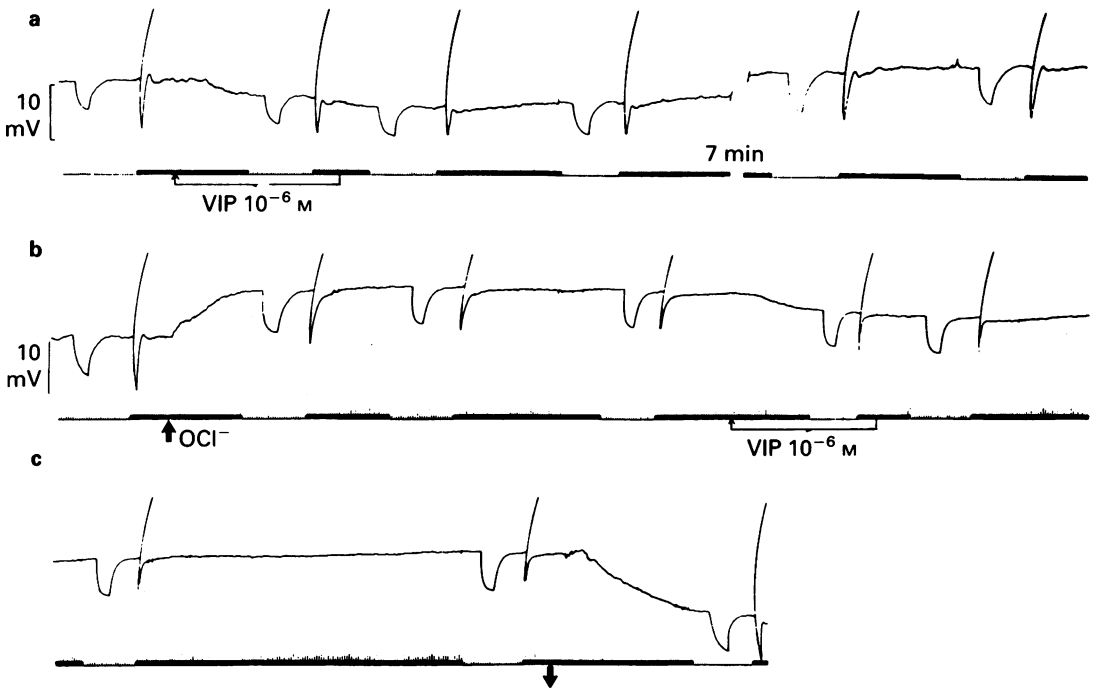

Figure 5 Comparison of the effects of chloride-free (OCl^-) bathing medium on inhibitory junction potentials (i.j.ps) and membrane responses to VIP (sucrose-gap recording). The upward deflection and the initial part of the i.j.p. are stimulus artefacts. (a) Shows effects of VIP (10^{-6} M) on the resting potential, on hyperpolarizing electrotonic potentials and on i.j.ps. In (b) the effects of substitution of isethionate for chloride are shown; a large (10 mV) and persistent depolarization leading to a gradual reduction in i.j.p. amplitude, hence in the peak negativity during the i.j.p. The applied electrotonic pulses (8–10 s) did not change in amplitude but had altered time-courses. VIP (10^{-6} M) still caused a similar (5 mV) hyperpolarization compared to control (a) although the i.j.p. was reduced, the peak negativity was the same as that before exposure to VIP, just as when Cl was present (a). In (c) which is continuous with (b), re-exposure to Cl-containing medium led to hyperpolarization to a resting potential slightly greater than the control membrane potential and to restoration of the i.j.p. amplitude without an alteration of the amplitude of electrotonic potentials. Note the varying time scale: the largest deflections show 1 min intervals, the second largest show 5 s intervals and the smallest (sometimes fused) show 1 s intervals.

exogenous VIP or endogenous VIP-like material; there was also a reversal potential near the chloride equilibrium potential for this material. Clearly, a different or additional mechanism must apply in the LOS since chloride replacement did not prevent the hyperpolarization to VIP. It was impossible to determine accurately the reversal potential of the small VIP-induced hyperpolarization in this muscle. Thus, the electrophysiological basis of the action of VIP in the LOS is unclear. If 9-AA is a potassium conductance blocker (Volle 1971; Volle & Henderson, 1972), then its ability to inhibit VIP-mediated relaxation suggests that a potassium conductance change was involved in the mechanism by which VIP acts.

There was also an obvious impairment of contractility in chloride-free media since the LOS relaxed despite persistent depolarization and carbachol contractions were smaller and less well sustained (Figure 1, Tables 1 and 2). This needs further analysis.

NANC-relaxation of gut muscle, whenever it has been analysed (Tomita, 1972; Bywater *et al.*, 1981; Jury *et al.*, 1985) is caused by increased potassium conductance. Our earlier studies (Jury *et al.*, 1985) showed that none of several established K-conductance blockers (apamin, tetraethylammonium, 4-aminopyridine or quinine) abolished the i.j.p. induced by NANC nerve stimulation in BCM, suggesting that a special postjunctional K-channel was involved. Since 9-AA does not block relaxant responses to field stimulation in concentrations effective against VIP-induced relaxation, it too, appears to be unable to affect these channels selectively. Whether VIP actions in other tissues are Cl-dependent or K-dependent remain to be determined.

Apart from the results with 9-AA, there is no evidence that VIP increases K-conductance; in contrast, we observed no change in the membrane conductance in the presence of VIP. An alternative explanation of the selective action of 9-AA on VIP induced relaxations is that 9-AA may have other actions as well as those reported (Volle, 1971; Volle & Henderson, 1972) on K-conductance.

The question arises, can these findings be reconciled with a role of VIP in NANC-mediated relaxation in the LOS. It is clear that the conductance changes underlying VIP-induced relaxation do not mimic those underlying NANC-nerve mediated responses and also their pharmacology differs. It is, therefore, difficult to maintain that the NANC nerve-mediated response in the LOS involves VIP as neurotransmitter acting directly on the smooth muscle cell membrane. Another role of VIP in the process is possible: as a secondary transmitter (Daniel *et al.*, 1983) it may add to the relaxation and lead to the subsequent 'off' contraction, as we have previously suggested. However, 'off' depolarization, which is thought to lead to 'off' contractions, was very small or absent in LOS. Alternatively, VIP may act mostly on interstitial cells of Cajal, which are major sites of innervation in oesophageal circular muscle (Daniel & Posey-Daniel, 1984) and there initiate events which are transmitted to smooth muscle via gap junction contacts ultimately causing relaxation (Daniel, 1985; Daniel *et al.*, 1985) by increasing potassium conductance. The present type of study did not provide clear evidence for VIP having such an indirect action on LOS. Future studies on this hypothesis will need to establish that VIP is present in the nerves associated with interstitial cells, is released to act on receptors on these cells, and initiates in them a process leading to a potassium conductance increase in smooth muscle cells.

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MEETINGS CALENDAR

21–23 March 1989

British Opioid Colloquium, Reading, U.K. (Dr. J.R. Traynor, Department of Chemistry, Loughborough University, Leics LE11 3TU, U.K.)

29 March–1 April 1989

International Symposium on Serotonin from Cell Biology to Pharmacology and Therapeutics, Florence, Italy (Secretariat, Fondazione Giovanni Lorenzini, Via Monte Napoleone 23, 20121 Milan, Italy).

2–5 April 1989

Seventh National Meeting of the Brain Research Association, Sheffield, U.K. (Dr. N. Payne, Dept. of Anatomy, Sheffield University, Sheffield, U.K.)

3–4 April 1989

British Neuroendocrine Group Meeting, Bristol, U.K. (Dr. C.D. Ingram, Department of Anatomy, The Medical School, University Walk, Bristol BS8 1TD).

5–7 April 1989

British Pharmacological Society Spring Meeting, Bristol, U.K. (Closed meeting for members and guests)

6–7 April 1989

International Symposium on Psychiatric Neuropathology and Neurochemistry, London, U.K. (Dr. R.W. Kerwin, Department of Neuropathology, Institute of Psychiatry, De Crespigny Park, London SE5).

7 April 1989

British Pharmacological Society. Symposium on the NMDA Receptor, Bristol, U.K. (Dr. G.L. Collingridge, Department of Pharmacology, The Medical School, University of Bristol, Bristol, BS8 1TD, U.K.)

21–22 April 1989

Satellite Symposium of Int. Soc. for Neurochemistry Congress, Adenosine Receptors, Agarne, Portugal (Professor J.A. Ribeiro, Laboratory of Pharmacology, Gulbenkian Institute of Science, 2781 Oeiras, Portugal).

30 April–5 May 1989

Fifth Int. Symposium on the Adrenal Chromaffin Cell as a Neuroendocrine model; from Basic to Clinical Aspects, Jerusalem, Israel (Dr. K. Rosenheck, Dept. of Membrane Research, Weizmann Institute of Science, Rehovot 76100, Israel).

8–12 May 1989

3rd International Conference on PAF and Structurally related Alkyl ether Lipids, Tokyo, Japan. (Professor S. Nojima, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Tsukin, Kanagawa 199, Japan).

9–12 May 1989

International group for Tryptophan Research. 6th International Meeting, Baltimore, USA. (Dr R. Schwarcz, Maryland Psychiatric Research Center, PO Box 21247, Baltimore, MD 21228, U.S.A.)

21–23 May 1989

Current and Future Trends in Anticonvulsant, Anxiety and Stroke Therapy, Princeton, USA. (Dr J. Emmett, Dialogue in Science, Packhorse Place, Kensworth, Beds LU6 3QU, U.K.)

22–26 May 1989

Second Meeting of the International Neurotoxicology Association, Barcelona, Spain. (Dr. E. Rodriquez Farre, Dept. of Pharmacology & Toxicology, CSIC, Jorge Girona Salgado, 18–26, E-08034, Spain).

25–27 May 1989

4th International Symposium on Calcium Antagonists, Florence, Italy. (Secretariat, Fondazione Giovanni Lorenzini, Via Monte Napoleone 23, 20121 Milan, Italy).

1–2 June 1989

International Workshop on Sensory Neuropeptides, Florence, Italy. (Miss A. Giannini, Istituto Farmacobiologico Malesci, S.p.A., Dept. Pharmacology, Via Porpora 22, 50144 Florence, Italy).

1–3 June 1989

International Symposium on Kidney, Proteins & Drugs, Montecatani Terme, Italy. (Dr. Claudio Bianchi, Centro Nefrologico, Clinica Medica Generale 2, University of Pisa, 56100 Pisa, Italy).

28 June–2 July 1989

Ethnicity & Cardiovascular Diseases Conference, Nairobi, Kenya. (Ms. C. Cate, Int. Society on Hypertension in Blacks, 69 Butler Street, S. E., Atlanta, GA 30303, U.S.A.).

12–14 July 1989

British Pharmacological Society Summer Meeting. Leeds, U.K. (Closed meeting for members and guests)

16–21 July 1989

Vth International Congress of Toxicology, Brighton, U.K. (IUTOX '89, Congress House, 65 West Drive, Sutton, Surrey SH2 7NB, U.K.)

23–25 July 1989

New Strategies in Pain Control, Princeton, USA. (Dr J. Emmett, Dialogue in Science, Packhorse Place, Kensworth, Beds, LU6 3QU, U.K.)

7–12 August 1989

Therapy with Amino Acids and Analogues, Ist International Congress, Vienna, Austria. (G. Lubec, University of Vienna, Dept. of Paediatrics, A-1090 Vienna, Währinger Gürtel 18, Austria).

1–3 September 1989

Second International FIP Symposium on Disposition & Delivery of Peptide Drugs, Leiden, The Netherlands. (Dr. J. Verhoef, Centre for Bio-Pharmaceutical Sciences, PO Box 9502, 2300 RA Leiden, The Netherlands).

13–15 September 1989

British Pharmacological Society Autumn Meeting. Manchester, U.K. (Closed meeting for members and guests)

17–20 September 1989

1st International $GABA_B$ Symposium, Cambridge, U.K. (Professor N.G. Bowery, Department of Pharmacology, School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX.)

19–21 September 1989

Electrochemical Detection, HPLC and *in vitro* monitoring in the Biosciences, Nottingham, U.K. (Dr I.A. MacDonald, Department of Physiology & Pharmacology, Medical School, Queen's Medical Centre, Nottingham, U.K.)

20–21 September 1989

9th Annual Meeting of the Bayliss and Starling Society. Peptides: Physiology, Pharmacology and Therapeutics, Nottingham, U.K. (Professor T. Bennett, Dept. of Physiology & Pharmacology, Medical School, Queen's Medical Centre, Nottingham, U.K.)

28 September 1989

Leukotrienes. London, U.K. (Society for Drug Research, c/o Institute of Biology, 20 Queensbury Place, London SW7 2DZ, U.K.)

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 Streed, New York, NY 10021, U.S.A.)

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.)

3–5 January 1990

British Pharmacological Society Winter Meeting. London, U.K. (Closed meeting for members and guests)

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.)

18–20 April 1990

British Pharmacological Society Spring Meeting. Sheffield, U.K. (Closed meeting for members and guests)

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).

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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PAPERS

507 OTERO LOSADA, M.E. Acute stress and GABAergic function in the rat brain

513 ELLIS, J.L. & FARMER, S.G. The effects of vasoactive intestinal peptide (VIP) antagonists, and VIP and peptide histidine isoleucine antisera on non-adrenergic, non-cholinergic relaxations of tracheal smooth muscle

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Erratum

Br. J. Pharmacol. (1989), **96,** 29–38.

'Actions of the $GABA_B$ agonist, (−)-baclofern, on neurones in deep dorsal horn of the rat spinal cord *in vitro*', by C.A. Allerton, P.R. Boden & R.G. Hill.

Point 4 of the summary (p. 29) should read as follows: 'The reversal potential of the hyperpolarizing event was estimated at −102 mV and was made less negative by increasing the external concentration of potassium ions.'