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SPECIAL REPORT

Coronary vasoconstriction *in vitro* in the hearts of polyarthritic rats: effectiveness of *in vivo* treatment with the endothelin receptor antagonist SB 209670

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Here we demonstrate that perfused hearts removed from polyarthritic rats develop a pronounced coronary vasoconstriction *ex vivo*. This vasoconstriction is almost entirely blocked by *in vivo* pretreatment of the rats with the endothelin receptor antagonist, SB 209670. Thus, inflammatory states may be associated with an increased activity of the endothelin system, leading to vascular dysfunction and vasoconstriction.

Keywords: Endothelin-1; endothelin receptor; endothelium; cytokines; inflammation; blood vessels

Introduction Cytokines, particularly tumour necrosis factor α (TNFα), enhance the release of endothelin-1 (ET-1) into the circulation (Vemulapalli *et al.*, 1991; Han *et al.*, 1994; Klemm *et al.*, 1995). In rats, this is associated with a striking coronary vasoconstriction, revealed when hearts are removed and perfused *in vitro* (Klemm *et al.*, 1995). To test whether there is such an effect in pathological conditions associated with elevated circulating levels of cytokines (Arai *et al.*, 1990; Arend & Dayer, 1990) we investigated changes in perfusion pressures in hearts removed from rats with avidin-induced polyarthritis (AP; Chang *et al.*, 1980).

Methods Adjuvant polyarthritis (AP) was induced in female Wistar rats (220–240g; Tuck, Essex) by a single intradermal injection at the base of the tail of 7 mg CP-20961 (N,N-dioctadecyl-N',N'-*bis*(2-hydroxyethyl)propanediamine; Central Pfizer, Groton, Conn., U.S.A.) dissolved in 0.1 ml Freund's incomplete adjuvant (Chang *et al.*, 1980). As a quantitative measure of arthritis the increase in the volume of both hindpaws was determined on days 0, 1, 2, 4, 7 and 12 by plethysmometry (Ugo Basile, Italy). On the same days, some animals were anaesthetized with thiopentone sodium (Trapanal; 120 mg kg^{-1}, i.p.) and their hearts rapidly removed and transferred to a Langendorff apparatus for perfusion (11–12 ml min^{-1}) with warmed (37°C) and gassed (95% O_2: 5% CO_2) Krebs buffer (pH 7.4). The perfusion pressure, reflecting coronary vascular resistance, was monitored via a pressure transducer linked to a chart recorder. A silicone balloon was introduced into the left ventricle via the mitral valve and inflated for the measurement of cardiac contractility via a pressure transducer linked to a chart recorder (as above). To investigate the effects of endothelin receptor antagonism in adjuvant-treated animals, the nonpeptide endothelin receptor antagonist, SB209670 [(+)-(1S,2R,3S)-3-(2-carboxymethoxy-4-methoxyhenyl)-1-(3,4-methylenedi-oxyphenyl)-5-(prop-1-yloxy)-indane-2-carboxylic acid] (Ohlstein *et al.*, 1994) was given i.p. (2.5 mg kg^{-1}) twice a day (Douglas *et al.*, 1994) on days 0–2 or on days 7–12.

Statistical analysis was performed by ANOVA followed by Bonferroni test for intergroup differences, or Student's *t* test when only 2 groups were compared, and $P<0.05$ taken as significant.

Results AP was associated with inflammatory reactions in the form of diffuse erythematous swelling of one or several paws between day 11 and 12 (mean hindpaw volume day 12: control rats, 1.59 ± 0.02 ml; AP rats 2.81 ± 0.14 ml, $P<0.05$, $n=10$). Hearts removed from control rats had initial perfusion pressures of 48 ± 2 mmHg ($n=6$) which changed little throughout the period of *in vitro* perfusion (Figures 1 and 2). However, hearts taken from AP rats developed a much greater coronary vasoconstriction (Figures 1 and 2). For instance, in hearts removed from AP rats on days 2, 4 and 12 the coronary perfusion pressures after 45 min of perfusion were 131 ± 9, 110 ± 8 and 164 ± 6 mmHg ($n=5-6$), respectively, compared with 64 ± 2 mmHg ($n=6$) in hearts taken from vehicle-treated animals. Treatment with SB 209670 did not affect the hindpaw swelling on day 12 (data not shown), but did, however, substantially inhibit the increases in coronary perfusion pressure seen in AP on days 2 and 12 (Figure 2a,b). During the period of *in vitro* perfusion there were no changes in contractility associated with any of the *in vivo* treatments.

Discussion We have shown that a model of chronic inflammatory disease is associated with a pronounced coronary vasoconstriction in the hearts perfused *in vitro* by the

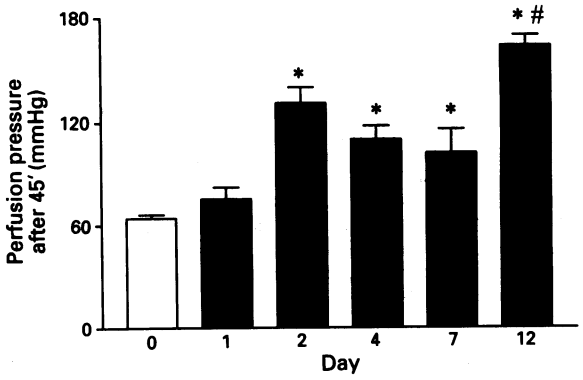

Figure 1 Perfusion pressures (mean ± s.e.mean) after 45 min *in vitro* of hearts removed from control rats ($n=6$, open column) and from AP rats ($n=4-6$, solid columns) on days 1, 2, 4, 7 and 12. *$P<0.05$ compared to control; #$P<0.05$ compared to day 2.

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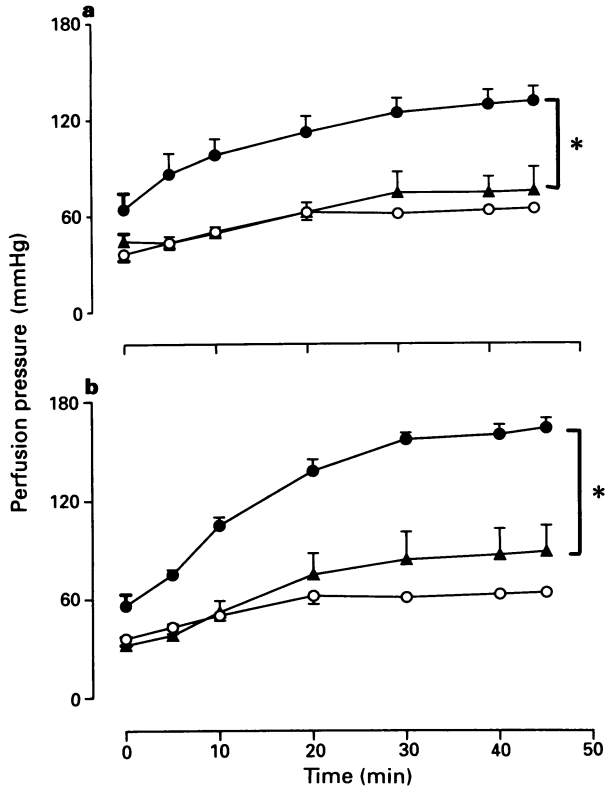

Figure 2 Time-courses of changes in coronary perfusion pressure (mean ± s.e.mean) *in vitro* of hearts removed from control rats (○, $n = 6$), and from avidin-induced polyarthritic (AP) rats treated with either SB 209670 (▲, 2.5 mg kg^{-1}, i.p., twice a day, $n = 5$) or vehicle (●, $n = 6$). (a) Hearts removed on day 2; (b) hearts removed on day 12. *$P < 0.05$ compared to control.

Langendorff method. This elevation in coronary vascular resistance was almost entirely prevented by prior administration of the endothelin receptor antagonist SB20970 (Ohlstein *et al.*, 1994), strongly suggesting that it was mediated by an increased production/release of ET-1.

Cytokines such as TNFα and interleukin-1β (IL-1β) have been implicated as important mediators of chronic inflammation and joint destruction in experimental models of rheumatoid arthritis (Arai *et al.*, 1990; Arend & Dayer, 1990). As exogenous and endogenous TNFα (Han *et al.*, 1994; Klemm *et al.*, 1995) and IL-1β (Vemulapalli *et al.*, 1991) increase ET-1 release *in vitro* and *in vivo* (Kanse *et al.*, 1991; Vemulapalli *et al.*, 1991; Klemm *et al.*, 1995) it is most likely that in AP rats it is these cytokines that induce increases in the production/release of ET-1, leading to the vasoconstriction we observed in the perfused hearts.

Interestingly, the increase in coronary perfusion pressure was greatest on days 2 and 12. On the earlier day this was not associated with any significant changes in hindpaw volumes (data not shown), indicating little joint inflammation. However, in this acute phase there is strong production of the pro-inflammatory cytokines TNFα, IL-1β, and interleukin-6 (Geiger *et al.*, 1992) which could elevate the production of ET-1. This early response is followed by changes such as the secretion of proteolytic enzymes which lead to joint destruction and impaired functionality (Arend & Dayer, 1990). These changes are associated with the second phase of our experimental model, when we observed the greatest coronary vasoconstriction. Of greater interest we found that the coronary vasoconstrictions associated with both phases were almost entirely blocked by *in vivo* treatment with SB 209670.

Thus, inflammatory states are associated with an increase in activity of the endothelin system, as detected in the isolated hearts. However, this in no way implies that such a response is limited to the coronary vasculature. Indeed it rather suggests that states in which there are increased levels of cytokines will be associated with an underlying tendency to vascular dysfunction and vasoconstriction caused by increased levels of ET-1.

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Functional dopamine D_2 receptors on rat vagal afferent neurones

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1 In the present study *in vitro* electrophysiology and receptor autoradiography were used to determine whether rat vagal afferent neurones possess dopamine D_2 receptors.

2 Dopamine (10–300 μM) elicited a temperature- and concentration-dependent depolarization of the rat isolated nodose ganglion preparation. When applied to the tissue 15 min prior to agonist, raclopride (10 μM), clozapine (10 μM) or a mixture of raclopride and clozapine (10 μM each) all produced a threefold parallel shift to the right of the dopamine concentration-response curve. In contrast, SCH 23390 (100 nM), phentolamine and propranolol (1 μM each) failed to antagonize the dopamine-mediated depolarization.

3 [^{125}I]-NCQ 298 (0.5 nM), a D_2 selective radioligand, bound topographically to sections of rat brainstem. Densitometric quantification of autoradiograms revealed 93.8 ± 0.5% specific binding of this salicylamide radioligand, as determined by raclopride (10 μM, $n = 10$ animals). Binding was highest in the nucleus tractus solitarius (NTS), particularly the medial and gelatinous subnuclei. In addition, specific binding was also observed in the interpolar spinal trigeminal nucleus and the inferior olive.

4 Unilateral nodose ganglionectomy caused a 36.6 ± 3.0% reduction in specific binding in the denervated NTS compared to the contralateral NTS. Furthermore, the loss of binding was confined to the dorsal aspect of the medial subnucleus of the NTS. Sham surgery had no effect on the binding of [^{125}I]-NCQ 298 in rat brainstem.

5 The present data provide evidence for the presence of functionally relevant dopamine D_2 receptors on both the soma and central terminals of rat vagal afferent neurones. In addition, the majority of D_2 receptors in the rat NTS appear to be located postsynaptically with respect to vagal terminals, and are presumably located either on ascending glossopharyngeal terminals, descending terminals from higher brain regions or on neuronal cell bodies within the NTS.

Keywords: Dopamine; [^{125}I]-NCQ 298; nodose ganglion; vagal afferent nerves; nucleus tractus solitarius; D_2 receptors; electrophysiology; autoradiography

Introduction

It has been shown by molecular biological techniques that dopamine receptors form a heterogeneous population of protein molecules (receptor subtypes). Current nomenclature describes D_1 through to D_5 receptors, some of which exhibit alternate splice variants (Civelli *et al.*, 1993). With the exception of the D_3 receptors, all other dopamine receptor subtypes appear to be coupled to G-proteins (Sokoloff *et al.*, 1990).

Historically, much interest has centred on the involvement of dopaminergic systems and dopamine receptors in neurodegenerative disorders such as Parkinson's disease (Birkmayer & Hornykiewicz, 1962) and psychoses (Seeman *et al.*, 1976); however, both dopamine and its receptors have been implicated in cardiovascular control and chemoreflex pathways (Fidone *et al.*, 1988; Van Den Buuse & De Jong, 1991). For example, activation of dopamine D_2 receptors in the periphery mediates a hypotensive response via the inhibition of noradrenaline release from noradrenergic terminals (Nagahama *et al.*, 1986). In contrast, central D_2 receptor activation leads to a pressor effect, and the receptors mediating the central pressor response have been localized to a discrete region of the posterior nucleus tractus solitarius (NTS) (Yang *et al.*, 1990). Furthermore, microiontophoretic application of dopamine into the NTS has been shown to inhibit neuronal activity in this brainstem nucleus (Granata & Woodruff, 1982). The NTS, which receives inputs from arterial baroreceptor and chemoreceptor primary afferent fibres via the IXth and Xth cranial nerves, is established as the pivotal relay nucleus in central cardiovascular control and chemoreflex pathways (Kalia, 1981).

Neuronal cell bodies of centrally projecting glossopharyngeal and vagal afferent neurones, including baroreceptor and chemoreceptor afferents, are located in the petrosal and nodose ganglia respectively (Kalia, 1981; Kumada *et al.*, 1990). Immunocytochemical studies have demonstrated the presence of the necessary enzymes for the synthesis of dopamine in petrosal ganglia cell bodies (Finley *et al.*, 1992). Furthermore, hypoxic insult causes an increase in dopamine release in the rabbit NTS (Goiny *et al.*, 1991). Taken together, these data provide strong evidence for dopamine as a neurotransmitter or modulator of chemoreceptor primary afferent neurones. In addition, neuronal perikarya of the carotid body, petrosal ganglion and superior cervical ganglion express the genetic information encoding the D_2 but not the D_1 receptor (Czyzyk-Krzeska *et al.*, 1992).

In contrast to the depth of information regarding the presence of dopamine and dopamine receptors in chemoreceptor afferent neurones, relatively little is known about the involvement of dopaminergic systems and dopamine receptors in baroreceptor primary afferents. The present study has therefore addressed this question in rats, using functional and anatomical techniques. In light of the documented ability of dopamine to depolarize mammalian primary afferent neurones (Zapata, 1975; Gallagher *et al.*, 1980; Monti-Bloch *et al.*, 1993), the isolated nodose/vagal grease-gap technique was employed to determine whether activation of dopamine receptors on the soma membranes of nodose ganglia could evoke an electrophysiological response.

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This methodology also gives an indication of the presence and properties of receptors on central vagal afferent terminals (Round & Wallis, 1986). In addition, the salicylamide [^{125}I]-NCQ 298, a selective radioligand for dopamine D_2 receptors (Hall *et al.*, 1991), was utilized in autoradiographic experiments to assess the distribution of D_2 binding sites in the rat brain stem. Specifically, the binding of [^{125}I]-NCQ 298 to sections of rat brain stem was studied following unilateral vagal deafferentation (nodose ganglionectomy) to determine whether labelled sites were pre- or post-synaptic in location with respect to central vagal afferent terminals.

Methods

All the experiments described here were performed in accordance with the Prevention of Cruelty to Animals Act 1986 under the guidelines of the NH & MRC Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

In vitro *electrophysiology*

Male Sprague–Dawley rats (250–350 g) were killed by cervical dislocation and the nodose ganglia with attached distal vagal trunk were removed and desheathed. The tissue was then placed in a twin chambered perspex bath with the nodose ganglion placed in one compartment of the bath and isolated from the vagal nerve trunk in an adjacent compartment by a silicone grease seal, as previously described (Widdop *et al.*, 1990). The preparation was superfused with Krebs buffer (36°C, 2 ml min^{-1}) of the following composition (in mM): NaCl 118, $NaHCO_3$ 24.9, KH_2PO_4 1.3, KCl 4.7, $CaCl_2$ 2.6, glucose 11, $MgSO_4$ 1.2, gassed with 95% O_2/5% CO_2, pH approximately 7.4. In experiments employing dopamine, the superfusate also contained ascorbic acid (0.11 mM) and EDTA (1 mM) which were present throughout the whole experimental period and at the concentration employed did not cause any depolarization. The d.c. potential between the two compartments following drug administration to the nodose ganglion was recorded by calomel electrodes connected to the preparation through agar-KCl bridges. The potential changes were amplified and displayed on a Grass Polygraph (Model 79D). Drugs were applied non-cumulatively and remained in contact with the tissue until apparent equilibrium was reached. This was followed by a washout and recovery period (10–20 min) to allow full repolarization prior to another drug addition. When used, antagonists were added to the superfusate 15 min before agonist application. This method allowed stable responses to be measured over a 5–6 h period. The magnitude of an observed depolarization was measured from a projection of the baseline preceding the response to account for any drift in the preparation. At the beginning and end of each experiment a positive control of a previously determined maximal concentration of 5-hydroxytryptamine (5-HT, 3 μM; Widdop *et al.*, 1992) was applied to the nodose ganglia in order to check the viability of the preparations.

Preparation of radioligand

[^{125}I]-NCQ 298 ((S)-3-iodo-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-5,6-dimethoxysalicylamide) was prepared from NCQ 634 (the corresponding des-iodo derivative) by the chloramine-T radioiodination technique and purified by paper chromatography, as described previously (Adams & Jarrott, 1982). The binding profile of this radioligand has been reported (Hall *et al.*, 1991).

Unilateral vagal deafferentation

Male rats (250–320 g) were anaesthetized with sodium methohexitone (60 mg kg^{-1}, i.p.) and placed on their back. A midline incision was made in the neck and the left nodose ganglion was exposed and excised ($n = 5$) including trunks of vagus, superior laryngeal and inferior pharyngeal nerves at the respective points of contact with the nodose ganglion. In a second group ($n = 5$), the left nodose ganglion was exposed but not removed (sham control). After a 14 day recovery period, the animals were re-anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and the rats were then immediately killed by decapitation and their brainstems removed. All tissue was frozen over liquid nitrogen at the time of removal and stored at −80°C until further processed.

Autoradiography

Cryostat-cut sections of brainstem (10 μm) were taken between bregma −14.3 mm to bregma −12.8 mm (Paxinos & Watson, 1986) and thaw-mounted onto gelatin/chrome alum/bovine serum albumin coated microscope slides. Autoradiographic experiments were carried out as previously described (Hall *et al.*, 1991). In short, tissue sections were allowed to warm to room temperature and then pre-incubated (30 min, room temperature) in Tris HCl buffer (170 mM, pH 7.6) containing NaCl 120 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM and ascorbic acid 0.001% w/v. The sections were then incubated with [^{125}I]-NCQ 298 (0.5 nM) for 60 min at room temperature in the same buffer used for the pre-incubation. Non-specific binding was defined as that remaining in the presence of raclopride (10 μM).

The slide-mounted sections were then washed in ice-cold Tris HCl buffer (170 mM, pH 7.6, 4 × 2 min) and finally rinsed in ice-cold distilled water (2 × 30 s). Following this, the sections were dried under a gentle stream of cool air and apposed to X-ray film (Kodak XAR-5) for 36 h at 4°C, in the presence of standard ^{125}I microscales (Amersham). Developed autoradiograms were quantified with an MCID M4 image analysis system (Imaging Research Inc., Canada), by comparison of the optical densities of autoradiographic images with those of the standard microscales. Brain regions were identified following microscopic examination of adjacent tissue sections stained with 0.1% thionin with reference to a stereotaxic atlas (Paxinos & Watson, 1986).

Materials

The salicylamide compounds NCQ 298, NCQ 634 and raclopride were gifts from Astra Pharmaceuticals, Sweden, Clozapine was a gift from Sandoz, Australia. Dopamine hydrochloride and 5-hydroxytryptamine creatinine sulphate were obtained from Sigma Chemicals, St Louis, MO, U.S.A. SCH 23390 was from Research Biochemicals Inc., U.S.A., phentolamine from Ciba-Geigy and propranolol from I.C.I. Other reagents were of either laboratory or analytical grade from various suppliers.

Statistics

A paired *t* test (two-tailed) was employed to analyse differences in [^{125}I]-NCQ 298 binding between the denervated and intact sides of the NTS. $P < 0.05$ was considered significant. Concentration-response curves on the isolated nodose ganglion preparation were analysed by potency ratios.

Results

In vitro *electrophysiology*

Addition of dopamine to the superfusate bathing the isolated nodose ganglia elicited a concentration-dependent depolarization (Figure 1). Furthermore, the action of dopamine on the isolated nodose ganglion was also temperature-dependent,

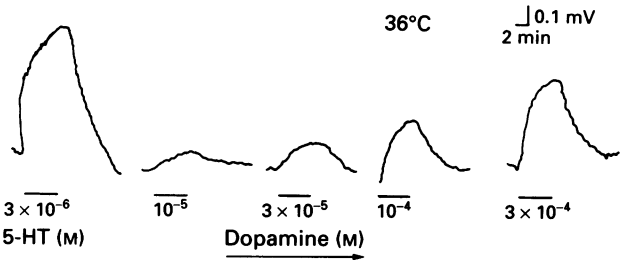

Figure 1 Polygraph trace showing a representative series of responses to different concentrations of dopamine applied to the rat isolated nodose ganglion preparation. The first depolarization represents an initial positive control of 5-hydroxytryptamine (5-HT, 3 μM). Scales as shown.

the catecholamine being ineffective at room temperature (~22°C). Over the concentration-range employed (10–300 μM), dopamine did not consistently achieve an apparent maximum response; however, higher concentrations were not applied due to the possibility of actions at non-dopamine receptors. Therefore, data have been quantified in terms of potency ratios and plotted in a similar manner to *in vitro* electrophysiological studies of cholecystokinin receptors on 5-hydroxytryptaminergic neurones of the dorsal raphe nucleus (Boden *et al.*, 1991). The D_2 antagonist, raclopride (10 μM, Köhler *et al.*, 1985) shifted the concentration-response curve for dopamine in a parallel fashion to the right by a factor of 2.96 ($n = 3$), yielding a potency ratio of 0.338 (Figure 2a). The D_4/D_2 antagonist clozapine (10 μM, Lahti *et al.*, 1993) had a similar effect, causing a 3.00 fold rightward shift of the dopamine concentration-response curve (potency ratio = 0.333; $n = 6$), as shown in Figure 2b. A combination of both raclopride (10 μM) and clozapine (10 μM) shifted the concentration-response curve for dopamine evoked depolarizations to the right by a factor of 2.89 (potency ratio = 0.346, $n = 6$, Figure 2c). In contrast to raclopride and clozapine, the selective D_1 antagonist, SCH 23390 (100 nM, Hyttel, 1983) had no effect on the dopamine concentration-response curve (Figure 2d). The ability of dopamine to depolarize the rat isolated nodose ganglion was unaffected by the addition of phentolamine and propranolol (1 μM each, $n = 3$; data not shown).

In vitro *autoradiography*

The binding of [^{125}I]-NCQ 298 has been fully characterized previously (Hall *et al.*, 1991) and in our hands this radioligand (0.5 nM) bound avidly to sections of rat brainstem. Raclopride (10 μM) markedly inhibited the binding of [^{125}I]-NCQ 298. Thus, densitometric quantification estimated specific binding to represent 93.8 ± 0.5% of total binding ($n = 10$ animals; minimum of 18 sections/animal analysed). Specific binding of [^{125}I]-NCQ 298 was predominantly localized to the NTS/dorsal motor nucleus of vagus, central/medial inferior olive and the interpolar spinal trigeminal nucleus (Figure 3). At most anatomical levels through the brainstem analysed, unilateral deafferentation of the vagus failed to have an effect on the density of raclopride-sensitive binding sites in the NTS. However, between bregma −13.4 and bregma −13.2, unilateral nodose ganglionectomy caused a 36.6 ± 3.0% reduction in the specific binding of [^{125}I]-NCQ 298 in the denervated (left) NTS (24 ± 3 d.p.m. mm^{-2}) compared to the contralateral (right) NTS (38 ± 4 d.p.m. mm^{-2}; $n = 7$ sections from 5 animals; $P = 0.0001$ paired t test, intact versus denervated NTS), as shown in Figure 3b. At this anatomical level, the specific binding of [^{125}I]-NCQ 298 in the rat NTS described a crescent-like shape, being restricted to the medial, gelatinous and lateral subnuclei and absent in the solitary tract itself. The loss of binding was isolated to the dorsal aspect of the medial NTS (Figure 3b). In contrast,

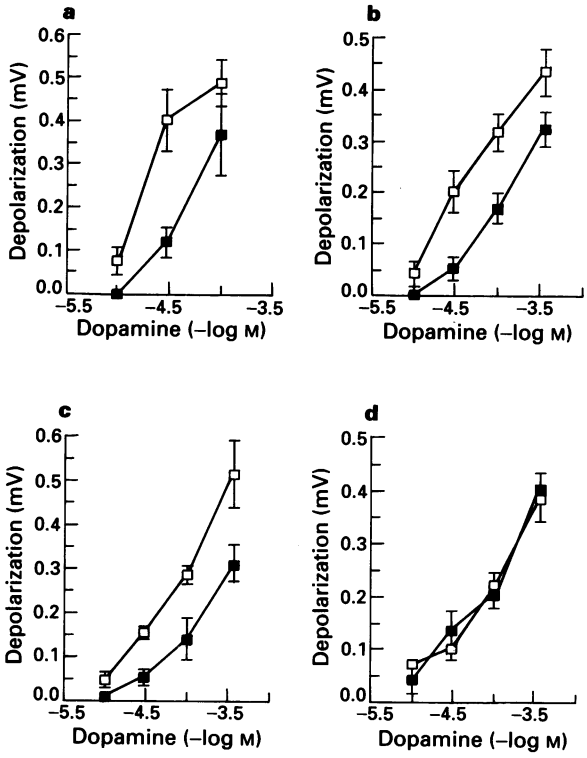

Figure 2 Concentration-response curves of dopamine on the rat isolated nodose ganglion preparation, in the absence (□) or presence (■) of: (a) raclopride (10 μM, $n = 3$); (b) clozapine (10 μM, $n = 6$); (c) a combination of raclopride and clozapine (10 μM each, $n = 6$); (d) SCH 23390 (100 nM, $n = 4$). Data points are mean ± s.e. of mean.

sham surgery did not affect raclopride-sensitive binding of [^{125}I]-NCQ 298 in the rats NTS ($n = 5$, Figure 3a). Figure 3c schematically demonstrates the anatomical subdivisions within the rat NTS. The medial NTS contains five subnuclei: medial, dorsal, intermediate, ventral and gelatinous whereas the lateral NTS is smaller and composed of four subnuclei: interstitial, lateral, ventrolateral and dorsolateral (Van Giersbergen *et al.*, 1992). A comparison between Figure 3b and Figure 3c demonstrates the loss of binding following unilateral nodose ganglionectomy is clearly restricted to the dorsal subdivision of the medial NTS.

Discussion

The present results demonstrate, for the first time, the presence of functional D_2 receptors on the soma membrane of rat vagal afferents. These findings are in agreement with the recent visualisation of D_2 binding sites on human inferior vagal ganglia (Lawrence & Jarrott, 1994). In addition, these data confirm the ability of dopamine to depolarize primary afferent neurones (Gallagher *et al.*, 1980; Monti-Bloch *et al.*, 1993). While the receptors mediating the dopamine-induced depolarization of the isolated rat nodose ganglia belong to the D_2 family, the current data cannot differentiate between the short and long forms of dopamine D_2 receptors (Monsma *et al.*, 1989). In addition to electrophysiological characterization, autoradiographic experiments in conjunction with unilateral vagal deafferentation confirm the presence of a population of dopamine D_2 receptors on central vagal afferent terminals within the rat NTS. Taken together, these observations suggest that D_2 receptors are synthesized in nodose perikarya and axoplasmically transported to the central nerve terminals in the medial subnucleus of the NTS.

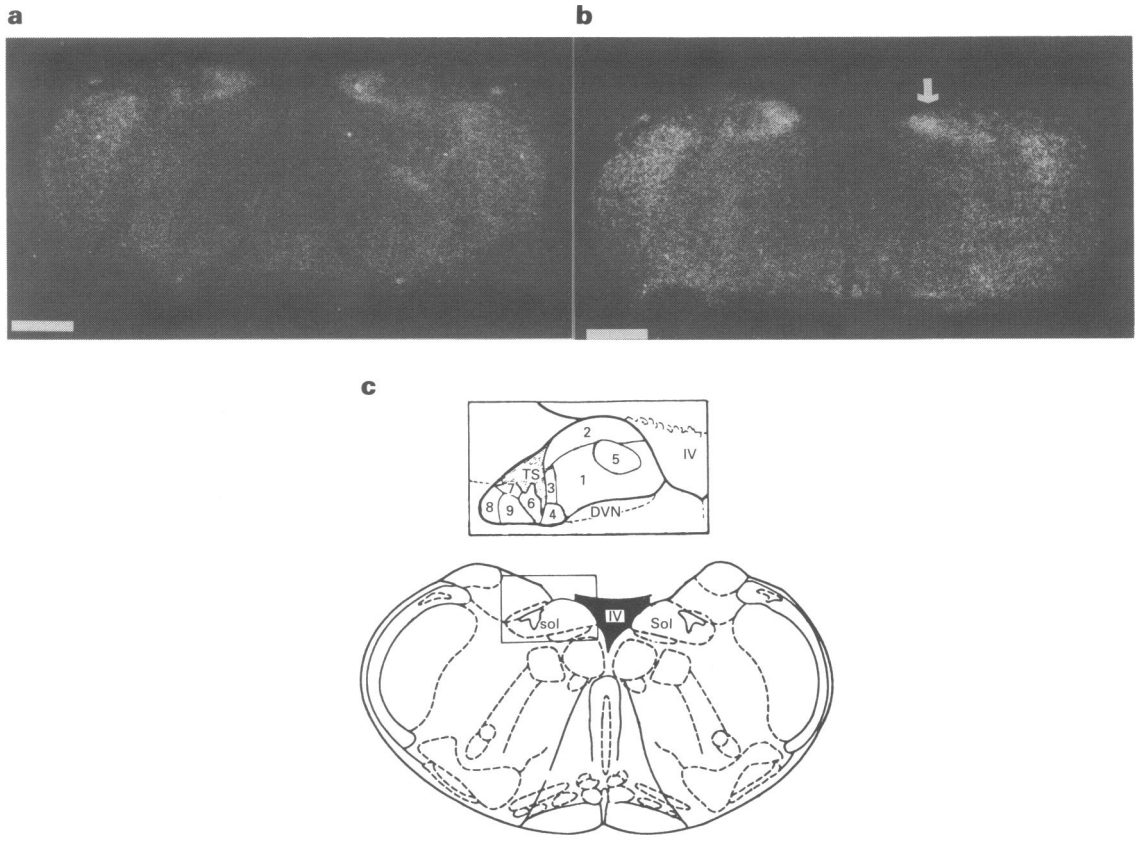

Figure 3 Autoradiograms of [^{125}I]-NCQ 298 binding to sections of rat brain stem. Specific binding represented >90% of total binding. (a) Sham nodose ganglionectomy control. Anatomical level shown corresponds to bregma −13.3 mm. D_2 binding sites are predominantly restricted to the medial and gelatinous subnuclei of the rat NTS. Less dense binding is apparent in the interpolar spinal trigeminal nucleus and the inferior olive. Scale bar = 1.04 mm. (b) Unilateral nodose ganglionectomy. Anatomical level shown corresponds to bregam −13.3 mm. A 36.6 ± 3.0% (n = 5) reduction in D_2 binding can be observed in the denervated NTS (arrowhead) compared to the contralateral NTS. Loss of binding is confined to the dorsal aspect of the medial subnucleus (arrowhead, see (c) for definition of NTS subdivisions). Scale as for (a). (c) Schematic diagram showing subdivisions of the rat NTS at the anatomical level of the autoradiograms. A complete section of brain stem shows the relative position of the NTS on the dorsal surface of the brain stem. The boxed area is shown enlarged above the main diagram to allow definition of NTS subnuclei. Annotation as follows: (1) medial; (2) dorsal; (3) intermediate; (4) ventral; (5) gelatinous; (6) interstitial; (7) dorsolateral; (8) lateral; (9) ventrolateral; DVN, dorsal vagal nucleus; TS (shaded area), solitary tract; IV, fourth ventricle. Adapted from Paxinos & Watson (1986) and Van Giersbergen *et al.* (1992).

The ability of dopamine to depolarize the isolated nodose ganglion was shifted to the right in a parallel manner by the selective D_2 antagonist raclopride (Köhler *et al.*, 1985) and the D_4/D_2 antagonist clozapine (Lahti *et al.*, 1993). The fact that both antagonists caused a threefold rightward shift, which was unchanged by use of a combination of the two drugs, suggests that the receptor involved is indeed a D_2 receptor and not a D_4 receptor. Furthermore, clozapine is reportedly less than three fold selective for the D_4 over the D_2 receptor in membrane binding assays (Lahti *et al.*, 1993). In contrast, the dopamine-induced depolarizations were insensitive to D_1 receptor blockade by SCH 23390 (Hyttel, 1983). Additionally, phentolamine and propranolol were ineffective in preventing the dopamine-evoked response, discounting the possibility of an action at α- or β-adrenoceptors. The temperature-dependence of agonist-induced depolarization of rat vagal afferents has been previously described for angiotensin II (Widdop *et al.*, 1990; 1992), supporting the notion that dopamine is stimulating the synthesis of a second messenger through an enzymatic process. While these data support the presence of D_2 receptors on the cell bodies of centrally-projecting vagal primary afferent neurones, one must remember that the isolated nodose ganglion technique measures a population response, thus the neurone-types involved, such as baroreceptor, cannot be distinguished.

The autoradiographic pattern of [^{125}I]-NCQ 298 binding in the rat brainstem is largely similar to that recently found with another D_2 selective radioligand [^{3}H]-YM-09151-2 (Yokoyama *et al.*, 1994), with the exception that the latter radioligand visualized a population of sites in the hypoglossal nucleus which was not observed in the present study. The reason for this difference is unclear; however, Yokoyama and colleagues (1994) were 'surprised' to observe specific binding of [^{3}H]-YM-09151-2 in a number of brainstem motor nuclei. Nonetheless, both studies describe high affinity D_2 binding in the rat NTS. The NTS is well established as the site of the first synapse for centrally-projecting vagal afferent neurones, including arterial baroreceptor afferents (Kumada *et al.*, 1990). While the majority of binding sites visualized appear to be postsynaptic to the vagal afferent terminals, the data presented herein demonstrate, for the first time, a population of presynaptic D_2 receptors on the central terminals of the vagus, particularly in the medial subnucleus of the NTS, the main location of primary arterial baroreceptor afferent terminals (Kumada *et al.*, 1990). Dopamine D_2 receptors in the rat NTS have been demonstrated to mediate a pressor response following microinjection of quinpirole (Yang *et al.*, 1990). This effect may, at least in part, be due to release-regulating D_2 receptors on baroreceptor afferent neurones. However, two thirds of the D_2 receptors in the NTS remain following nodose ganglionectomy, suggesting that postsynaptic receptors may also be involved in the central cardiovascular actions of dopamine. It is unlikely that the incomplete loss of D_2 binding sites in the NTS represents only a partial

denervation of the vagal projections. We have previously used this technique to demonstrate the presynaptic location of adenosine A_{2a} receptors (Castillo-Meléndez *et al.*, 1994); angiotensin II receptors (Lewis *et al.*, 1986) and vasopressin V_1 receptors (Gao *et al.*, 1992) in the rat NTS. In addition, nodose ganglionectomy has also been employed to study the cellular localization in the NTS of glutamate receptors (Lewis *et al.*, 1988), neurotensin receptors (Kessler & Beaudet, 1989), cholecystokinin receptors (Ladenheim *et al.*, 1988), muscarinic cholinoceptor and delta-opioid receptors (Leslie *et al.*, 1989), 5-HT_3 receptors (Merahi *et al.*, 1992) and substance P receptors (Manaker & Zucchi, 1993).

The dopamine D_2 receptors located postsynaptic to vagal afferent terminals in the NTS are likely to represent a combination of soma-membrane receptors and receptors on glossopharnygeal terminals. Dopamine-immunoreactive cell-bodies have been demonstrated in the rat NTS (Dahlström & Fuxe, 1964; Hökfelt *et al.*, 1984), while arterial baro- and chemoreceptor afferents also project through the glossopharyngeal nerve from the carotid sinus and petrosal ganglia (Kalia, 1981). Furthermore, 96% of carotid body afferent neurones in the petrosal ganglion are immunopositive for tyrosine hydroxylase while only 2% of such neurones stain for tyrosine hydroxylase in the nodose ganglion (Finley *et al.*, 1992). Indeed, dopamine is well-established as an important neuromodulator of chemoreceptor afferent neurones (Zapata, 1975; Fidone *et al.*, 1988; Goiny *et al.*, 1991; Czyzyk-Krzeska *et al.*, 1992; Finley *et al.*, 1992; Marchal *et al.*, 1992; Monti-Bloch *et al.*, 1993; Bairam *et al.*, 1993).

In conclusion, the present study has determined the ability of dopamine to depolarize rat vagal afferent neurones in a concentration- and temperature-dependent manner. Furthermore, the action of dopamine on the isolated nodose ganglion appears to be mediated by activation of D_2 receptors. In addition, the binding of [^{125}I]-NCQ 298 to slide-mounted sections of rat brainstem has provided anatomical confirmation of the presence of presynaptic D_2 receptors on central afferent terminals of the vagus nerve in the rat NTS. Whether these presynaptic D_2 receptors are involved in the central cardiovascular actions of dopamine remains to be elucidated.

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Co-induction of nitric oxide synthase and cyclo-oxygenase: interactions between nitric oxide and prostanoids

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1 Lipopolysaccharide (LPS) co-induces nitric oxide synthase (iNOS) and cyclo-oxygenase (COX-2) in J774.2 macrophages. Here we have used LPS-activated J774.2 macrophages to investigate the effects of exogenous or endogenous nitric oxide (NO) on COX-2 in both intact and broken cell preparations. NOS activity was assessed by measuring the accumulation of nitrite using the Griess reaction. COX-2 activity was assessed by measuring the formation of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) by radioimmunoassay. Western blot analysis was used to determine the expression of COX-2 protein. We have also investigated whether endogenous NO regulates the activity and/or expression of COX *in vivo* by measuring NOS and COX activity in the lung and kidney, as well as release of prostanoids from the perfused lung of normal and LPS-treated rats.

2 Incubation of cultured murine macrophages (J774.2 cells) with LPS (1 $\mu g\,ml^{-1}$) for 24 h caused a time-dependent accumulation of nitrite and 6-keto-$PGF_{1\alpha}$ in the cell culture medium which was first significant after 6 h. The formation of both 6-keto-$PGF_{1\alpha}$ and nitrite elicited by LPS was inhibited by cycloheximide (1 μM) or dexamethasone (1 μM). Western blot analysis showed that J774.2 macrophages contained COX-2 protein after LPS administration, whereas untreated cells contained no COX-2.

3 The accumulation of 6-keto-$PGF_{1\alpha}$ in the medium of LPS-activated J774.2 macrophages was concentration-dependently inhibited by chronic (24 h) exposure to sodium nitroprusside (SNP; 1–1000 μM). Sodium nitroprusside (1–1000 μM) also acutely (30 min) inhibited COX-2 activity in broken cell preparations of LPS-activated (12 h) J774.2 macrophages, in a similar concentration-dependent manner. Addition of adrenaline (5 mM) and glutathione (0.1 mM) increased the activity of COX-2 in broken cell preparations. In the presence of these co-factors, SNP inhibited prostanoid production only at the highest concentration used (1 mM). When J774.2 cells were incubated in the presence of LPS (1 $\mu g\,ml^{-1}$) and N^G-monomethyl-L-arginine (L-NMMA: 1 mM) for 12 h, SNP at the highest concentration used (1 mM) acutely (30 min) inhibited the activity of COX-2 in cell homogenates with co-factors. However, when J774.2 macrophages were incubated for 24 or 12 h with LPS (1 $\mu g\,ml^{-1}$) and L-NMMA (1 mM), the addition of SNP (0.001–1000 μM) increased in a concentration-dependent manner the accumulation of 6-keto-$PGF_{1\alpha}$ in intact cells (measured at 24 h) and COX-2 activity in cell homogenates in the presence of co-factors (determined at 12 h). SNP (1 mM; together with LPS for 12 h) decreased the amount of COX-2 protein induced by LPS in J774.2 macrophages.

4 Indomethacin (30 μM) abolished the formation of 6-keto-$PGF_{1\alpha}$ by LPS-activated macrophages, but had no effect on the release of nitrite. Conversely, L-NMMA, at the highest concentrations used (1 and 10 mM), increased the release of 6-keto-$PGF_{1\alpha}$, an effect which was reversed by excess L-arginine (3 mM) but not by D-arginine. Similarly, the decrease in nitrite formation caused by L-NMMA was partially reversed by L-arginine (3 mM), but not by D-arginine. L-NMMA (10 mM; together with LPS for 12 h) increased the amount of COX-2 protein induced by LPS in J774.2 macrophages.

5 In separate experiments, J774.2 macrophages were activated with LPS (1 $\mu g\,ml^{-1}$), and L-NMMA (10 mM) was added for various times (0.5–24 h) before the collection of medium at 24 h. L-NMMA enhanced the release of 6-keto-$PGF_{1\alpha}$ in a time-dependent manner, with the maximal enhancement seen when the NOS inhibitor was incubated with the cells for 24 h.

6 In experiments on male Wistar rats, we investigated the effect of L-NMMA on the release of prostanoids (6-keto-$PGF_{1\alpha}$, prostaglandin E_2, thromboxane B_2) elicited by arachidonic acid (AA, 30 nmol) from *ex vivo* perfused kidneys and lungs. The release from the organs from normal and LPS-treated rats was unaffected by L-NMMA intraperitoneally (30 $mg\,kg^{-1}$) for 6 h together with LPS (5 $mg\,kg^{-1}$) or LPS vehicle. Similarly, acute (15 min) *in vitro* exposure to L-NMMA (1 mM) of the perfused organs from control and LPS-treated animals did not change the release of prostanoids elicited by AA (30 nmol).

7 These results show that LPS causes the induction of iNOS and COX-2 in J774.2 macrophages. The co-release of NO and PGI_2 induced by LPS is dependent on protein synthesis and occurs after a lag-time of 6–12 h. The formation of COX metabolites has no effect on NOS activity whereas NO inhibits both COX-2 activity and induction. These results demonstrate that NOS and COX can be co-induced *in vitro* and that under these conditions large amounts of NO inhibit the degree of COX expression and activity. In the absence of endogenous NO, lesser amounts of exogenous NO increase the activity of COX-2. In those situations *in vivo* when the level of NO induction is relatively low, NO does not regulate the increased activity of COX.

Keywords: Nitric oxide; nitric oxide synthase; prostacyclin; cyclo-oxygenase; N^G-monomethyl-L-arginine; N^G-nitro-L-arginine; cytokine; endotoxin

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Introduction

Nitric oxide (NO) is formed from L-arginine by NO synthase (NOS) in a wide variety of cells (see Förstermann *et al.*, 1991; Nathan, 1992). Several isoforms of NOS have now been isolated, purified, cloned and expressed (see, Förstermann *et al.*, 1991; Janssens *et al.*, 1992; Lamas *et al.*, 1992; Lowenstein *et al.*, 1992; Nishida *et al.*, 1992). The constitutive isoform in endothelial cells (eNOS) is a 135 kD protein located predominantly in the membrane fraction (Pollock *et al.*, 1991; Sessa *et al.*, 1992). Bacterial lipopolysaccharide (LPS) or cytokines induce macrophages, vascular smooth muscle and other cells to express a different isoform of NOS (see Förstermann *et al.*, 1991; Nathan, 1992). The formation of large amounts of NO by the inducible isoform of NOS (iNOS) accounts for the cytotoxicity of activated macrophages and plays an important role in the circulatory failure associated with septic (Thiemermann & Vane, 1990; Julou-Schaeffer *et al.*, 1990; Wright *et al.*, 1992; Szabó *et al.*, 1993) and haemorrhagic shock (Thiemermann *et al.*, 1993). Although eNOS and iNOS have the same co-factor requirements for NADPH and tetrahydrobiopterin, and contain/require FAD/FMN (see Förstermann *et al.*, 1991; Pollock *et al.*, 1993), they differ in that eNOS is calcium-dependent, where iNOS is not. Both are inhibited, although to different degrees, by N^G-monomethyl-L-arginine (L-NMMA) or other N^G-substituted analogues of L-arginine (see Thiemermann, 1994).

Prostaglandins, a family of mediators, have numerous cardiovascular and inflammatory effects (see Botting & Vane, 1990). Cyclo-oxygenase (COX), the first enzyme in the pathway of prostaglandin and thromboxane A_2 formation from arachidonic acid, is localized primarily in the endoplasmic reticulum and exists as a dimer of 70 kD subunits (Helmer *et al.*, 1976). COX also exists in both constitutive (COX-1) and cytokine/LPS-inducible (COX-2) isoforms (see Xie *et al.*, 1992).

Endothelial cells contain eNOS (Pollock *et al.*, 1991; Sessa *et al.*, 1992) and COX-1 (Mitchell *et al.*, 1993a) and hence release both NO and prostacyclin (PGI_2) when activated by receptor-stimulating agonists such as bradykinin or ADP (De Nucci *et al.*, 1988; Mitchell *et al.*, 1991a; 1992). Similarly, induced iNOS (Stuehr *et al.*, 1991) and COX-2 may also co-exist in some cells after exposure to LPS or cytokines (Szabó *et al.*, 1993; Mitchell *et al.*, 1993a; Corbett *et al.*, 1993). In endothelial cells inhibition of NO release potentiates the coupled release of PGI_2 (Keen *et al.*, 1990), whereas exogenous NO (Doni *et al.*, 1988) or sodium nitroprusside (Keen *et al.*, 1990; Matthews *et al.*, 1993) inhibits the release of PGI_2. However, in other cell types, inhibitors of NO formation reduce the release of prostanoids (Stadler *et al.*, 1991; Rettori *et al.*, 1993; Corbett *et al.*, 1993; Salvemini *et al.*, 1993; 1994; Franchi *et al.*, 1994). We have, therefore, investigated how inhibition of endogenous NO formation in LPS-activated J774.2 macrophages affects COX-2 induction and the release of PGI_2. Furthermore, to investigate this crosstalk further, we have also examined whether inhibition of COX with indomethacin affects NO formation by these cells. Some of these results have been published in abstract form (Mitchell *et al.*, 1993b).

Methods

Cell Culture

Murine J774.2 macrophages were obtained from the European Collection of Animal Cell Culture (Salisbury, Wilts) and cultured in 96-well plates in Dulbecco's Modified Eagle's Medium (DMEM; ICN Biomedical Limited, High Wycombe, Bucks) containing 10% foetal calf serum (Gibco BRL, Uxbridge, Middlesex) and 2 mM L-glutamine (200 µl/well; B.D.H., Dagenham, Essex) until 80–100 % confluent. The culture medium was replaced with fresh medium at the beginning of each experiment. All drugs were dissolved in distilled water and sterilised by filtration through a 0.2 µm filter before being added to the cells under sterile conditions. Cells were incubated at 37°C in a humidified incubator.

Measurement of nitrite and 6-keto-$PGF_{1\alpha}$

Nitrite accumulation, an indicator of NO synthase activity, was measured in the culture medium by the Griess reaction adapted for a 96-well plate reader (Gross *et al.*, 1991). Nitrite was measured by adding 100 µl of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamine in 5% phosporic acid) to 100 µl samples of cell culture medium. The optical density at 550 nm (OD_{550}) was measured with a Molecular Devices micro plate reader (Richmond, CA, U.S.A.). Nitrite concentrations were calculatd by comparison with the OD_{550} of standard solutions of sodium nitrite prepared in culture medium. The detection limit for nitrite measurement in culture medium was 1 µM. The accumulation of 6 keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$, the breakdown product of PGI_2), was measured as an indicator of COX activity by radioimmunoassay. [^{3}H]-6-keto-$PGF_{1\alpha}$ was obtained from Amersham International (Buckinghamshire). Antibodies to 6-keto-$PGF_{1\alpha}$ were purchased from Sigma Chemical Company (Poole, Dorset).

NOS and COX activity were induced in J774.2 macrophages by incubation with *E. coli* LPS (1 ng ml^{-1}–10 µg ml^{-1}) for 24 h. For time-course experiments, LPS was added for 0, 3, 6, 12 or 24 h, after which time the medium was removed for nitrite and 6-keto-$PGF_{1\alpha}$ determinations. In some experiments L-NMMA (0.1 µM – 10 mM; Calbiochem, La Jolla, CA, U.S.A.), D-NMMA (0.1 µM – 10 mM), dexamethasone (1 µM), sodium nitroprusside (0.001–1000 µM), indomethacin (30 µM) or cycloheximide (1 µM) were added together with LPS for 24 h after which time the medium was removed for the measurement of nitrite or 6-keto-$PGF_{1\alpha}$.

Broken cell preparations

J774.2 macrophages were cultured in T175 flasks until confluent. LPS (1 µg ml^{-1}) was added for 12 h, after which time the cells were washed and scraped into ice-cold phosphate buffered saline (pH 7.4). The cells were then centrifuged (1,000 *g* for 10 min) and the cell pellet was homogenized with a glass-Teflon homogeniser in Tris buffer (50 mM; pH 7.4) containing phenylmethylsulphonyl fluoride (1 mM), pepstatin A (1.5 mM) and leupeptin (0.2 mM). The broken cell preparation was incubated at 37°C with sodium nitroprusside (0.1–1000 µM) for 30 min. Arachidonic acid (30 mM) was added and the incubations continued for a further 15 min until stopped by boiling. The incubates were centrifuged (10,000 *g* for 30 min) and the concentrations of 6-keto-$PGF_{1\alpha}$ measured in the supernatant as indicators of COX activity. In separate experiments, COX activity was measured in the presence of the co-factors adrenaline (5 mM) and glutathione (0.1 mM) together with different concentrations of sodium nitroprusside (0.1–1000 µM) as above. In some experiments, J774.2 cells were incubated for 12 h with LPS (1 µg ml^{-1}) and L-NMMA (1 mM), in the presence or absence of SNP (0.01–1000 µM), and the activity of COX in cell homogentates was measured as described above.

Western blot analysis

J774.2 macrophages were cultured in T175 flasks until confluent. Cells were treated with LPS (1 µg ml^{-1}) and either L-NMMA (10 mM) or SNP (1 mM) or vehicle (distilled water) for 12 h. Cells were washed with PBS (pH 7.4) and incubated (10 min) with 2–3 ml of extraction buffer [Tris, 50 mM; EDTA, 10 mM; Triton X-100, 1 % v/v; phenylmethylsul-

phonyl fluoride (PMSF; 1 mM), pepstatin A (50 μM) and leupeptin (0.2 mM)] with gentle shaking. The cell extract was then boiled (10 min) in a ratio of 1:1 with gel loading buffer (Tris, 50 mM; SDS, 10 %; glycerol, 10 % and bromphenol blue, 2 mg ml^{-1}). The samples were loaded onto gradient gels (4–12 % Tris-glycine; Novex) and subjected to electrophoresis (1.5 h at 125 mV). The separated proteins were transferred to nitrocellulose (BIORAD; 1 h at 200 mV). After transfer to nitrocellulose, the blot was primed with a selective rabbit antibody raised to murine COX-2 (Cayman Chemical Company, MI, U.S.A.). The blot was then incubated with an anti-rabbit IgG developed in sheep, which was linked to alkaline phosphatase conjugate. The blot was then developed with premixed solution (pH 9.2) containing 5-bromo-4-chloro-3-indolyl phosphate (0.56 mM), nitro blue tetrazolium (0.48 mM), Tris (10 mM) and $MgCl_2$ (59.3 mM).

Measurement of cell viability

Cell respiration, an indicator of cell viability, was assessed by the mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Gross *et al.*, 1991). Cells in 96-well plates were incubated at 37°C with MTT (200 μg ml^{-1}) for 60 min. Culture medium was removed by aspiration and the cells solubilized in dimethylsulphoxide (DMSO; 100 μl). The extent of reduction of MTT to formazan within cells was quantitated by measurement of OD_{550}.

Whole organ experiments

Interactions between the NOS and COX systems were investigated in male Wistar rats (250–300 g). Rats were injected intraperitoneally (i.p.) with LPS (5 mg kg^{-1}) or vehicle and 6 h later killed by an overdose of pentobarbitone, at which time kidneys or lungs were removed and perfused *ex vivo*. Kidneys were perfused through the renal artery at 10 ml min^{-1}, whereas lungs were inflated via the trachea and perfused through the pulmonary artery at 5 ml min^{-1}, with warmed and oxygenated Krebs solution. The Krebs solution had the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.17, $CaCl_2$ 2.5, $NaHCO_3$ 2.5 and glucose 5.6, pH 7.4. After an equilibration period of 30 min, organs were infused with L-NMMA (1 mM) or vehicle and 15 min later arachidonic acid (AA, 30 nmol) was injected. Effluent was collected at intervals of 1 min. The concentrations of 6-keto-$PGF_{1\alpha}$, prostaglandin E_2 and thromboxane B_2 in the effluent were determined by radioimmunoassay. In separate experiments, rats were injected i.p. with L-NMMA (30 mg kg^{-1}) at the same time as LPS (5 mg kg^{-1}) or vehicle, and then at 6 h organs taken and perfused *ex vivo* as above. The activity of NOS was determined *ex vivo* by the ability of organ homogenates to convert [^{3}H]-L-arginine to [^{3}H]-L-citrulline (Mitchell *et al.*, 1991b). iNOS was distinguished from the constitutive NOS by performing experiments in the presence of Ca^{2+} (2 mM) or in the absence of Ca^{2+} (EGTA, 1 mM). COX activity was determined by the ability of organ homogenates to metabolize AA (30 μM) to prostacyclin (measured as 6-keto-$PGF_{1\alpha}$) as measured by radioimmunoassay.

Statistics

Results are shown as mean ± s.e.mean for n experiments. One way analysis of variance was used and P-values of $P<0.05$ (* or +), $P<0.01$ (**) or $P<0.001$ (***) taken as significant.

Materials

All compounds were obtained at the highest quality from Sigma Chemical Company, Poole, Dorset, unless otherwise stated.

Results

Accumulation of nitrite

Bacterial LPS (1 ng ml^{-1} – 10 μg ml^{-1}; for 24 h) caused concentration-dependent increases in nitrite in the culture medium of J774.2 macrophages ($n=9$ for each, data not shown), with a maximum effect being caused by 1 μg ml^{-1} of LPS. As treatment of the cells with this concentration of LPS did not affect cell viability ($n=3$; data not shown) and also caused maximal stimulation of COX-2 formation, LPS was used at this concentration in all further experiments.

Activation of macrophages with LPS (1 μg ml^{-1} for 24 h) caused a time-dependent increase in nitrite, which was significant at 12 h and 24 h (Figure 1). Dexamethasone (1 μM) or cycloheximide (1 μM), added 30 min prior to LPS, attenuated the increase measured at 24 h by 89 ± 5 % and 92 ± 4 %, respectively ($n=3$ for each).

Accumulation of 6-keto-$PGF_{1\alpha}$

Treatment of J774.2 macrophages with LPS (1 μg ml^{-1}) caused a time-dependent accumulation of 6-keto-$PGF_{1\alpha}$ in the culture medium which was first significant ($P<0.05$) at 6 h after the addition of LPS (Figure 1; $n=9$). The formation of 6-keto-$PGF_{1\alpha}$ at 24 h was attenuated by dexamethasone (1 μM; 80 ± 3 %) or cycloheximide (1 μM; 94 ± 4 %), added 30 min before LPS ($n=3$). Indomethacin (30 μM) abolished the release of 6-keto-$PGF_{1\alpha}$ at all time points, but did not affect nitrite release from LPS-activated cells, measured after 3, 6, 12 or 24 h ($n=9$ for each; data not shown).

Effect of sodium nitroprusside on the release of 6-keto-$PGF_{1\alpha}$

Sodium nitroprusside (0.1–1000 μM), added together with LPS for 24 h, produced a concentration-dependent inhibition of the formation of 6-keto-$PGF_{1\alpha}$ by J774.2 cells (Figure 2a; $n=12$) with no significant effect on cell viability (data not shown). A broken cell preparation of COX-2 (LPS-activated J774 macrophages) was used to assess any direct effects of SNP on COX-2 activity. Broken cell preparations of J774.2 macrophages exposed to LPS (1 μg ml^{-1} for 12 h) converted arachidonic acid (30 μM) to 6-keto-$PGF_{1\alpha}$; an effect that was inhibited by more than 80 % by indomethacin (30 μM; data not shown). Incubations of broken cell preparations with sodium nitroprusside (0.1–1000 μM) for 30 min produced a

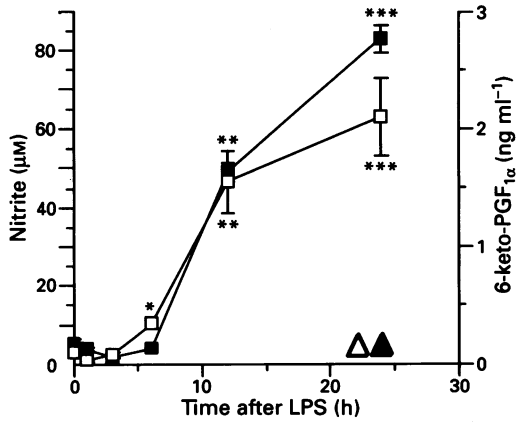

Figure 1 Time-course for the accumulation of nitrite (■) and 6-keto-$PGF_{1\alpha}$ (□) in the culture medium of J774.2 macrophages incubated with LPS (1 μg ml^{-1}). The amounts of nitrite (▲) or 6-keto-$PGF_{1\alpha}$ (Δ) in the culture medium of untreated cells after 24 h incubation are indicated. Each point represents the mean ± s.e.mean for 12 determinations performed on 4 separate experimental days. Significance of asterisks, see text.

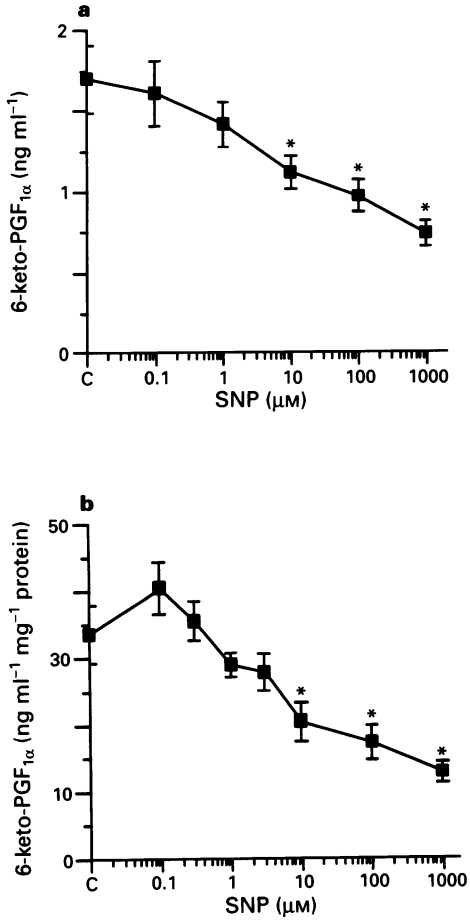

Figure 2 The effect of sodium nitroprusside on the accumulation of 6-keto-$PGF_{1\alpha}$ (a) or COX-2 activity (b) in J774.2 macrophages exposed to LPS (1 μg ml^{-1}) for 24 or 12 h, respectively. (a) Sodium nitroprusside (SNP; 0.1–1000 μM), co-incubated with LPS for 24 h, caused a concentration-dependent inhibition of 6-keto-$PGF_{1\alpha}$ accumulation in the medium of intact J774.2 macrophages. The figure shows the mean ± s.e.mean for 12 determinations performed on 4 separate experimental days. (b) SNP (0.1–1000 μM), incubated for 30 min with homogenates of LPS-treated (12 h) J774.2 macrophages, concentration-dependently inhibited the ability of cell homogenates to form 6-keto-$PGF_{1\alpha}$ in response to exogenous arachidonic acid (30 μM). The data represents the mean ± s.e.mean from 12 determinations performed on 4 separate enzyme preparations. *Denotes significant differences in the production of 6-keto-$PGF_{1\alpha}$ (compared to control [C]-cells treated in the absence of SNP). Similar results were obtained when PGE_2 was measured as an index of COX-2 activity ($n = 12$; data not shown).

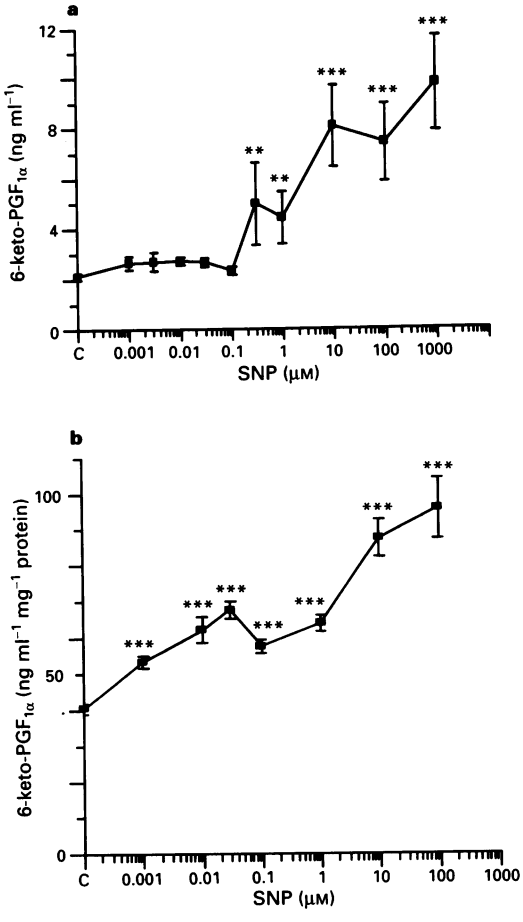

Figure 3 The effect of sodium nitroprusside on the accumulation of 6-keto-$PGF_{1\alpha}$ (a) or COX-2 activity (b) in J774.2 macrophages exposed to LPS (1 μg ml^{-1}) for 24 or 12 h, respectively, in the presence of L-NMMA (1 mM). (a) Sodium nitroprusside (SNP; 0.001–1000 μM), co-incubated with the cells in the presence of LPS (1 μg ml^{-1}) and L-NMMA (1 mM) for 24 h, caused a concentration-dependent increase in the accumulation of 6-keto-$PGF_{1\alpha}$ in the medium of intact J774.2 macrophages. The figure shows the mean ± s.e.mean ($n = 8$). (b) SNP (0.01–1000 μM), co-incubated with the cells in the presence of LPS (1 μg ml^{-1}) and L-NMMA (1 mM) for 12 h, caused a concentration-dependent increase in the ability of cell homogenates to form 6-keto-$PGF_{1\alpha}$ in response to exogenous arachidonic acid (30 μM) administered with the co-factors (adrenaline, 5 mM; glutathione, 0.1 mM). The figure shows the mean ± s.e.mean ($n = 9$). *Denotes significant differences in the production of 6-keto-$PGF_{1\alpha}$ (compared to control [C]-cells treated in the absence of SNP).

concentration-dependent inhibition of COX-2 activity which was significant at 10, 100 and 1000 μM (Figure 2b; $n = 12$). When haemoglobin (10 μM) was added together with SNP, only the highest concentration used caused a significant inhibition of COX-2 activity ($n = 6$; data not shown). COX-2 activity was greatly enhanced when the co-factors, adrenaline (5 mM) and glutathione (0.1 mM), were added to the incubations. This combination of co-factors maximally potentiates the activity of COX (Swierkosz, unpublished observations). SNP at the highest concentration of 1 mM also inhibited COX-2 activity in broken cell preparations carried out in the presence of adrenaline and glutathione ($n = 12$; data not shown).

When J774.2 macrophages were incubated with LPS (1 μg ml^{-1}) and L-NMMA (1 mM) for 12 h, SNP at the highest concentration used (1 mM; $n = 6$; data not shown) acutely (30 min) inhibited in the presence of co-factors the activity of COX-2 in cell homogenates. However, when J774.2 macrophages were incubated for 24 h with LPS (1 μg ml^{-1}) and L-NMMA (1 mM), the addition of SNP (0.001–1000 μM) to the culture medium for 24 h increased in a concentration-dependent manner the accumulation of 6-keto-$PGF_{1\alpha}$ in the medium of intact cells (Figure 3a; $n = 8$-32). Similarly, when J774.2 macrophages were incubated for 12 h with LPS (1 μg ml^{-1}) and L-NMMA (1 mM), the addition of SNP (0.01–1000 μM) to the culture medium for 12 h increased in a concentration-dependent manner the activity of COX-2 in cell homogenates containing co-factors (Figure 3b; $n = 9$). In the J774.2 cells which were not treated with LPS, production of 6-keto-$PGF_{1\alpha}$ was undetectable but became detectable when the cells were incubated with SNP (1 mM; $n = 4$; data not shown).

Effect of NO synthase inhibition on the accumulation of nitrite and 6-keto-$PGF_{1\alpha}$

L-NMMA (1 μM – 10 mM) caused a concentration-dependent inhibition of nitrite accumulation and a concentration-

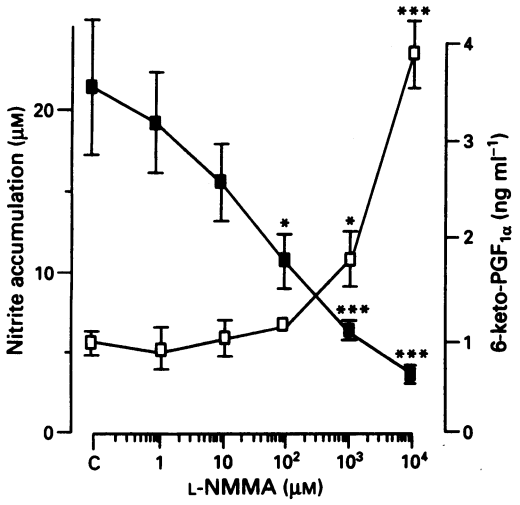

Figure 4 The effect of L-NMMA on nitrite (■) and 6-keto-$PGF_{1\alpha}$ (□) accumulation in the culture medium of J774.2 macrophages treated with LPS (1 μg ml^{-1}). L-NMMA was added together with LPS and incubated for 24 h. The points represent the mean ± s.e.mean for 9 determinations performed on 3 experimental days. *Denotes significant differences in the production of 6-keto-$PGF_{1\alpha}$ or in the production of nitrite (compared to control [C]-cells treated with LPS alone).

Table 1 Reversal by L-arginine of the effects of N^G-monomethyl-L-arginine (L-NMMA) on the formation of nitrite and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) in LPS-treated (1 μM for 24 h) J774.2 macrophages

	LPS	*LPS + L-NMMA*	*LPS + L-NMMA + L-Arg*
Nitrite (μM)	49 ± 1	8 ± 1***	20 ± 3+
6-keto-$PGF_{1\alpha}$ (ng ml^{-1})	1.7 ± 0.1	3.1 ± 0.3*	1.7 ± 0.2+

L-Arginine (L-Arg 3 mM) reversed by approximately 50% the inhibitory effect of L-NMMA (1 mM) on the formation of nitrite and completely reversed the potentiation induced by L-NMMA (1 mM) on the formation of 6-keto-$PGF_{1\alpha}$. The data show the mean ± s.e.mean ($n = 3$). *Denotes significant differences between the LPS + L-NMMA group and the LPS group. +Denotes significant differences between the LPS + L-NMMA + L-Arg group and the LPS + L-NMMA group.

dependent increase in 6-keto-$PGF_{1\alpha}$ accumulation (Figure 4; $n = 9$). The inhibitory effect of L-NMMA (1 mM) on nitrite release from LPS-activated J774.2 macrophages was partially reversed by L-arginine (3 mM; Table 1; $n = 3$). Similarly, the potentiation of the release of 6-keto-$PGF_{1\alpha}$ from LPS-activated J774.2 macrophages caused by L-NMMA was prevented by L-arginine (3 mM; Table 1; $n = 3$). In contrast, D-NMMA (1 μM – 10 mM) had no significant effect on the LPS-stimulated formation of either nitrite or 6-keto-$PGF_{1\alpha}$ ($n = 3$; data not shown).

In separate experiments, J774.2 macrophages were activated with LPS (1 μg ml^{-1}; for 24 h), and L-NMMA (10 mM) was added to the cells for various times (0.5–24 h) before the collection of medium at 24 h (Figure 5). Addition of L-NMMA for 30 min before the collection of medium at 24 h after LPS did not affect the release of 6-keto-$PGF_{1\alpha}$ (Figure 5). However, addition of L-NMMA for 12, 18, 21 and 24 h caused significant and time-dependent increases in 6-keto-$PGF_{1\alpha}$ accumulation measured at 24 h. The maximal (3 fold) enhancement was seen when L-NMMA was incubated with LPS for the entire 24 h period (Figure 5; $n = 9$).

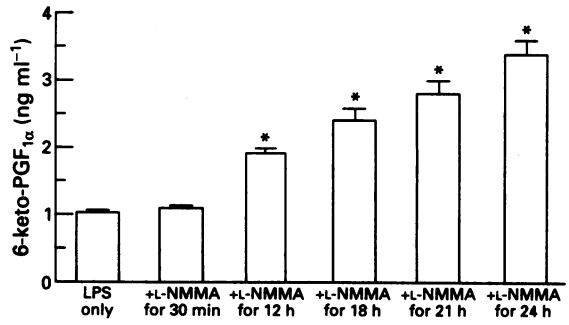

Figure 5 Accumulation of 6-keto-$PGF_{1\alpha}$ in LPS-treated J774.2 macrophages after incubation with L-NMMA for various times before the collection of medium. L-NMMA caused a time-dependent increase in 6-keto-$PGF_{1\alpha}$ accumulation when J774.2 macrophages were treated with LPS (1 μg ml^{-1}) for 24 h and L-NMMA (10 mM) (30 min–24 h) before the collection of medium. The enhancement by L-NMMA of the release of 6-keto-$PGF_{1\alpha}$ was maximal when L-NMMA was incubated with LPS for the entire 24 h period. The data represent the mean ± s.e.mean of 9 separate determinations. *Denotes significant differences from control (cells incubated with LPS only).

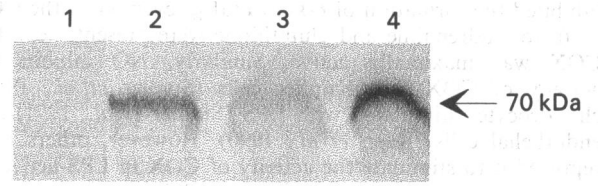

Figure 6 Effect of sodium nitroprusside (SNP) and L-NMMA on COX-2 induction in LPS-activated J774.2 macrophages. Cell extracts from untreated J774.2 macrophages contained no COX-2 (lane 1). However, extracts of J774.2 macrophages exposed to LPS (1 μg ml^{-1}) for 12 h contained COX-2 which migrated as a protein of approximately 70 kDa (lane 2). The amount of COX-2 present 12 h after LPS administration was decreased when SNP (1 mM) was co-incubated with LPS (lane 3) and increased when L-NMMA (10 mM) was co-incubated with the LPS (lane 4). The figure represents the data from a single experiment. Similar results were obtained with cell extracts in 5 other experiments.

Effect of SNP and L-NMMA on induction of COX-2 protein

Cell extracts from J774.2 macrophages incubated with LPS (1 μg ml^{-1} for 12 h) contained COX-2 protein ($n = 6$, Figure 6). The amount of COX-2 protein induced by LPS was increased approximately 2 fold by L-NMMA (10 mM, $n = 6$) and decreased by SNP (1 mM, $n = 3$; Figure 6). No COX-2 protein was detected in unstimulated cells ($n = 6$, Figure 6).

Effect of L-NMMA in whole organs

In the lung, LPS (5 mg kg^{-1}, i.p., for 6 h) increased NOS and COX activity, as measured in organ homogenates, as well as the release of thromboxane B_2 measured from perfused organs (NOS activity, pmol L-Cit $30\ min^{-1}\ mg^{-1}$ protein: control, 2.7 ± 0.6; LPS, 28 ± 20; COX activity, ng 6-keto-$PGF_{1\alpha}$ $30\ min^{-1}\ mg^{-1}$ protein: control, 14.7 ± 3.3; LPS, 30 ± 2.9; thromboxane release, ng ml^{-1} $5\ min^{-1}$: control, 5.93 ± 1.6; LPS, 10.1 ± 0.9). L-NMMA (30 mg kg^{-1}), given i.p. together with LPS (5 mg kg^{-1}) for 6 h or administered to perfused organs acutely for 15 min, did not affect any of the parameters measured above. Intraperitoneal (i.p.) administration of LPS (5 mg kg^{-1}, for 6 h) to rats increased the basal perfusion pressure of the kidney measured *ex vivo* (control, 36 ± 4; LPS, 63 ± 5 mmHg) but this was not associated with

any significant changes in the release of prostaglandins elicited by 30 nmol AA ($n = 3$).

Discussion

Here we show that endogenous or exogenous NO inhibits the expression of the COX-2 enzyme and that exogenous NO also inhibits its activity.

Sodium nitroprusside attenuated the accumulation of 6-keto-$PGF_{1\alpha}$ in the medium of intact J774.2 macrophages and reduced the expression of COX-2 protein within the same cells. This accords directly with the known inhibitory effects of SNP or NO on the release of PGI_2 from endothelial cells (Doni *et al.*, 1988; Keen *et al.*, 1990; Mathews *et al.*, 1993). More interestingly, endogenous NO had a similar inhibitory role in the cells, for the addition of L-NMMA, at a concentration that reduced the accumulation of nitrite from approximately 20 μM to 1 μM, caused a significant increase in PGI_2 release and an increase in the expression of COX-2 protein.

In addition to the inhibitory effect of NO on the expression of COX-2, we also most generally found it to reduce the activity of COX-2. For instance, in homogenates of cells treated with LPS for 12 h, addition of SNP for 30 min inhibited the formation of 6-keto-$PGF_{1\alpha}$, even when the COX co-factors adrenaline and glutathione were present, i.e. when COX was maximally active. Similarly, NO inhibits the activity of COX-2 in Kupffer cells (Stadler *et al.*, 1993), chondrocytes stimulated with LPS (Stadler *et al.*, 1991) and endothelial cells (Keen *et al.*, 1990). However, others have reported it to stimulate the activity of COX in LPS-activated RAW macrophages (Salvemini *et al.*, 1993), vascular smooth muscle cells (Inoue *et al.*, 1993), homogenates of COX-1 or COX-2 transfected cells (Salvemini *et al.*, 1993), islets of Langerhans (Corbett *et al.*, 1993), hypothalamic neurones (Rettori *et al.*, 1993), uterine smooth muscle (Franchi *et al.*, 1994) and chondrocytes stimulated with LPS and cytokines (Stadler *et al.*, 1991). None of these results distinguishes between the activity of COX and the expression of the enzyme. How can we explain these apparent conflicts? Perhaps there are different amounts of NO in the various assay systems used, as L-NMMA, for instance, suppresses the release of PGE_2 from interleukin-1-treated chondrocytes, when endogenous NO production is low, but elevates it when endogenous NO production is high (Stadler *et al.*, 1991). In our experiments, the endogenous production of NO by J774.2 macrophages certainly attained levels that inhibit the activity of COX-2. When the cells were co-incubated with LPS plus L-NMMA, to eliminate endogenously produced NO, together with SNP to provide controlled amounts of exogenous NO (Feelisch, 1991), we found that it enhanced the accumulation of 6-keto-$PGF_{1\alpha}$ measured at 24 h, and increased the acute formation of 6-keto-$PGF_{1\alpha}$ at 12 h after the addition of LPS.

The influence of NO on prostanoid synthesis may, therefore, vary in different cell types with variations in the rate of prostanoid synthesis, the state of cell activation, and the amount of iNOS and COX-2 proteins present. This hypothesis also answers the long standing controversy of whether nitrovasodilators release prostacyclin from the endothelium (Mehta *et al.*, 1983a,b; Schrör *et al.*, 1988; Levin *et al.*, 1981; Nakabayashi *et al.*, 1985) or not (Mehta *et al.*, 1983b; Fitzgerald *et al.*, 1984; Levin *et al.*, 1982; De Caterina *et al.*, 1985; Bennett *et al.*, 1987; Brotherton, 1986).

Thus, NO has a dual (potentiating in small amounts and inhibiting in large amounts) effect on the activity of COX-2 and an inhibitory effect on the expression of COX-2 protein. Mechanistically, this inhibition of COX-2 activity can be explained by the ability of NO to reduce the ferric-active form of COX to the ferrous-inactive form (Kanner *et al.*, 1992), or to nitrosylate tyrosine groups within COX (Shimokawa *et al.*, 1990). Conversely, there is not a good mechanistic explanation for the suggestion that NO directly stimulates the activity of COX (Stadler *et al.*, 1991; Corbett *et al.*, 1993; Rettori *et al.*, 1993; Salvemini *et al.*, 1993; Franchi *et al.*, 1994).

The dual modulatory effects of NO on COX activity may also be related to the levels of other radicals formed by macrophages, such as superoxide anions or hydrogen peroxide (Beckman *et al.*, 1990; Heinzel *et al.*, 1992). The concentrations of these agents will also vary greatly between different cell types, providing an alternative explanation of the conflicting observations described above (see Lands, 1985).

In contrast to the inhibitory effects of endogenously released NO on COX-2, the accompanying release of prostanoids did not affect the activity of NOS, even though exogenously applied PGE_2 or iloprost (a stable analogue of PGI_2) inhibit nitrite accumulation in LPS-treated J774.2 cells (Marotta *et al.*, 1992). This difference is explained by the endogenous release of prostanoids from the J774.2 macrophages being insufficient to produce this effect.

We also investigated the interactions between the NOS and COX systems *in vivo*, where, for instance, in septic or haemorrhagic shock elevated levels of cytokines lead to the induction of NOS and COX, and the formation of large amounts of NO (Thiemermann *et al.*, 1993). No evidence was found, however, for any cross regulation in our *ex vivo* organ studies, although this does not preclude the possibility that in other situations, when the induction of iNOS is more pronounced, NO may influence COX. For instance, in lungs from LPS-treated rats (Sautebin & Di Rosa, 1994) or in the rabbit perfused hydronephrotic kidney (Salvemini *et al.*, 1994), the activity of COX is stimulated by endogenous NO, although the mechanism of this effect is unclear. The lack of a regulatory effect of NO on the activity of COX found in our whole organ study is in line with the recent findings of Tsai *et al.* (1994) who questioned the existence of significant interactions of the COX haem with NO under conditions likely to be encountered *in vivo*. They showed, using electron paramagnetic resonance measurements, that NO reacted strongly with COX-1, forming an NO-haem complex and so breaking the bond between the protein and the haem iron. However, this was only in anaerobic conditions, which would not allow normal cyclo-oxygenase or peroxidase catalysis.

We conclude that iNOS and COX-2 can be co-induced *in vitro* and that under these conditions NO inhibits the degree of both COX-2 expression and activity. In the absence of endogenous NO, exogenous NO can activate COX-2. In situations *in vivo*, in which the level of NO induction is relatively low, NO does not regulate the increased activity of COX.

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Substance P-induced inflammatory responses in guinea-pig skin: The effect of specific NK_1 receptor antagonists and the role of endogenous mediators

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1 The sensory neuropeptide substance P (SP), when released from sensory nerves, has been implicated in the development of neurogenic inflammation. In the present study, using an *in vivo* model system, we have characterized and investigated the mechanisms underlying SP-induced leukocyte accumulation and oedema formation in the guinea-pig.

2 Intradermally injected SP (i.d., 10^{-13}–10^{-9} mol per site), induced a dose- and time-dependent accumulation of ^{111}In-neutrophils, ^{111}In-eosinophils and oedema formation as measured by the local accumulation of i.v. injected ^{125}I-albumin. The leukocyte accumulation evoked by SP was significant at 10^{-10} and 10^{-9} mol per site, whereas oedema formation was significant at the lowest dose tested (10^{-13} mol per site).

3 The NK_1 receptor antagonists, CP-96,345 (1 mg kg^{-1}, i.v.) and RP-67,580 (10 μg per site, i.d.), significantly attenuated the oedema formation induced by the lower doses of SP. Oedema formation and leukocyte accumulation induced by 10^{-9} mol per site SP were unaffected by either antagonist.

4 SP-elicited responses were not significantly affected by the platelet activating factor (PAF) receptor antagonist, UK-74,505 (2.5 mg kg^{-1}, i.v.) or the H_1 histamine receptor antagonist, chlorpheniramine (10^{-8} mol per site, i.d.). However, the ^{111}In-eosinophil accumulation, but not the ^{111}In-neutrophil accumulation or oedema formation, induced by SP was significantly inhibited by the specific 5-lipoxygenase (5-LO) inhibitor, ZM-230,487 (10^{-8} mol per site, i.d.).

5 The accumulation of both ^{111}In-neutrophils and ^{111}In-eosinophils induced by SP was abolished in guinea-pigs treated i.v. with an anti-CD18 monoclonal antibody 6.5E F(ab')$_2$ (2.5 mg kg^{-1}). The oedema response was unaffected in these animals.

6 These results suggest that SP-induced inflammatory events may be mediated via two mechanisms involving NK_1 receptor-dependent and independent pathways. Oedema formation induced by the lower doses of SP may be mediated via the direct activation of NK_1 receptors whilst, at higher doses, oedema formation and leukocyte accumulation may be mediated via the release of secondary mediators, possibly mast cell derived, with 5-LO products playing an important role in the leukocyte infiltration. The leukocyte accumulation, but not the oedema induced by SP, is dependent on the expression of the CD18 antigen on leukocytes.

Keywords: Substance P; eosinophils; neutrophils; oedema formation; inflammation; CP-96,345; RP-67,580; chlorpheniramine; UK-74,505; ZM-230,487

Introduction

Substance P (SP), an 11 amino acid neurotachykinin present in sensory nerve fibres, is believed to be a mediator of neurogenic inflammation (Lembeck & Holzer, 1979). This response, which can be induced by electrical or chemical stimulation of sensory neurones, involves a number of inflammatory events such as local vasodilatation, oedema formation and leukocyte accumulation. These components of the inflammatory response can be elicited by administration of SP. In man, SP causes a classic wheal and flare response (Hagermark *et al.*, 1978; Foreman & Jordan, 1983; Foreman *et al.* 1983) as well as eliciting granulocyte infiltration (Smith *et al.*, 1993) and modulating the activity of another neuropeptide calcitonin gene-related peptide (CGRP) (Brain & Williams, 1988). In addition, SP-induced oedema formation and leukocyte accumulation has been studied in a number of experimental animals (Matsuda *et al.*, 1989; Yano *et al.*, 1989; Lembeck *et al.*, 1992; Wilsoncroft *et al.*, 1994).

The inflammatory properties of SP appear to be primarily mediated via its interaction with the neurokinin$_1$ (NK_1) receptor (Guard & Watson, 1991) which is a member of the G-protein-coupled receptor superfamily (Hershey & Krause, 1990; Takeda *et al.*, 1991). The availability of specific, non-peptide, NK_1 receptor antagonists has allowed direct investigations into the role of endogenous SP in different inflammatory reactions. The NK_1 receptor antagonist, CP-96,345 (Snider *et al.*, 1991) has been shown to inhibit SP-induced plasma extravasation in guinea-pig dorsal skin (Nagahisa *et al.*, 1992) and neurogenic inflammation in rats (Lembeck *et al.*, 1992). Similarly, the NK_1 receptor antagonist, RP-67,580 (Garret *et al.*, 1991) inhibits oedema formation induced by SP and in response to a passive cutaneous anaphylaxis reaction in guinea-pig skin (Wilsoncroft *et al.*, 1994).

A number of mediators have been implicated in the inflammatory responses elicited by SP. In particular, it has been suggested that mast cell-derived mediators such as histamine, play an important role in the induction of SP-induced responses (Hagermark *et al.*, 1978; Foreman *et al.*, 1983; Foreman & Jordan, 1983). In addition, leukotriene B_4, (LTB_4) mediates in part the SP-induced leukocyte infiltration into mouse skin (Iwamoto *et al.*, 1993) and SP selectively induces the expression of messenger RNA for tumour necrosis factor-α (TNF-α) in a mast cell line (Ansel *et al.*, 1993).

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The effects of SP on cutaneous vascular permeability have previously been described in the guinea-pig and a role for NK_1 receptors identified (Iwamoto & Nadel, 1989; Wilsoncroft *et al.*, 1994). The aim of the present study was to extend these findings by addressing the role of NK_1 receptors in SP-induced neutrophil and eosinophil accumulation in guinea-pig skin. In addition, we have investigated the contributions of endogenously formed histamine, PAF and 5-lipoxygenase (5-LO) products in these reactions. Since these responses involve a close interaction between leukocytes and endothelial cells, we have also investigated the role of the leukocyte adhesion molecule, CD18.

Methods

Animals

Female ex-breeder Dunkin-Hartley guinea-pigs (800–900 g) and recipient guinea-pigs of either sex (300–400 g) were purchased from Harlen Olac, Bicester, Oxon, U.K.

Preparation of ^{111}In-labelled guinea-pig neutrophils and eosinophils

Guinea-pig peritoneal neutrophils and eosinophils were purified and radiolabelled with ^{111}In chelated to 2-mercaptopyridine-*N*-oxide as previously described (Faccioli *et al.*, 1991; Sanz *et al.*, 1994). Briefly, to elicit neutrophils, ex-breeder guinea-pigs were injected i.p. with 15 ml of 5% casein solution 18 h before they were killed. To elicit eosinophils, ex-breeder guinea-pigs were injected i.p. with 1 ml horse serum every second day, at least 5 times. The final injection was given 18 h before they were killed. Animals were killed by asphyxiation with CO_2 and the peritoneal cavity was lavaged with 30 ml of heparinized saline (10 u ml^{-1}). Both eosinophils and neutrophils were purified over discontinuous percoll gradients (1.080, 1.085, 1.090, 1.095, 1.100 g ml^{-1}). Cell populations were used only when the purity of the eosinophil preparation was above 95% and the purity of the neutrophil preparation was above 99%. The cells were then incubated with ^{111}In (100 μCi in 10 μl) chelated to 2-mercaptopyridine-*N*-oxide (40 μg in 0.1 ml of 50 mM PBS) for 15 min, washed twice in Ca^{2+}- and Mg^{2+}-free HBSS containing 10 mM HEPES and 10% platelet poor plasma (pH 7.3) and resuspended at a final cell concentration of 10^7 cells ml^{-1}.

Measurement of ^{111}In-leukocyte accumulation and oedema formation

Recipient guinea-pigs (300–400 g) were anaesthetized with 0.1 ml Hypnorm and their dorsal skin shaved. Either radiolabelled neutrophils or eosinophils (5×10^6) were mixed with ^{125}I-HSA (^{125}I-albumin, 5 μCi kg^{-1}) and injected i.v. via an ear vein in a volume of 0.5 ml. After 5 min, test agents were injected i.d. in volumes of 0.1 ml. At the end of the 2 h *in vivo* test period, animals were anaesthetized with i.p. sodium pentobarbitone and a 3 ml cardiac blood sample collected. The animals were then killed by an overdose of anaesthetic, the dorsal skin removed and the injection sites punched out. Skin, blood and plasma samples were counted in a gamma counter. Eosinophil and neutrophil accumulation was expressed as the number of labelled leukocytes per site and oedema formation, as measured by the local accumulation of ^{125}I-albumin, was expressed as μl of plasma per site (Faccioli *et al.*, 1991; Sanz *et al.*, 1994).

The kinetics of responses induced by SP were investigated using a cumulative time-course protocol. The animals were injected with ^{111}In-cells and ^{125}I-albumin as described above. Over a test period of 2 h, SP (10^{-9} mol per site) was injected i.d. at 2, 1, 0.5 and 0 h before the animals were killed. The inflammatory responses induced by i.d. SP (10^{-13}–10^{-9} mol per site) were investigated using 2 specific non-peptide NK_1 receptor antagonists, CP-96,345 (1 mg kg^{-1}, i.v.) and RP-67,580 (10 μg per site, equivalent to 2.0×10^{-8} mol per site, i.d.). In addition, the effects of the specific H_1 receptor antagonist, chlorpheniramine (10 μg per site, equivalent to 2.5×10^{-8} mol per site, i.d.), the PAF receptor antagonist, UK-74,505 (2.5 mg kg^{-1}, i.v.), the 5-lipoxygenase inhibitor, ZM-230,487 (10 μg per site, equivalent to 2.4×10^{-8} mol per site, i.d.) and the anti-CD18 monoclonal antibody 6.5E $F(ab')_2$ (2.5 mg kg^{-1}, i.v.) on the SP-induced responses were investigated.

Materials

Substance P, histamine, 2-mercaptopyridine-N-oxide, BSA were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Horse serum, sterile Hanks Balanced Salt Solution (HBSS, without Ca^{2+} and Mg^{2+} 10 ×) and HEPES (1 M) were obtained from Gibco Limited, Paisley, Renfrewshire, U.K. ^{125}I-human serum albumin (^{125}I-albumin, 20 mg ml^{-1} of sterile saline, 50 μCi ml^{-1}), ^{111}Indium chloride ($^{111}InCl_3$; 10 mCi ml^{-1} sterile pyrogen-free 0.04 N hydrochloric acid) were obtained from Amersham International, Amersham, Buckinghamshire, U.K. Percoll was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Chlorpheniramine (10 mg ml^{-1}) was obtained from Allen and Hanburys Ltd., London, U.K. LTB_4 was obtained from Cascade Biochem Ltd., Reading, Berkshire, U.K. PAF was obtained from Bachem Ltd., Saffron Waldon, Essex, U.K.

The following substances were kind gifts: RP-67,580 (2-[1-imino-2-(methoxy phenyl) ethyl]-7,7 diphenyl-4 perhydro-isoindolone (3aR, 7aR)) was from C. Garret, Rhone-Poulenc Rorer, Vitry, France. CP-96,345 (the dihydrochloride salt of (2S,3S)-*cis*-2-(diphenylmethyl)-*N*-((2-methoxyphenyl)methyl)-1-azabicyclo[2.2.2]octan-3-amine) and its 2R,3R enantiomer CP-96,344 were from Pfizer Incorporation, Groton, U.S.A. and UK-74,505 (4,-(2-chlorophenyl)-1,4-dihydro-3-ethoxy-carbonyl-6-methyl-2-[4-(2-methylimidazol[4,5-c]pyrid-1-yl) phenyl]-5-[N-(2-pyridylcarbamoyl] pyridine) was from Dr John Parry, Pfizer Central Research, Sandwich, U.K. Monoclonal antibody 6.5E $F(ab')_2$ was from Dr Martyn Robinson, Celltech, Slough, U.K. ZM-230,487 (1-ethyl-6-[fluoro-5-(4methoxy-3,4,5,6-tetrahydro-2H-pyran-4-yl)phenoxy] methyl-quinol-2-one) was from Dr Roger McMillan, Zeneca Pharmaceuticals, Macclesfield, Cheshire, U.K.

Data analysis

Results are expressed as the means ± s.e.mean for *n* animals where each datum unit is the average of responses in duplicate sites. Data were analysed by two way analysis of variance (ANOVA) of log transformed data and statistical significance determined with the Neuman-Keuls procedure for repeated comparisons (Snedecor & Cochran, 1967).

Results

Substance P-induced inflammatory responses in guinea-pig skin

The i.d. injection of SP caused a dose-dependent increase in ^{111}In-neutrophil (Figure 1a), ^{111}In-eosinophil (Figure 1b) and oedema formation (Figure 1c) above levels detected in saline injected sites in guinea-pig skin. Oedema formation was significant at 10^{-13} mol per site SP, the lowest dose tested, and increased in a dose-dependent manner. However, significant ^{111}In-neutrophil and ^{111}In-eosinophil accumulation occurred only at the top two doses of SP, namely 10^{-10} and 10^{-9} mol per site ($P < 0.05$).

Cumulative time-course experiments indicated that following the i.d. administration of SP, leukocyte accumulation

(Figure 2a,b) and oedema formation (Figure 2c) reached a maximal level within the first hour.

Effect of NK_1 receptor antagonists on SP-induced responses

The effects of two specific NK_1 receptor antagonists, CP-96,345 and RP-67,580, on the inflammatory responses elicited by SP were examined. Injection of CP-96,345 at 1 mg kg^{-1}, i.v. 30 min prior to injection of labelled cells and ^{125}I-albumin significantly attenuated the oedema formation induced by the lower doses of SP, (10^{-13}–10^{-10} mol per site), but had no significant effect on the response to the highest dose (10^{-9} mol per site; Figure 3a). This antagonist had no significant effect on ^{111}In-neutrophil or ^{111}In-eosinophil accumulation induced by SP; 10^{-9} mol per site SP induced 1404 ± 88 and 1720 ± 195 ^{111}In-neutrophils per site in control and CP-96,345-treated animals respectively, $n = 7$ animal pairs, and 10^{-9} mol per site SP induced 4246 ± 1112 and 5580 ± 1557 ^{111}In-eosinophils per site in control and CP-96,345-treated animals respectively, $n = 8$ animal pairs. SP, 10^{-10} mol per site, induced 1293 ± 269 and 837 ± 147 ^{111}In-neutrophils per site in control and CP-96,345-treated animals respectively, $n = 7$ animal pairs, and 1430 ± 200 and 1805 ± 264 ^{111}In-eosinophils per site in control and CP-96,345-treated animals respectively, $n = 8$ animal pairs. The inactive isomer of CP-96,345, CP-96,344, also given at 1 mg kg^{-1} i.v., had no significant effect on oedema formation (Figure 3a) or cell accumulation in response to SP (data not shown) at any of the doses tested.

Similarly, a second, structurally different NK_1 receptor antagonist, RP-67,580, this time when co-injected i.d.

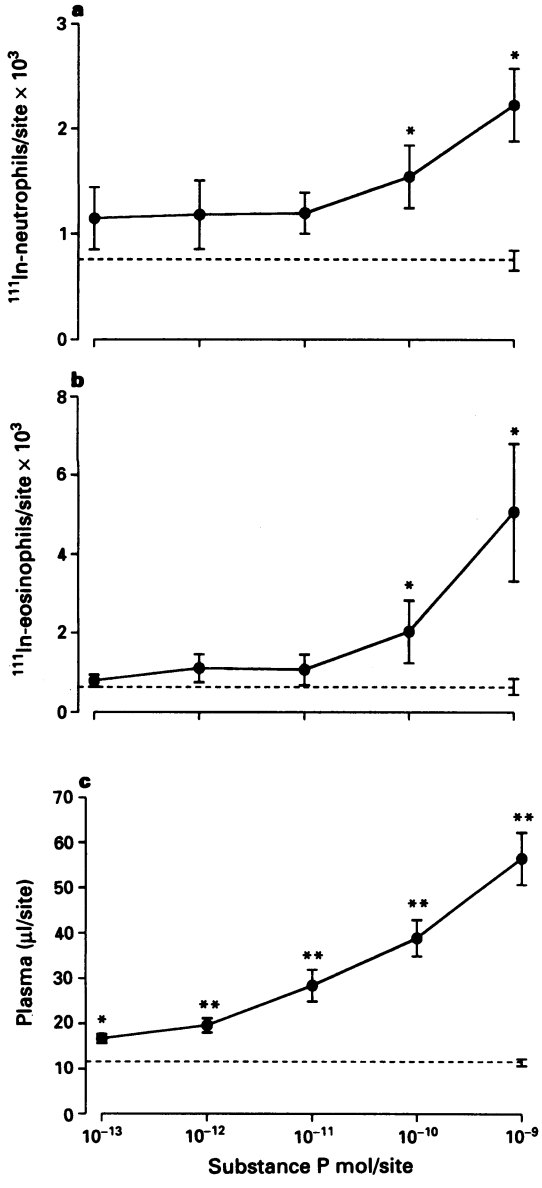

Figure 1 Dose-response relationship of substance P (SP)-induced (a) ^{111}In-neutrophil accumulation, (b) ^{111}In-eosinophil accumulation and (c) oedema formation in guinea-pig skin. Responses were measured over 2 h following i.d. SP (10^{-13}–10^{-9} mol per site, 100 µl per site). The dashed line represents levels detected in sites injected with saline/BSA (0.1% w/v). Results are expressed as the mean ± s.e.mean for $n = 6$ (for neutrophils) and $n = 8$ (for eosinophils) experiments. Asterisks indicate a significant difference from levels in sites injected with saline/BSA: $^*P < 0.05$; $^{**}P < 0.01$.

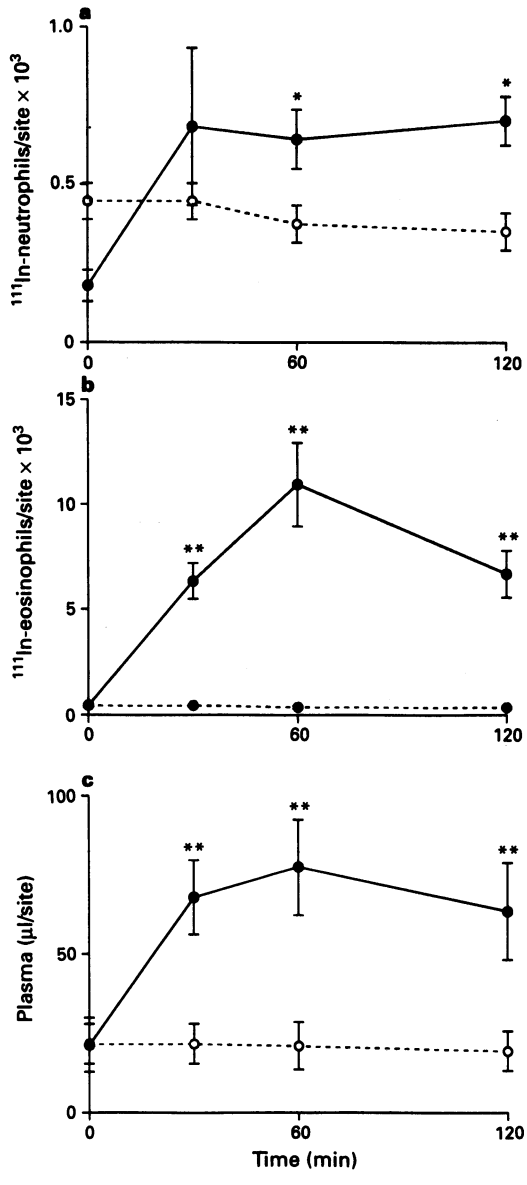

Figure 2 Time course of substance P (SP)-induced (a) ^{111}In-neutrophil accumulation, (b) ^{111}In-eosinophil accumulation and (c) oedema formation in guinea-pig skin. Labelled cells and ^{125}I-albumin were injected i.v. into recipient guinea-pigs and SP (10^{-9} mol per site) was injected i.d. (100 µl per site) at different time points after injection of cells as indicated on the axis. Closed symbols represent the responses to SP 10^{-9} mol per site while open symbols show the effect of saline/BSA injections. Results are the mean ± s.e.mean for $n = 6$ experiments. Asterisks indicate a significant difference from levels in sites injected with saline/BSA: $^*P < 0.05$, $^{**}P < 0.01$.

(2.5×10^{-8} mol per site) with SP, significantly suppressed the oedema formation induced by the lower doses of SP (10^{-13} and 10^{-11} mol per site, Figure 3b). As found with CP-96,345, RP-67,580 had no effect on SP-induced ^{111}In-neutrophil accumulation (10^{-9} mol per site induced 1601 ± 196 cells per site and 1601 ± 364 cells per site in SP- and SP + RP − 67,580-treated sites respectively, 10^{-10} mol per site induced 1295 ± 269 and 1118 ± 233 cells per site in SP- and SP + RP − 67,580 sites respectively, $n = 7$ animals) or ^{111}In-eosinophil accumulation (10^{-9} mol per site induced 5372 ± 1359 cells per site and 3658 ± 1157 in SP- and SP + RP − 67,580-treated sites respectively while 10^{-10} mol per site induced 2401 ± 591 and 2219 ± 590 cells per site in SP- and SP + RP − 67,580 treated sites respectively, $n = 6$ animals). Neither antagonist affected inflammatory responses elicited by other test agents such as 30% zymosan-activated plasma (ZAP), used as a source of C5a des arg, LTB_4 (10^{-10} mol per site) or PAF (10^{-9} mol per site) (data not shown).

Effects of chlorpheniramine, UK-74,505 and ZM-230,487 on responses induced by SP

The roles of histamine, PAF and 5-LO products in SP-induced responses were investigated by use of specific antagonists and inhibitors.

Chlorpheniramine (10 μg per site), co-injected locally with SP (10^{-9} mol per site), had no significant effect on the oedema formation or the leukocyte accumulation induced by the neuropeptide (Table 1). The antihistamine did, however, significantly inhibit the oedema formation induced by histamine (Table 1) and ZAP (30%). The response to ZAP was reduced from 41.9 ± 8.1 to 25.9 ± 5.6 μl plasma ($P < 0.05$, $n = 7$). Chlorpheniramine did not affect the cell accumulation or oedema formation induced by PAF or LTB_4 (data not shown).

The specific and long acting PAF antagonist, UK-74,505 (Alabaster *et al.*, 1991; Parry *et al.*, 1994), when given i.v. at 2.5 mg kg^{-1}, abolished the leukocyte accumulation and oedema formation induced by i.d. PAF (10^{-9} mol per site; Table 1). The antagonist did not, however, inhibit the responses elicited by i.d. SP (10^{-9} mol per site). UK-74,505 had no effect on the responses elicited by LTB_4 (10^{-10} mol per site) or 30% ZAP (data not shown).

To investigate the role of 5-LO products in the inflammatory responses elicited by SP, the effect of the non-redox 5-LO inhibitor, ZM-230,487 (Crawley *et al.*, 1993) was

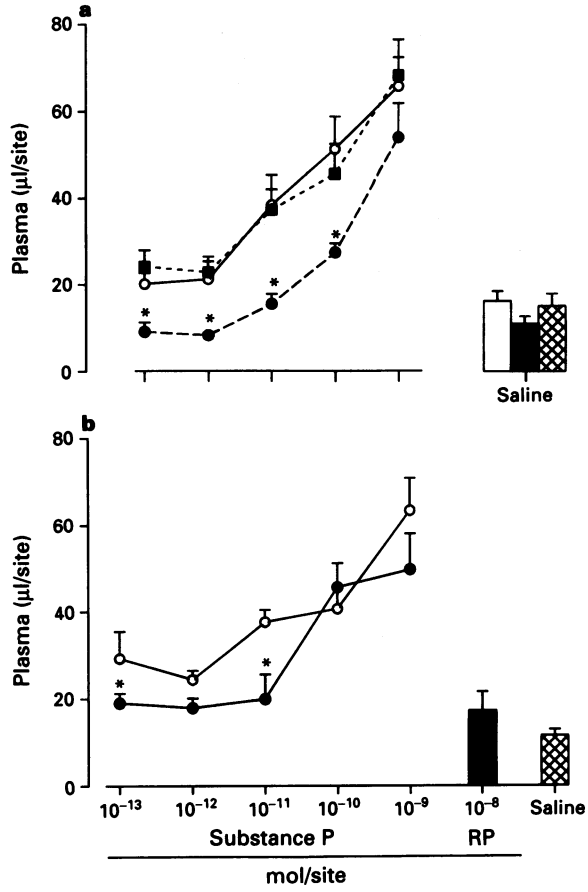

Figure 3 The effect of the NK_1 receptor antagonists, CP-96,345 and RP-67,580, on oedema formation induced by substance P (SP) after a 2 h period. (a) Shows the effects of CP-96,345 and its inactive isomer CP-96,344. Both drugs were given i.v. at 1 mg kg^{-1}, 30 min prior to injection of labelled cells and ^{125}I-albumin; (○) control responses to SP alone: (●) effect of CP-96,345; (■) effect of CP-96,344. Levels detected in saline-injected sites are shown by the columns: open column for control; solid column for CP-96,345; hatched column for CP-96,344-treated animals. (b) Shows the effect of RP-67,580, 10 μg per site co-injected with SP: (●) effect of the drug on SP-induced oedema; solid column effect of RP-67,580 when injected alone. Results are the mean ± s.e.mean for $n = 8$ (a) and $n = 7$ (b). Asterisks indicate a significant difference from SP injected sites: $^*P < 0.05$.

Table 1 The effect of chlorpheniramine and UK-74,505 on substance P-induced inflammatory responses in guinea-pig skin

	Plasma (μl per site)	^{111}*In-neutrophils* (per site)	^{111}*In-eosinophils* (per site)
Substance P (10^{-9} mol per site)	29.3 ± 4.8 (7)	1914 ± 366 (4)	1536 ± 687 (7)
Substance P + chlorpheniramine (10 μg per site)	20.0 ± 2.3 (7)	1767 ± 384 (4)	1487 ± 415 (7)
Histamine (10^{-8} mol per site)	38.7 ± 4.3 (7)	164 ± 76 (4)	91 ± 31 (7)
Histamine + chlorpheniramine (10 μg per site)	7.3 ± 1.9* (7)	192 ± 116 (4)	71 ± 27 (7)
Substance P (10^{-9} mol per site)	50.2 ± 8.7 (6)	1412 ± 312 (6)	2763 ± 730 (6)
Substance P + UK-74,505 (2.5 mg kg^{-1})	49.7 ± 7.5 (6)	1379 ± 292 (6)	4077 ± 1252 (6)
PAF (10^{-9} mol per site)	51.1 ± 5.8 (6)	2217 ± 561 (6)	3045 ± 570 (6)
PAF + UK-74,505 (2.5 mg kg^{-1})	0.0 ± 1.9* (6)	500 ± 245* (6)	70 ± 7* (6)

The effect of the H_1 histamine receptor antagonist, chlorpheniramine (10 μg per site), and the PAF antagonist, UK-74,505 (2.5 mg kg^{-1}, i.v.) on substance P-induced ^{111}In-neutrophil, ^{111}In-eosinophil and ^{125}I-albumin accumulation in the guinea-pig skin. Values are expressed as mean ± s.e.mean after subtraction of saline responses. Asterisks indicate a significant difference from PAF and histamine treated sites: $^*P < 0.05$. Number of experiments in parentheses.

studied. This compound, when co-injected i.d. at 10^{-8} mol per site with SP, significantly inhibited the accumulation of ^{111}In-eosinophils (Figure 4b) but had no effect on the SP-induced oedema formation (Figure 4c). Whilst in these experiments the SP-induced ^{111}In-neutrophil accumulation did not reach statistical significance, ZM-230,487 did not appear to affect this response (Figure 4a). At the dose used, as previously reported (Teixeira & Hellewell, 1994), ZM-230,487 inhibited the leukocyte accumulation, but not the oedema formation, induced by arachidonic acid (3×10^{-8} mol per site; Figure 4).

Effect of the anti-CD18 mAb 6.5E

The role of the CD18 adhesion molecule in SP-induced inflammatory responses was investigated by using the anti-CD18 monoclonal antibody 6.5E F(ab')$_2$ (Andrew *et al.*, 1993). This antibody has previously been shown to block both neutrophil and eosinophil accumulation in the guinea-pig without affecting the number of circulating leukocytes (Teixeira *et al.*, 1994). When given i.v. at 2.5 mg kg^{-1}, 6.5E F(ab')$_2$ greatly attenuated both ^{111}In-neutrophil (Figure 5a) and ^{111}In-eosinophil (Figure 5b) accumulation in response to

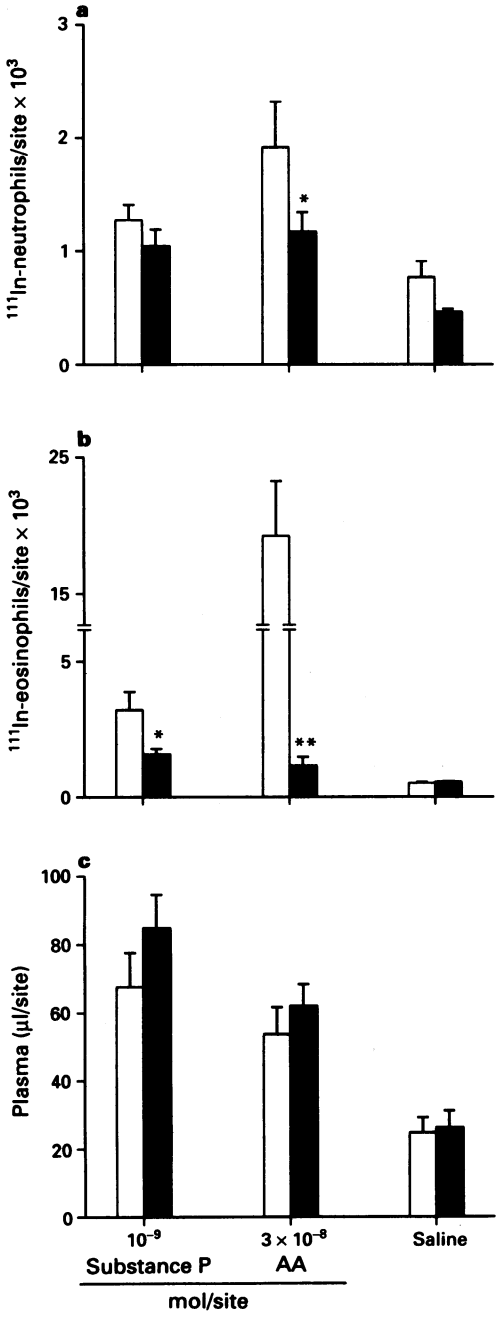

Figure 4 The effect of ZM-230,487 on substance P (SP, 10^{-9} mol per site)- and arachidonic acid (AA, 3×10^{-8} mol per site)-induced (a) ^{111}In-neutrophil accumulation, (b) ^{111}In-eosinophil accumulation and (c) oedema formation after a 2 h period. Open columns show the control response while the solid columns show the effect of ZM-230,487 when co-injected at 10^{-8} mol per site with SP, AA or saline. All data are mean ± s.e.mean for $n = 7$ experiments. Asterisks indicate a significant difference from control responses: $^*P < 0.05$, $^{**}P < 0.01$.

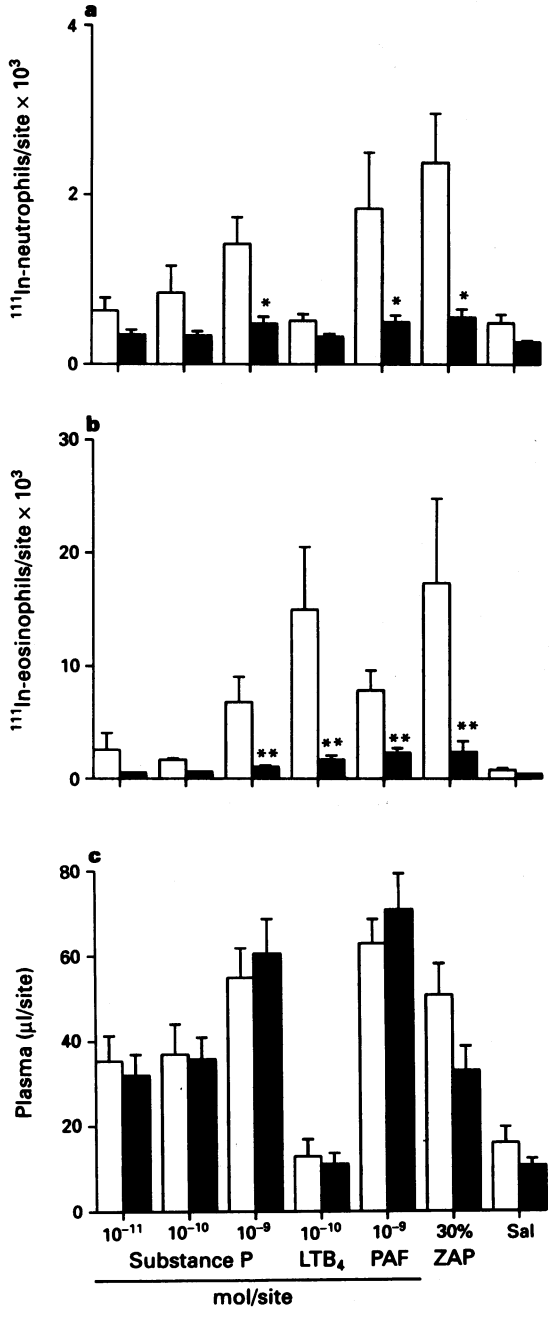

Figure 5 The effect of the anti-CD18 monoclonal antibody 6.5E F(ab')$_2$ on substance P (SP)-induced (a) ^{111}In-neutrophil accumulation, (b) ^{111}In-eosinophil accumulation and (c) oedema formation after a 2 h period. The antibody (solid columns) was given i.v. at 2.5 mg kg^{-1}, 5 min prior to the injection of labelled cells and ^{125}I-albumin. Open columns represent the control responses following the i.d. injections of different mediators. Data presented are mean ± s.e.mean for $n = 6$ experiments. Asterisks indicate a significant difference from untreated controls: $^*P < 0.05$, $^{**}P < 0.01$.

SP and other preformed mediators such as LTB_4, ZAP and PAF. The antibody did not, however, affect the oedema formation induced by any of the mediators tested (Figure 5c). Two control monoclonal antibodies, 1E6 and MOPC 21, have previously been shown not to affect leukocyte accumulation in the present model (Weg *et al.*, 1993; Teixeira *et al.*, 1994).

Discussion

The aim of the present study was to investigate and characterize the ability of SP to induce leukocyte accumulation and oedema formation *in vivo*. We have shown that the intradermal administration of SP caused the accumulation of ^{111}In-neutrophils, ^{111}In-eosinophils and local oedema formation in guinea-pig skin. Interestingly, the dose-range over which these effects occurred differed. Leukocyte accumulation was significant only at the highest doses tested, 10^{-10} and 10^{-9} mol per site whilst oedema formation was significant over the entire dose-range of 10^{-13}–10^{-9} mol per site. In addition, it appeared that SP was more effective in stimulating oedema formation than leukocyte accumulation. The leukocyte accumulation induced by SP was relatively small as compared to the leukocyte accumulation induced by other stimuli, e.g. ZAP, tested in our model. A similar pattern of results has been reported by Yano and colleagues in mice (1989). In our study, the dissociation between the dose-response relationship for leukocyte accumulation and oedema formation suggests differences in the regulatory mechanisms associated with these responses. Cumulative time-course studies indicated that the maximal level of leukocyte accumulation and oedema formation induced by SP was achieved within the first 30–60 min after administration of the peptide.

To investigate the role of NK_1 receptors in the induction of SP-induced inflammatory events in the present study, we investigated the effects of two structurally different non-peptide NK_1 receptor antagonists, CP-96,345 (Snider *et al.*, 1991) and RP-67,580 (Garret *et al.*, 1991). Both CP-96,345 and RP-67,580 significantly inhibited oedema formation induced by the lower doses of SP (10^{-13}–10^{-11} mol per site), but at the higher doses tested, there was no significant reduction. In addition, the antagonists had no significant effect on ^{111}In-neutrophil or ^{111}In-eosinophil accumulation induced by SP. A similar profile of effects was obtained when CP-96,345 was tested at higher doses of 5–10 mg kg^{-1} (data not shown). These results suggest that the oedema formation induced by low doses of SP ($<10^{-10}$ mol per site) is mediated via the activation of NK_1 receptors, the preferred receptor type for this neuropeptide. At higher doses however, when in addition to oedema formation, SP can induce leukocyte accumulation, the responses do not appear to be entirely NK_1 receptor-mediated. At high doses, the SP elicited responses may be partly mediated via the activation of NK_2 receptors. Such a possibility may be investigated by the use of specific NK_2 receptor agonists or antagonists.

Hence, our results suggest that oedema formation induced by SP may be primarily mediated directly via the activation of specific SP receptors. This conclusion was also reached by Iwamoto & Nadel (1989) who demonstrated that the NK_1 receptor is stimulated by its preferred agonist, SP to cause an increase in vascular permeability in guinea-pig skin. Indeed, functional NK_1 receptors, mediating SP-induced rapid elevations in cytoplasmic levels of Ca^{2+}, have been detected on human venular endothelial cells (Greeno *et al.*, 1993). Whilst there is much evidence showing that SP is an activator of cutaneous mast cells (Hagermark *et al.*, 1978; Foreman & Jordan, 1983; Foreman *et al.*, 1983), the SP-induced oedema formation does not appear to be mast cell-dependent (Kowalski & Kaliner, 1988; Baraniuk *et al.*, 1990; Kowalski *et al.*, 1990). This is clearly indicated in the study of Kowalski & Kaliner (1988) where, using a model of cutaneous neurogenic inflammation induced by electrical nerve stimulation in rats and mice, a temporal dissociation was found between increased vascular permeability and mast cell degranulation. Interestingly, the dependency of the SP-induced oedema formation on mast cells may be governed by its route of administration. Kowalski *et al.* (1990) found that mast cell-deficient mice exhibited normal changes in vascular permeability in response to i.v. SP, whilst in a similar study, Yano *et al.* (1989) showed that i.d. administered SP did not induce oedema formation in mast cell-deficient mice. In our study, however, we found that whilst the H_1 receptor antagonist, chlorpheniramine, had a small inhibitory effect on SP-induced oedema, this effect did not reach statistical significance, indicating that this response is not dependent on mast cell-derived histamine. Such discrepancies may be related to animal species. However, in contrast to the oedema formation in our study, the SP-induced leukocyte accumulation which was not inhibited by NK_1 receptor antagonists, may be mediated by mast cell-derived factors as reported in other models (Matsuda *et al.*, 1989; Yano *et al.*, 1989; Iwamoto *et al.*, 1992).

Mast cells are a source of numerous inflammatory mediators including histamine and PAF. To investigate the contribution of these mediators in the SP-induced leukocyte accumulation, we studied the effects of the H_1 receptor antagonist, chlorpheniramine and the PAF antagonist, UK-74,505 (Alabaster *et al.*, 1991; Parry *et al.*, 1994). These drugs had no effect on responses elicited by SP, suggesting that histamine and PAF do not play major roles in the induction of the inflammatory events induced by the neuropeptide in the present model. Another PAF-antagonist, CV-6209, has been reported to be without effect on granulocyte accumulation into mouse skin induced by SP (Iwamoto *et al.*, 1993). However, the potent and specific 5-LO inhibitor, ZM-230,487, did significantly reduce the eosinophil accumulation response, induced by SP. The most likely 5-LO product involved in this response is LTB_4, which is an effective inducer of eosinophil accumulation in guinea-pig skin (see Figure 5 and Faccioli *et al.*, 1991). In this context, it has recently been found that LTB_4 is an important mediator of SP-induced granulocyte accumulation in mice (Iwamoto *et al.*, 1993). The lack of effect of ZM-230,487 on ^{111}In-neutrophil accumulation may be due to the fact that LTB_4 is not a very effective inducer of neutrophil accumulation in the guinea-pig (see Figure 5). Hence, as the 5-LO inhibitor had no effect on neutrophil accumulation and only partially suppressed eosinophil accumulation, the results suggest that in addition to leukotrienes, other inflammatory mediators are generated or released in response to SP. Whilst mast cells contain an array of inflammatory cytokines such as interleukin-1 (IL-1), IL-6 and IL-8 (Gordon *et al.*, 1990; Möller *et al.*, 1993), SP appears to stimulate selectively the induction of tumour necrosis factor-α (TNF-α) gene expression in a murine mast cell line (Ansel *et al.*, 1993). The mRNA for MIP-1α and MIP-1β has also been demonstrated in mast cells (Schall, 1991) and the possible involvement of these and other chemokines such as RANTES (Schall, 1991) in the inflammatory responses elicited by neuropeptides needs to be investigated.

The process of leukocyte accumulation at sites of inflammation is mediated by a cascade of adhesive events involving a number of adhesion molecules (Springer, 1994). The well characterized adhesion molecules on leukocytes include L-selectin and the integrins CD11a/CD18, CD11b/CD18, CD11c/CD18 and VLA-4. On the endothelium, the important adhesion molecules are intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and P-selectin and E-selectin. The adhesive mechanisms involved in the recruitment of leukocytes in response to SP are not yet fully understood. In particular, it is not yet clear whether SP acts on the leukocytes and/or endothelial cells to trigger this inflammatory response. *In vitro*, SP induces mast cell-dependent expression of E-selectin

on human microvascular endothelium in organ cultures of neonatal human foreskins (Matis *et al.*, 1990). These findings are in agreement with the *in vivo* results of Smith *et al.* (1993), where intradermal administration of SP was found to induce infiltration of neutrophils and eosinophils in human skin as well as increasing the endothelial cell surface expressions of E- and P-selectin. Interestingly, in the same study, it was noted that there was no detectable change in the expression of the endothelial cell adhesion molecules ICAM-1 and VCAM-1. SP has also been reported to act directly on neutrophils to stimulate adhesion and to enhance the leukocytes cell surface expression of CD11b (Shipp *et al.*, 1991; DeRose *et al.*, 1994). In contrast to the above, Zimmerman *et al.* (1992) reported that whilst SP enhanced the adhesion of human neutrophils to cultured endothelial cells, this response was not affected by monoclonal antibodies to CD18, L-selectin, ICAM-1 or E-selectin. Our results, however, clearly demonstrate that an anti-CD18 antibody inhibits the neutrophil and eosinophil accumulation induced by SP and other inflammatory stimuli in guinea-pig skin. The discrepancies between our results and the findings of the above studies could be due to difference in species, assay systems and/or concentrations of SP employed. The results of the present investigation are in agreement with previous findings showing that anti-CD18 antibodies can very effectively suppress neutrophil accumulation in a number of inflammatory models (reviewed in Nourshargh, 1992). *In vitro* studies have implicated CD18 in the stimulated adhesion of neutrophils and eosinophils to endothelial cells and their transendothelial cell migration across endothelial cell barriers (Lamas *et al.*, 1988; Smith *et al.*, 1988; 1989; Ebisawa *et al.*, 1992). However, it is not clear from our studies at what stage of their accumulation *in vivo* the anti-CD18 antibody is acting. Interestingly, blockade of the CD18 antigen had no effect on the oedema formation induced by SP, clearly differentiating the mechanisms involved in cell accumulation and oedema formation elicited by the neuropeptide in this species.

In summary, we have demonstrated that SP can induce a dose- and time-dependent accumulation of granulocytes and oedema formation in an *in vivo* model system and that these two effects can be separated by specific NK_1 receptor antagonists. Our results suggest that SP may act through two independent mechanisms, (1) a direct NK_1 receptor-dependent event to cause oedema formation and (2) an NK_1 receptor-independent event, involving 5-LO products with respect to eosinophils, to induce a CD18-dependent accumulation of granulocytes and to stimulate a component of the oedema response.

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Selective modification of rat hepatic microsomal fatty acid chain elongation and desaturation by fibrates: relationship with peroxisome proliferation

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1 The time-course of the effect of clofibrate (CFB), bezafibrate (BFB) and gemfibrozil (GFB) on lipid plasma levels and palmitoyl-, palmitoleoyl- and γ-linolenoyl-CoA elongase, Δ-9, Δ-6 and Δ-5 desaturase activities, and microsomal electron transport chains, as well as the correlation with the peroxisomal proliferation phenomenon have been studied in male Sprague-Dawley rats.

2 As reported in our previous work, the three drugs behave as peroxisomal proliferators (the order of potency was BFB > CFB ≥ GFB) and induced a clear reduction in both plasma cholesterol and triglyceride levels.

3 Palmitoyl-CoA elongation activity was increased by the three drugs (BFB = GFB > CFB), whereas palmitoleoyl-CoA elongation activity was only enhanced by GFB. Elongation activity was not modified by fibrates when γ-linolenoyl-CoA was used as substrate. These results are in accordance with the existence of three different elongation systems for saturated, mono- and polyunsaturated fatty acids.

4 Δ-9, Δ-6 and Δ-5 desaturase activities were increased by the three fibrates, with an order of potency BFB > CFB = GFB for Δ-9 and Δ-5, and GFB > BFB = CFB for Δ-6.

5 Of the enzyme activities integrated in the microsomal electron transport chains, NADH cytochrome b_5 reductase was not affected by fibrate treatment, NADPH cytochrome c reductase activity was enhanced (BFB = GFB > CFB), whereas NADH cytochrome c reductase activity was reduced by CFB and BFB.

6 The increase in Δ-9 and Δ-5 desaturase activities was highly dependent on the peroxisomal proliferation phenomena, whereas the increase in Δ-6 desaturase activity and the decrease in NADH cytochrome c reductase was mainly independent. The modifications of palmitoyl-CoA elongase and NADPH cytochrome c reductase activities, as well as plasma lipid levels, were partially correlated with peroxisomal β-oxidation, but the r^2 values obtained point to the existence of additional independent mechanisms.

7 As man is assumed to be a species refractory to peroxisomal proliferation, only those fibrate effects not absolutely related to this phenomenon are expected to appear after fibrate therapy.

Keywords: Fibrates; peroxisomal proliferation; β-oxidation; elongases; desaturases

Introduction

Fibric acid derivatives constitute a well known group of hypolipidemic drugs, mainly used in the treatment of mixed hyperlipoproteinemia and hypertriglyceridemia (Shepherd *et al.*, 1991). They share three characteristic features: (a) they behave as potent peroxisome proliferators in mice and rats (Sirtori *et al.*, 1992); (b) their hypolipidemic mechanism of action is not well established (Catapano, 1992), and (c) they increase the content of mono-unsaturated fatty acids (mainly palmitoleic and oleic) and decrease the content of polyunsaturated fatty acids (mainly linoleic) in the cholesteryl ester, triglyceride, and phospholipid plasma fractions from hyperlipidemic-treated subjects (Vessby & Lithell, 1990; Agheli & Jacotot, 1991; Tavella *et al.*, 1993). The mechanism underlying this latter effect is, at present, unknown.

In the last decade, a considerable body of information has been gathered regarding the influence of the different species of fatty acid containing glycerolipid molecules, on lipoprotein metabolism. We are aware that the type of fatty acid, especially the length and number of unsaturations present in their structure, modulates the enzyme activities directly related to the handling of lipoproteins in the organism, e.g. lipoprotein lipase (Wang *et al.*, 1992) or lecithin-cholesterol acyltransferase (Hida *et al.*, 1993). In consequence, we have been interested in the effect of fibric acid derivatives on the enzyme activities related to hepatic fatty acid synthesis, either *in vitro* (Alegret *et al.*, 1991; Sánchez *et al.*, 1992a,b; 1993a,b) or *in vivo* (Vázquez *et al.*, 1993; Alegret *et al.*, 1994). We have also studied the possible relationship between these effects and peroxisome proliferation; given that man is assumed to be resistant to the induction of these cellular organelles (Lake & Gray, 1985; Hawkins *et al.*, 1987). Many of the studies about the effects of fibrates on fatty acid metabolism are based on only one drug, clofibrate, accepted as a fibrate prototype, but which is gradually being replaced by more efficient drugs, like bezafibrate and gemfibrozil. Thus, here we compare the effect of three fibric acid derivatives, clofibrate (CFB), bezafibrate (BFB) and gemfibrozil (GFB) on the fatty acid chain elongation and desaturation systems *in vivo*. The microsomal electron transport chains were also studied, as they are directly involved in providing reducing power to such systems (Keyes & Cinti, 1980). Further, we have studied the temporal evolution of these activities and their relationship with peroxisome induction. The results obtained provide evidence of a selective modification of the elongation activity produced by fibrate treatment in rats and also an increase of Δ-5, Δ-6, and Δ-9 desaturase activities. In the case of Δ-6 desaturase, the mechanism may be indepen-

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dent of the peroxisomal proliferation. These modifications may help to explain some of the alterations in plasma fatty acid composition observed in humans after fibrate therapy.

The abbreviations used in this paper are as follows: CFB, clofibrate; BFB, bezafibrate; GFB, gemfibrozil; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, γ-linolenic acid; 20:3, dihomo-γ-linolenic acid.

Methods

Animals

Male Sprague-Dawley rats (150) from Letica (Barcelona, Spain), weighing around 120 g, were maintained in a 12 h light-dark cycle under conditions of constant humidity and temperature (22 ± 2°C) and were fed standard Panlab diet (Barcelona, Spain) for five days prior to the beginning of the studies. The animals were distributed randomly according to their weight in four groups of 36. Each group was fed, respectively, a control diet or a diet containing 0.3% w/w CFB, 0.45% w/w BFB or 0.3% w/w GFB (equimolar doses). The six remaining rats were used as controls on day 0 of treatment to establish basal values. Throughout the study the weight and daily food intake of the animals was measured. The 36 rats of each group were killed in random groups of 6 after 1, 2, 4, 7, 10 and 15 days of treatment. The diets were prepared as described previously (Alegret *et al.*, 1994).

Experimental

The animals were killed on the assigned day by decapitation between 08 h 00 min and 09 h 00 min, to avoid circadian variations of the enzyme activities (Dato *et al.*, 1972). Samples of blood were collected from the neck and plasma was obtained by centrifugation at 3000 *g* for 10 min. Livers were removed, perfused with ice-cold 0.9% NaCl, weighed and homogenized in eight volumes of 0.25 M sucrose, 50 mM Tris-HCl buffer, pH 7.4. Microsomal and cytosolic fractions were obtained by differential centrifugations as described previously (Nagi *et al.*, 1989), and the protein content was determined by the method of Bradford (1976) with bovine serum albumin used as standard. Liver subcellular distribution was determined by assaying marker enzyme activities, i.e. NADPH cytochrome c reductase for microsomes, succinate dehydrogenase for mitochondria and glucose-6-phosphate for cytosol (Alegret *et al.*, 1994).

Synthesis of γ-linolenoyl-CoA and [1-^{14}C]-stearoyl-CoA

18:3 CoA and [1-^{14}C]-18:0 CoA were synthesized by the mixed anhydride procedure as described elsewhere (Sánchez *et al.*, 1993b). Concentrations of the CoA derivatives were determined by the method of Ellman (1959) after cleavage of the thioester bond with hydroxylamine.

Enzyme assays

Microsomal fatty acid elongation was assayed by the measurement of [2-^{14}C]-malonyl-CoA incorporation into exogenous acyl-CoAs essentially as described previously (Sánchez *et al.*, 1992b), with some modifications. Briefly, the assay medium contained: 100 mM Tris-HCl, pH 7.4, 500 μM NADPH, 40 μM acyl-CoA and 200 μg of microsomal protein, freshly obtained, all in a final volume of 0.5 ml. After 2 min preincubation at 37°C, the reaction was started by adding 60 μM malonyl-CoA (containing 0.037 μCi [2-^{14}C]-malonyl-CoA). After 5 min incubation at 37°C the reaction was stopped by adding 1 ml 15% KOH in methanol, followed by saponification at 65°C for 45 min. After acidification (pH 1.5) with ice-cold 5 N HCl, the free fatty acids were extracted three times with 2 ml hexane (total volume 6 ml). The pooled hexane fractions were placed in scintillation vials and dried under nitrogen, and after addition of 7 ml scintillation mixture, the radioactivity incorporated was counted in a scintillator counter. Blanks were carried out by adding KOH before malonyl-CoA. Under our assay conditions, fatty acid elongation activity was proportional to the amount of protein added (up to 800 μg ml^{-1}), and was linear for at least 15 min.

Δ9 desaturase activity was determined according to Laguna *et al.* (1989), with 200 μg of microsomal protein, freshly obtained, in a final volume of 0.5 ml. Blanks were performed by adding 18:0 CoA after stopping the reaction.

To determine the Δ6 desaturase activity, the assay medium contained, in a final volume of 0.5 ml, 100 mM Tris-HCl buffer pH 7.4, 150 μM GSH, 7.5 mM $MgCl_2$, 5 mM ATP, 200 μM CoA, 500 μM NADH and 500 μg of microsomal protein and 125 μg of cytosolic protein, freshly obtained. The reaction was started, after 2 min preincubation, by adding an ethanolic solution of 18:2 (containing 0.05 μCi of [1-^{14}C]-18:2) to a final concentration of 25 μM. The volume of ethanol added did not exceed 1% of the total volume. The assay continued as described (Sánchez *et al.*, 1993b), except for the content of $AgNO_3$ of the thin layer chromatography plates, which was 20%, and the solvent system used to develop them, which was diethyl ether/petroleum ether 1:1 (v/v).

Δ5 desaturase activity was determined essentially as described (Sánchez *et al.*, 1993b), but using 20:3 (containing 0.05 μCi of [1-^{14}C]-20:3) in a final concentration of 50 μM as a substrate, and 20% $AgNO_3$ plates in order to achieve better resolution.

The enzyme activities related to the microsomal electron transport chains, namely NADH and NADPH cytochrome c reductase and NADH cytochrome b_5 reductase, and also the peroxisomal β-oxidation activity were determined as described in Alegret *et al.* (1991, 1994).

Chemicals

Malonyl-CoA, palmitoyl-CoA (16:0 CoA), palmitoleoyl-CoA (9–16:1 CoA), γ-linolenic acid (6,9,12–18:3), stearoyl-CoA (18:0 CoA), linoleic acid (6,9–18:2), dihomo-γ-linolenic acid (8,11,14–20:3), NAD(P)H, fatty acid-free bovine serum albumin (BSA), GSH, ATP, CoA and Trizma were obtained from Sigma (St. Louis, MO, U.S.A.). [2-^{14}C]-malonyl-CoA (50 mCi mmol^{-1}), [1-^{14}C]-stearic acid (18:0) (58 mCi mmol^{-1}), [1-^{14}C]-linoleic acid (18:2) (50 mCi mmol^{-1}) and [1-^{14}C]-dihomo-γ-linolenic acid (20:3) (47 mCi mmol^{-1}) were purchased from New England Nuclear (Boston, MA, U.S.A.) and Amersham Iberica (Madrid, Spain). Scintillation fluids (Co 136 and Co 36) were from Scharlau Co. (Barcelona, Spain). $AgNO_3$ (20%) silica gel G thin layer chromatography plates were from Alltech (Deerfield, IL, U.S.A.). General chemicals were obtained from commercial sources and were of analytical grade.

Drugs

Clofibrate (CFB) was a generous gift from ICI-Farma (Pontevedra, Spain), bezafibrate (BFB) from Boehringer Mannheim (Barcelona, Spain) and gemfibrozil (GFB) from Parke-Davis (Barcelona, Spain).

Plasma cholesterol and triglyceride concentration

Cholesterol was determined by the colorimetric test Monotest Cholesterol CHODPAP No 290319, and triglyceride concentration was assayed by means of a Peridochrom Triglyceride GPO-PAP No 701882 test, both from Boehringer Mannheim (Barcelona, Spain).

Statistical evaluation

Results are expressed as means ± s.e.mean of six experiments performed in duplicate. Statistical differences were established by a two way ANOVA test and the *a posteriori* multiple comparison Duncan test, using the FOUNDS computer programme. Differences with $P<0.05$ were considered significant. Correlations between two variables were performed by linear regression using the GPIP computer programme.

Results

Non-enzymatic parameters

Body weight and daily food intake of the rats were measured throughout the treatment. The body weight of treated rats was similar to that of control rats up to the seventh day of treatment. From that time on, treated rats tended to gain weight slower than control rats (data not shown). Daily food intake was not significantly changed by the treatment, although BFB- and GFB-treated animals showed a tendency to consume more than the corresponding controls (e.g. on day 14 the daily food intake was 32.6 ± 1.4 g day^{-1} for control animals, and 32.3 ± 0.9, 36.2 ± 2.7, and 36.2 ± 2.3 g day^{-1} for CFB-, BFB- and GFB-treated animals, respectively) in accordance with our previous paper (Alegret *et al.*, 1994).

Liver/body weight ratio and peroxisomal β-oxidation activity are suitable markers for the peroxisomal proliferation phenomena (Vázquez *et al.*, 1993; Alegret *et al.*, 1994; Lake & Gray, 1985). As expected, and in agreement with our previous results (Alegret *et al.*, 1994), both parameters were greatly increased in rats treated with CFB, BFB and GFB. The increase in liver/body weight ratio began early, on the second day of treatment with each of the three drugs (Figure 1). In rats treated with BFB, the most potent drug in this respect, a maximal ratio was achieved on day 10 of treatment (77% increase).

As can be seen in Figure 2, BFB was also the most potent inducer of peroxisomal β-oxidation activity, followed by CFB and GFB. Differences with respect to control β-oxidation activity were significant from day 2 of treatment (day 1 in the case of the BFB group). Maximal increase in β-oxidation activity was achieved on day 7 for BFB-treated (9 fold induction), on day 7 for GFB-treated rats (3.3 fold induction) and on day 15 for CFB-treated rats (7.3 fold induction).

Treatment of rats with fibric acid derivatives led to a marked hypolipidemic effect, which is reflected in the reduction of plasma cholesterol and triglyceride levels (Figure 3a and b). The hypocholesterolemic effect was statistically significant on the first day of treatment. While CFB and BFB exhibited similar potency in reducing cholesterol levels (maximal reductions about 40% on days 10–15 of treatment), the effect of GFB was less significant. In fact, on days 10 and 15 there was no difference between the values obtained in GFB-treated and control animals (Figure 3a). Regarding the reduction of plasma triglyceride levels (Figure 3b), the most effective drugs were BFB and GFB, while the decreases attributable to CFB were slighter, although still significant. The hypotriglyceridemic effect was significant from the first day of treatment with all three drugs. Maximal effect (about 60% reduction after treatment with BFB or GFB) was reached on day 7 and maintained for the following days.

Microsomal electron transport chains

The three enzyme activities assayed, namely NADH and NADPH cytochrome c reductase and NADH cytochrome b_5 reductase, behaved differently after fibrate administration. NADH cytochrome b_5 reductase was unaffected throughout fibrate treatment (data not shown). For example, on day 15,

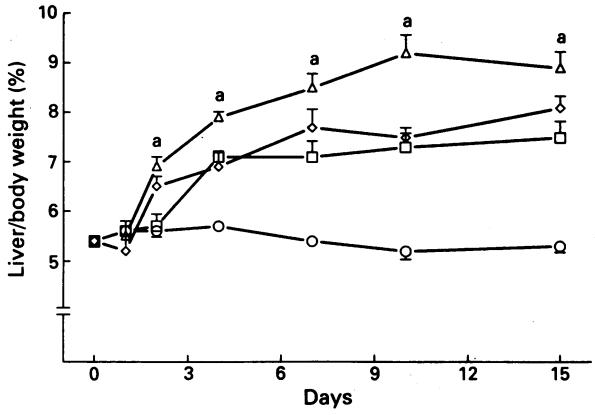

Figure 1 Time-course of the effect of a standard diet (○) or a diet supplemented with 0.3% w/w CFB (□), 0.45% w/w BFB (△), or 0.3% w/w GFB (◇) on liver/body weight ratio (%) of male Sprague-Dawley rats. Results are means ± s.e.mean of 6 experiments performed in duplicate. [a]Values for CFB, BFB and GFB are all significantly different from controls, $P<0.05$.

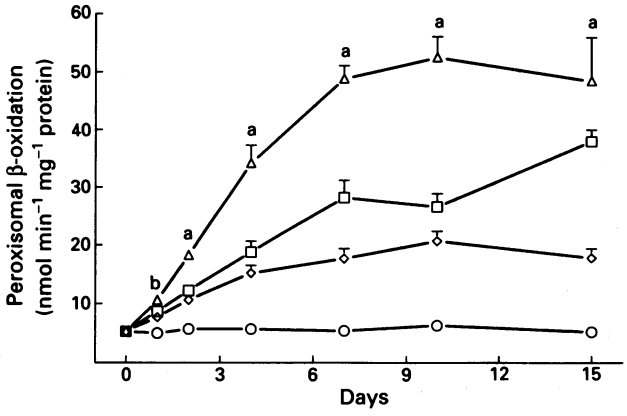

Figure 2 Time-course of the effect of a standard diet (○) or a diet supplemented with 0.3% w/w CFB (□), 0.45% w/w BFB (△), or 0.3% w/w GFB (◇) on cyanide-insensitive peroxisomal β-oxidation determined in the postnuclear fraction of livers from male Sprague-Dawley rats. Results are means ± s.e.mean of 6 experiments performed in duplicate. [a]Values for CFB, BFB and GFB are all significantly different from controls, $P<0.05$; [b]values for BFB are significantly different from controls, $P<0.05$.

the activity values were 3.3 ± 0.2, 3.5 ± 0.1, 3.4 ± 0.2 and 3.5 ± 0.2 μmol min^{-1} mg^{-1} microsomal protein for control, CFB, BFB and GFB-treated animals. NADH cytochrome c reductase activity was significantly reduced from the second day of treatment with CFB and BFB, as shown in Figure 4 (mean reductions of 37 and 39% respectively). In contrast, GFB administration resulted in no modification of this activity. NADPH cytochrome c reductase activity was increased by the administration of all three drugs, and reached maximal values at day 7 (Figure 5). BFB and GFB produced the same degree of increase (79% and 77%, mean increase versus control values); CFB was somewhat less effective (64% mean increase).

Microsomal chain elongation

Microsomal chain elongation was assayed using exogenously added CoA esters of saturated, monounsaturated and polyunsaturated fatty acids as substrates, namely 16:0, 16:1, and 18:3 CoAs. The variations of the elongase activity upon drug treatment depended on the substrate used. As shown in Figure 6a, 16:0 CoA elongation activity was significantly increased from days 1–2 of treatment by the three drugs

assayed. BFB and GFB exhibited similar potency, with about 4 fold increases from day 4. CFB was less effective, leading to maximal induction (2.3 fold) on day 4.

16:1 CoA elongation activity (Figure 6b) was less affected in comparison to the changes in the activity using the saturated substrate (i.e. maximal induction, caused by GFB after 4 days of treatment, was about 2 fold). GFB treatment significantly induced 16:1 CoA chain elongation activity from day 2 of treatment, while CFB failed to cause any significant induction. BFB showed intermediate behaviour and caused a slight increase in activity after day 4.

On the other hand, when 18:3 CoA was used, none of the drugs assayed caused significant changes in the elongase activity (Figure 6c).

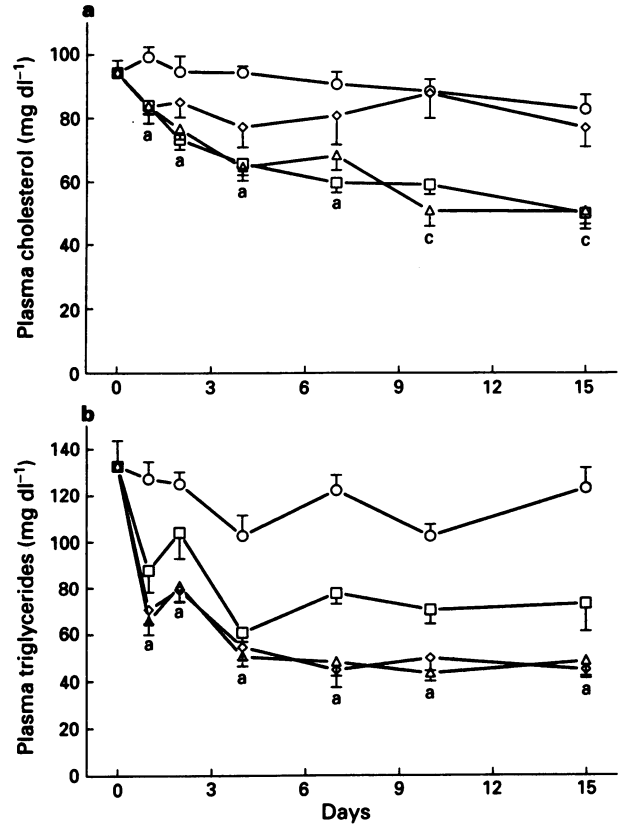

Figure 3 Time-course of the effect of a standard diet (○) or a diet supplemented with 0.3% w/w CFB (□), 0.45% w/w BFB (△), or 0.3% w/w GFB (◇) on (a) plasma cholesterol levels, and (b) plasma triglyceride levels of male Sprague-Dawley rats. Results are means ± s.e.mean of 6 experiments performed in duplicate. [a]Values for CFB, BFB and GFB are all significantly different from controls, $P<0.05$; [c]values for CFB and BFB are significantly different from controls, $P<0.05$.

Microsomal desaturases

The three desaturases here studied, Δ9, Δ5 and Δ6, showed marked increases in their specific activities in response to the treatment of rats with fibric acid derivatives. However, the potency order of the three drugs assayed was not the same in all cases.

Δ9 desaturase activity was increased by the treatment of rats with CFB, BFB and GFB (Figure 7a). The increase caused by CFB and GFB was similar, while BFB was slightly more potent. Thus, maximal effect of the drugs was observed on day 7, with 2.3, 2.4, and 3 fold increase of Δ9 desaturase activity in CFB-, GFB-, and BFB-treated rats, respectively. Δ5 desaturase response to drug treatment (Figure 7b) was similar to that of Δ9; this enzyme activity was clearly enhanced and the order of potency of the drugs for this effect was also the same, i.e. BFB > CFB = GFB. Inductions were significant from day 2 with BFB or GFB (day 4 for the CFB group), and maximal effect was achieved after 15 days of treatment with BFB (about 3 fold induction).

In the case of Δ6 desaturase, GFB showed clearly higher inductive ability than CFB and BFB, which were of similar potency. Values from GFB-treated rats were statistically different from control values from the first day of treatment, and maximal increase, found on day 10, was about 4 fold (Figure 7c).

Discussion

The three drugs studied clearly acted as peroxisome inducers in rats, producing strong hepatomegaly and an evident increase in the peroxisomal β-oxidation activity. The order of potency as peroxisomal inducers (BFB > CFB ≥ GFB) agrees with the reported structural requisites for the manifestation

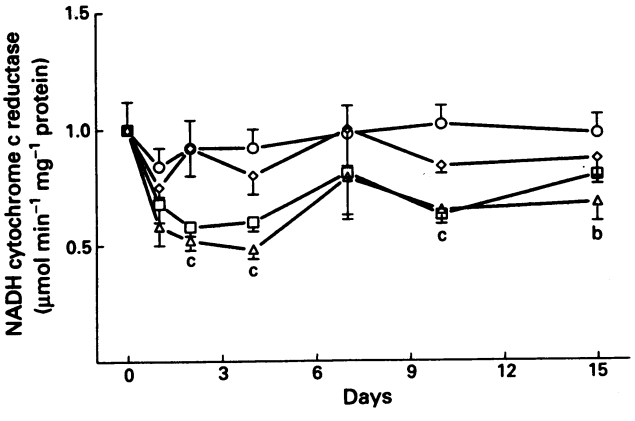

Figure 4 Time-course of the effect of a standard diet (○) or a diet supplemented with 0.3% w/w CFB (□), 0.45% w/w BFB (△), or 0.3% w/w GFB (◇) on NADH cytochrome c reductase activity in the microsomal fraction of livers from male Sprague-Dawley rats. Results are means ± s.e.mean of 6 experiments performed in duplicate. [b]Values for BFB are significantly different from controls, $P<0.05$; [c]values for CFB and BFB are significantly different from controls, $P<0.05$.

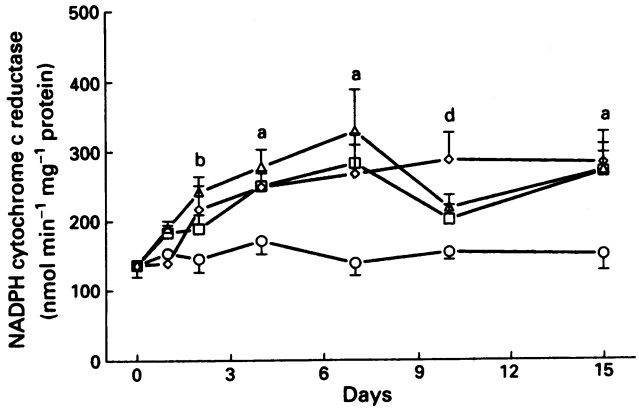

Figure 5 Time-course of the effect of a standard diet (○) or a diet supplemented with 0.3% w/w CFB (□), 0.45% w/w BFB (△), or 0.3% w/w GFB (◇) on NADH cytochrome c reductase activity in the microsomal fraction of livers from male Sprague-Dawley rats. Results are means ± s.e.mean of 6 experiments performed in duplicate. [a]Values for CFB, BFB and GFB are all significantly different from controls, $P<0.05$; [b]values for BFB are significantly different from controls, $P<0.05$; [d]values for GFB are significantly different from controls, $P<0.05$.

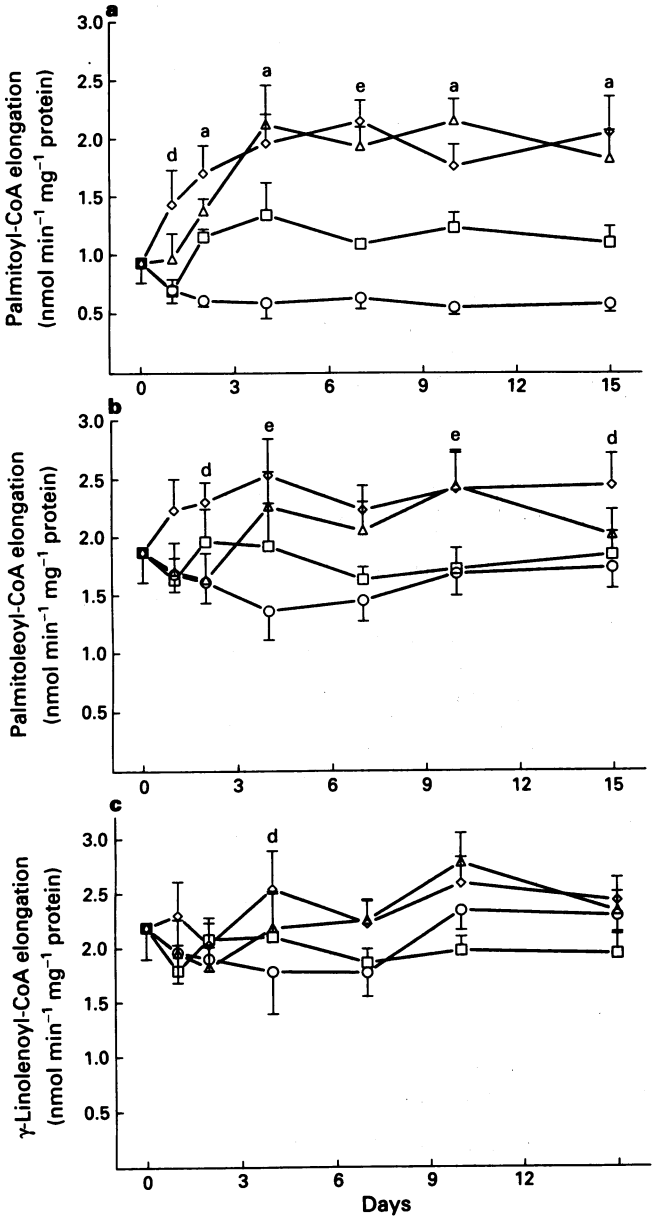

Figure 6 Time-course of the effect of a standard diet (○) or a diet supplemented with 0.3% w/w CFB (□), 0.45% w/w BFB (△), or 0.3% w/w GFB (◇) on (a) palmitoyl-CoA elongation, (b) palmitoleoyl-CoA elongation, and (c) γ-linolenoyl-CoA elongation activities on the microsomal fraction of livers from male Sprague-Dawley rats. Results are means ± s.e.mean of 6 experiments performed in duplicate. [a]Values for CFB, BFB and GFB are all significantly different from controls, $P<0.05$; [d]values for GFB are significantly different from controls, $P<0.05$; [e]values for BFB and GFB are significantly different from controls, $P<0.05$.

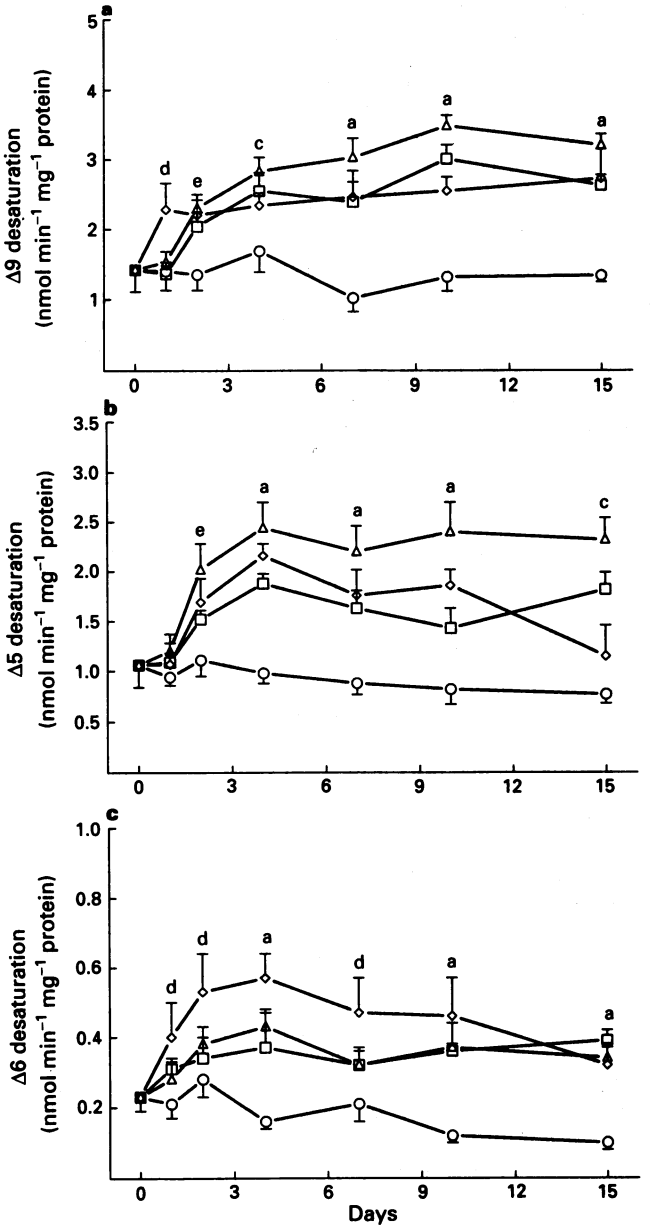

Figure 7 Time-course of the effect of a standard diet (○) or a diet supplemented with 0.3% w/w CFB (□), 0.45% w/w BFB (△), or 0.3% w/w GFB (◇) on (a) Δ9 desaturase, (b) Δ5 desaturase, and (c) Δ6 desaturase activities on the microsomal fraction of livers from male Sprague-Dawley rats. Results are means ± s.e.mean of 6 experiments performed in duplicate. [a]Values for CFB, BFB and GFB are all significantly different from controls, $P<0.05$; [c]values for CFB and BFB are significantly different from controls, $P<0.05$; [d]values for GFB are significantly different from controls, $P<0.05$; [e]values for BFB and GFB are significantly different from controls, $P<0.05$.

of this effect (McGuire *et al.*, 1991). Moreover, there is a strong direct correlation between liver/body weight ratio and the increase in peroxisomal activity ($r^2=0.66$, $P<0.001$; Table 1). Regarding the hypolipidemic activity, the correlation values for cholesterol or triglyceride levels and peroxisomal β-oxidation activity (Table 1), point to some involvement of peroxisomal proliferation in the hypolipidemic activity of fibrates in rats, although the r^2 values found (0.26 and 0.33 for triglycerides and cholesterol respectively) leave a major part of the hypolipidemic response unexplained. Thus, the proliferation of peroxisomes and their associated enzyme activities can be viewed as an additional contribution to an independent hypolipidemic mechanism. Moreover, it should be borne in mind that in guinea-pigs (Vázquez *et al.*, 1993) and presumably in man (Lake & Gray, 1985; Hawkins *et al.*, 1987), the hypolipidemic response to treatment with fibrates is not accompanied by peroxisomal proliferation.

The results obtained in this study concerning the weight evolution, daily food intake, liver/body weight ratio, peroxisomal β-oxidation activity and hypolipidemic profile are in accordance with those reported in our previous study *in vivo* (Alegret *et al.*, 1994), where a more extensive discussion of the modifications of these parameters can be found.

Kawashima *et al.* (1983; 1986; 1989) demonstrated that microsomal cytochrome b_5 levels are not modified by the treatment of rats with different peroxisome proliferators. Further, the same authors reported that NADH cytochrome

b_5 reductase activity was not modified, while NADH cytochrome c reductase was decreased in rats after feeding a 0.5% w/w CFB-diet (Kawashima *et al.*, 1983; 1989; 1990). Our results with the three fibrates agree with those previous reports. The decrease in NADH cytochrome c reductase activity does not appear to be related to the proliferation of peroxisomes, as there is no significant correlation between the reductase and the peroxisomal β-oxidation activities (Table 1). The decrease in activity could be related to the hindering of electron flow from the flavoprotein to the cytochrome b_5 produced by fibrates *in vitro* (Alegret *et al.*, 1991). In contrast to NADH cytochrome c reductase, the NADPH-dependent reductase activity is increased in the fibrate-treated rats. Although the r^2 value for the correlation between the reductase and the peroxisomal β-oxidation activities is low (Table 1), the induction of the NAPDH cytochrome c reductase activity may be directly associated to the activation of cytochrome P450IVA1 genes (Milton *et al.*, 1990) produced by fibrates, like other cytochrome P450 inducers, such as phenobarbitone (Waxman & Azaroff, 1992). Moreover, the increase in activity cannot be ascribed to a direct interaction of the drugs with the reductase, as the activity of this enzyme is not modified *in vitro* by the addition of any of the three fibrates studied (Alegret *et al.*, 1991).

It should be pointed out that the activities of reductases present in microsomes from control or treated-animals are about two orders of magnitude greater than those of elongases or desaturases, more than sufficient to supply reducing power to support these enzyme activities.

The microsomal fatty acid chain elongation system is composed of four independent enzyme activities, of which the first, the condensing enzyme, is the rate limiting step (Bernert & Sprecher, 1979). As far as we know, this protein has not been purified to homogeneity from mammalian sources (Cinti *et al.*, 1992). Nevertheless, Sprecher (Bernert & Sprecher, 1977), and Cinti's group in particular (Prasad *et al.*, 1986), have provided indirect evidence for the existence of at least three different condensation/elongation systems responsible for the elongation of saturated, monounsaturated and polyunsaturated fatty acids. Accordingly, we have assayed the microsomal elongation activity using three different substrates, 16:0, 16:1, and 18:3 CoAs. The differentiated effects produced by the fibrates on these three activities are in good agreement with Cinti's proposal (Prasad *et al.*, 1986). Thus, CFB induced the elongation of 16:0, while it was ineffective in modifying 16:1 elongation, supporting the hypothesis of two, differentiated enzymes for saturated and monounsaturated fatty acids. Moreover, GFB induced 16:1 elongation while not affecting 18:3 elongation, suggesting the existence of two different enzymes for monounsaturated and polyunsaturated fatty acids.

Conflicting results appeared in the literature concerning the effect of fibric acid derivatives on fatty acid chain elongation activity. For example, Landriscina *et al.* (1977), found a 47% decrease in elongation activity after 14 days of CFB treatment. In contrast, Kawashima *et al.* (1984) described an induction of palmitoyl-CoA elongation activity after CFB treatment, ascribing the discrepancy with Landriscina's results to the fact that the latter had used acetyl-CoA and not 16:0 CoA as a primer. Our results are in good agreement with those of Kawashima *et al.* (1990), not only because 16:0 CoA elongation activity is increased, but also because they found that 18:3 CoA elongation activity was not modified by CFB treatment. As far as we know, no studies have been carried out *in vivo* on the effect of other fibric acid derivatives, such as BFB and GFB, on microsomal chain elongation activity. From the data presented here, it is clear that these two drugs are more potent than CFB as chain elongation inducers. Kawashima *et al.* (1990) reported that the induction of palmitoyl-CoA elongation produced by CFB was directly related to the proliferation of peroxisomes. Nevertheless, the proliferation of peroxisomes explains, at most, only 29% of the increase in 16:0 CoA elongation (Table 1). Moreover, GFB, which is the weakest peroxisome proliferator of the three fibrates tested, is the only one capable of inducing the 16:1 CoA elongation activity. In addition, no correlation was found between the values for this activity in GFB-treated rats and the corresponding peroxisomal β-oxidation activities. Thus, from our own results, the induction of the elongation system of fibrates is mainly independent of the activity of these compounds as peroxisome proliferators.

Table 1 Correlation between enzymatic and non enzymatic parameters and peroxisomal proliferation (measured as peroxisomal β-oxidation activity)

	Peroxisomal β-oxidation	
	n	r^2
Liver/body weight	142	0.664*
Plasma triglycerides	142	0.260*†
Plasma cholesterol	142	0.331*†
Palmitoyl-CoA elongase	141	0.292*
Palmitoleoyl-CoA elongase	33	0.000
Δ9 desaturase	141	0.443*
Δ9 desaturase (only for bezafibrate-treated animals)	34	0.580*
Δ5 desaturase	134	0.425*
Δ6 desaturase	136	0.014†
NADH cytochrome c reductase	149	0.023†
NADPH cytochrome c reductase	149	0.286*

Enzymatic and non enzymatic parameters were measured as described under Methods. *n* are the number of pairs of data analysed. *r^2 values statistically significant ($P<0.001$). †Negative correlation.

We have previously shown that fibrates (Sánchez *et al.*, 1993b), especially GFB (Sánchez *et al.*, 1992b), behave as potent inhibitors of the microsomal fatty acid chain elongation *in vitro*, irrespective of the degree of unsaturation of the substrate used. Therefore, the induction elicited by fibrates *in vivo* should be regarded as a compensatory response by the cell, in order to override the drug inhibition. This behaviour has been reported for other drugs acting as inhibitors of key lipogenic enzymes, such as statins for hydroxymethylglutaryl-CoA reductase (Endo, 1986).

It is known that feeding a fat-free, carbohydrate rich diet to 24 h starved rats greatly increases the activity of the elongation system (Cinti *et al.*, 1992). With such dietary manipulation, in our laboratory we have obtained activities between 2–2.4 nmol min^{-1} mg^{-1}, irrespective of the substrate used (Sánchez *et al.*, 1992b), which means that, in our experimental conditions, 18:3 CoA elongation is already fully expressed in control animals (see values in Figure 6c). Further, the 'fat-free diet activities' practically matched the induced activities for saturated and monounsaturated substrates obtained after fibrate treatment. Taking all these data together, it seems that, at least with respect to GFB, the selectivity for the three elongation systems is a consequence of the different basal levels of expression of these systems.

The three fibrates tested here acted as inducers of the three desaturase activities assayed, namely Δ9, Δ5, and Δ6 desaturase. Nevertheless, from the results shown in Figure 7, it seems that the behaviour of Δ9 and Δ5 desaturase consistently differs from that of Δ6 desaturase. Kawashima *et al.* (1986, 1989) reported that not only CFB, but other structurally unrelated peroxisome proliferators such as tiadenol, are capable of inducing the Δ9 desaturase activity. Indeed, the same authors (Kawashima *et al.*, 1990) relate the induction of Δ9 directly to peroxisome proliferation. Our results seem to confirm Kawashima's hypothesis: First, the order of potency in inducing Δ9 desaturase activity (BFB > CFB ≥ GFB) matched the order of peroxisome inductive activity. Second, no inhibition of Δ9 desaturase can be obtained *in vitro*, even with supraphysiological concentrations of fibrates

(Sánchez *et al.*, 1993b). Third, there is a fairly good correlation between Δ9 desaturase activity and peroxisomal β-oxidation ($r^2 = 0.44$; $P < 0.001$). When analysing the data for BFB, the most potent peroxisomal inducer, the correlation is even better ($r^2 = 0.58$). The same points outlined for Δ9 desaturase can be argued for Δ5 desaturase behaviour after fibrate treatment. Although in this case there is some inhibition of Δ5 desaturase activity in the presence of fibrates *in vitro* (Sánchez *et al.*, 1993b), the effect is obtained at such high concentrations that a compensatory response *in vivo* appears unlikely.

Although the three fibrates evoked an induction of Δ6 desaturase activity, in this case GFB, the strongest inducer of this desaturase is the least potent in inducing peroxisome proliferation. Consistently, there is a lack of correlation between Δ6 desaturase and peroxisomal β-oxidation activities (see Table 1). Thus, for this enzyme activity, the effect of fibrates seems to be independent of the peroxisomal proliferation.

The modification of fatty acid elongation or desaturation activities by fibrates may be reflected in changes in the nature of acyl chains in the microsomal phospholipid fraction or, further, in plasma lipoproteins (Kawashima *et al.*, 1984; Agheli & Jacotot, 1991). Those modifications that are unrelated to peroxisomal proliferation, such as the increase in Δ6 desaturase activity shown in the present work, are more likely to occur in man and may be responsible for the decrease in linoleic acid content in lipoproteins reported for human subjects after fibrate treatment (Vessby & Lithell, 1990; Agheli & Jacotot, 1991; Tavella *et al.*, 1993). Nevertheless, the same authors also report an increase in monoenoic species. In view of the results reported here (Δ9 desaturase is dependent of peroxisomal proliferation), these data can be explained in two ways. First, although human subjects are generally considered refractory to peroxisomal proliferation (Lake & Gray, 1985; Hawkins *et al.*, 1987), this phenomenon has been identified to a certain extent in several studies (Hanefeld *et al.*, 1983; Reddy *et al.*, 1984). Second, the increase in substrates for the Δ9 desaturase provided by the induction of the elongation of saturated fatty acids, which is independent of peroxisomal proliferation, could result in an increased proportion of monoenoic products. This second possibility is currently under investigation.

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Blockade by polyamine NMDA antagonists related to ifenprodil of NMDA-induced synthesis of cyclic GMP, increases in calcium and cytotoxicity in cultured neurones

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1 Antagonists acting at the polyamine site of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor, including a number of heterocyclic aminoalcohols related to ifenprodil, were investigated to establish their functional interaction at the NMDA receptor and their neuroprotective profile.

2 In murine cultured neocortical neurones, NMDA (100 μM)-stimulated production of guanosine 3':5'-cyclic monophosphate (cyclic GMP) was blocked by N-1([thienyl]-cyclohexyl)-piperidine (1 μM) and by the nitric oxide (NO) synthase inhibitor N^G-nitro-L-arginine (100 μM). Ifenprodil and structurally related heterocyclic aminoalcohols inhibited in a concentration-dependent manner the NMDA-stimulated, NO-dependent production of cyclic GMP; rank potency order was: ifenprodil > 2309 BT > tibalosine > threo-tibalosine > 840S.

3 All of the polyamine NMDA antagonists blocked NMDA (300 μM)-stimulated increases in intracellular calcium concentrations as measured by changes in the fluorescence of pre-loaded fluo-3-acetoxy methyl ester. Rank potency order was: ifenprodil > 2309 BT > 840S > tibalosine > threo-tibalosine.

4 In a series of experiments to evaluate the effectiveness of the polyamine NMDA antagonists as blockers of NMDA-induced cytotoxicity, all of the drugs were found to inhibit the leakage of lactate dehydrogenase after the exposure of the murine neocortical cultures to NMDA (100 μM, 5 h). Rank potency order was: 2309 BT > ifenprodil > tibalosine > threo-tibalosine > 840S.

5 These results provide direct evidence that polyamine NMDA antagonists produce a functional blockade of the NMDA receptor complex. The heterocyclic aminoalcohols described herein, like ifenprodil, block NMDA-mediated elevation of intracellular NO and calcium, two key events in the excitotoxic cascade, and are cytoprotective.

Keywords: NMDA receptors; polyamines; ifenprodil analogues; cultured neurones; excitotoxicity; calcium; cyclic GMP; cytoprotection

Introduction

Antagonists at the polyamine domain of the N-methyl-D-aspartate (NMDA) receptor complex, unlike competitive antagonists and channel blockers (Wong & Kemp, 1991), have a relatively inert behavioural profile at doses that are neuroprotective in focal ischaemia and head trauma (Carter *et al.*, 1990; 1991). Ifenprodil and a synthetic analogue, SL82.0715 (eliprodil, (+)-(4-chlorophenyl)-4-[(4-fluorphenyl) methyl]-1-piperidine-ethanol), are two such NMDA antagonists which appear to block the receptor non-competitively via its polyamine domain (Carter *et al.*, 1988; 1990; 1991). This unique site of the multi-domained NMDA receptor has been poorly characterized, although apparently linked to the receptor ion channel and functional *in vivo* (Ransom & Stec, 1988; Singh *et al.*, 1990; Williams *et al.*, 1991). The polyamines, spermine and spermidine enhance the binding of NMDA channel ligands by acting as agonist modulators (Ransom & Stec, 1988), and may increase the duration of the channel open state or increase the frequency of channel opening (Sprosen & Woodruff, 1990; Williams *et al.*, 1991). Both ifenprodil and SL82.0715 inhibit polyamine-stimulated binding of NMDA channel ligands (Carter *et al.*, 1989; 1991; Beart *et al.*, 1992a). Other evidence that this unique class of drugs produces a functional antagonism at the NMDA receptor includes data demonstrating that ifenprodil and SL82.0715 blocked (a) NMDA-evoked depolarization of cultured spinal cord neurones, (b) NMDA-stimulated release of [^{3}H]-acetylcholine from striatal slices, and (c) NMDA-stimulated production of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in cerebellar slices (Carter *et al.*, 1988; 1991).

Recently, the characteristics of the polyamine domain of the NMDA receptor complex have been further examined by use of the ligands [^{3}H]- and [^{125}I]-ifenprodil (Schoemaker *et al.*, 1990; Beart *et al.*, 1991; 1992a,b; Dana *et al.*, 1991; Benavides *et al.*, 1992; Mercer *et al.*, 1993). Our laboratory has extended the range of available polyamine NMDA antagonists by describing a series of heterocyclic aminoalcohols (Figure 1), which, like ifenprodil and SL82.0715, fully inhibited all polyamine-sensitive binding of [^{125}I]-ifenprodil in cerebral cortical membranes (Beart *et al.*, 1992b; Mercer *et al.*, 1993). Additionally, this new class of polyamine NMDA antagonists inhibited the polyamine-stimulated binding of [^{3}H]-(N-1-[thienyl]cyclohexyl)-piperidine (TCP) to cortical synaptic membranes (Beart *et al.*, 1992b). At the present time direct evidence does not exist as to whether these heterocyclic aminoalcohols are NMDA antagonists and we sought to describe their functional interaction at the NMDA receptor. Since these ifenprodil-like molecules are likely to have therapeutic potential in excitotoxic neuronal injury, we have also investigated their potential as cytoprotective agents. A preliminary account of these studies has been presented (Beart *et al.*, 1993b).

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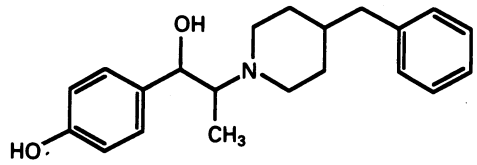
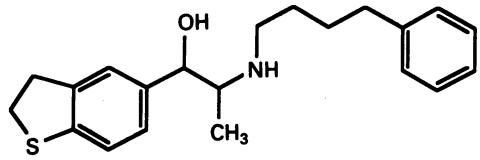
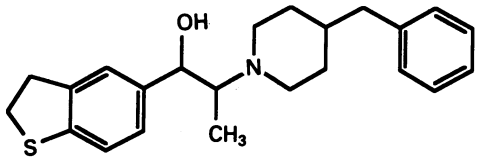


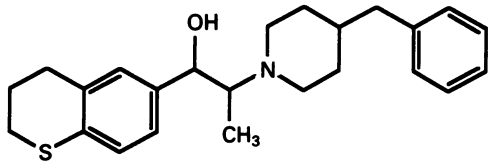
Ifenprodil



Tibalosine (804S)



2309BT



840S

Figure 1 Structures of the various heterocyclic aminoalcohols.

Methods

Cell cultures

Murine cerebral cortical neurones were cultured essentially as described by Dichter (1978) and Frandsen & Schousboe (1987). Cells were seeded in 35 mm Petri dishes in 2 ml for toxicity and cyclic GMP experiments, and in 100 µl in 96 multiwells for measurements of $[Ca^{2+}]_i$, all at a density of 3×10^6 cells ml^{-1}. Astocytic proliferation was curtailed by addition of the mitotic inhibitor cytosine arabinoside (20 µM) after 48 h of culture and onwards during the entire culture period (Dichter, 1978; Larsson *et al.*, 1985). All experiments were carried out on neurones cultured for 7 days.

NMDA-stimulated production of cyclic GMP

Experimental procedures were essentially as described previously (Frandsen *et al.*, 1992). Before experiments were performed, the culture medium was exchanged for 2 ml of Mg^{2+}-free phosphate-buffered saline (composition, mM): NaCl 137, KCl 2.7, Na_2HPO_4 7.3, KH_2PO_4 1.5, $CaCl_2$ 0.9 (pH 7.4) containing 6 mM glucose. The neurones were incubated at 37°C for 2 min with NMDA (100 µM) in the presence or absence of various drugs (Frandsen *et al.*, 1992). Incubations were terminated by rapid vacuum aspiration of the buffer and the immediate addition of 500 µl of hot 5 mM Tris-EDTA pH 7.4, with subsequent boiling for 3 min. Neurones were harvested and the cyclic GMP content of 100 µl portions of the supernatant was determined by radioimmunoassay as detailed by Frandsen *et al.* (1992). This radioimmunoassay was linear within the range from 0 to 8 pmol cyclic GMP per 100 µl sample. In preliminary experiments, NMDA (10–333 µM) produced a concentration-dependent generation of cyclic GMP in the cultured cerebral cortical neurones (EC_{50} value 100 µM; Frandsen *et al.*, 1992); 100 µM NMDA was employed in all subsequent experiments because it produced a submaximal stimulation of cyclic GMP production. Pellets from the centrifugations were used for protein determination (Lowry *et al.*, 1951) with bovine serum albumin as the standard.

NMDA-stimulated increases in $[Ca^{2+}]_i$

Fluo-3-acetoxy methyl ester loading and fluorescence measurements were performed according to the procedure of Wahl *et al.* (1989) and Frandsen & Schousboe (1992). In order to allow calculations of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) cells were incubated with the ionophore A-23187 (10 µM in HEPES-buffered saline (composition, mM): NaCl 135, KCl 5, $MgSO_4$ 0.62, $CaCl_2$ 1.8, HEPES 10 and glucose 6, pH 7.4) allowing sufficient influx of Ca^{2+} to attain the saturation level of binding with the intracellularly trapped Fluo-3 ligand (F_{max}). Subsequently, the fluorescence was quenched with 2 mM $CuCl_2$ dissolved in 0.9% (w/v) NaCl plus 10 µM A-23187 to obtain the minimum fluorescence signal (F_{min}). Fluorescence was blanked against unloaded cells. The observed relative fluorescence values for the cells were used in the following equation to calculate the free cytosolic Ca^{2+} concentration: $[Ca^{2+}]_i = K_D(F-F_{min})/(F_{max}-F)$, where the K_D is 450 nM and F the observed fluorescence, which increases upon binding of Ca^{2+} without shifts in excitation or emission wavelengths (Minta *et al.*, 1989). The neurones were exposed to NMDA (300 µM) alone or plus the compounds in HEPES buffered saline for 90 s.

NMDA-induced leakage of lactage dehydrogenase

Lactate dehydrogenase (LDH) activity for quantitative assessment of cytotoxic damage was determined as detailed previously (Frandsen & Schousboe, 1987; 1990; Frandsen *et al.*, 1989; 1992). Aliquots were taken just before change of medium, after replacement of the culture medium to the exposure medium with NMDA (100 µM) added, and after 5 h of exposure. The cellular residual LDH activity was determined by the following procedure: after discarding the exposure medium and addition of 1 ml phosphate-buffered saline, the cells were harvested and subsequently centrifuged. An aliquot of the supernatant was taken for determination of the residual LDH activity. The LDH activity in the medium after exposure to the different experimental conditions was expressed relative (in %) to the total LDH present in the cells at the beginning of the experiment, i.e. equivalent to the LDH activity in the cells plus the medium at the end of the experiment.

Materials

Pregnant mice (15th gestational day) were supplied by the Panum Institute, (Copenhagen, Denmark). Plastic culture dishes were purchased from NUMC A/S, Denmark, and foetal calf serum from Sera-Lab Ltd., Crawley Down, England. Insulin was from NOVO-Nordisk (Denmark), and penicillin was from Leo (Denmark). Poly-L-lysine (mol.wt.> 300,000), trypsin, trypsin inhibitor, DNase, NMDA and N^G-nitro-L-arginine from Sigma, U.S.A. Ifenprodil was from Research Biochemicals, U.S.A., and NMDA from Tocris Neuramin, Bristol, England. Enzyme and coenzymes were from Boehringer, Germany. SL82.0715 and TCP were

generous gifts from LERS Synthelabo, France, and Lundbeck, Denmark respectively. Tibalosine and other heterocyclic aminoalcohols were obtained from Searle Europe, Belgium. All chemicals used were the highest commercial grade.

Data analysis

Results obtained from experiments where NMDA-stimulated production of cyclic GMP was determined were examined by simultaneous analysis of all data for the various drugs by iterative curve-fitting using the FLEXIFIT programme (Guardabasso *et al.*, 1988). Inhibition in the absence of drug was constrained to the same value, the logistic transformation was used and all curves were monotonic. These analyses provided estimates of IC_{50} values and s.e. for the drugs under investigation. Data from the investigations examining NMDA-induced LDH leakage and $[Ca^{2+}]_i$ were analysed by iterative curve-fitting using the ENZFITTER programme (Leatherbarrow, 1987) to provide estimates of the IC_{50} and s.e. for the various drugs.

Results

NMDA-stimulated production of cyclic GMP

In these experiments, the aim was to demonstrate that the heterocyclic aminoacohols related to ifenprodil could antagonize NMDA-stimulated synthesis of cyclic GMP in cultured cortical neurones (as previously described for the competitive NMDA antagonist 2-amino-5-phosphonopentanoate; Frandsen *et al.*, 1992), thus revealing their direct interaction at the NMDA receptor complex. The NMDA (100 μM)-stimulated production of cyclic GMP, 9.36 ± 0.65 pmol mg^{-1} protein ($n = 8$) (mean ± s.e.mean and number of observations), was completely blocked by the inclusion of 1 μM TCP (1.99 ± 0.04 pmol mg^{-1} protein ($n = 18$); $P < 0.005$) or the NO synthase inhibitor N^G-nitro-L-arginine (100 μM; 1.38 ± 0.26 pmol mg^{-1} protein, $n = 3$; $P < 0.005$, ANOVA with Dunnett's t test). Ifenprodil and the heterocyclic aminoalcohols were able to inhibit fully the NMDA-stimulated, NO-dependent production of cyclic GMP in a concentration-dependent manner. Figure 2 shows the inhibition by ifenprodil and the structurally related aminoalcohols of NMDA-stimulated synthesis of cyclic GMP in cultured cerebrocortical neurones. The rank order of potency was ifenprodil > 2309 BT > tibalosine > threo-tibalosine > 840S. Computer-assisted analyses of the inhibition data provided estimates of the IC_{50} value (see Table 1). Spermidine (100 μM) failed to reverse the blockade by ifenprodil of NMDA-specific cyclic GMP production (data not shown).

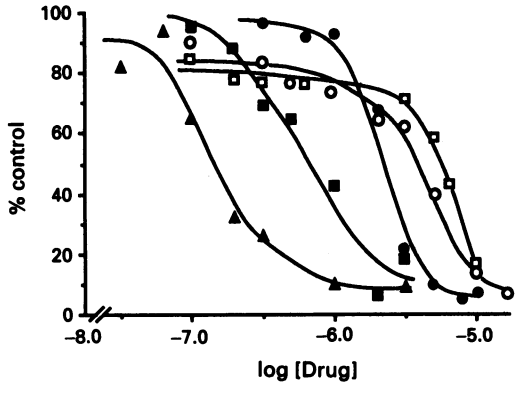


Figure 2 Inhibition of NMDA (100 μM)-stimulated production of cyclic GMP by various aminoalcohols. Murine cerebral cortical neurones were cultured for 6–8 days before experiments were performed. Values are expressed as a percentage of the specific NMDA-stimulated production of cyclic GMP (non-specific levels defined by 1 μM TCP were subtracted) and are from multiple experiments ($n = 3$ or 4) which employed 7–10 drug concentrations and 14–21 individual cultures. Cyclic GMP levels in each culture were determined in duplicate assays. Curves are those obtained by simultaneous analysis of all data obtained from individual cultures: (▲) ifenprodil; (■) 2309BT; (●) tibalosine; (○) 804S/T; (□) 840S.

NMDA-stimulated increases in $[Ca^{2+}]_i$

All of the heterocyclic aminoalcohols were also tested for their ability to prevent rises in $[Ca^{2+}]_i$ which occur subsequent to the activation by NMDA of the receptor-gated calcium channel (Frandsen & Schousboe, 1993; Wahl *et al.*, 1989; Wong & Kemp, 1991). The molecules all blocked the NMDA-induced increases in $[Ca^{2+}]_i$ in a dose-dependent manner. The rank order of potency was ifenprodil >2309 BT > 840S > tibalosine > threo-tibalosine (Figure 3, Table 1).

NMDA-induced leakage of LDH

Since the heterocyclic aminoalcohols were found to be effective NMDA antagonists, they were evaluated for their cytoprotective activity in the model examining the NMDA-induced release of the intracellular enzyme, LDH. Under the experimental conditions described, NMDA (100 μM, 5 h)

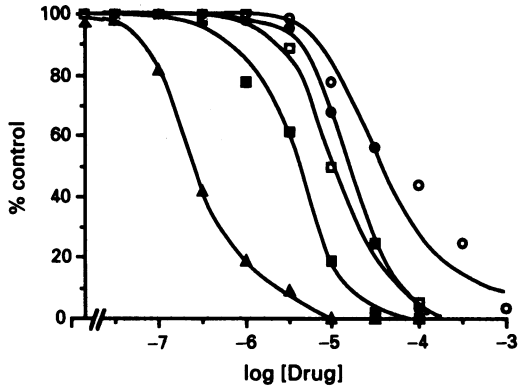


Figure 3 Inhibition by various aminoalcohols of the NMDA (300 μM)-stimulated increases in $[Ca^{2+}]_i$ in 7 day old murine neocortical neurones. Stimulation by NMDA alone led to an increase in $[Ca^{2+}]_i$ of 662 ± 24 nM, and basal levels never exceeded 105 ± 14 nM. Results are the mean of 18–24 individual cultures at each dose of drug. Curves are those obtained by iterative curve-fitting: (▲) ifenprodil; (■) 2309BT; (□) 840S; (●) tibalosine; (○) 804S/T.

Table 1 Potency of ifenprodil and related heterocyclic alcohols as antagonists of NMDA on cultured cerebral cortical neurones

	IC_{50} (μM)		
	NMDA-stimulated synthesis cyclic GMP	*NMDA-induced increases $[Ca^{2+}]_i$*	*NMDA-induced leakage LDH*
Ifenprodil	0.14 ± 0.01	0.25 ± 0.33	0.80 ± 0.13
2309BT	0.63 ± 0.10	4 ± 0.73	0.27 ± 0.13
Tibalosine	2.3 ± 0.10	15 ± 2.9	0.99 ± 0.25
804S/T*	4.5 ± 0.64	37 ± 1.8	10 ± 0.34
840S	6.3 ± 0.53	10 ± 1.2	15 ± 3.0

For details of individual assays see text. IC_{50} values are the estimates ± s.e. from iterative curve-fitting of data obtained from experiments employing the following number of cultures: cyclic GMP $n = 14–21$, $[Ca^{2+}]_i$ $n = 144$-216 and LDH $n = 48$-72. Abbreviations are given in the text. *Tibalosine = 804S, 804S/T = threo-tibalosine.

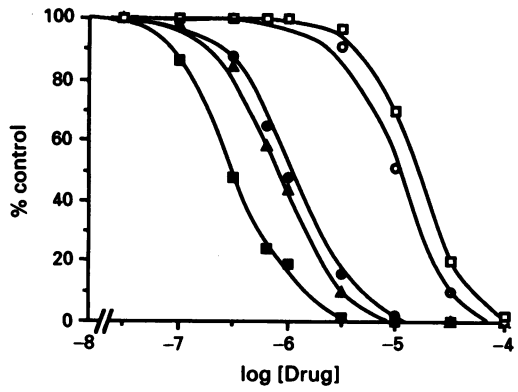


Figure 4 Dose-response curves for the neuroprotective effects of various aminoalcohols against cytotoxicity induced by exposure of 7 day old murine neocortical cultures to NMDA (100 μM, 5 h) in Mg^{2+}-free phosphate-buffered saline. Exposure of the neurones to NMDA alone led to a leakage of LDH activity of 65 ± 4% and to 13 ± 1% in unexposed neurones. Results are the mean of 6 individual cultures for each dose of drug. Curves are those obtained by iterative curve-fitting: (■) 2309BT; (▲) ifenprodil; (●) tibalosine; (○) 840S/T; (□) 840S.

produced a leakage of LDH activity of 65 ± 4% ($n = 6$) of the total activity present in the cultures. The leakage of LDH activity in control cultures was 13 ± 1% ($n = 6$) of the total activity in the cultures. All of the heterocyclic aminoalcohols, like ifenprodil, were able to block fully the cytodestructive action of NMDA (Figure 4). The rank order of potency was 2309 BT > ifenprodil > tibalosine > threo-tibalosine > 840S. Tibalosine (erythro conformation) was found to be approximately ten fold more active than its threo-enantiomer (Figure 4, Table 1).

Discussion

Our investigations have revealed a novel series of heterocyclic aminoalcohols that are able to produce functional antagonism of the NMDA receptor. Whilst we have previously shown that these molecules inhibited polyamine-sensitive binding of [^{125}I]-ifenprodil and of [^{3}H]-TCP (Beart *et al.*, 1992b; Mercer *et al.*, 1993), evidence that the heterocyclic aminoalcohols blocked both NMDA-stimulated production of cyclic GMP and NMDA-induced rises in $[Ca^{2+}]_i$ in cultured neocortical neurones demonstrated their direct action at the NMDA receptor-complex. The heterocyclic aminoalcohols are structural analogues of ifenprodil and SL 82.0715, two polyamine NMDA antagonists, which unlike competitive and channel NMDA antagonists do not possess untoward behavioural profiles at doses that are neuroprotective (Carter *et al.*, 1991). In other experiments, the heterocyclic aminoalcohols effectively protected the neuronal cultures from NMDA-induced cytotoxicity, with the most potent member of the series, 2309BT, being more neuroprotective than ifenprodil itself.

The present results demonstrate that in cultured murine neocortical neurones, and under the experimental conditions employed, the NMDA-stimulated production of cyclic GMP is totally dependent on NO synthesis. NMDA receptor-mediated production of NO is now well documented (Garthwaite, 1991) and thus cultured neocortical neurones represent a convenient model system to investigate the NO/cyclic GMP system and to evaluate the ability of putative NMDA antagonists to block the production of this potentially cytotoxic free radical (Dawson *et al.*, 1991; Garthwaite, 1991; Frandsen *et al.*, 1992; Lipton *et al.*, 1993). Polyamine NMDA antagonists were able to inhibit completely the NMDA-mediated, NO-dependent production of cyclic GMP with ifenprodil being more potent than the heterocyclic aminoalcohols. These data thus support an older body of work that ifenprodil blocked NMDA-stimulated production of cyclic GMP in cerebellar slices (Carter *et al.*, 1988) and attenuated NMDA-dependent, cerebellar cyclic GMP responses *in vivo* (Rao *et al.*, 1991). This inhibition probably occurs subsequent to the blockade of the NMDA receptor-gated calcium channel (Reynolds & Miller, 1989) as the enzyme for NO biosynthesis, NO synthase, is known to be calcium-dependent (Garthwaite, 1991). The inability of spermidine to reverse the blockade was somewhat unexpected, although interestingly the polyamine recognition site is apparently constitutively occupied by endogenous polyamines under normal culture conditions (Rodriguez *et al.*, 1993). The interaction between polyamines and ifenprodil congeners was not the focus of this investigation, but this issue requires further investigation. Certainly polyamines reverse the effects of ifenprodil in a number of model systems (e.g. Carter *et al.*, 1991; Williams *et al.*, 1991), although, there is evidence for discrete, but interacting, ifenprodil and polyamine sites within this domain of the NMDA receptor (Beart *et al.*, 1992a; Carter *et al.*, 1991). Whilst there is a close polyamine/ifenprodil link, the relationship is complex and may involve multiple polyamine sites, which might be both extra- and intracellular (Sprosen & Woodruff, 1990; Williams *et al.*, 1991; Rodriguez *et al.*, 1993). The actions of ifenprodil described here more closely resemble in terms of potency the high affinity, voltage-independent action of ifenprodil (IC_{50} 340 nM) to block NMDA responses at heteromeric NMDA receptors expressed in oocytes (see below; Williams, 1993). Indeed, this latter effect of ifenprodil appeared to be extracellular (Legendre & Westbrook, 1991) and was consistent with a considerable body of evidence at native NMDA receptors, where the effects of ifenprodil were mechanistically different from those of the use-dependent channel blockers, TCP and dizocilipine (Reynolds & Miller, 1989; Legendre & Westbrook, 1991; Williams *et al.*, 1993).

The other culture models employed in our study are also very useful for predicting the neuroprotective potential of NMDA antagonists. All heterocyclic aminoalcohols were active in the three NMDA-related model systems, although there were some minor differences in potency; moreover, the data correlate quite well with their IC_{50} values versus polyamine-sensitive binding of [^{125}I]-ifenprodil (Beart *et al.*, 1992b). 840S was more active than expected against NMDA-induced increases in $[Ca^{2+}]_i$, and whether this result reflects any contribution attributable to the Ca^{2+} channel activity (Adeagbo & Magbageola, 1985; Bovy *et al.*, 1986) of these molecules is unknown. Interestingly, 2309 BT appeared to be more potent than ifenprodil at inhibiting NMDA-induced leakage of LDH from the neocortical neurones, perhaps due to its greater lipophilicity. This model examining the NMDA-induced leakage of LDH provided the clearest indication that the NMDA-directed activity of this series of heterocyclic aminoalcohols resides in the erythro-conformer; tibalosine (erythro conformation) was some 10 fold more potent than its threo-enantiomer whereas this separation was less clear in other systems although tibalosine appeared to display lower IC_{50} values than its threo enantiomer. Moreover, it is pertinent to recall that ifenprodil itself, has been defined as possessing the erythro conformation. Differences in potency between the three systems may be attributable to the relative time of drug exposure employed in the various experimental procedures (e.g. 2 min in cyclic GMP studies versus 5 h in LDH leakage). SL 82.0715 possessed an IC_{50} value of 0.60 ± 0.13 μM against the NMDA-induced increase in $[Ca^{2+}]_i$ (data not shown). In co-cultures of neurones and glia, ifenprodil (IC_{50} 200 nM) and SL 82.0715 (IC_{50} 4 μM) have been shown in a preliminary report to antagonize NMDA- but not kainate- or quisqualate-induced leakage of LDH (Graham *et al.*, 1992).

The new NMDA antagonists described here are likely to be of interest because they may have therapeutic usefulness

like ifenprodil and SL 82.0715, which are both neuroprotective in animal models of focal ischaemia and head trauma (Choi, 1990; Gotti *et al.*, 1990; Carter *et al.*, 1988; 1991) at doses that do not produce untoward behavioural effects (Carter *et al.*, 1991). Moreover, unlike some NMDA antagonists (Olney *et al.*, 1989; Nehls *et al.*, 1990), SL 82.0715 does not have the limitations of causing vacuolization in posterior cortex (Duval *et al.*, 1992) or of elevating limbic glucose utilisation (Scatton *et al.*, 1991). Additionally, we have confirmed the cytoprotective properties of the heterocyclic aminoalcohols, as demonstrated here in the NMDA/LDH model, by investigating their actions in the methamphetamine-induced-model of nigrostriatal degeneration (Sonsalla *et al.*, 1991). Like ifenprodil and SL 82.00715 (Sonsalla *et al.*, 1991), all of the heterocyclic aminoalcohols reported herein were active in this model with 2309BT being particularly efficacious (Beart *et al.*, 1993a). Ifenprodil-like NMDA antagonists have a particular advantage over other classes of NMDA antagonists that in addition to effecting non-competitive receptor blockade, they also may block the co-agonist-like actions of polyamines, which are known to be released in neuronal trauma such as excitotoxicity (Paschen *et al.*, 1988; Carter *et al.*, 1991), and which may exacerbate the NMDA receptor-mediated excitotoxic cascade.

Finally, the unique pharmacological profile of ifenprodil-like NMDA antagonists has to be re-appraised in the light of recent advances in our understanding of the molecular properties of the NMDA receptor-complex. In view of findings that the NMDA receptor is hetero-oligomeric (Hollman & Heinemann, 1994) and that the existence of multiple NMDA receptor subtypes or splice variants may underlie the diversity of functions associated with NMDA receptor activation (Durand *et al.*, 1992), the apparent functional selectivity of the polyamines and ifenprodil for the NMDAR2B subunit (Durand *et al.*, 1992; Carter *et al.*, 1993; Williams, 1993; Williams *et al.*, 1993) is of appreciable interest. High affinity, polyamine-sensitive binding sites for [^{3}H]-ifenprodil appear to co-localize with the NMDAR2B subunit (Carter *et al.*, 1993), which is concentrated forebrain. The NMDAR2B subunit when expressed with the NMDAR1 transcript in *Xenopus* oocytes yields a functional hetero-oligomeric channel where polyamines potentiate the action of NMDA (Durand *et al.*, 1992) and where ifenprodil is an effective NMDA antagonist (Williams *et al.*, 1993). In terms of the results obtained in the present study with polyamine NMDA antagonists, the NMDAR2B, unlike the 2A subunit, it strongly expressed in cortical neurones at 6–8 days in culture (Zhong *et al.*, 1993). Perhaps the distribution of NMDA receptors containing the NMDAR2B subunit in forebrain (Standaert *et al.*, 1994) may be related to the effectiveness of ifenprodil and SL 82.0715 in focal ischaemia and head trauma (Carter *et al.*, 1991). In such a scenario, the new series of polyamine NMDA antagonists reported here is of some significance – these heterocyclic aminoalcohols are more lipophilic than ifenprodil, and 2309BT is more cytoprotective in the LDH model and *in vivo* in the methamphetamine model (Beart *et al.*, 1993a). Clearly these new polyamine NMDA antagonists appear worthy of attention as potential, therapeutically useful neuroprotective agents.

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Neuroprotective profile of lifarizine (RS-87476) in rat cerebrocortical neurones in culture

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1 The ability of the neuroprotective agent, lifarizine (RS-87476), to mitigate veratridine-, cyanide- and glutamate-induced toxicity in rat embryonic cerebrocortical neurones in primary culture has been compared with that of tetrodotoxin (TTX), nitrendipine, (+)-MK-801 and (−)-MK-801. Lactate dehydrogenase (LDH) released into the culture medium was used as the indicator of cell viability.

2 Incubation of cultures for 16 h in a medium containing veratridine (10^{-4} M), sodium glutamate (10^{-3} M) or sodium cyanide (10^{-3} M) resulted in consistent elevations of LDH activity in the culture medium. The ability of compounds to attenuate these elevations was expressed as the concentration required to inhibit the increases in LDH release by 50% (IC_{50}).

3 Neurotoxicity induced by veratridine was inhibited by lifarizine ($IC_{50} = 4 \times 10^{-7}$ M), TTX ($IC_{50} = 3 \times 10^{-8}$ M) and nitrendipine ($IC_{50} = 3 \times 10^{-5}$ M). In contrast, (+)-MK-801 (up to 3×10^{-5} M) was ineffective against this insult.

4 Glutamate-induced neurotoxicity was inhibited by (+)-MK-801 ($IC_{50} = 1.4 \times 10^{-8}$ M) and to a lesser extent by (−)-MK-801 ($IC_{50} = 1 \times 10^{-7}$ M), but was unaffected by lifarizine, TTX or nitrendipine (up to 10^{-6} M).

5 (+)-MK-801 was effective against sodium cyanide-induced neurotoxicity ($IC_{50} = 1.9 \times 10^{-8}$ M), whereas lifarizine and TTX (up to 10^{-6} M) and nitrendipine (up to 3×10^{-6} M) were without protective activity against this insult.

6 The results demonstrate that lifarizine potently protects rat cortical neurones *in vitro* against a neurotoxic insult that requires activation of sodium channels for its expression, and that the compound is ineffective against insults mediated by *N*-methyl-D-aspartate receptor activation. The weak efficacy of nitrendipine against veratridine-induced cell death argues against the involvement of L-type calcium channels in this insult. These data are consistent with the notion that the neuroprotective activity of lifarizine observed *in vivo* may be mediated by inhibition of neuronal sodium currents.

Keywords: Lifarizine (RS-87476); drug effects; neurotoxicity; neuroprotection; stroke; cerebral ischaemia; sodium channels; excitatory amino acids; cerebrocortical neurones

Introduction

The identification of neuroprotective agents that may be of potential benefit in the clinical management of stroke is ultimately based on efficacy in *in vivo* animal models of cerebral ischaemia (for review see Karpiak *et al.*, 1989). However, these models inevitably blur potentially important structure-activity relationships between compounds due to their different fates in the complex *in vivo* environment (brain uptake, metabolism, plasma protein binding, and so on). Moreover, side-effects (for example, hypotension and hypothermia) may obscure or even enhance any intrinsic neuroprotective activity of candidate compounds. These considerations have led a number of workers to search for *in vitro* test systems both to complement the *in vivo* models and to enhance understanding of the mechanisms involved in neuronal and glial cell damage in stroke.

Lifarizine (RS-87476-190; Figure 1) is a diphenylpiperazine analogue that possesses marked neuroprotective efficacy in both focal and global models of cerebral ischaemia: rat 4-vessel occlusion model (Alps *et al.*, 1990), cat middle cerebral artery occlusion model (Kucharczyk *et al.*, 1991), gerbil unilateral carotid artery occlusion (Brown *et al.*, 1993), mouse middle cerebral artery occlusion (C.M. Brown *et al.*, 1994).

The present experiments were designed in an effort to gain further insight into the mechanism of action of lifarizine by examining the ability of the compound to protect against rat cortical neuronal death *in vitro* mediated by a number of neurotoxic stimuli (veratridine, glutamate and cyanide) and by comparing its actions with those of other agents with well-established pharmacological properties: the specific sodium channel blocker, tetrodotoxin, the stereoselective NMDA channel blockers, (+)- and (−)-MK-801, and the dihydropyridine calcium entry blocker, nitrendipine.

Methods

Cell culture

Neurones were obtained from the cerebral cortices of 18 day old embryos taken from pregnant Sprague-Dawley rats and

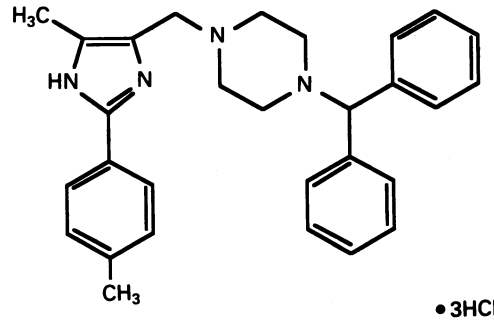

Figure 1 Structure of lifarizine: 1-[(2-(4-methylphenyl)-5-methyl)-1H-imidazol-4-yl-methyl]-4-diphenylmethyl-piperazine trihydrochloride.

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cultured according to the method described by Pauwels *et al.* (1989). Briefly, the isolated cortices were finely chopped and incubated in Hank's balanced salt solution (HBSS; Gibco-BRL) containing trypsin (0.25%; Sigma Type III) and DNase (0.05%; Sigma Type I) at 37°C for 12 min. Following addition of trypsin inhibitor (0.25%; Sigma Type III-O), the tissue was centrifuged (540 *g*; 5 min) and the resulting supernatant discarded and replaced by HBSS containing 0.025% DNase. The tissue was then gently triturated with a flame-polished Pasteur pipette and then centrifuged (540 *g*, 10 min). The supernatant was discarded and the pellet resuspended by agitation into HBSS and centrifuged at 540 *g* for a further 10 min. The supernatant was again discarded and the pellet resuspended in culture medium consisting of 1:2 Ham's F12/Dulbecco's MEM supplemented with donor horse serum (10%; Sigma), L-glutamine (2×10^{-3} M; Gibco-BRL), penicillin (0.25 iu ml^{-1}) and streptomycin (0.1 mg ml^{-1}) (penicillin/streptomycin solution; Gibco-BRL), and the cell density adjusted to 10^6 ml^{-1}. The cell suspension was plated out onto poly-D-lysine (0.01 mg ml^{-1}; Sigma)-coated 4-well tissue culture plates (NUNC) at a density of 10^6 cells per 15 mm well.

After 24 h the medium was replaced by a serum-free chemically-defined medium (CDM) consisting of Dulbecco's MEM supplemented with insulin (10 μg ml^{-1}; Sigma), human apotransferrin (0.2 mg ml^{-1}; Sigma), progesterone (12.6 ng ml^{-1}; Sigma), putrescine (32.22 μg ml^{-1}; Sigma), selenium (15.78 ng ml^{-1}; Sigma), triiodo-L-thyronine (19.5 ng ml^{-1}; Sigma), corticosterone (0.2 μg ml^{-1}; Sigma), bovine serum albumin (0.01%; fraction V, fatty acid free; Sigma), penicillin (0.25 iu ml^{-1}) and streptomycin (0.1 mg ml^{-1}) (penicillin/streptomycin solution; Gibco-BRL), and L-glutamine (2 mM; Gibco-BRL).

The cultures were maintained for 7 to 9 days in a humidified CO_2 incubator (37°C) before being used for the neurotoxicity experiments. The above conditions were shown by immunostaining with anti-neurofilament and anti-glial fibrillary acid protein antibodies to produce cultures enriched in neuronal cells (results not shown).

Exposure to neurotoxic insults

The experimental protocol of Pauwels *et al.* (1989) was followed throughout this study. Briefly, the medium in the 4-well plates was exchanged twice with fresh medium and then a chemically-defined medium (CDM) was added to give a volume of 0.2 ml in each well. The CDM contained one of the following agents: veratridine (10^{-4} M; Sigma V5754), sodium glutamate (10^{-3} M; Sigma) or sodium cyanide (10^{-3} M). The cultures were then returned to the incubator and left for 16 h in the neurotoxic media after which the medium in each well was assayed for lactate dehydrogenase (LDH) activity (see below).

Estimation of cell viability

Cell viability following exposure to the neurotoxic insults was assessed according to the method of Pauwels *et al.* (1989), using LDH release as a marker of cell death. Briefly, extracellular LDH activity was determined from a 0.15 ml sample of CDM taken from each well at the end of the 16 h exposure period. Following two washes with HBSS, the remaining intact cells in each well were lysed by adding 0.2 ml water to each well and mechanically rupturing them by repeatedly drawing and expelling them through the tip of a micropipette. A 0.15 ml sample of the resulting solution was then assayed to give an estimation of intracellular LDH activity. The amount of LDH released by the neurotoxic stimulus was defined as follows:

$$\text{Specific LDH release} = \left\{\frac{\text{extracellular LDH activity}}{\text{total LDH activity}}\right\} - \text{basal LDH release}$$

where 'specific LDH release' is the LDH released in response to the neurotoxic stimulus, 'total LDH activity' is the sum of the extra- and intracellular activities and 'basal LDH release' is the ratio of extracellular to total LDH activity in time-matched control cultures not subjected to a neurotoxic insult.

LDH activity was determined on the day of experimentation with an automated optimetric system (BM Hitachi 805) for following NADH oxidation at 340 nm.

Measurement of neuroprotective activity of drugs

Cultures were pretreated for 30 min with drug or vehicle at 37°C in a humidified CO_2 incubator. Drug (or vehicle) was present throughout the subsequent 16 h exposure period to the insult. The degree of neuroprotection was expressed as the percentage inhibition of LDH release and was estimated in the following way:

$$\%\ \text{inhibition of specific LDH release} = \left\{1 - \frac{\text{specific LDH release in the presence of neurotoxic stimulus + drug}}{\text{specific LDH release in the presence of neurotoxic stimulus + vehicle}}\right\} \times 100$$

Analysis of data

The concentration of drug producing 50% inhibition of specific LDH release (IC_{50}) was used as an index of neuroprotective potency. Data are expressed as mean ± s.e.mean.

Drugs and solutions

Lifarizine trihydrochloride (Syntex) and (+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)cyclohepten-5, 10-imine maleate (+)-MK-801 or (−)-MK-801 maleate (RBI) stock solutions (10^{-2} M) were made up in sterile water and diluted in CDM to give the appropriate final concentrations. Nitrendipine (synthesized by Syntex) and veratridine (Sigma) were dissolved initially in ethanol and diluted in CDM or water, respectively. Tetrodotoxin (Sigma) was made up as a 10^{-3} M stock solution in sterile water. Sodium cyanide (Sigma) and sodium glutamate (Sigma) were made up as 10^{-2} M stock solutions in sterile water. Sources for other materials are indicated elsewhere in the Methods section.

Results

Neurotoxic stimuli

Exposure of neuronal cultures for 16 h to veratridine (10^{-4} M), glutamate (10^{-3} M) or cyanide (10^{-3} M) produced significant amounts of LDH release above those observed in unstimulated time-matched control cultures (Figure 2). The specific LDH released in response to exposure to veratridine, glutamate and cyanide (expressed as a % of total LDH) was 15%, 16% and 12% respectively.

Protection against veratridine-induced neurotoxicity

Veratridine-induced LDH release was attenuated by tetrodotoxin, lifarizine and nitrendipine (concentration-response curves shown in Figure 3). The IC_{50} concentrations for inhibiting the toxic effects of veratridine were 3×10^{-8} M, 4×10^{-7} M and 3×10^{-5} M for tetrodotoxin, lifarizine and nitrendipine, respectively. However, (+)-MK-801 (up to 3×10^{-5} M) failed to achieve 50% inhibition of specific LDH release under these experimental conditions (Figure 3).

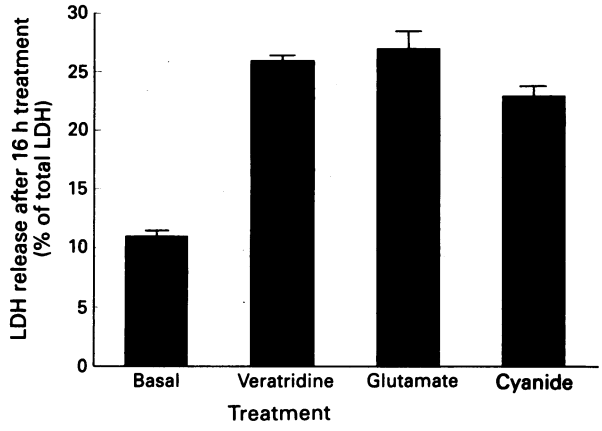

Figure 2 Lactate dehydrogenase (LDH) release induced by 16 h exposure to veratridine (10^{-4} M), sodium glutamate (10^{-3} M) or sodium cyanide (10^{-3} M).

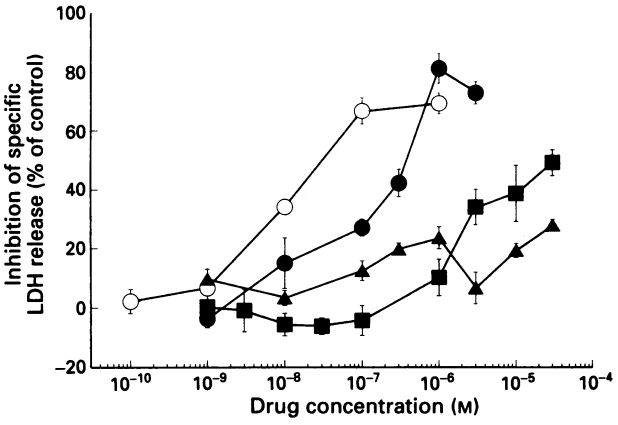

Figure 3 Specific lactate dehydrogenase (LDH) release induced by 16 h exposure to veratridine (10^{-4} M) was inhibited by tetrodotoxin (○), lifarizine (●) and nitrendipine (■) ($IC_{50} = 3 \times 10^{-8}$, 4×10^{-7} and 3×10^{-5} M, respectively). (+)-MK-801 (▲) failed to achieve 50% inhibition of LDH release.

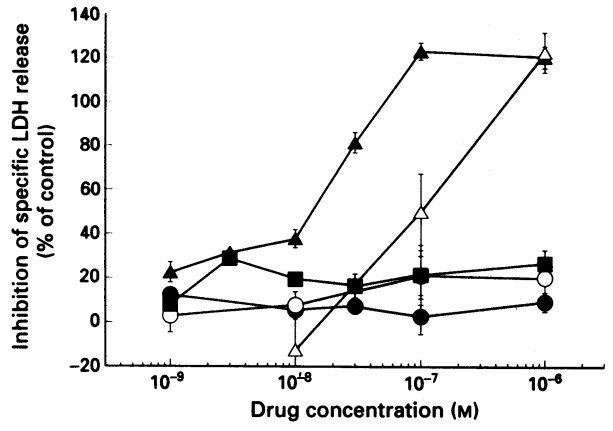

Figure 4 Specific lactate dehydrogenase (LDH) release induced by 16 h exposure to sodium glutamate (10^{-3} M) was inhibited stereoselectively by (+)-MK-801 (▲) and (−)-MK-801 (△). Tetrodotoxin (○), lifarizine (●) and nitrendipine (■) (up to 10^{-6} M) were ineffective.

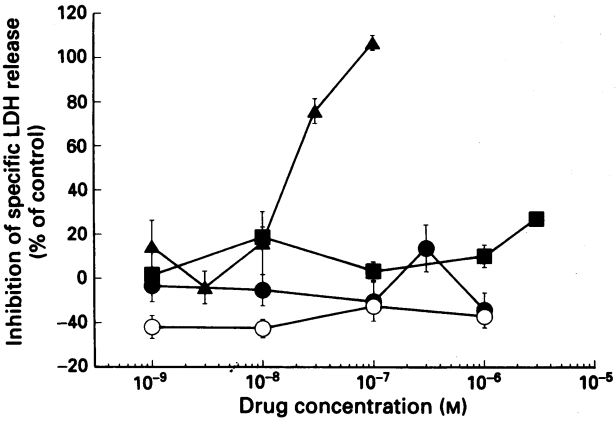

Figure 5 Specific lactate dehydrogenase (LDH) release induced by 16 h exposure to sodium cyanide (10^{-3} M) was inhibited by (+)-MK-801 (▲) but not by lifarizine (●) or tetrodotoxin (○) (up to 10^{-6} M) or by nitrendipine (■) (up 3×10^{-6} M).

Protection against sodium glutamate-induced neurotoxicity

Both isomers of MK-801 provided protection against glutamate-induced neurotoxicity, with the (+)-isomer being about 7 fold more potent than the (−)-isomer ($IC_{50} = 1.4 \times 10^{-8}$ M and 1×10^{-7} M for (+)-and (−)-MK-801, respectively). In contrast, tetrodotoxin, lifarizine and nitrendipine (up to 10^{-6} M) were ineffective against cell death induced by glutamate (concentration-response curves shown in Figure 4).

Protection against sodium cyanide-induced neurotoxicity

Cyanide-induced neurotoxicity was inhibited by (+)-MK-801 ($IC_{50} = 1.9 \times 10^{-8}$ M) but was unaffected either by tetrodotoxin and lifarizine (up to 10^{-6} M) or by nitrendipine (up to 3×10^{-6} M) (concentration-response curves shown in Figure 5).

Discussion

Primary cultures of embryonic rat cerebrocortical neurones have been exposed to three neurotoxic stimuli (veratridine, glutamate and cyanide), and the neuroprotective profile of lifarizine has been compared with that of four pharmacologically well-defined agents: the specific sodium channel blocker, tetrodotoxin, the dihydropyridine L-type calcium channel blocker, nitrendipine, the *N*-methyl-D-aspartate channel blocker, (+)-MK-801, and its less potent enantiomer, (−)-MK-801.

The results show that lifarizine potently protected against neurotoxicity induced by the sodium channel activator, veratridine, but that it was ineffective against either glutamate- or cyanide-induced neurotoxicity.

Tetrodotoxin was clearly the most potent inhibitor of veratridine-induced cell death and this, together with the known pharmacological specificity of the toxin for the sodium channel, underlines the pivotal role played by sodium channel activation in the expression of this neurotoxic insult. That lifarizine was protective against veratridine-induced neurotoxicity is likely to result from an inhibitory interaction of the compound with the sodium channel. Thus, lifarizine has been shown in ligand binding studies potently to displace the sodium channel marker, [^{3}H]-batrachotoxin, from rat cortical synaptosomes ($IC_{50} = 5.5 \times 10^{-8}$ M; C.M. Brown *et al.*, personal communication). Furthermore, in electrophysiological experiments lifarizine voltage-dependently inhibits whole-cell sodium currents in voltage clamped neuroblastoma N1E-115 cells, and this arises predominantly from an interaction of the compound with the inactivated state of the sodium channel (Sheridan *et al.*, 1991; N.A. Brown *et al.*, 1994; McGivern *et al.*, 1994a,b). Consistent with this interaction with the inactivated state was the finding that the potency of lifarizine at inhibiting sodium currents

varied according to the membrane potential at which the cells were held. Thus, at a holding potential of -100 mV the IC_{50} for inhibition of the sodium current was 7.3×10^{-6} M, whereas at a more physiological holding potential of -60 mV the IC_{50} was 3×10^{-7} M (McGivern *et al.*, 1994b). The latter value is near to the IC_{50} value obtained in the present experiments for inhibition of veratridine-induced LDH release ($IC_{50} = 4 \times 10^{-7}$ M), and lends further support to the notion that block of neuronal sodium currents is important for the protective effect of lifarizine to be expressed in this test system.

Although previous studies have indicated that lifarizine possesses some L-type calcium channel antagonistic activity (Fraser & Spedding, 1991), the poor protective activity of nitrendipine against veratridine-induced neurotoxicity suggests that it is highly unlikely that an L-type calcium channel interaction plays a part in the neuroprotective action of lifarizine *in vitro*. Indeed, it is probable that the weak effect of nitrendipine is mediated by an interaction of the dihydropyridine with sodium channels, as nitrendipine at concentrations much higher than those required to inhibit L-type calcium currents is known also to inhibit sodium currents (Yatani & Brown, 1985; Yatani *et al.*, 1988). A similar lack of potency of dihydropridine calcium channel antagonists against veratridine-induced LDH release has been observed in rat hippocampal neurones in primary culture by Pauwels *et al.* (1990). The latter authors also presented a good correlation between the abilities of a series of dihydropyridines to inhibit veratridine-induced LDH release and their abilities to displace [^{3}H]-batrachotoxin from rat cortical synaptosomes, thus providing further evidence that a sodium channel interaction is the underlying mechanism by which the calcium antagonists protect against the veratridine insult.

The lack of protection afforded by (+)-MK-801 against veratridine-induced neurotoxicity indicates the lack of involvement of NMDA receptor activation in this insult and provides indirect evidence that the protection conferred by lifarizine is not mediated by blockade of the NMDA ionophore.

The tetrodotoxin-sensitive neurotoxic action of veratridine in primary cultures of rat brain is presumed to be mediated (at least initially) via the persistent activation of sodium channels (Pauwels *et al.*, 1989). It is unclear, however, whether the neurotoxicity is generated by the entry of sodium *per se*, or whether events occurring downstream of this are responsible. Based on the observation that chelation of extracellular calcium by EGTA prevents the neurotoxic effects of veratridine, Pauwels *et al.* (1989) put forward five candidate mechanisms by which the neurotoxicity could be induced: calcium entry through veratridine-modified sodium channels, calcium entry resulting from an impaired or even reversed operation of the plasmalemmal sodium/calcium exchanger, calcium entry through non-specific membrane leak, raised intracellular calcium by release from internal stores, and calcium entry through voltage-operated calcium channels activated by veratridine-induced depolarization of the membrane. As the present experiments were not designed to elucidate the mechanisms responsible for veratridine-induced neurotoxicity, we cannot speculate any further on this matter other than to state that calcium entry through L-type calcium channels or through the NMDA ionophore is unlikely to be involved.

The stereoselective protection by the NMDA channel blocker, MK-801, against glutamate-induced neurotoxicity underlines the crucial role played by NMDA receptor activation in the expression of this insult and confirms the findings made by others using similar *in vitro* test systems (Choi *et al.*, 1988; Pauwels *et al.*, 1989; Shalaby *et al.*, 1992). The lack of effect of tetrodotoxin against glutamate-induced LDH release is in agreement with the findings of others (Pauwels *et al.*, 1989) and confirms that sodium channel activation is not a feature of this form of neurotoxicity. The failure of nitrendipine to protect against glutamate neurotoxicity would suggest that L-type calcium channels are not involved in the evolution of this insult under the present experimental conditions, although a report by Weiss *et al.* (1990) suggests that under certain conditions a protective effect of the dihydropyridine, nifedipine, against excitatory amino acid exposure may be observed. It may be that the duration of the glutamate exposure used in the present study was too long to permit a dihydropyridine-sensitive component to be detected. The lack of protection against glutamate neurotoxicity provided by lifarizine implies that the latter compound possesses no NMDA antagonistic properties, and this is consistent with the contrasting actions of (+)-MK-801 and lifarizine in the veratridine toxicity experiments.

The third and final neurotoxic stimulus employed in the present series of experiments was the inhibitor of oxidative phosphorylation, sodium cyanide. (+)-MK-801 potently protected against this insult, thereby demonstrating the involvement of NMDA receptor activation in the production of this insult and confirming the observations made by Pauwels *et al.* (1989). On the other hand, the manifest ineffectiveness of lifarizine, tetrodotoxin and nitrendipine against cyanide-induced neurotoxicity argues against the involvement of sodium and L-type calcium channels in the expression of the neurotoxicity induced by cyanide.

Interestingly, in both the cyanide- and glutamate-induced neurotoxicity experiments, (+)-MK-801 produced greater than 100% inhibition of toxin-stimulated LDH release. We attribute this apparently paradoxical finding to an inhibition by MK-801 of LDH release under basal (non-toxin-stimulated) conditions, although this was not explicitly controlled for in the present study. It does, however, suggest the presence of an on-going neurotoxic glutamatergic 'tone' in the untreated cultures.

What is the relevance of the various neurotoxic stimuli used in the present experiments to the events occurring in human stroke? This is perhaps the most difficult question to address. A wide variety of pharmacological agents have been shown to reduce the neuronal damage produced by cerebral ischaemia in animal models. These agents include NMDA antagonists (Park *et al.*, 1988; Gill *et al.*, 1991), non-NMDA excitatory amino acid receptor antagonists (Sheardown *et al.*, 1990; but see DeGraba *et al.*, 1994), calcium and sodium channel inhibitors (Steen *et al.*, 1985; Alps *et al.*, 1988; Pauwels *et al.*, 1991) and inhibitors of lipid peroxidation (Hall *et al.*, 1988; Young *et al.*, 1988). However, despite the promise held out by animal experiments, none of the agents which has so far reached the stage of clinical testing has yet been shown unequivocally to be of benefit in the management of stroke. This is undoubtedly due partly to the post-stroke intervention time and partly to the fact that stroke is not a homogeneous entity, producing ischaemic lesions of widely varying size and severity. Given this failure to demonstrate clinically a clear therapeutic benefit of any particular pharmacological principle it is difficult, if not impossible, to judge the immediate relevance of the *in vitro* findings.

The involvement of sodium channel activation in ischaemic neuronal damage is supported by a number of studies. Thus, Prenen *et al.* (1988) reported a tetrodotoxin-sensitive change in interstitial electric potential in rat striatum following induction of cardiac arrest, while Yamasaki *et al.* (1991) described a protective action of intrahippocampally injected tetrodotoxin on the histological outcome in area CA1 following four-vessel occlusion in the rat. Boening *et al.* (1988) observed a protective effect of tetrodotoxin on the recovery of electrical activity following anoxia in the rat hippocampal slice preparation, while Milusheva (1992), who examined the release of dopamine from rat striatal slices under conditions of simulated ischaemia, found that tetrodotoxin abolished the ischaemia-induced increase in dopamine release. More recently, Vornov *et al.* (1994) described a protective effect of tetrodotoxin against histologically-assessed damage produced by metabolic inhibition in organotypic hippocampal slice cultures. The anticonvulsant agent and sodium channel

inhibitor, phenytoin, has been shown to enhance recovery of synaptic transmission in rat hippocampal slices under conditions of simulated ischaemia (Kenny & Sheridan, 1992). Taken together, these observations provide a rationale for the use of sodium channel blockers in cerebral ischaemia. The clear advantage of lifarizine over tetrodotoxin is that its blocking actions are voltage-dependent: block is enhanced at depolarized potentials and removed at hyperpolarized potentials due to a selective interaction with the inactivated state of the channel (Sheridan *et al.*, 1991; McGivern *et al.*, 1994a,b), whereas tetrodotoxin interacts indiscriminately with the resting, open and inactivated states of the channel.

The neuroprotective profile of lifarizine in the present experiments is very similar to that of the difluorinated diphenylpiperazine derivative, flunarizine; flunarizine protects embryonic rat hippocampal neuronal cultures against veratridine-induced cell death, but is ineffective against glutamate and cyanide toxicity (Pauwels *et al.*, 1989). Moreover, flunarizine, like lifarizine, displaces [^{3}H]-batrachotoxin from rat cortical synaptosomes (Grima *et al.*, 1986), exerts potent sodium current blocking actions in neuronal cells (Kiskin *et al.*, 1993), and is neuroprotective in various animal stroke models (Pauwels *et al.*, 1991). In view of these similarities between lifarizine and flunarizine it is conceivable that the *in vivo* neuroprotective action of both compounds derives from their common ability to inhibit voltage-dependent sodium channels.

In conclusion, the present study demonstrates that lifarizine potently protects *in vitro* against veratridine-induced neurotoxicity but fails to protect against the neurotoxicity induced by either glutamate or cyanide. The similar neuroprotective profiles of lifarizine and tetrodotoxin may suggest that both agents exert their protective effects by inhibiting neuronal sodium channels, thereby preventing the toxic sequelae of excessive sodium influx. This action of lifarizine on sodium channels is supported by data from electrophysiological and ligand binding experiments and constitutes a mechanism by which the compound could provide neuroprotection in animal models of cerebral ischaemia.

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Influence of chronic treatment with a nitric oxide donor on fatty streak development and reactivity of the rabbit aorta

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1 The influence of chronic treatment with molsidomine, pro-drug of the nitric oxide (NO) donor, 3-morpholino-sydnonimine (SIN-1), on fatty streak development and release of NO and prostacyclin (PGI_2) was studied in the aorta of normal and cholesterol-fed rabbits.

2 Groups of 10 rabbits received standard diet (150 g day^{-1}), or diets with 0.3% cholesterol, with 0.02% molsidomine or with the combination of cholesterol and molsidomine for 16 weeks. Lesion area and thickness, maximum change in isometric force (E_{max}) and sensitivity ($-\log EC_{50}$ or pD_2) to constricting and relaxing agonists were assessed in segments of arch, thoracic and abdominal aorta. Bioassay was used to assess NO release.

3 Cholesterol-induced fatty streaks tapered off towards the abdominal aorta. Area, thickness, weight and cholesterylester content of the lesions were augmented by the NO donor, whereas the hypercholesterolaemia remained unchanged. The exacerbation was attributed to co-release of superoxide anion from the sydnonimine.

4 As fatty streaks progressed, amplitude and pD_2 of acetylcholine (ACh)-induced relaxations decreased, whereas cyclic GMP and cyclic AMP second messenger systems were not influenced, since E_{max} and sensitivity to SIN-1 and forskolin remained unchanged. However, extensive lesions apparently trapped some NO, as the pD_2 of authentic NO decreased.

5 The fatty streaks curtailed the biosynthesis of PGI_2 and the overflow of NO from the perfused thoracic aorta. The latter defect was not restored by L-arginine and appears to be consistent with a functional change of the endothelial muscarinic receptors.

6 The NO donor desensitized the aorta to cyclic GMP-mediated relaxations (ACh, SIN-1 and NO), without affecting cyclic AMP-mediated relaxation to forskolin or constrictor responses to phenylephrine and 5-hydroxytryptamine.

7 The drug also suppressed the ACh-induced overflow of NO, without changing PGI_2 release. This selective reduction of endothelial NO release and the desensitization of cyclic GMP-mediated relaxations occurred independently of fatty streak formation.

8 The results indicate that chronic exposure to exogenous NO downregulates endothelial NO release and cyclic GMP-mediated relaxations, and provide evidence for the existence of negative feed-back regulations of the L-arginine NO pathway under *in vivo* conditions.

Keywords: Atherosclerosis; fatty streak; endothelium; 5-hydroxytryptamine; L-arginine; prostacyclin; molsidomine; SIN-1; nitric oxide release; rabbit aorta.

Introduction

Rabbits that are fed diets supplemented with cholesterol develop intimal lesions in conduit arteries, which bear a superficial resemblance to fatty streaks in human arteries (Clarkson *et al.*, 1974). As these experimental 'atherosclerotic lesions' develop, endothelium-dependent relaxations (Verbeuren *et al.*, 1986b; Freiman *et al.*, 1986; Bossaller *et al.*, 1987; Jayakody *et al.*, 1987; Minor *et al.*, 1990) and NO release (Moncada *et al.*, 1991), assessed by bioassay of the 'overflow' of NO into the lumen of the vessel (Guerra *et al.*, 1989; Verbeuren *et al.*, 1990; Minor *et al.*, 1990), become progressively suppressed. The NO defect is presumably the result, rather than the cause of the vascular lesions, since endothelium-dependent responses are preserved in adjacent normal areas and arteries which do not develop fatty streaks (Verbeuren *et al.*, 1986b; Senaratne *et al.*, 1991; Galle *et al.*, 1991). The latter findings are hard to reconcile with the proposal that hypercholesterolaemia as such, suppresses endothelial NO release (Minor *et al.*, 1990). However, one cannot exclude that progressive reduction of NO release aggravates fatty streak development (Cayatte *et al.*, 1994; Naruse *et al.*, 1994), and it has been proposed that treatment with L-arginine restores cholinergic-mediated relaxation (Cooke *et al.*, 1991) and suppresses intimal thickening (Cooke *et al.*, 1992) in this model. Therefore, we investigated whether long-term supplementation with a donor of exogenous NO delayed the development of fatty streaks and the concomitant abnormalities in vascular reactivity in hypercholesterolaemic rabbits.

Classical organic nitrates, glyceryltrinitrate (GTN), or isosorbide dinitrate, which require biotransformation in the vessel wall, would be inappropriate for this purpose because of the rapidly developing tolerance. An important aspect of this tolerance is the compromised biotransformation in the smooth muscle cells (Ahlner *et al.*, 1991). To circumvent the problem of biotransformation we selected molsidomine, the pro-drug of 3-morpholino-sydnonimine (SIN-1), which releases NO non-enzymatically (Kukovetz & Holzmann, 1985) through a mechanism which involves stoichiometric formation of superoxide anion (Feelisch, 1991). Sixteen weeks later, the reactivity of isolated rings of the aorta to vasodilators operating via guanosine 3':5'-cyclic monophosphate (cyclic GMP) (SIN-1, NO and ACh) or cyclic AMP (forskolin, Laurenza *et al.*, 1989) and contractile responses to phenylephrine and 5-hydroxytryptamine (5-HT) were evaluated in organ chambers. Furthermore, the receptor-mediated overflow of NO and PGI_2 was assessed. As the development and the severity of the fatty streaks in the

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rabbit aorta decrease downstream towards the bifurcation (Clarkson *et al.*, 1974), three well-defined areas (aortic arch, central part of thoracic aorta and central part of the abdominal aorta) were investigated in order to delineate the contribution of lesion size and/or hypercholesterolaemia to the alterations in reactivity. Part of the results have been presented to the Royal Society, London (Bult *et al.*, 1990).

Methods

Induction of fatty streaks

New Zealand rabbits (2.34 ± 0.02 kg, $n = 40$, Borrey Farm, Belgium) were randomly divided into four groups to receive the following diets (150 g day^{-1}) for 16 weeks: (1) control diet; (2) a diet with 0.3% cholesterol; (3) a diet with 0.02% molsidomine; (4) a diet containing both molsidomine and cholesterol. The dose of molsidomine (10 mg kg^{-1} day^{-1}) was equivalent to the clinical dose (0.1 to 0.2 mg^{-1} kg^{-1} day^{-1}), since rabbits require about 100 times as much molsidomine to induce a blood pressure fall (data provided by manufacturer, Cassella AG, Frankfurt, Germany). After fasting overnight the rabbits were anaesthetized (sodium pentobarbitone, 30 mg kg^{-1}, i.v.), heparinised (100 units kg^{-1}) and a catheter was placed in the carotid artery for blood collection. Thereafter the animals were killed by an overdose of pentobarbitone.

In a second experiment, two groups of eight rabbits received 150 g diet containing 0 or 0.02% molsidomine per day. On the seventh day, two hours after feeding, the dorsal skin of one ear was sprayed with lignocaine (10%), a catheter was inserted in the central ear artery, connected to a pressure transducer and blood pressure was evaluated during 5 min.

Preparation of vascular segments

After killing the rabbit, the chest was opened, the aorta exposed and trimmed free of fat. Throughout the procedures care was taken to preserve the endothelium. The intercostal arteries were thermocoagulated before cutting, in order to avoid collapse of the vessel, and the segment (3 cm, D) immediately following the descending arch was cannulated and used for the perfusion study (see below). Thereafter, the arch, the thoracic aorta and the abdominal aorta were carefully removed and immediately immersed in physiological salt solution. Sets of three adjacent rings (3 mm long) were cut from aortic arch, central thoracic aorta (about 2 cm cranial of the diaphragm) and abdominal aorta (about 2 cm caudal of the diaphragm). One of these segments (A) was used for histology, the others (B and C) were cleaned of loose connective tissue and used to measure vascular reactivity. In the remaining segment of the lower thoracic aorta hydroxyfatty acids were determined; these results have been published (De Meyer *et al.*, 1991a).

Organ chamber studies

Fine stainless-steel wire clips were inserted through the lumen of the rings, allowing them to be suspended for recording of isometric contraction (Verbeuren *et al.*, 1986b; De Meyer *et al.*, 1991b). The rings were mounted in organ chambers filled with 25 ml Krebs-Ringer solution containing 3 μM indomethacin, maintained at 37°C and aerated continuously with 95% O_2–5% CO_2. Tension was measured by strain gauges (Statham UC2). The rings were gradually stretched to a force of 8 g, which brings both control and atherosclerotic aortae to the optimal length for isometric contraction (Verbeuren *et al.*, 1986b). The preparations were then allowed to equilibrate for 45 min prior to experimentation. In the B segments cumulative dose-response curves to SIN-1 (10^{-9}–1.6×10^{-5} M), ACh (10^{-9}–4×10^{-6} M) and NO (10^{-9}–1.6×10^{-5} M) were made after contraction with 3.5×10^{-7} M phenylephrine. In the C segments, cumulative dose-contraction curves to 5-HT (10^{-9}–10^{-5} M) and phenylephrine (10^{-8}–10^{-5} M) were made, followed by a relaxation curve to forskolin (10^{-8}–10^{-5} M) after contraction with 3.5×10^{-7} M phenylephrine. Although PGI_2 is devoid of vascular activity in the rabbit aorta (Moncada *et al.*, 1976), indomethacin (3 μM) remained present throughout these experiments.

Release of NO and prostacyclin by the thoracic aorta

The upper thoracic segment (D, 3 cm) was mounted horizontally in a heated chamber and perfused as described (Bult *et al.*, 1988). A ring (2 mm), obtained from the lower abdominal aorta of a rabbit that had not been included in the study, was used as recipient tissue. It was superfused with physiological salt solution and contracted with 0.1 μM noradrenaline. The sensitivity of the recipient was tested by injecting 0.02, 0.2 and 2 nmol GTN. Thereafter, 0.3, 3 and 30 nmol ACh was injected into the donor tissue. Both ACh and GTN were administered in random-order at 20 min intervals. L-Arginine (1 mM) was then added to the perfusate and after 30 min the whole protocol was repeated in the presence of L-arginine. Aortic effluent was collected in the presence of indomethacin (10 μM) between 90 and 30 s before, and from 30 to 90 s after each injection of ACh. PGI_2 was determined by radioimmunoassay of 6-oxo-$PGF_{1\alpha}$ as described by Beetens *et al.* (1986) and Bult *et al.* (1988).

Evaluation of the fatty streaks

The A segments were directly fixed in 4% neutral formalin and stained with haematoxylin-eosin. One cross-section of each segment was examined under a microscope by an independent observer, using the following score: (0) no plaques; (1) plaques of modest thickness (less than 20% of media); (2) medium thickness (20–50% of media); (3) great thickness (more than 50% of media). All other segments (B, C, D) were opened longitudinally upon completion of the organ chamber or perfusion study and intimal lesions were evaluated on a scale from zero to five by two independent observers using 4 × binocular magnification (Verbeuren *et al.*, 1986b; Beetens *et al.*, 1986). A zero was attributed to segments without lesions, a segment completely covered by fatty streaks received a five. The tissues were then stored in 4% neutral formalin. After staining (0.5% Fat Red 7B (Gurr, BDH Chemicals) in 70% ethanol) for 20 min, the preparations were briefly placed in 70% ethanol and rinsed with tap water for 20 min. The segments were examined under a microscope using low power magnification. The stained areas were measured with a digitizing tablet and software (Sigmascan, Jandel Scientific, Corte Madera, CA, U.S.A.) and expressed as a percentage of the total surface.

Cholesterol extraction and determination

Plasma was stored at −20°C and after appropriate dilution (10 to 200 fold) total cholesterol was determined (Omodeo-Sale *et al.*, 1984). The upper thoracic aorta (donor, segment D, stored at −20°C) was cut into small pieces, and homogenized in 5 ml 0.1 N acetic acid. After lipid extraction free and total cholesterol were determined (Omodeo-Sale *et al.*, 1984).

Materials

The diets were prepared by Altromin (Lage, Germany). ACh, L-arginine, atropine sulphate, *o*-dianisidine dihydrochloride, forskolin and L-isoprenaline-D-bitartrate dihydrate were obtained from Sigma (St. Louis, MO, U.S.A.); indomethacin

sodium tetrahydrate from Merck, Sharp & Dohme (Brussels, Belgium); glyceryltrinitrate (1% solution in ethanol) from Merck (Darmstadt, Germany); A-23187 from Calbiochem (La Jolla, CA, U.S.A.); (-)-noradrenaline-L-hydrogentartrate from Fluka (Buchs, Switzerland); phenylephrine chloride from Winthrop (Brussels, Belgium); 5-hydroxytryptamine creatinine sulphate from Janssen Chimica (Beerse, Belgium); sodium pentobarbitone from Psyphac (Brussels, Belgium); cholesterol esterase, cholesterol oxidase and horseradish peroxidase from Boehringer Mannheim (Mannheim, Germany) and heparin Leo from Therabel Pharma (Brussels, Belgium). SIN-1 and molsidomine were gifts from Therabel Research (Brussels, Belgium). The physiological salt solution contained (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, Ca EDTA 0.025 and glucose 11.1. Saturated NO solutions (estimated to contain 2 mM) were freshly prepared by gassing argon-deoxygenated 0.9% NaCl with NO (Air Liquide, Belgium) for 5 min and diluted with argon-deoxygenated 0.9% NaCl.

Statistical analysis

Relaxations (g) were expressed as a percentage of the initial tension (g) induced by 3.5×10^{-7} M phenylephrine, and contractions as percentage of the maximum contraction to phenylephrine. All data are given as mean ± s.e.mean. The negative logarithm of the concentration ($-\log EC_{50}$ or pD_2) that produced the half maximal effect (E_{max}) of that agonist was calculated, using linear regression analysis (Tallarida & Murray, 1981). The Spearman rank correlation coefficient (r_s) was used to determine whether correlations existed between reactivity parameters (E_{max} and pD_2) and lesion area (Fat Red, %) or plasma cholesterol level (log M) in the combined cholesterol fed groups (2 and 4). A factorial analysis of variance (ANOVA) was used to compare the four treatments, with cholesterol (2 levels, added to diet or not) and exogenous NO (2 levels, molsidomine in diet or not) as between rabbit factors. Plasma cholesterol (log M) or the lesion size (Fat Red, %) were added as covariate in an analysis of covariance (ANCOVA), if the Spearman test indicated a statistically significant ($P<0.01$) correlation in the cholesterol-fed rabbits (Sokal & Rohlf, 1981). If variances of groups were unequal (plasma cholesterol, prostacyclin) the logarithm of the values was analysed. Non-parametric methods (Mann-Whitney and Friedman's test) were used for the comparison of ordinal data (scores of fatty streaks) (Sokal & Rohlf, 1981). A 5% level of significance was selected. All data were analysed with the SPSS (Release 5) software (SPSS Inc., Chicago, IL, U.S.A.).

Results

Body weight, plasma cholesterol and blood pressure

Rabbits on either molsidomine diet gained more weight in the first four weeks. The difference was maintained until the end of the experiment (Table 1). Hypercholesterolaemia (plasma cholesterol > 1 mM) developed in 15 out of 20 rabbits on cholesterol-rich diets, and was accompanied by increased plasma triglycerides (Table 1). Molsidomine did not influence the incidence of the hypercholesterolaemia, or plasma levels of cholesterol or triglycerides. In a second experiment two groups of eight rabbits received diets containing 0 or 0.02% molsidomine. After one week there was a tendency to a fall in mean blood pressure (control 97 ± 4 and molsidomine 90 ± 3 mmHg), but the difference was not statistically significant.

Fatty streaks

Light microscopy of transverse sections revealed the presence of endothelial cells in all segments examined. Only in aortic segments of the hypercholesterolaemic (plasma cholesterol > 1 mM) rabbits in groups 2 and 4 were foam cell rich intimal plaques of variable thickness observed (Table 2). Lipid infiltration was also visible macroscopically and after staining with Fat Red. All parameters indicated that lesions were most extensive in the aortic arch and decreased gradually towards the abdominal aorta. In rabbits on the combined diet (cholesterol plus NO donor) these indices gave larger values in all three segments of the aorta (Table 2). However, the increase was not statistically significant as the ranges of both groups overlapped completely when all rabbits were examined. Restricting the analysis to hypercholesterolaemic (plasma cholesterol > 1 mM) rabbits indicated that the NO donor increased the area (thoracic aorta) and thickness (thoracic and abdominal aorta) of the fatty streaks (Table 2).

The wet weight of the thoracic segment used in the bioassay was raised by the cholesterol-rich diet and not influenced by molsidomine alone. However, the combination of NO donor and cholesterol led to a synergistic, eight fold further increase of the wet weight (indicated by interaction in ANOVA, Table 3). The normalized protein content was not influenced by any diet. Cholesteryl esters were undetectable in aortae of control and molsidomine-treated rabbits, but accumulated together with free cholesterol after cholesterol feeding (Table 3). The combination with the NO donor led to a synergistic four fold further increase of the content of

Table 1 Body weight and plasma cholesterol and triglycerides

	Control $n=10$	*Cholesterol* $n=10$	*NO donor* $n=10$	*NO + cholesterol* $n=10$	*ANOVA* *Cholesterol* $F_{(1,36)}$	*NO* $F_{(1,36)}$	*NO by Cholesterol* $F_{(1,36)}$
Body weight start (kg)	2.31 (0.03)	2.34 (0.05)	2.35 (0.04)	2.35 (0.05)	1.60	0.00	0.105
Body weight end (kg)	3.61 (0.06)	3.50 (0.07)	3.79 (0.06)	3.85 (0.09)	0.13	14.8***	1.55
Hypercholesterolaemia, plasma cholesterol > 1 mM	0/10	7/10	0/10	8/10			
Plasma:							
Cholesterol (mM)	0.5 (0.1)	6.0 (2.0)	0.2 (0.1)	5.8 (1.3)			
Cholesterol, log (mM)	− 0.4 (0.1)	0.5 (0.2)	− 0.7 (0.1)	0.6 (0.1)	67.5***	0.41	2.52
Triglycerides (mg dl^{-1})	27 (11)	159 (133)	16 (5)	86 (42)			
Triglycerides log (mg dl^{-1})	1.1 (0.2)	1.4 (0.2)	1.0 (0.2)	1.6 (0.2)	5.74*	0.05	0.44

Values given as mean and (s.e.mean); *$P<0.05$, ***$P<0.001$, significance of F ratio.

esterified cholesterol, as indicated by the interaction in the ANOVA (Table 3). The effect of the NO donor on cholesterol and cholesteryl ester accumulation was not statistically significant after normalisation for the increased wet weight of the tissues (Table 3).

In the cholesterol-fed animals all lesion parameters (wet weight and cholesterol content of thoracic aorta, scores of area and thickness, and area stained with Fat Red) showed highly significant, positive correlations to each other ($r_s > 0.83$; $P < 0.001$) and also when the analysis was restricted to hypercholesterolaemic (plasma cholesterol > 1 mM) rabbits, results not shown. Scatter diagrams showed that correlations between plasma cholesterol and these lesion parameters were much less clear ($0.57 < r_s < 0.70$, $0.01 < P < 0.05$) and were mainly due to the presence of five rabbits without hypercholesterolaemia, which did not develop fatty streaks. Indeed, none of the lesion parameters was correlated to plasma cholesterol ($r_s < 0.41$, $P > 0.1$) when the analysis was restricted to hypercholesterolaemic rabbits (plasma cholesterol > 1 mM, results not shown).

Vascular reactivity: contractile responses

The response curve to phenylephrine was slightly displaced to the right (about two fold) in the aortic arch and thoracic aorta of cholesterol-fed rabbits (Table 4), but not in the abdominal aorta, a segment with less lipid infiltration. Negative correlations existed between lesion area (% Fat Red) and pD_2 (r_s −0.74, $P < 0.001$) and to a smaller extent between plasma cholesterol and pD_2 (r_s −0.50, $P < 0.001$) when all segments ($n = 60$) of cholesterol-fed rabbits were analysed. The ANOVA pointed to lesion size as the factor responsible for the shift of the dose-response curve in aortic arch and thoracic aorta. Molsidomine, either alone or in combination with cholesterol did not change the sensitivity to phenylephrine (Table 4). The E_{max} (11.3 ± 0.4, 10.2 ± 0.5 and 12.6 ± 0.6 g, $n = 40$, respectively in arch, thoracic and abdominal aorta) was not altered by any treatment (results not shown).

The two cholesterol-rich diets affected responses towards 5-HT profoundly (Figure 1, Table 4) and E_{max} and pD_2 were augmented in the aortic arch and thoracic aorta, but not in the less affected abdominal segment. E_{max} (r_s 0.51, $P < 0.001$) and pD_2 (r_s 0.33, $P < 0.01$) showed positive correlations to the logarithm of the plasma cholesterol content when all segments ($n = 60$) of cholesterol-fed rabbits were pooled. Associations with lesion area were absent (E_{max}) or less clear (pD_2 all segments r_s 0.29, $P = 0.02$, $n = 60$), as they appeared to be non-linear: small lesions were already often associated with large pD_2 values. In the ANCOVA plasma cholesterol was the most significant factor for the sensitization (Table 4) and augmented E_{max} in arch ($F_{1,34}$ 26.2, $P < 0.001$) and thoracic aorta ($F_{1,34}$ 12.7, $P = 0.001$), but not in the abdominal aorta ($F_{1,34}$ 1.0). After this correction, the NO

Table 2 Evaluation of fatty streaks in the aortic arch, the thoracic and the abdominal aorta in both groups of cholesterol-fed rabbits

	All rabbits			*Hypercholesterolaemic rabbits (plasma cholesterol > 1 mM)*	
	Cholesterol $n = 10$	*NO donor by cholesterol* $n = 10$	*Friedman*	*Cholesterol* $n = 7$	*NO donor + cholesterol* $n = 8$
*Area, macroscopic score**					
Arch	2.9 ± 0.6	3.6 ± 0.6	$P < 0.001$	3.3 ± 0.6	4.4 ± 0.3
Thorax	2.0 ± 0.5	3.1 ± 0.6		2.2 ± 0.5	3.8 ± 0.5†
Abdomen	1.0 ± 0.3	1.7 ± 0.6		1.2 ± 0.3	2.1 ± 0.6
Thickness, score					
Arch	1.7 ± 0.4	2.2 ± 0.4	$P = 0.008$	2.0 ± 0.5	2.8 ± 0.6
Thorax	1.0 ± 0.4	1.9 ± 0.4		1.3 ± 0.4	2.4 ± 0.3†
Abdomen	0.7 ± 0.3	1.4 ± 0.3		0.8 ± 0.3	1.8 ± 0.3†
Area, Fat Red (%)					
Arch	70 ± 11	78 ± 11	$P < 0.001$	78 ± 10	92 ± 3
Thorax	24 ± 11	53 ± 13		30 ± 13	66 ± 13
Abdomen	19 ± 9	36 ± 10		23 ± 11	42 ± 12

*See Methods. Both scores and the Fat Red area were zero in all rabbits of groups 1 and 3. Values given as mean ± s.e.mean. †$P < 0.05$, Mann-Whitney test.

Table 3 Wet weight, protein and cholesterol content and lipid-rich area in the thoracic segments used in the perfusion bioassay

	Control $n = 10$	*Cholesterol* $n = 10$	*NO donor* $n = 10$	*NO + cholesterol* $n = 10$	*ANOVA* *Cholesterol* $F_{(1,36)}$	*NO* $F_{(1,36)}$	*NO by Cholesterol* $F_{(1,36)}$
Wet weight (mg)	101 (16)	130 (18)	94 (7)	236† (46)	10.60**	3.54	4.61*
Protein (mg g^{-1} tissue)	42 (5)	36 (4)	40 (3)	40 (5)	0.57	0.04	0.73
Cholesterol#	0.5	4	0.5	17	22.0***	3.17	3.13
μmol	(0.05)	(2)	(0.04)	(6)			
μmol g^{-1} tissue	6 (0.4)	13 (3)	6 (0.4)	25 (6)	23.5***	2.63	0.14
Cholesteryl esters#	− 0.1	2	0.0	9†	8.7**	2.17	4.23*
μmol	(0.09)	(1)	(0.1)	(3)			
μmol g^{-1} tissue	− 0.1 (0.2)	10 (6)	0.1 (0.2)	27 (8)	7.17*	2.35	0.46
Area fatty streaks, score	0 (0)	1.7 (0.4)	0 (0)	3.2† (0.5)			

Values given as mean and (s.e.mean); *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significance of F ratio.
#Because of the significant heterogeneity of variances logarithmically transformed values were used for cholesterol and cholesteryl esters. †Significantly different from cholesterol alone, Mann-Whitney test, $P < 0.05$.

donor and cholesterol were without overall effects in any region (Table 4). However, significant interactions were present in the aortic arch: the NO donor alone increased pD_2 (Table 4) and E_{max} to 5-HT ($F_{1,18}$ 6.29, $P = 0.02$), an effect not seen in the presence of cholesterol (Figure 1). Analysis of the raw data (g contraction) yielded similar results (data not shown).

Vascular reactivity: relaxations

The average initial contractions to 0.35 μM phenylephrine (g, $n = 40$) were 5.8 ± 0.4 and 3.7 ± 0.2 (aortic arch, B and C segments), 6.1 ± 0.4 and 5.3 ± 0.3 (thoracic aorta, B and C segments) and 8.7 ± 0.7 and 7.2 ± 0.5 (abdominal aorta, B and C segments) and were not different among the four groups, although they tended to be smaller in the arch of cholesterol-fed rabbits (groups 2 and 4).

ACh caused 70% (aortic arch) to 90% (abdominal aorta) relaxation. The cholesterol-rich diet led to insignificant (abdominal aorta), moderate (thoracic aorta) or severe (aortic arch) reductions of amplitude (Figure 2) and two (abdominal aorta) to three (aortic arch) fold right ward shifts of the dose-response curves (Table 5). Both E_{max} ($r_s -0.89$, $P<0.001$, $n = 60$) and pD_2 ($r_s -0.53$, $P<0.001$, $n = 49$) were negatively correlated to lesion size when all segments of cholesterol-fed rabbits were pooled, the associations with plasma cholesterol were less strong (E_{max}, $r_s -0.49$, $P<0.001$; pD_2, $r_s -0.35$, $P = 0.02$). After correction for lesion area, both cholesterol and the NO donor still shifted the dose-response curves in all segments (Table 5). In thoracic and abdominal aorta both factors were additive. In the aortic arch the cholesterol effect was so pronounced (complete loss of relaxation in 3 rabbits) that the effect of the NO donor was not additive, as indicated by the interaction (Table 5). Lesion size was the major determinant for the reduced amplitude of the ACh-induced relaxations, but in addition, the NO donor reduced the amplitude as well, independently of the lesion area. This effect of the NO donor was not statistically significant in abdominal segments (Figure 2, Table 5). Although Figure 2 suggests that the NO donor and cholesterol synergistically suppressed the amplitude of the relaxations in the thoracic aorta, the ANCOVA clearly showed the additive effect of the NO donor when the lesion size was taken into consideration.

Table 4 Influence of fatty streaks, plasma cholesterol and treatment with exogenous NO on pD_2 values of the contractile responses to phenylephrine and 5-hydroxytryptamine

	Aortic arch	*Thoracic aorta*	*Abdominal aorta*
Phenylephrine, pD_2			
Control (−logM)	6.60 ± 0.04	6.78 ± 0.03	7.06 ± 0.06
Cholesterol (−logM)	6.37 ± 0.03	6.68 ± 0.08	7.00 ± 0.13
NO donor (−logM)	6.71 ± 0.05	6.84 ± 0.12	7.28 ± 0.14
Cholesterol + NO donor (−logM)	6.36 ± 0.08	6.61 ± 0.11	7.03 ± 0.10
ANCOVA: area Fat Red $F_{1,35}$	41.8***	23.7***	2.83
cholesterol $F_{1,35}$	3.03	3.27	0.09
NO donor $F_{1,35}$	1.90	0.14	0.20
NO by cholesterol $F_{1,35}$	1.18	1.13	0.09
5-HT, pD_2			
Control (−logM)	6.41 ± 0.08	6.15 ± 0.08	6.51 ± 0.11
Cholesterol (−logM)	6.74 ± 0.03	6.43 ± 0.08	6.77 ± 0.08
NO donor (−logM)	6.50 ± 0.05	6.28 ± 0.05	6.63 ± 0.10
Cholesterol + NO donor (−logM)	6.69 ± 0.07	6.46 ± 0.08	6.82 ± 0.13
ANCOVA: plasma cholesterol $F_{1,35}$	10.2**	12.15***	0.71
cholesterol $F_{1,35}$	0.55	0.15	0.45
NO donor $F_{1,35}$	0.50	0.96	3.85
NO by cholesterol $F_{1,35}$	5.56*	1.28	0.09

Values given as mean ± s.e.mean; factorial analysis of variance with cholesterol and NO donor as between rabbit factors and area Fat Red or plasma cholesterol (log M) as covariate.
*$P<0.05$, **$P<0.01$, ***$P<0.001$, significance of F ratio.

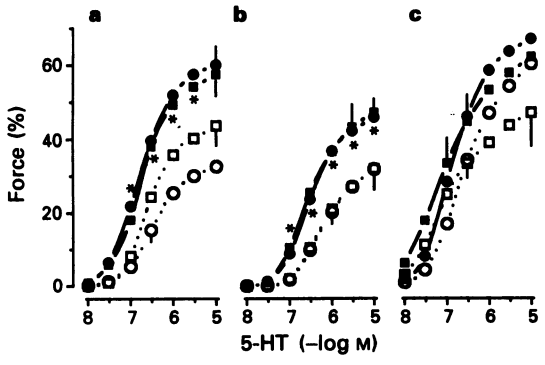

Figure 1 Increased contractions to 5-hydroxytyptamine (5-HT) in rings of aortic arch (a) and thoracic aorta (b) but not in the abdominal aorta (c) of rabbits treated with cholesterol or cholesterol plus NO donor. Force expressed as percentage of the maximum response to phenylephrine. (○) Control; (□) NO donor; (●) cholesterol, (■) cholesterol plus NO donor, $n = 10$. For reasons of clarity only the largest s.e.mean (vertical bar) shown in each curve. *Different from corresponding group without cholesterol ($P<0.05$, analysis of variance and Bonferroni test).

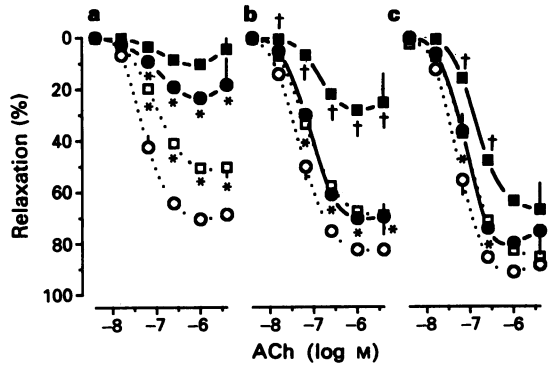

Figure 2 Relaxation curves to acetylcholine (ACh) in the aortic arch (a), the thoracic (b) and abdominal aorta (c) of rabbits exposed to a control diet (○) or diets with cholesterol (●), exogenous NO (□) or NO donor plus cholesterol (■). Tension expressed as percentage of the initial phenylephrine contraction ($n = 8-10$). For reasons of clarity only the largest s.e.mean (vertical bar) shown in each curve. *Different from control group; †Different from cholesterol group ($P<0.05$, analysis of variance and Bonferroni test).

Table 5 Influence of fatty streaks, cholesterol and NO donor on pD_2 and E_{max} (% of phenylephrine contraction) of acetylcholine-induced relaxation in the rabbit aorta

	Aortic arch	*Thoracic aorta*	*Abdominal aorta*
Acetylcholine, pD_2			
Control (−logM)	7.25 ± 0.03	7.28 ± 0.08	7.34 ± 0.08
Cholesterol (−logM)	6.77 ± 0.13†	7.05 ± 0.07†	7.08 ± 0.06
NO donor (−logM)	7.02 ± 0.06	7.13 ± 0.06	7.00 ± 0.11
Cholesterol + NO donor (−logM)	6.89 ± 0.11†	6.82 ± 0.06†	6.77 ± 0.07
ANCOVA: area Fat Red $F_{1,35}$	9.96**	11.7**	3.20
cholesterol $F_{1,35}$	0.05	5.51*	5.34*
NO donor $F_{1,35}$	0.47	8.90**	11.9**
NO by cholesterol $F_{1,35}$	7.59**	0.36	0.00
Acetylcholine, E_{max}			
Control (%)	67 ± 4	84 ± 2	91 ± 2
Cholesterol (%)	27 ± 9	71 ± 6	87 ± 6
NO donor (%)	52 ± 3	70 ± 4	87 ± 3
Cholesterol + NO donor (%)	13 ± 6	31 ± 10	83 ± 3
ANCOVA: area Fat Red $F_{1,35}$	107.3***	78.6***	0.71
cholesterol $F_{1,35}$	0.53	2.18	0.45
NO donor $F_{1,35}$	29.4***	4.93*	3.85
NO by cholesterol $F_{1,35}$	1.20	0.29	0.09

†Only segments in which acetylcholine induced relaxation could be analysed. Values given as mean ± s.e.mean; factoral analysis of variance with cholesterol and NO donor as between rabbit factors and area Fat Red as covariate. *$P<0.05$, **$P<0.01$, ***$P<0.001$, significance of F ratio.

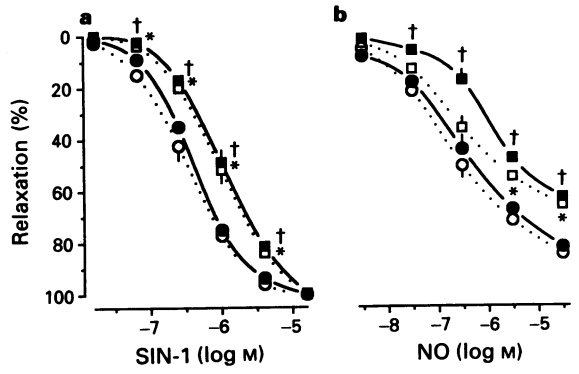

Figure 3 Right ward shifts of the concentration-response curves for 3-morpholino-sydnonimine (SIN-1) (a) and NO (b) in the thoracic aorta of rabbits treated with exogenous NO (molsidomine) or NO donor plus cholesterol. Tension expressed as percentage of the initial phenylephrine contraction ($n = 8-10$). For reasons of clarity only the largest s.e.mean (vertical bar) shown in each curve. (○) Control; (□) NO donor; (●) cholesterol; (■) cholesterol plus NO donor, $n = 10$. *Different from control group; †Different from cholesterol group ($P<0.05$, analysis of variance and Bonferroni test).

SIN-1 caused complete relaxation of all segments, irrespective of the treatment (Figure 3). The EC_{50} was about 3×10^{-7} M and was not altered by severe (aortic arch) to moderate (abdominal aorta) fatty streak formation (Figure 3, Table 6). However, small but highly significant rightward shifts of the dose-response curves were present in all aortic segments taken from molsidomine-fed rabbits (Figure 3, Table 6).

Nitric oxide caused transient, fast relaxations, followed by smaller, more sustained relaxations at higher concentrations. Fatty streaks and the NO donor displaced the fast responses to the right and slightly increased their amplitude (Figure 3, Table 6). The pD_2 (r_s −0.79, $P<0.001$, $n = 60$) and amplitude (E_{max}, r_s 0.63, $P<0.001$ $n = 60$) were correlated to the lesion size when all segments of cholesterol-fed rabbits were combined. The ANCOVA indicated that cholesterol was without effect when lesion size was taken into account, whereas the NO donor caused highly significant reductions of both amplitude and sensitivity in the three aortic regions after this correction (Table 6).

Forskolin produced complete relaxation of the three regions of the aorta. Neither cholesterol feeding nor the chronic treatment with exogenous NO induced changes of pD_2 (Table 6) or E_{max} (results not shown) to forskolin in any segment of the aorta. Finally, none of the pD_2 or E_{max} values showed any correlation to the hydroxyfatty acid content of the thoracic aorta (De Meyer *et al.*, 1991a) of molsidomine-treated rabbits (groups 3 and 4, data not shown).

Release of prostacyclin and nitric oxide

Prostacyclin was measured in the effluent of the perfused aorta, before and 1 min after injection of 0.3, 3 or 30 nmol ACh. The three base-line measurements yielded very similar concentrations (0.75 ± 0.03, 0.74 ± 0.04 and 0.84 ± 0.08 nM, mean ± s.e.mean, $n = 38$), which were not affected by any diet. The logarithms of the concentrations in the samples taken after ACh injection were analysed in a factorial analysis of variance. ACh caused a dose-dependent ($F_{2,62} = 206$, $P<<0.001$), 30 fold stimulation of the biosynthesis of prostacyclin (Figure 4), which was 50 to 60% depressed by the presence of fatty streaks ($F_{1,31} = 8.65$, $P = 0.006$), but not influenced at all by the NO donor ($F_{1,31} = 0.02$).

The initial tension (7.6 ± 0.3 g) and the noradrenaline-induced contraction (6.2 ± 0.4 and 6.4 ± 0.3 g, respectively before the first series of GTN and ACh injections) of the bioassay detector tissue showed no differences among the four groups. The tension was not changed when ACh (30 nmol) was injected directly over the tissues. However, transient, dose-dependent relaxations occurred after injection of 0.3 to 30 nmol ACh into the donor aortae of control rabbits (Figure 5). The aortae of all other groups required ten times more ACh to initiate relaxation of the recipient tissue and the amplitude was about 50% (group 2) to 70% (groups 3 and 4) reduced at the top dose (Figure 5). ANOVA of the raw data (minimum tension of recipient, g) showed that relaxations of the detector tissue were dose dependent ($F_{2,62} = 62.5$, $P<<0.001$), attenuated by the cholesterol diet ($F_{1,31} = 5.29$, $P<0.05$), and profoundly reduced by the NO donor ($F_{1,31} = 10.10$, $P<0.01$). The effects of the NO donor and cholesterol (interaction $F_{1,31} = 10.87$, $P<0.01$) were not additive, since the NO donor alone abolished the overflow of NO completely in several donors, making it impossible to detect further suppression with the combination.

Analysis of the dose-dependent relaxations to GTN

Table 6 Influence of fatty streaks and treatment with exogenous NO on the pD_2 values of 3-morpholino-sydnonimine (SIN-1), NO and forskolin in the different segments of the rabbit aorta

	Aortic arch	*Thoracic aorta*	*Abdominal aorta*
NO, pD_2			
Control (−logM)	6.46 ± 0.08	6.82 ± 0.10	7.05 ± 0.08
Cholesterol (−logM)	6.21 ± 0.12	6.83 ± 0.13	6.88 ± 0.13
NO donor (−logM)	6.27 ± 0.08	6.64 ± 0.06	6.77 ± 0.16
Cholesterol + NO donor (−logM)	5.95 ± 0.05	6.14 ± 0.12	6.69 ± 0.13
ANCOVA: area Fat Red $F_{1,35}$	15.7***	35.0***	5.44*
cholesterol $F_{1,35}$	1.79	0.66	0.05
NO donor $F_{1,35}$	19.8***	13.7***	7.05**
NO by cholesterol $F_{1,35}$	0.10	2.36	0.71
NO, E_{max}			
Control (−logM)	79 ± 2	85 ± 2	89 ± 2
Cholesterol (−logM)	86 ± 3	83 ± 3	95 ± 4
NO donor (−logM)	63 ± 3	68 ± 4	79 ± 5
Cholesterol + NO donor (−logM)	74 ± 4	66 ± 2	83 ± 4
ANCOVA: area Fat Red $F_{1,35}$	9.82**	25.3***	9.52**
cholesterol $F_{1,35}$	1.67	0.28	0.35
NO donor $F_{1,35}$	26.5***	30.0***	12.9***
NO by cholesterol $F_{1,35}$	0.01	0.0	0.41
SIN-1, pD_2			
Control (−logM)	6.36 ± 0.05	6.82 ± 0.10	7.05 ± 0.08
Cholesterol (−logM)	6.32 ± 0.09	6.83 ± 0.13	6.88 ± 0.13
NO donor (−logM)	5.99 ± 0.05	6.64 ± 0.06	6.77 ± 0.16
Cholesterol + NO donor (−logM)	6.08 ± 0.04	6.14 ± 0.12	6.69 ± 0.13
ANOVA: cholesterol $F_{1,36}$	0.11	1.97	0.47
NO donor $F_{1,36}$	26.11***	42.2***	22.8***
NO by cholesterol $F_{1,36}$	1.23	0.14	0.97
Forskolin, pD_2			
Control (−logM)	6.52 ± 0.09	6.58 ± 0.10	6.72 ± 0.12
Cholesterol (−logM)	6.40 ± 0.08	6.42 ± 0.08	6.58 ± 0.11
NO donor (−logM)	6.33 ± 0.08	6.25 ± 0.12	6.42 ± 0.11
Cholesterol + NO donor (−logM)	6.40 ± 0.08	6.36 ± 0.10	6.63 ± 0.12
ANOVA: cholesterol $F_{1,36}$	0.09	0.08	1.28
NO donor $F_{1,36}$	1.11	3.60	1.89
NO by cholesterol $F_{1,36}$	1.28	1.14	2.53

Values given as mean ± s.e.mean; factorial analysis of variance with cholesterol and NO donor as between rabbit factors and area Fat Red as covariate. *$P<0.05$, **$P<0.01$, ***$P<0.001$, significance of F ratio.

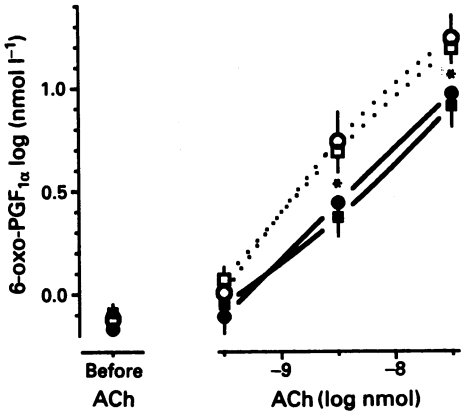

Figure 4 In contrast to fatty streaks, the chronic NO treatment did not reduce the acetylcholine (ACh) induced prostacyclin biosynthesis. Prostacyclin measured by radioimmunoassay of 6-oxo-$PGF_{1\alpha}$ (nM, logarithmic scale) in effluent of the perfused thoracic aorta before and after injection of 0.3, 3 and 30 nmol ACh. The rabbits had been exposed to a control diet (○) or diets with cholesterol (●), NO donor (□), and NO donor plus cholesterol (■). *Concentration significantly ($P<0.01$) reduced in comparison to the corresponding diets without cholesterol.

confirmed the absence of differences in sensitivity among the detector tissues used for the four groups (Figure 5). Hence, normalisation of the ACh-induced relaxations for initial contraction of the detector tissue (as shown in Figure 5) or the sensitivity of the detector tissue to GTN yielded identical conclusions (results not shown).

Subsequently L-arginine (1 mM) was added to the perfusate, and after 30 min the donor tissue was exposed to the same sequence of ACh and GTN doses. The initial tension of the detector tissue decreased slowly (change −0.8 ± 0.2 g, after 2 h) without differences among the four diets. The ACh-induced overflow of NO was not influenced by L-arginine, either when raw data (minimum contraction or change from base line, Figure 6), or normalised data (relaxation expressed as percent of the initial tension or of the response to the GTN standards) were analysed. After the L-arginine treatment the aortae of cholesterol- or molsidomine-fed rabbits still failed to liberate vasodilator activity at the threshold bolus (data not shown), and relaxations to the medium (3 nmol, Figure 6) and top (30 nmol, results not shown) bolus administrations of ACh were not improved.

Discussion

Molsidomine and fatty streak formation

The absence of aortic lesions in cholesterol-fed rabbits which maintained a fairly normal cholesterol level (less than 1 mM), confirmed that development of hypercholesterolaemia is crucial for the development of fatty streaks in this model (Clarkson *et al.*, 1974; Daugherty *et al.*, 1986). The lack of associations between plasma cholesterol and the dimensions of the vascular lesions in hypercholesterolaemic rabbits (plasma cholesterol > 1 mM) may seem to be paradoxical. However, in addition to the plasma cholesterol level, the cholesterol deposition in arteries also depends on vessel wall injury (Moore, 1989), on the rate of very low density lipo-

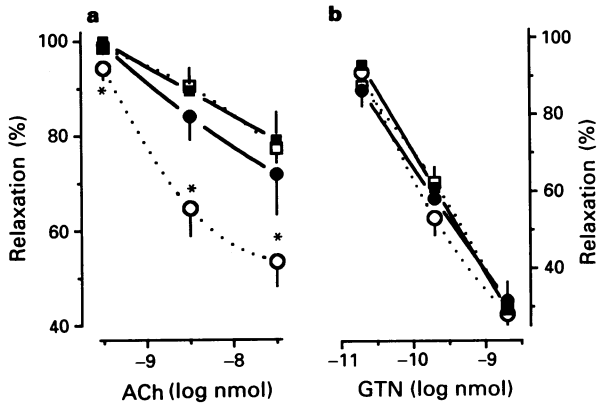

Figure 5 Fatty streaks and the chronic NO treatment reduce the overflow of endothelium-derived NO released in response to 0.3, 3 and 30 nmol acetylcholine (ACh, a) from perfused thoracic aortae of rabbits exposed to a control diet (○), or diets containing cholesterol (●), NO donor (□) or cholesterol plus NO donor (■). The bioassay results give the minimum contraction of the recipient tissue as percentage of the tension immediately prior to the ACh injection. Panel (b) shows the minimum contractions of the recipient tissues in response to glyceryltrinitrate (GTN) (0.02, 0.2 and 2 nmol) injections into the perfusate. *Control group different from all other groups ($P<0.05$, analysis of variance and Bonferroni test).

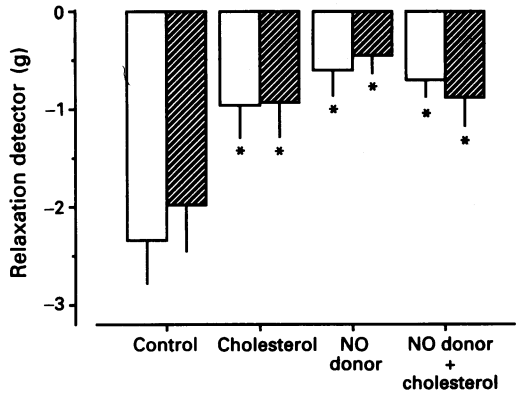

Figure 6 L-Arginine failed to restore the acetylcholine (ACh)-induced overflow of endothelium-derived NO released by perfused thoracic aortae of rabbits exposed to a control diet or diets with cholesterol, the NO donor or cholesterol plus NO donor. Relaxation of the recipient tissue (g) after injection of 3 nmol ACh into the donor, before (open columns) and after (hatched columns) infusion of 1 mM L-arginine. *Different from control group ($P<0.05$, analysis of variance and Bonferroni test).

protein synthesis (Daugherty *et al.*, 1986), on biliary secretion of cholesterol and bile acids by the liver (Havel, 1985) and on cholesterol transport to other tissues, in which cholesterol accumulates earlier, and to a greater extent than in arteries (Clarkson *et al.*, 1974). A single plasma cholesterol determination can thus not be used to predict the severity of fatty streaks in hypercholesterolaemic rabbits.

The present experiments did not support the hypothesis that supplementation with exogenous NO might retard atherosclerosis. In fact, the NO donor promoted fatty streak formation, as it increased wet weight and cholesteryl-ester content of the thoracic aorta. Furthermore, less sensitive indices (scores of area and thickness of fatty streaks) were doubled in the thoracic aorta, if the analysis was restricted to rabbits with hypercholesterolaemia. It is important to note that molsidomine elevated neither extent, nor incidence of the hypercholesterolaemia, unlike NO synthase inhibitors (Cayatte *et al.*, 1994; Naruse *et al.*, 1994). Hence, augmented plasma cholesterol levels can be ruled out as an explanation for the accelerated atherosclerosis with the NO donor.

Although the dose of molsidomine (10 mg kg^{-1} day^{-1}) was 70 to 100 times as high, it was pharmacologically equivalent to the dose used in the clinic and we noted that the rabbits maintained a normal arterial pressure. However, the increased body weight pointed to fluid retention as an adaptation to the prolonged exposure to a vasodilator via activation of compensatory neurohumoral systems (Dupuis *et al.*, 1990; Packer, 1990). Yet, it is unlikely that these adaptations could have accelerated the fatty streak formation, as it is not observed with other vasodilator drugs (Habib *et al.*, 1986; Verbeuren *et al.*, 1986a; Sugano *et al.*, 1986; Chobanian *et al.*, 1990).

Previously we found increased contents of 13-L-hydroxy-octadecadienoic acid and 15-L-hydroxyeicosatetraenoic acid, metabolites of the peroxides of linoleic acid and arachidonic acid, in the lower thoracic aorta of both molsidomine-treated goups (De Meyer *et al.*, 1991a). This indicates that the NO donor favoured lipid peroxidation in the vessel wall and it is conceivable that this accelerated atherosclerosis. NO release from SIN-1 is accompanied by the production of superoxide anion, reacting with NO to yield peroxynitrite, which decomposes to form the highly reactive hydroxyl radical (Feelisch, 1991; Lipton *et al.*, 1993). SIN-1 can initiate peroxidation of lipids and proteins in low density lipoproteins (Darley-Usmar *et al.*, 1992), which are important inducers of foam cell formation (Ylä-Herttuala *et al.*, 1989; Sparrow *et al.*, 1992). The combined data thus strongly suggest that peroxynitrite-induced lipid peroxidation took place in the rabbit aorta during *in vivo* syndnonimine treatment. This was not sufficient to induce fatty streaks as such, but it appeared to accelerate cholesterol-induced atherosclerosis.

Fatty streaks and vascular reactivity

In spite of the deliberate inclusion of rabbits without hypercholesterolaemia, the modest desensitization to the α_1-adrenoceptor agonist, phenylephrine (Habib *et al.*, 1986; Mügge *et al.*, 1991), the increased sensitivity and contractility to 5-HT (Henry & Yokoyama, 1980; Verbeuren *et al.*, 1986b; Wines *et al.*, 1989) and the diminished ACh-induced relaxations (Verbeuren *et al.*, 1986b; Freiman *et al.*, 1986; Habib *et al.*, 1986; Jayakody *et al.*, 1987; Bossaller *et al.*, 1987) confirmed the altered reactivity of arteries with fatty streaks. Since indomethacin was present throughout the organ chamber studies, and since PGI_2 fails to cause relaxation of the rabbit aorta (Moncada *et al.*, 1976), these alterations are not explained by the reduced biosynthesis of PGI_2 (see below). The lesions and all changes in vascular reactivity became less pronounced towards the abdominal aorta, indicating that the presence of fatty streaks rather than the hypercholesterolaemia was responsible for the alterations. With the exception of 5-HT, all reactivity changes were indeed more clearly correlated to the lesion size than to the plasma cholesterol level. It thus appears to be essential to document lesion size and some of the controversies in the literature may be due to the lack of this crucial information. Only the augmented responses to 5-HT seem to precede fatty streak development (Wines *et al.*, 1989; Galle *et al.*, 1991), but this could also be due to failure of the staining techniques to detect the very first lesions, i.e. subendothelial accumulation of unesterified cholesterol (Kruth, 1985). The early hyperreactivity to 5-HT is seen in other models as well and has been attributed to increased expression of contractile 5-HT receptors (Heistad *et al.*, 1984; Wines *et al.*, 1989) or decreased uptake or metabolism by the endothelium (Verbeuren *et al.*, 1988). It is not due to depressed endothelial NO release (Shimokawa & Vanhoutte, 1989; Manderson *et al.*, 1989), since NO synthase inhibitors do not

influence responses to 5-HT in the normal and atherosclerotic rabbit aorta (Galle *et al.*, 1991).

An important conclusion from the unaltered E_{max} to phenylephrine was that the fatty streaks did not interfere with the contractile capacity of the segments in any aortic region. For this reason phenylephrine had been selected as the constrictor agonist in the relaxation studies. The unaltered relaxations to SIN-1 and forskolin also excluded the possibility that the fatty streaks interfered mechanically with the dilator capacity in any region. As these agonists stimulate cyclic GMP and cyclic AMP formation directly, either through spontaneous release of NO (SIN-1, Feelisch, 1991) or by adenylate cyclase activation (forskolin, Laurenza *et al.*, 1989), the results further exclude that biosynthesis, efficacy and/or degradation of both cyclic nucleotides in the vascular smooth muscle cells were modified by the lesions. This confirms that exogenous NO, delivered from nitrates (Harrison *et al.*, 1987; Bossaller *et al.*, 1987) or SIN-1 (this study), still produces normal relaxation even when atherosclerosis interferes significantly with the endogenous, receptor-mediated NO pathway. Only advanced fatty streaks appear to interfere with the dilator activity of organic nitrates and other dilators (Verbeuren *et al.*, 1986b; 1990).

In contrast to SIN-1, relaxations to threshold concentrations of authentic NO became depressed in segments with pronounced lipid infiltration, although the maximum relaxation was not reduced or even enhanced. This apparent discrepancy between NO and SIN-1 is presumably explained by kinetic differences. NO is quickly flushed away and/or converted to NO_2^- after injection into the organ bath. The fast, but brief, relaxations to authentic NO presumably reflect this transient concentration peak. The same inactivation pathways are operating for NO released from SIN-1, but the sustained release from this molecule will establish a less temporary steady state concentration in bath and tissue. Only the transient exposure to authentic NO revealed that fatty streaks limited the access of NO to the smooth muscles to some extent. As the shift decreased in segments with smaller lesions, the alteration again appeared to be unrelated to the hypercholesterolaemia as such. Conversely, accumulation of the lipophilic NO in fatty streaks, serving as a depot for sustained release, could explain the unaltered or even increased E_{max} when higher concentrations of the gas were injected into the bath. Taken together, the shift of the responses to authentic NO, but not to SIN-1, confirms suggestions that fatty streaks (Jayakody *et al.*, 1987; Verbeuren *et al.*, 1990), oxidized lipoproteins (Jacobs *et al.*, 1990; Galle *et al.*, 1991; Chin *et al.*, 1992) in those lesions (Ylä-Herttuala *et al.*, 1989), or excess generation of oxygen-derived free radicals (Mügge *et al*, 1991) trap and/or inactivate NO on passage from the endothelium to the smooth muscle cells.

In addition to this barrier, the reduced overflow of endothelium-derived NO confirmed that diminished biosynthesis (Guerra *et al.*, 1989; Verbeuren *et al.*, 1990) or enhanced metabolism of NO or a nitroso compound (Minor *et al.*, 1990; Mügge *et al.*, 1991) is a principal cause for the decreased endothelium-dependent responses to ACh. Endothelial cells were present in all sections examined, confirming that this is not due to their loss (Kruth, 1985; Verbeuren *et al.*, 1986b; Freiman *et al.*, 1986; Jayakody *et al.*, 1987). Indeed, focal endothelial denudation could explain the decreased amplitude, but would be difficult to reconcile with the shift of the ACh curves in organ chamber as well as bioassay. Furthermore, the unaltered endothelium-dependent relaxations in the segments without fatty streaks confirms other reports (Verbeuren *et al.*, 1986b; Galle *et al.*, 1991; Senaratne *et al.*, 1991) and argues against the proposal that hypercholesterolaemia as such alters the release of nitrogen oxides (Minor *et al.*, 1990). It implies that the reduced endothelium-dependent relaxation does not precede lipid infiltration and that it is a consequence rather than a cause of fatty streaks.

The decreased availability of NO was apparently not due to a shortage of L-arginine, since this precursor failed to restore NO release in the bioassay. This is in accordance with the failure of L-arginine to correct the endothelial dysfunction of the aorta of cholesterol-fed (Bult *et al.*, 1990) and Watanabe heritable hyperlipidaemic rabbits (Caparotta *et al.*, 1993) *in vitro*, and in conduit arteries of hypercholesterolaemic patients *in vivo* (Drexler *et al.*, 1991). These findings are at variance with a report that high doses of L-arginine given immediately prior to killing partly normalize endothelium-dependent responses of the isolated aorta (Cooke *et al.*, 1991). Possible explanations for this discrepancy are (1) differences in lesion severity, which is rather variable (this study and Clarkson *et al.*, 1974) and was not reported for placebo or arginine-treated rabbits (Cooke *et al.*, 1991); (2) induction of non-endothelial NO synthase (Moncada *et al.*, 1991) as lesions progress further (Verbeuren *et al.*, 1993); or (3) the potent hormonal secretagogue activity of arginine (Barbul, 1990), which could have influenced vascular reactivity in general, as suggested by the similar shift of endothelium-independent relaxations after *in vivo* L-arginine treatment (Cooke *et al.*, 1991).

Moreover, the diminished biosynthesis of PGI_2 (Beetens *et al.*, 1986) proves that the endothelial alteration is not restricted to the arginine-NO pathway. Indeed, calcium ionophore-induced NO release may be preserved after induction of fatty streaks (Bossaller *et al.*, 1987) or a neo-intima (De Meyer *et al.*, 1991b), when muscarinic responses have become depressed. The shift of the ACh-induced relaxation curves in the organ chamber further points to a defect at the level of the muscarinic receptor, as it is difficult to reconcile with a shortage of L-arginine or co-factors of NO synthase. These three lines of evidence indicate that the function of endothelial muscarinic receptors becomes depressed as fatty streaks develop (Bossaller *et al.*, 1987; Caparotta *et al.*, 1993), a finding which is consistent with another model of intimal thickening (De Meyer *et al.*, 1991b).

Prolonged NO exposure and vascular reactivity

It was often essential to add lesion size as covariate to the ANOVA in order to discriminate the direct effects of the NO donor on vascular reactivity, because (1) fatty streaks display heterogeneity among and within rabbits, (2) the NO donor increased fatty streaks and (3) the lesion size was directly related to most reactivity changes. Relaxations induced by NO, GTN or SIN-1 are not influenced by endothelial cells in the rabbit aorta (Bult *et al.*, 1991). The shifts of their dose-response curves therefore indicate that the NO donor desensitized the smooth muscle cells to agonists operating via cyclic GMP (ACh, SIN-1 and NO), without affecting the cyclic AMP-mediated (Laurenza *et al.*, 1989) relaxation to forskolin. The latter finding, the unaltered endothelial PGI_2 release and the unchanged constrictor responses argue against a non-specific drug toxicity.

Complete relaxation to SIN-1 was always achieved and even after 16 weeks exposure to exogenous NO the shift was modest and not comparable to the tolerance following a brief exposure to organic nitrates (Kukovetz & Holzmann, 1985; Mülsch *et al.*, 1989). Since SIN-1 does not require biotransformation to release NO, and since a similar shift was observed for authentic NO, the desensitization must be due to another mechanism. Indeed, *in vitro* data suggest that the cyclic GMP system may become depressed upon exposure to NO, either due to diminished guanylate cyclase activity, and/or increased cyclic GMP specific phosphodiesterase activity (Schröder *et al.*, 1988; Mülsch *et al.*, 1989; Ahlner *et al.*, 1991). ACh-induced relaxations are mediated by NO and it is thus not surprising that a similar rightward shift of its dose-response curve was seen. Tolerance to GTN is not accompanied by this cross desensitization to SIN-1 or endothelium-derived NO (Stewart *et al.*, 1987; Mülsch *et al.*, 1989), partly because the blunted biotransformation (Ahlner *et al.*, 1991;

Feelisch, 1991) quickly curtails the exposure to exogenous NO.

Furthermore, the chronic NO treatment decreased the amplitude of the endothelium-dependent relaxations. The suppression was inversely related to the E_{max} in control segments, i.e. most significant in aortic arch, intermediate in thoracic aorta and not seen in abdominal aorta. The results point to reduced NO release, which becomes apparent when the contribution of NO to endothelium-dependent relaxation increases, whereas K^+ channels, in addition to NO, contribute considerably to ACh-induced relaxations in the abdominal aorta (Cowan *et al.*, 1993). The reduced release of NO was clearly confirmed by the bioassay. Inactivation of NO by superoxide anion released from SIN-1 present in the tissue is unlikely to explain this reduction. The rabbits were examined 24 h after consuming the last dose of molsidomine, at a time when SIN-1 was absent (less than 2 ng ml^{-1}, results not shown) from the circulation of most (18 out of 20) molsidomine-treated rabbits due to its short biological half-life. Moreover, the reduction persisted after a washout period of several hours (cf, Figure 6), during which the aorta was continuously perfused. Again, the decline was not due to a shortage of substrate, as it could not be restored by the high concentration of L-arginine, which was consistent with the failure of L-arginine to restore responsiveness of isolated vascular rings (Bult *et al.*, 1990). Furthermore, the effect was specific, since the biosynthesis of PGI_2 was not affected. Hence, density, characteristics or coupling of the muscarinic receptors were not altered by the NO donor. The data thus provide evidence for the negative feedback regulation of the L-arginine-NO pathway (Assreuy *et al.*, 1993; Buga *et al.*, 1993) under *in vivo* conditions.

The effects of fatty streaks (desensitization of muscarinic receptors and trapping of NO) and treatment with exogenous NO (desensitization of smooth muscle cells and reduced endothelial NO release) were additive in the three regions of the aorta studied. Hence, the strongest inhibition of the endothelium-dependent relaxations was observed in the arch and thoracic aorta of rabbits treated with both cholesterol and the NO donor. The two effects of the sydnonimine are presumably related to release of NO rather than superoxide anion, since they occurred to the same extent in normal and atherosclerotic vessels and were not correlated to the hydroxy fatty acid content of the aorta.

In conclusion, the prolonged molsidomine treatment enhanced fatty streak development in hypercholesterolaemic rabbits. In addition, the NO donor desensitized aortic smooth muscle cells to vasodilators acting via the cytoplasmic guanylate cyclase. Furthermore, it selectively suppressed the capacity of aortic endothelial cells to release NO in response to muscarinic receptor stimulation. This shows for the first time that chronic exposure to exogenous NO down-regulates the endothelial NO release, thereby providing evidence for the *in vivo* existence of a negative feedback control of the L-arginine – NO pathway (Assreuy *et al.*, 1993; Buga *et al.*, 1993).

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Contractile activity of endothelin precursors in the isolated gallbladder of the guinea-pig: presence of an endothelin-converting enzyme

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1 We have compared the activities of the endothelin precursors (human big ET-1_{1-38}, porcine big ET-1_{1-39}, big ET-2_{1-38} and big ET-$3_{1-41\ amide}$) and their respective mature 21 amino acid peptides as contractors of isolated gallbladder strips of the guinea-pig. We have also used different protease inhibitors and/or epithelial cell removal to investigate the nature and the location of the endothelin-converting enzyme (ECE) activity responsible for the conversion of porcine big ET-1_{1-39} in this isolated preparation. In addition, we have conducted binding studies to investigate whether porcine big ET-1_{1-39} interacts directly with ET receptors.

2 ET-1, ET-2 and ET-3 were equipotent at causing 50% of the contractions of the isolated strips (22.9 ± 6.8, 39.5 ± 9.9 and 35.9 ± 3.7 ($\times 10^{-9}$ M), respectively), as estimated with contractions to 3×10^{-7} M taken as 100%. Big ET-1_{1-38} was equipotent to ET-1 while big ET-1_{1-39} was one fifth as potent as ET-1, and big ET-2_{1-38} was one fifth as potent as ET-2. Precursors of ET-1 and ET-2 induced similar contractions at 3×10^{-7} M. Conversely, the contraction induced by big ET-$3_{1-41\ amide}$ (3×10^{-7} M) was only 14% of that induced by the same concentration of ET-3 (287 ± 37% of 5 μM histamine).

3 The kinetics of the responses induced by single additions of 3×10^{-7} M of the endothelin isopeptides were compared. A single addition of 3×10^{-7} M ET-1, ET-2 or ET-3 produced contractions that reached a steady state in 28.2 ± 4.2, 21.1 ± 1.3 and 24.0 ± 3.8 min, respectively and took 2.7 ± 0.4, 2.1 ± 0.1 and 1.6 ± 0.1 min to reach half of this steady-state response.

4 Contractions induced by 3×10^{-7} M big ET-1_{1-38} or big ET-1_{1-39} reached a plateau in 38.5 ± 3.6 and 35.6 ± 3.3 min, respectively, and half of these responses were attained in 12.0 ± 2.5 and 7.1 ± 1.1 min. Thus, these contractions developed more slowly than those induced by ET-1. Contractions induced by 3×10^{-7} M big ET-2_{1-38} were also much slower to develop than those to ET-2, for these took 49 ± 2 min to reach plateau and 19.4 ± 2.1 min to attain half that response. Contractions induced by 3×10^{-7} M big ET-$3_{1-41\ amide}$ took 50.2 ± 3.7 min to reach a plateau and 27.3 ± 3.0 min to reach half of this response.

5 Phosphoramidon (0.1, 1 and 3×10^{-4} M) inhibited contractions induced by big ET-1_{1-39}. For instance, the contractions induced by 3×10^{-7} M big ET-1_{1-39} were inhibited by 10^{-4} M or 3×10^{-4} M of phosphoramidon by 62.8 ± 6.7% or 74.5 ± 4.6%, respectively. Similarly, contractions induced by ET-2_{1-38} were inhibited by 91.3 ± 5.4% and the small response induced by big ET-$3_{1-41\ amide}$ was abolished by 3×10^{-4} M phosphoramidon. Conversely, the neutral endopeptidase (EC 24.11) inhibitor DL-thiorphan (3×10^{-4} M) had no effect. Captopril (10^{-5} M), pepstatin A (10^{-5} M), phenylmethylsulphonylfluoride (PMSF, 10^{-3} M), aprotinin (10^{-5} M), E-64 (10^{-5} M), cystatin (10^{-6} M), leupeptin (10^{-4} M), chymostatin (10^{-4} M), or bestatin (10^{-5} M) did not inhibit but rather increased to a similar, but small degree the contractions induced by 3 to 30×10^{-9} M big ET-1_{1-39}. Only captopril (10^{-5} M) or leupeptin (10^{-4} M) increased the contraction induced by 3×10^{-7} M big ET-1_{1-39}. Phosphoramidon (10^{-4} M), pepstatin (10^{-5} M) or PMSF (10^{-3} M) did not affect contractions induced by ET-1.

6 Removal of the epithelium increased by 70% the size of the contraction induced by 5 μM histamine (1.08 ± 0.05 g; $n = 160$ to 1.84 ± 0.14 g; $n = 12$) but did not affect, in absolute terms, the contraction induced by ET-1 (as a % of the response to histamine, these responses were, of course, apparently depressed). Epithelium removal did, however, increase the size of the contractions induced by 3 to 30×10^{-9} M big ET-1_{1-39} which was very similar to the effect of the protease inhibitors.

7 In competition binding studies on membranes prepared from the guinea-pig gallbladder, 10^{-11} M ET-1 inhibited by 76.9 ± 3.1% the binding of [^{125}I]-ET-1 while porcine big ET-1_{1-39} caused no inhibition (0.7 ± 3.0; $n = 3$). ET-1 (10^{-6} M) inhibited binding by 95.7 ± 1.1% ($n = 3$) while at this much higher concentration, big ET-1_{1-39} inhibited binding by only 16.8 ± 4.2% ($n = 3$). This clearly suggests that big ET-1_{1-39} does not bind directly to ET receptors.

8 Thus, a phosphoramidon-sensitive endothelin-converting enzyme (ECE), different from neutral endopeptidase (NEP; EC 24.11) and not located on the epithelium, converts big ET-1 into ET-1 in the gallbladder of the guinea-pig. This ECE appears to act preferentially on big ET-1 or big ET-2 over big ET-3.

Keywords: Endothelins; big-endothelins; endothelin-converting enzyme (ECE); guinea-pig gallbladder; protease inhibitors; phosphoramidon

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Introduction

Endothelin-1 (ET-1) is a 21 amino acid peptide, first isolated from the culture medium of aortic endothelial cells (Yanagisawa *et al.*, 1988). ET-1 and the related peptides ET-2, ET-3 and the sarafotoxins (SX6a, SX6b, SX6c, SX6d) cause vasoconstriction, vasodilatation, mitogenesis and various renal, cardiac and endocrine effects (see Huggins *et al.*, 1993). ET/SX peptides are also potent contractors of non-vascular smooth muscle preparations such as airways (Battistini *et al.*, 1992, 1994c; Hay *et al.*, 1993), the urinary bladder (Maggi *et al.*, 1989b; Saenz de Tejada *et al.*, 1992; Traish *et al.*, 1992), the uterus (Bousso-Mittler *et al.*, 1989; Svane *et al.*, 1993; Rae *et al.*, 1993a,b), the stomach fundus (Wood *et al.*, 1992; Warner *et al.*, 1993a), the ileum (Maggi *et al.*, 1989a; Wollberg *et al.*, 1991; Guimaraes & Rae, 1992; Miasiro *et al.*, 1993; Warner *et al.*, 1993b), the colon (Lin & Lee, 1990; Takahashi *et al.*, 1990) and the gallbladder (Maggi *et al.*, 1990; Moummi *et al.*, 1992; Battistini *et al.*, 1994b). In this last preparation, ET-1 causes a slowly-developing contraction (EC_{50}: 10 nM) and is 20 and 40 times more potent than carbachol and histamine, respectively, although 5 times less potent than cholecystokinin (Moummi *et al.*, 1992). The response to ET-1 is not affected by tetrodotoxin, atropine or ω-conotoxin, but is reduced by nicardipine, diltiazem or removal of calcium (Moummi *et al.*, 1992). We have recently identified two receptors, an ET_B receptor and an additional ET receptor not conforming to the established ET_A/ET_B receptor subtype classification, that mediate contractions to the endothelins in isolated gallbladder strips of the guinea-pig (Battistini *et al.*, 1994b). Interestingly, human gallbladder epithelial cells in primary culture secrete endothelin, both intrahepatic and extrahepatic bile duct epithelial cells appear to contain big ET-1 and the release of ET is increased by physiological concentrations of cholecystokinin (Housset *et al.*, 1993). Thus, ET-1 is produced locally, and by a paracrine route could play a role in choledochal motility and gallbladder contraction. Alteration of this pathway could be associated with the formation of gallstones. It is known that ET-1 is formed by a putative endothelin converting enzyme (ECE) from a 38 (human) or 39 (porcine)-amino acid precursor by cleavage between residues Trp^{21} and Val^{22} (Yanagisawa *et al.*, 1988; Inoue *et al.*, 1989). A similar process may well form the other two endothelins, ET-2 and ET-3, from their precursors big ET-2_{1-37}, big ET-2_{1-38} or big ET-$3_{1-41\ amide}$ (Bloch *et al.*, 1989; 1991; Onda *et al.*, 1990). Numerous candidates have been proposed as the ECE for the conversion of big ET-1, but a membrane-bound phosphoramidon-sensitive neutral metalloprotease is the one most studied (see Opegenorth *et al.*, 1992). Such an enzyme has been purified from the rat lung (Takahashi *et al.*, 1993) and the membrane fraction of porcine aortic endothelium (Ohnaka *et al.*, 1993) and cloned from rat endothelial cells (Shimada *et al.*, 1994).

Here, we have compared mechanical responses to human big ET-1_{1-38}, porcine big ET-1_{1-39}, big ET-2_{1-38}, big ET-$3_{1-41\ amide}$, ET-1, ET-2 and ET-3 to assess the ECE activity in the gallbladder. We have also used several protease inhibitors and removal of the epithelium to characterize the nature and location of the ECE involved in the conversion of porcine big ET-1_{1-39}. In addition, receptor binding studies were employed to demonstrate whether this precursor could bind directly to ET receptors.

A preliminary account of some of these data was presented at the Spring meeting of the British Pharmacological Society (Battistini *et al.*, 1994a).

Methods

Preparations of the tissues for organ bath experiments

Male Hartley guinea-pigs (300–400 g) were killed by cervical dislocation. The gallbladder was rapidly excised, placed in warmed (37°C) and oxygenated (95% O_2: 5% CO_2) Krebs-Henseleit solution (comparison, mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.18, $NaHCO_3$ 25, $MgSO_4.7H_2O$ 1.18 and glucose 5.6, pH 7.4) and trimmed free of connective tissue and fat. Each gallbladder was opened along its longitudinal axis and two muscle strips were prepared by cutting parallel to the long axis. The strips were mounted in 10 ml organ baths under tensions of 1.0 g and connected to Grass FT.03 force-displacement isometric transducers coupled to a Grass 79D pen recorder (Grass Instrument Co., Quincy, MA, U.S.A.). Following an equilibration period (60 min), tissues were contracted 3 times with histamine (5×10^{-6} M) with a 30 min washout period between contractions.

Experimental protocols for organ bath experiments

In the first set of experiments, concentration-response curves (0.001 to 3×10^{-7} M) to ET-1, ET-2, ET-3, human big ET-1_{1-38}, porcine big ET-1_{1-39}, big ET-2_{1-38} and big ET-$3_{1-41\ amide}$ were constructed in the isolated strips. Concentrations of any endothelin peptides higher than 3×10^{-7} M were not tested because of the cost of the peptide. In a second set of experiments, the tissues were incubated with one of several protease inhibitors (phosphoramidon (10^{-5} M, 10^{-4} M, 3×10^{-4} M), an inhibitor of some metalloendopeptidases; 1,10-phenanthroline (10^{-4} M), an inhibitor of metalloproteases (chelator); DL-thiorphan (3×10^{-4} M), an inhibitor of metalloprotease; captopril (10^{-4} M), an angiotensin-converting enzyme inhibitor; pepstatin A (10^{-5} M), an inhibitor of some aspartic proteases (like cathepsin D, pepsin, renin); PMSF (10^{-3} M), an irreversible inhibitor of serine protease; aprotinin (10^{-6} M), a serine protease inhibitor; E-64 (10^{-5} M), a cysteine protease inhibitor; cystatin (10^{-6} M), a cysteine protease inhibitor leupeptin (10^{-4} M), a trypsin-like serine and cysteine protease inhibitor; chymostatin (10^{-4} M), a chymotrypsin-like and cysteine protease inhibitor; or, bestatin (10^{-5} M), an inhibitor of aminopeptidases) for 30 min before addition of cumulative concentrations of either big ET-1_{1-39} or ET-1. The concentrations of inhibitors used in the present set of experiments was based on effective concentrations reported by Beynon & Salvesen (1989). In a third set of experiments, a single addition of either 3×10^{-7} M ET-1, ET-2, ET-3, big ET-1_{1-38}, big ET-1_{1-39}, big ET-2_{1-38} or big ET-$3_{1-41amide}$ was given to equilibrated preparations. In a final set of experiments, the epithelium was gently removed by rubbing with a wet cotton swab and concentration-dependent response curves (0.001 to 3×10^{-7} M) to ET-1 or porcine big ET-1_{1-39} were constructed. Removal of the epithelium was assessed by light microscopical examinations. Responses to agonists, in the presence or absence of inhibitors or the epithelium, were not compared in paired tissues. In all experiments, only one response or response curve to a single agonist was recorded in each strip. The contractile responses to each agonist were standardized by comparison to the contraction induced by 5×10^{-6} M histamine.

Preparation of membranes for competition binding studies

Gallbladders were removed as before, minced to small pieces and homogenized for 10 min (Polytron homogenizer) in 10 volumes of ice-cold assay buffer (Tris-HCl 50 mM, EDTA 1 mM, phenylmethylsulphonylfluoride (PMSF) 100 μg ml^{-1}, captopril 10 μM, pepstatin 1 μM, at pH 7.4). The homogenate was centrifuged (Beckman, model GS-6R) for 15 min at 1,500 *g* (4°C), the supernatant collected and centrifuged (Beckman, model Optima L80) for 60 min at 100,000 *g* (4°C). The pellet formed was resuspended in 3 ml of ice-cold assay buffer (as above) and its protein content determined by the method of Bradford (1976). The membranes were stored at −70°C until used.

Competition binding assays

Membranes (20 μg of protein) were incubated in binding buffer (EDTA 1 μM, PMSF 100 μg ml^{-1}, $MgCl_2$ 1 mM, $CaCl_2$ 100 μM, pepstatin 1 μM, captopril 10 μM, Tris-HCl 50 mM, BSA 0.1%, at pH 7.4) with [^{125}I]-ET-1 (2000 Ci mmol^{-1}) in the presence of 10^{-11} M or 10^{-6} M ET-1 or big ET-1_{1-39}, in the presence or absence of phosphoramidon (100 μM). After 240 min at room temperature, the binding was stopped by filtering the incubate through a Whatman glass filter followed by washing with 3 × 4 ml of ice-cold binding buffer. The amount of radioactivity present on the filter was measured in a gamma counter (Hydrogamma 16, Innotron, U.K.) for 60 s.

Agonists and peptidase inhibitors

ET-1 was synthesized at the INRS-Santé (Montréal, P.Q., Canada). ET-2, ET-3, big ET-1_{1-39}, big ET-1_{1-38}, big ET-2_{1-38} and big ET-$3_{1-41\ amide}$ were obtained from Peptide Institute, Inc. (Scientific Marketing Associates, Barnet, Herts, U.K.). All peptidase inhibitors (see Table 2) and histamine hydrochloride were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). All agonists were dissolved in distilled water and stored at −20°C in aliquots of 5×10^{-5} M. Further dilutions were made in Krebs buffer. Peptidase inhibitors were dissolved in water, methanol or dimethylsulphoxide (DMSO), according to their solubility (Beynon & Salvesen; 1989). The final concentrations of vehicle in the baths were ≤ 0.1%.

Statistical analysis

All values in the text and tables are expressed as mean ± s.e.mean. Statistical analysis of the data was performed with an unpaired, two-tailed, Mann-Whitney U test and $P < 0.01$ to 0.05 were considered significant. Although full concentration-response curves could not be obtained, the relative potencies of the agonists was estimated by taking the response to 3×10^{-7} M to be 100%. Significance between concentration-response curves (10^{-10} to 3×10^{-7} M) induced by big ET-1_{1-39} in the presence or absence of a protease inhibitor was calculated by a two-way, with replication, analysis of variance using the Pharmacological Calculation System (v. 4.2).

Results

Effect of big endothelins

ET-1, ET-2 and ET-3 (10^{-10} to 3×10^{-7} M) were equipotent in causing slowly-developing concentration-dependent contractions of the isolated gallbladder strips and produced similar increases in tension at 3×10^{-7} M (Battistini *et al.*, 1994a,b). Human big ET-1_{1-38} also caused concentration-dependent contractions of the preparations which were similar to those induced by ET-1, while porcine big ET-1_{1-39} was one fifth as potent as ET-1 (Figure 1a). Big ET-2_{1-38} was one fifth as potent as ET-2 (Figure 1b) while big ET-$3_{1-41\ amide}$ (3×10^{-7} M) induced 40 ± 15% of the response induced by 5×10^{-6} M histamine, which was equivalent to only 14% of the concentration induced by ET-3 (3×10^{-7} M) (Figure 1c).

Comparative effects of a single addition of endothelin precursors or corresponding mature peptides

Single addition of ET-1, ET-2 or ET-3 (3×10^{-7} M) caused long-lasting contractions that took 21 to 28 min to reach plateau but less than 3 min to reach half-maximum values (Figure 2a; Table 1). Big ET-1_{1-38} and big ET-1_{1-39} (3×10^{-7} M) induced much more slowly developing contractions that took 36 and 39 min to reach a plateau. Half of the responses were reached within 8 and 12 min (Figure 2a-b; Table 1). Big ET-2_{1-38} (3×10^{-7} M) induced an even slower developing contraction than big ET-1 (Figure 2c; Table 1). The response to big ET-$3_{1-41\ amide}$ was small and took more than 27 min to reach half the level achieved at 50 min (Figure 2d; Table 1).

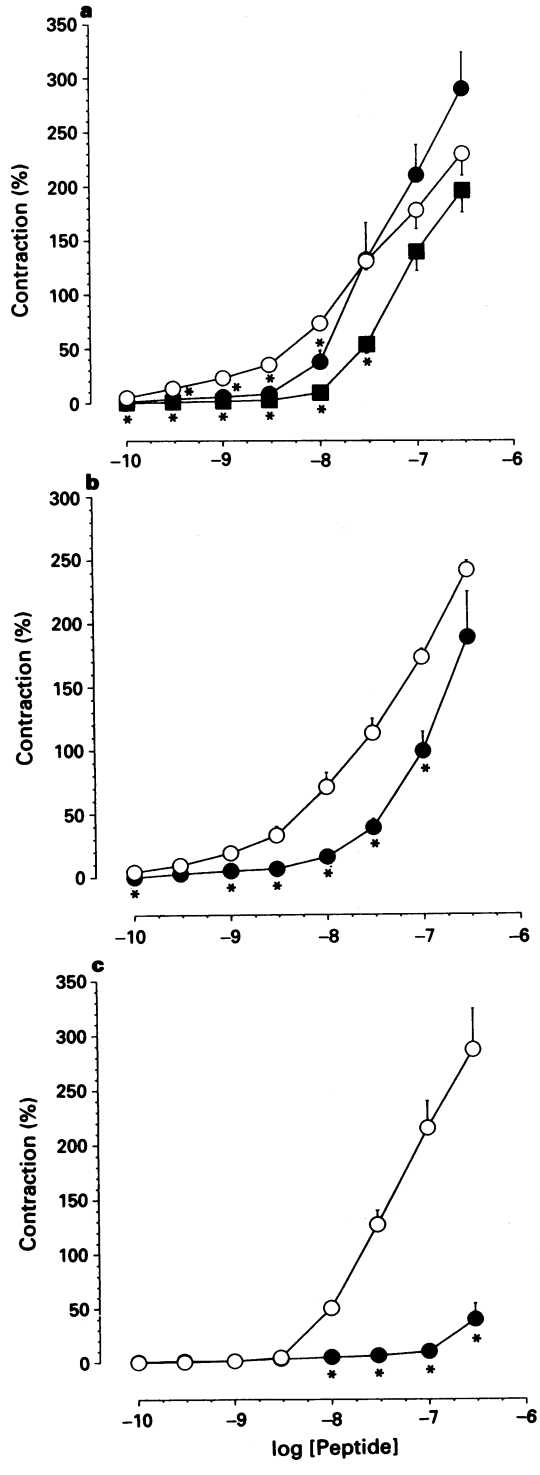

Figure 1 Contractions of strips of the isolated gallbladder of the guinea-pig induced by (a) endothelin-1 (ET-1) (○), big ET-1_{1-38} (●) or big ET-1_{1-39} (■); (b) ET-2 (○) or big ET-2_{1-38} (●); (c) ET-3 (○) or big ET-$3_{1-41\ amide}$ (●); n = 5-12. Results are expressed as the percentage of the response to histamine at 5×10^{-6} M, and are means with s.e.mean. *$P < 0.05$ between agonists (compared by an unpaired, two-tailed, Mann-Whitney U test).

Effects of protease inhibitors on the contractile activity of big ET-1_{1-39}

Contractions induced by big ET-1_{1-39} were inhibited by 1,10-phenanthroline (10^{-4} M) and concentration-dependently by phosphoramidon (10^{-5} to 3×10^{-4} M) (Table 2). They were not affected, however, by DL-thiorphan (3×10^{-4} M) (Table 2). Captopril (10^{-5} M), pepstatin A (10^{-5} M), PMSF (10^{-3} M), aprotinin (10^{-5} M), E-64 (10^{-5} M), cystatin (10^{-6} M), leupeptin (10^{-4} M), chymostatin (10^{-4} M) or bestatin (10^{-5} M) potentiated the contractions, especially those big ET-1_{1-39} at concentrations of 3×10^{-9} to 3×10^{-8} M, such that they were similar to the responses induced by ET-1 (Table 2).

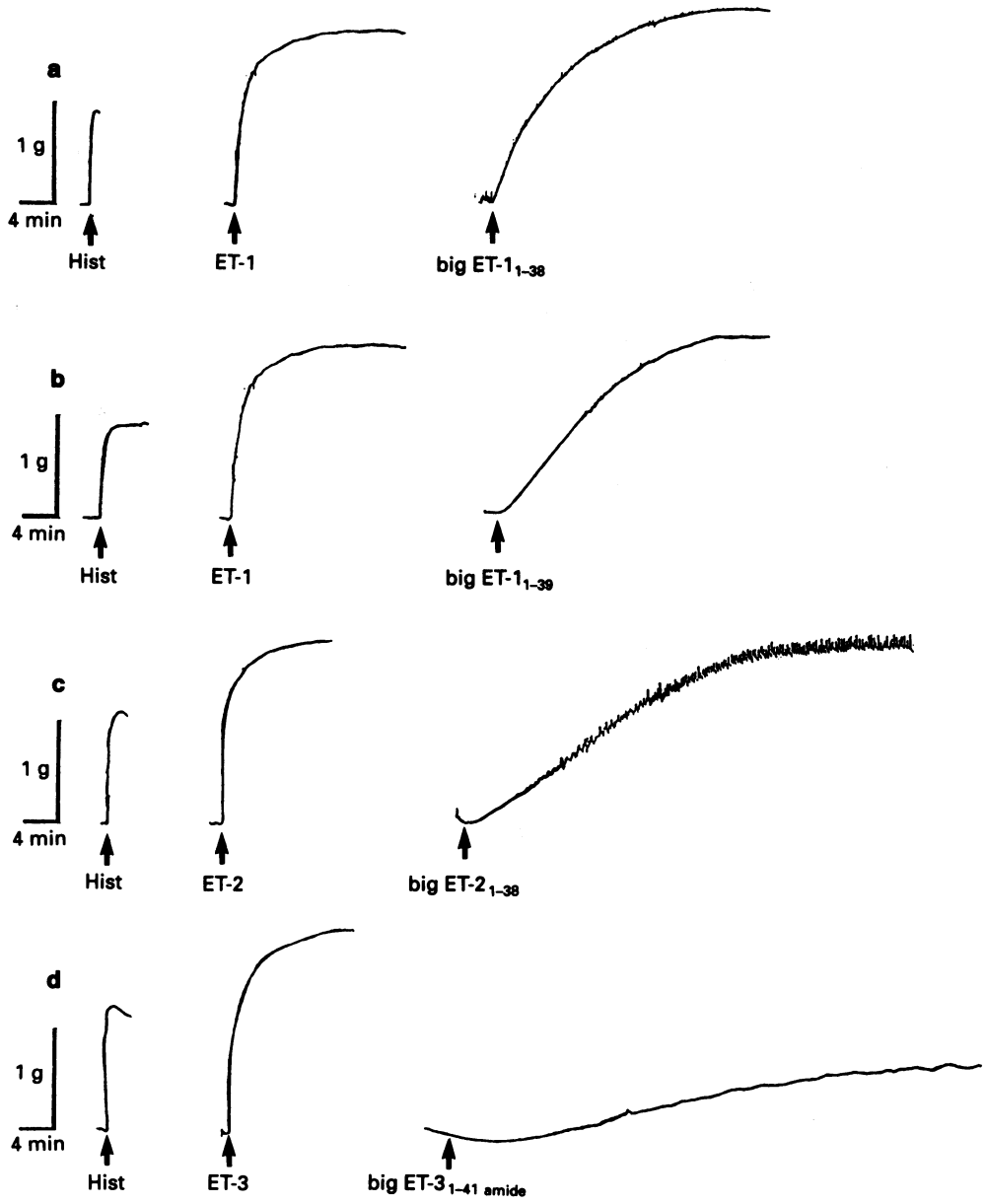

Figure 2 Representative tracings of contractions of strips of the isolated gallbladder of the guinea-pig induced by a single addition of 3×10^{-7} M endothelin-1 (ET-1), ET-2, ET-3, big ET-1_{1-38}, big ET-1_{1-39}, big ET-2_{1-38} or big ET-$3_{1-41\ amide}$. The first arrow is for the contraction induced by addition of histamine (5×10^{-6} M).

Table 1 Effect of a single addition of 3×10^{-7} M endothelin-1 (ET-1), ET-2, ET-3 or their respective precursors in isolated gallbladder strips of the guinea-pig

Agonists	n	*Contraction* (% 5 μM histamine)		t_{max} (min)		$t_{1/2}$ (min)	
ET-1	5	254.7 ± 36.4	Control	28.2 ± 4.2	Control	2.7 ± 0.4	Control
ET-2	3	237.2 ± 14.7	NS	21.1 ± 1.3	NS	2.1 ± 0.1	NS
ET-3	8	151.9 ± 9.6	*	24.0 ± 3.8	NS	1.6 ± 0.1	*
big ET-1_{1-39}	3	285.2 ± 45.2	NS	38.5 ± 3.6	NS	12.0 ± 2.5	*
big ET-1_{1-38}	8	220.5 ± 19.9	NS	35.6 ± 3.3	NS	7.1 ± 1.1	*
big ET-2_{1-38}	8	133.6 ± 10.0	‡	49.0 ± 2.0	*‡	19.4 ± 2.1	*‡
big ET-$3_{1-41amide}$	5	32.4 ± 7.7	‡	50.2 ± 3.7	*‡	27.3 ± 3.0	*‡

*$P<0.05$, compared to ET-1. ‡$P<0.05$, compared to the respective mature peptide. NS, non-significant as compared to ET-1.

Table 2 Effect of various protease inhibitors on contractions of isolated gallbladder strips of the guinea-pig induced by big ET-1_{1-39}

Inhibitors	Concentration (μM)	n	Concentrations at 3×10^{-7} M (% 5 μM histamine)		EC_{50}[1] (nM)	Shift of the C/R curve (x-fold)	
Control	–	11	196 ± 20	–	114.7 ± 31.5	1.0	–
Captopril	1	6	346 ± 45	$P<0.05$	21.6 ± 3.3	5.3	$P<0.01$
Pepstatin A	10	5	266 ± 52	NS	17.4 ± 3.4	6.6	NS
PMSF	1 mM	5	254 ± 30	NS	33.5 ± 7.8	3.4	$P<0.05$
Aprotinin	1	7	240 ± 33	NS	31.5 ± 7.9	3.6	NS
E-64	10	6	222 ± 19	NS	26.0 ± 7.7	4.4	$P<0.01$
Cystatin	1	5	189 ± 22	NS	52.9 ± 34.0	2.2	NS
Leupeptin	100	6	288 ± 19	$P<0.05$	20.3 ± 1.8	5.7	$P<0.01$
Chymostatin	100	4	236 ± 49	NS	48.5 ± 23.4	2.4	NS
Bestatin	10	6	197 ± 29	NS	24.9 ± 5.6	4.6	NS
1,10-Phenantroline	100	4	113 ± 12	$P<0.05$	247.5 ± 120.8	0.5	$P<0.01$
DL-Thiorphan	300	6	193 ± 15	NS	69.4 ± 15.6	1.7	NS
Phosphoramidon	300	3	50 ± 7	$P<0.01$	469.2 ± 80.8	0.2	$P<0.01$
	100	5	73 ± 19	$P<0.01$	241.1 ± 51.2	0.5	$P<0.01$
	10	3	197 ± 25	NS	108.6 ± 18.3	1.1	NS

All values are mean ± s.e.mean.
[1]The apparent EC_{50} is an estimate of the effective concentration causing 50% of the response induced by 3×10^{-7} M of big ET-1_{1-39}. $P<0.01$; $P<0.05$; statistically different. NS, non-significant as compared to control.

Effects of phosphoramidon on the contractile activity of big ET-2_{1-38} or big ET-$3_{1-41\ amide}$

Contractions induced by big ET-2_{1-38} (188.0 ± 35.7% of 5 μM histamine at 3×10^{-7} M; $n = 7$) were inhibited by 3×10^{-4} M phosphoramidon (16.3 ± 10.4% of 5 μM histamine at 3×10^{-7} M; $n = 3$). Similarly, the small response to big ET-$3_{1-41\ amide}$ (39.5 ± 14.6%; $n = 6$) was abolished (3.2 ± 3.2; $n = 3$).

Effects of phosphoramidon, 1, 10-phenanthroline, PMSF or pepstatin on contractions induced by ET-1

Phosphoramidon (10^{-4} M; $n = 3$), pepstatin A (10^{-5} M; $n = 5$) or PMSF (10^{-3} M; $n = 6$) did not affect contractions induced by ET-1 whereas 1, 10-phenanthroline (10^{-4} M) inhibited by 40% the contraction induced by 3×10^{-7} M ET-1 ($n = 6$; $P<0.05$) and shifted the ET-1 concentration-response curve to the right. Protease inhibitors (up to 3×10^{-4} M) or their vehicles did not affect the basal tone of isolated gallbladder strips or contractions induced by 5×10^{-6} M histamine (1.08 ± 0.05 g; $n = 160$).

Effects of epithelium removal on contractions induced by histamine, ET-1 or porcine big ET-1_{1-39}

Removal of the epithelium increased by 70% the contraction induced by 5×10^{-6} M histamine (1.08 ± 0.05 g, $n = 160$ to 1.84 ± 0.14 g, $n = 12$). Consequently, the calculated responses to either ET-1 or porcine big ET-1_{1-39} were attenuated. However, expressed as the direct response (g), removal of the epithelium did not affect the contractions induced by ET-1 (Figure 3a). Conversely, it increased the contractions induced by 3 to 30×10^{-9} M big ET-1_{1-39} (Figure 3b).

Competition binding studies

Total binding of [^{125}I]-ET-1 was 39.8 ± 2.5% of total counts ($n = 16$). ET-1 (10^{-11} M) caused a 76.9 ± 3.1% inhibition of total binding while porcine big ET-1_{1-39} (10^{-11} M) caused no inhibition (0.7 ± 3.0; $n = 3$). A concentration of 10^{-6} M ET-1 inhibited [^{125}I]-ET-1 binding further (95.7 ± 1.1%, $n = 3$), while at the same concentrations, big ET-1_{1-39} caused only a 16.8 ± 4.2% inhibition ($n = 3$). The presence of phosphoramidon (100 μM) did not affect the binding of either peptide.

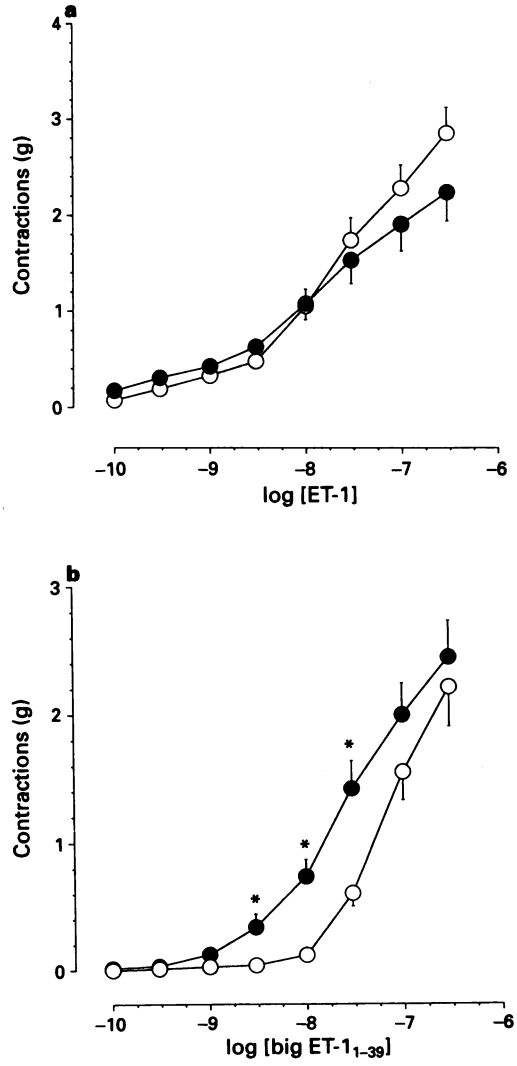

Figure 3 Contractions of strips of the isolated gallbladder of the guinea-pig induced by (a) endothelin-1 (ET-1) ($n = 6$-7) or (b) big ET-1_{1-39} ($n = 6$-11), in the presence (○) or absence (●) of the epithelium. Results are expressed in grams and are means with s.e.mean *$P<0.05$ (compared by an unpaired, two-tailed, Mann-Whitney U test).

Discussion

Here we demonstrate that the gallbladder of the guinea-pig contains ECE activity. Human and porcine big ET-1 caused similar concentration-dependent contractions to those produced by ET-1, although these responses developed more slowly. In addition, the response to porcine big ET-1_{1-39} was inhibited by the endopeptidase inhibitor, phosphoramidon, which is known to inhibit ECE and NEP, but not by thiorphan which inhibits NEP but not ECE. Contractions induced by big ET-2_{1-38} or big ET-$3_{1-41\ amide}$ were also sensitive to phosphoramidon. The ECE activity in this tissue resembles, therefore, the phosphoramidon-sensitive ECE described in endothelial cells, vascular smooth muscle cells and within the circulation (see Opgenorth *et al.*, 1992).

Human big ET-1_{1-38} was equipotent with ET-1 while porcine big ET-1_{1-39} was one fifth as potent as ET-1 in causing concentration-dependent contractions. Comparable observations have been made *in vivo* where these ET-1 precursors have similar potencies to ET-1 as pressor agents (Kashiwabara *et al.*, 1989; Fukuroda *et al.*, 1990; Matsumura *et al.*, 1990; D'Orleans-Juste *et al.*, 1990; 1991). Conversely, ET-1 is much more potent than big ET-1 at contracting isolated vascular preparations such as the rat pulmonary artery (Hirata *et al.*, 1990), the rat thoracic aorta (Kashiwabara *et al.*, 1989; Nishikori *et al.*, 1991), or the porcine coronary artery (Kimura *et al.*, 1989). Despite their equal potency in our experiments, both ET-1 precursors caused more slowly developing contractions than did ET-1 which may be attributed to the requirement for initial enzyme-dependent conversion. A similar observation was reported by Fukuroda *et al.* (1990) who found that a single addition of ET-1 (3 nM) in isolated porcine coronary arteries caused a response that took 15 min to reach half of the peak contraction whereas the response to big ET-1_{1-38} (30 nM) took 35 min to reach half of the peak. Thus, the gallbladder contains an ECE activity which converts human or porcine big ET-1 and big ET-2_{1-38} such that contractions induced by these precursors are similar to those induced by the mature peptides. These contractile effects are not explained by a direct effect on endothelin receptors, for binding assays demonstrated that big ET-1 displaced [^{125}I]-ET-1 only very poorly, as has been noted before (Hirata *et al.*, 1990). The most interesting aspect of this study is that, unlike isolated vascular preparations where big ET-1 is almost always 10–100 times less potent than ET-1, big ET-1 is equipotent to ET-1 in the guinea-pig gallbladder. This is similar to the human bronchus (Advenier *et al.*, 1992), the rat uterus (Rae *et al.*, 1993a, b) and the rat vas deferens (Télémaque & D'Orleans-Juste, 1991). This is highly suggestive that these non-vascular preparations are particularly rich in ECE, supporting the idea of extra-vascular roles for endothelin.

In the present study, big ET-2_{1-38} was found to be approximately equipotent to porcine big ET-1_{1-39} in producing contractions, and the similar speed of these contractions was suggestive of similar rate of conversion. Big ET-3, on the other hand was almost entirely without effect. Thus, the phosphoramidon-sensitive ECE activity present in the guinea-pig gallbladder is selective for big ET-1 or big ET-2 over big ET-3 as is the case in cultured bovine endothelial cells (Okada *et al.*, 1990; Takada *et al.*, 1991), in rat or human brain (Warner *et al.*, 1992a, b), in rat microvessels (Lawrence & Brain, 1993), in the circulation of the guinea-pig (D'Orleans-Juste *et al.*, 1991), in guinea-pig lungs (D'Orleans-Juste *et al.*, 1991) and in the rat vas deferens (Télémaque & D'Orléans-Juste, 1991). Conversely, it has been shown that big ET-3 is converted to its active metabolite by a phosphoramidon-sensitive ECE in porcine cultured aortic endothelial cells (Matsumura *et al.*, 1992), and that big ET-3 produces a phosphoramidon-sensitive elevation in blood pressure in anaesthetized rats (Matsumura *et al.*, 1993; Pollock *et al.*, 1993). Thus, there may be varied phosphoramidon-sensitive or insensitive ECEs in different tissues and species. However, as we found that contractions of isolated gallbladder strips caused by big ET-1_{1-39} were not inhibited by inhibitors of angiotensin-converting enzyme or aspartic, serine or cysteine proteases, we may exclude these enzymes as ECEs in this preparation. Similarly, the ECE present in endothelial cells is not inhibited by other endopeptidase inhibitors such as thiorphan or captopril (Matsumura *et al.*, 1990; Okada *et al.*, 1990). The relationship between the ECE in the gallbladder and that in endothelial cells may well become easier to define now that a phosphoramidon-sensitive ECE has been purified from rat lung (Takahashi *et al.*, 1993) and the membrane fraction of porcine aortic endothelium (Ohnaka *et al.*, 1993), and cloned in rat endothelial cells (Shimada *et al.*, 1994).

Contractions induced by big ET-1_{1-39} were potentiated, albeit to a small degree, by the protease inhibitors, such that contractions induced by porcine big ET-1_{1-39} became similar to those induced by ET-1. Most probably, this is due to the prevention of nonspecific degradation of the peptidic precursor and/or its mature peptide, i.e. ET-1, following the slow conversion. The peptidase inhibitors did not affect the contractions induced by ET-1. This may well be because ET-1 induces contraction very rapidly and in this short time is less susceptible to degradation. The nature of the following responses suggest that much of these metabolic processes are epithelially localised, as contractions induced by big ET-1_{1-39} were potentiated in a manner similar to those in the presence of protease inhibitors, while contractions induced by ET-1 were not affected. These experiments also demonstrated that the ECE activity is not associated with the epithelium and that the conversion may take place on the underlying smooth muscle cells.

In conclusion, the guinea-pig gallbladder is a rich source of a phosphoramidon-sensitive ECE that catalyzes selectively the conversion of big ET-1 to ET-1, as observed in cultured endothelial cells (Ohnaka *et al.*, 1990). This is a most interesting enzyme because phosphoramidon interferes with the pharmacological effects of exogenous big-ET-1 *in vivo* (Okada *et al.*, 1990; Matsumura *et al.*, 1990; Ikegawa *et al.*, 1990), decreases the size of the infarct following myocardial ischaemia reperfusion (Grover *et al.*, 1993) and reduces cerebral vasospasm after subarachnoid haemorrhage (Matsumura *et al.*, 1991). Thus, the study of ECE may lead to the development of specific inhibitors which could be effective agents in blocking the production of ET-1 and so the deleterious effects of ETs in many pathogenic states (see Battistini *et al.*, 1993; Warner *et al.*, 1994). Isolated gallbladder strips of the guinea-pig could therefore provide a useful bioassay to test novel ECE inhibitors, for this preparation has an ECE activity similar to that in endothelial or vascular smooth muscle cells.

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Bronchodilator and pre-protective effects of urodilatin in bovine bronchi *in vitro*: comparison with atrial natriuretic peptide

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1 This study examined the activity and mechanisms of action of urodilatin in bovine bronchi. For comparison, the ability of urodilatin to evoke bronchodilatation or protect against subsequent challenge was compared to that of the closely related peptide α-human atrial natriuretic peptide (ANP).

2 Urodilatin reversed methacholine-evoked contraction in a concentration-dependent manner in bovine bronchi. In the absence of any attempt to prevent degradation by neutral endopeptidases, urodilatin was more potent than ANP in this tissue.

3 The bronchodilator properties of urodilatin were significantly augmented by the neutral endopeptidase inhibitor, phsophoramidon (3.68×10^{-5} M). This provides evidence for at least partial degradation of urodilatin by neutral endopeptidases. With phosphoramidon present, urodilatin and ANP were equipotent.

4 In the presence of phosphoramidon (3.68×10^{-5} M), pre-incubation with urodilatin (10^{-6} M) had a protective effect against subsequent methacholine-induced contraction. This action of urodilatin was quantitatively similar to that of ANP in the presence of this endopeptidase inhibitor.

5 The actions of urodilatin appear to involve ATP-sensitive K^+ channels since tolbutamide (10^{-6}–10^{-5} M) significantly attenuated the relaxations induced by this peptide.

6 Small conductance Ca^{2+}-activated K^+ channels seem likewise to be implicated in the actions of urodilatin since blockade of these channels with apamin (10^{-7}–10^{-6} M) resulted in a marked attenuation of urodilatin-evoked responses.

7 The presence of charybdotoxin (10^{-9} M–10^{-7} M) had no significant effect on subsequent responses to urodilatin suggesting that large conductance Ca^{2+}-activated K^+ channels are not involved in the relaxations evoked by this peptide.

8 In the presence of phosphoramidon (3.68×10^{-5} M), urodilatin (10^{-6} M) evoked elevation of cyclic GMP levels within bovine bronchial tissue. Levels of cyclic GMP increased significantly within 5–10 s in response to this peptide and preceded the initiation of relaxant responses. Maximum increases in cyclic GMP levels were reached within 5 min; the time required for maximal relaxation evoked by this peptide.

9 In conclusion, urodilatin, like ANP reversed and protected against, subsequent methacholine-induced bronchoconstriction; an action enhanced by the presence of phosphoramidon (3.68×10^{-5} M). Associated with these actions of urodilatin was a rise in cyclic GMP levels as well as the opening of ATP-sensitive K^+ and small conductance Ca^{2+}-activated K^+ channels.

Keywords: Urodilatin; bovine bronchi; bronchodilatation; natriuretic peptides; cyclic GMP; potassium channels

Introduction

Urodilatin is a 32-amino acid peptide, isolated from human urine, closely related to α-human atrial natriuretic peptide (ANP), but with four additional residues at the NH_2-terminus. While little is known about the effect of urodilatin on airway smooth muscle function, recent investigations have looked at the effect of ANP in this area. For example, it has been reported that infused ANP reduces airway resistance in normal subjects (Hulks *et al.*, 1990) and, in asthmatic patients it produces a significant bronchodilator response (Hulks *et al.*, 1989; Chanez *et al.*, 1990; Angus *et al.*, 1993). Inhaled ANP attenuates the bronchial responsiveness to histamine and methacholine (Hulks & Thomson 1992; Angus *et al.*, 1994a). Several studies examining ANP *in vitro* have also been undertaken and ANP was shown to confer protection against and reverse methacholine-induced contraction in human and bovine bronchial rings (Angus *et al.*, 1994b). However, these promising effects of ANP are only short lived, possibly due to the degradation of this peptide by neutral endopeptidases.

In contrast to ANP, urodilatin has been reported to be resistant to hydrolysis by dog kidney membranes (which contain neutral endopetidase-24.11) (Gagelmann *et al.*, 1988). In addition, an *in vivo* study showed that prior treatment of rats with a specific neutral endopeptidase inhibitor (SQ-28,603) decreased the clearance of ANP but was without significant effect on the clearance of urodilatin (Abassi *et al.*, 1992). Evidence for at least partial degradation of urodilatin by this mechanism was however provided by the results of Kenny and co-workers (1993) which suggest degradation of this related peptide at about half the rate of ANP.

Little is known about the mechanism of action of urodilatin, although, once again, there have been some studies examining this question using ANP. Ishihera *et al.* (1985) proposed that ANP causes vasodilatation by modulating the transmembrane mobilisation of Ca^{2+} via activation of guanosine 3':5'-cyclic monophosphate (cyclic GMP)-dependent protein kinase (protein kinase G). Alternative suggestions include K^+ channel opening either directly or as a result of protein kinase G activation, as suggested by Winquist *et al.* (1984) and Ohlstein & Berkowitz (1985) who

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reported that ANP relaxes vascular tissue through either membrane hyperpolarisation or synthesis of cyclic GMP. It is tempting to speculate that urodilatin may act through similar mechanisms.

In this study we examined whether urodilatin can reverse and protect against subsequent methacholine-induced contraction in bovine bronchi and whether neutral endopeptidases inactivate urodilatin in airways. We have also examined the role for K^+ channels in the activity of this peptide, by the use of several selective K^+ channel blockers, tolbutamide, apamin and charybdotoxin, which block ATP-sensitive and small and large conductance Ca^{2+}-activated K^+ channels, respectively (for review see Black & Barnes, 1990).

Methods

Tissue preparation

Bovine bronchi were obtained from cattle from the abattoir within 30 min of slaughter and dissected free of connective tissue and fat. Lengths of third to sixth order bronchi were cut into rings and maintained in oxygenated (95% O_2; 5% CO_2) Krebs-Henseleit solution of the following composition mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2 and glucose 11.1.

Measurement of mechanical response

Mechanical responses were measured isometrically from bronchial rings suspended in vertical organ baths (5 ml) containing oxygenated (95% O_2; 5% CO_2) Krebs-Henseleit solution. Tension (2 g wt.) was applied via two platinum wires inserted into the lumen. One was anchored while the other was attached to a force displacement transducer (Grass FT303). Tissues were allowed to equilibrate for approximately 2 h, during which time tone was readjusted and the tissues were washed out several times with Krebs-Henseleit solution.

The action of urodilatin and ANP on methacholine-evoked contractions

Tissues were pre-constricted with 3×10^{-6} M methacholine, this being equivalent to the EC_{25-30} (i.e. the concentration producing 25–30% of the maximum contraction) of methacholine. Once contractions had reached a plateau, cumulative concentration-response curves were constructed to ANP or urodilatin. Relaxations evoked were expressed as a % of the initial contraction to methacholine. Experiments were then repeated in the presence of phosphoramidon (3.68×10^{-5} M).

In a second set of experiments, bronchi were preincubated with 10^{-6} M urodilatin or ANP in the absence or presence of phosphoramidon. Approximately 15 min later, concentration-response curves to methacholine were obtained. All subsequent experiments were undertaken with phosphoramidon (3.68×10^{-5} M) present.

Effect of K^+ channel blockers on urodilatin-evoked relaxations

Subsequent to raising the tone with methacholine, concentration-response curves to urodilatin (in the presence of phosphoramidon (3.68×10^{-5} M)) were obtained in the presence and absence of tolbutamide, apamin and charybdotoxin which respectively block ATP-sensitive and small and large conductance Ca^{2+}-activated K^+ channels. Antagonists were added approximately 15 min before methacholine-induced preconstriction.

In each of the above experiments, one ring of bovine bronchi acted as a time control.

Preparation of samples for cyclic GMP assay

Rings of tissue were equilibrated in oxygenated (95% O_2; 5% CO_2) Krebs-Henseleit solution (37 ± 0.5°C) for 30–45 min. They were then stimulated with phosphoramidon (3.68×10^{-5} M) alone, or urodilatin both in the presence and absence of phosphoramidon (3.68×10^{-5} M) for the required time (0, 5, 10, 20, 30, 60, 120 and 300 s). Reactions were stopped by immersing the tissue in liquid nitrogen. The rings were pulverised while frozen with a custom built crusher and transferred to a test tube containing 1 ml of ice cold 4% perchloric acid solution. Samples were collected in duplicate. The homogenate was vortexed and then sonicated for 20 min at 4°C prior to removal of a 200 µl sample for determination of protein content by the method of Peterson (1977).

The remaining solution was treated for removal of perchloric acid by neturalising with potassium hydroxide (1.5 M)/HEPES (0.6 M)/universal indicator solution and centrifuged for 5 min at 3000 *g*. The supernatant was removed for determination of cyclic GMP levels using the commercially available cyclic GMP ^{125}I radioimmunoassay kit (Du Pont). This technique is adapted from the procedures of Steiner *et al.* (1972). Control (without urodilatin) tissues were prepared in the same way. The assays were carried out with acetylation and performed in duplicate. Results are expressed as fmol cyclic GMP μg^{-1} protein.

Materials

The following drugs were used: ANP (α-human atrial natriuretic peptide 28-amino acid) (Bachem); apamin (Sigma); cyclic GMP ^{125}I radioimmunoassay kits (Du Pont); charybdotoxin (Sigma); tolbutamide (Sigma); methacholine chloride (Sigma); N-(arhamnopyranosyl oxyhydroxyphosphinyl Leu-Trp (phosphoramidon, Sigma); tolbutamide (Sigma) and urodilatin (Sigma).

Drugs, with the exception of charybdotoxin and tolbutamide, were dissolved primarily in distilled water. Charybdotoxin and tolbutamide were reconstituted in 50% and 100% alcohol respectively. In each case subsequent dilutions of stock solutions were made in Krebs-Henseleit solution. Where appropriate, time controls contained the appropriate amount of ethanol (0.001–0.01%).

Analysis of results

Statistical analysis of data sets was performed by two-way analysis of variance. Results have been expressed in terms of mean maximum inhibition (% inhibition of the initial methacholine contraction evoked by 10^{-6} M ANP or urodilatin) or the $-\log EC_{50}$ (the negative log of the concentration effecting 50% of the control maximum response). Significance between mean maximum inhibition, $-\log EC_{50}$ or points on cyclic GMP time courses was analysed by Student's *t* test. Results are expressed as mean ± s.e.mean. A probability level of $P<0.05$ was considered significant and number of observations (*n*) refers to the number of animals from which tissue was obtained.

Results

Bronchodilator effects of ANP and urodilatin

ANP (10^{-9}–10^{-6} M) evoked a small reversal of methacholine-induced bronchoconstriction. The presence of phosphoramidon (3.68×10^{-5} M) markedly potentiated these concentration-dependent bronchodilator effects (mean maximum inhibition; 48.6 ± 9.0 in presence of phosphoramidon; 13.2 ± 1.7% in the presence of ANP alone, $P<0.001$, $n = 6$, Figure 1). Urodilatin (10^{-9}–10^{-6} M) evoked a more marked reversal of methacholine-induced tone than ANP when they were each present alone (mean maximum inhibition

29.02 ± 9.2% with urodilatin, compared with 13.21 ± 1.68% with ANP at 10^{-6} M level). Following preincubation with phosphoramidon (3.68 ± 10^{-5} M), the effect of urodilatin was significantly augmented (mean maximum inhibition; 45.8 ± 5.0% in presence of phosphoramidon, compared with 29.02 ± 9.2% in the presence of urodilatin alone ($P<0.001$, $n = 6$)). The augmentation of the maximum ANP response in the presence of the endopeptidase inhibitor phosphoramidon was comparatively greater, than that of urodilatin (Figure 1).

With phosphoramidon present, ANP and urodilatin were approximately equipotent.

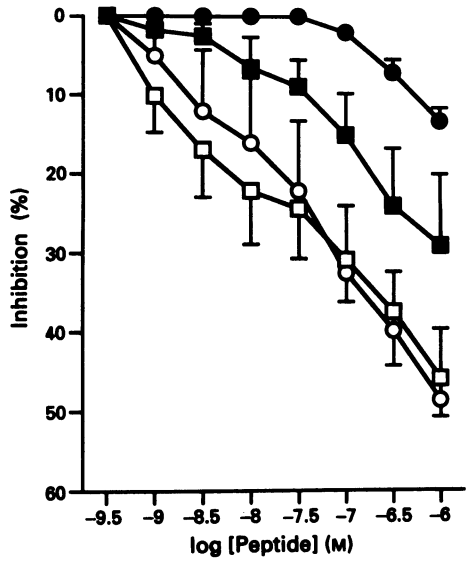

Figure 1 The effects of atrial natriuretic peptide (ANP, 10^{-9}–10^{-6} M), in the presence (○) and absence (●) of phosphoramidon (3.68×10^{-5} M) and the effects urodilatin (10^{-9}–10^{-6} M) likewise with phosphoramidon present (□) or absent (■), on methacholine-induced tone. ANP evoked a concentration-dependent relaxation which was enhanced by phosphoramidon. Urodilatin evoked a greater degree of relaxation than ANP alone; however, these agonists were equipotent in the presence of phosphoramidon. Number of observations (n) = 6 in each case.

Protective effect of ANP and urodilatin

After pre-incubation of the bronchi with 10^{-6} M urodilatin there was an insignificant reduction in subsequent methacholine-evoked contractions (10^{-9}–3×10^{-4} M) (Figure 2). In the presence of phosphoramidon (3.68×10^{-5} M), however, there was a marked attenuation in the responses evoked by methacholine ($-\log EC_{50}$, 4.24 ± 0.50 compared with control $-\log EC_{50}$; 5.27 ± 0.09 and 4.43 ± 1.15 in the presence of urodilatin but absence of phosphoramidon) ($P<0.001$). Only 62.2% ± 14.0 of the control maximum contraction was evoked in the presence of phosphoramidon compared with 78.7% ± 25.6 in its absence (Figure 2, $n = 6$ in each case). ANP (10^{-6} M) alone similarly did not evoke a significant attenuation of the concentration-response curves evoked by methacholine. In the presence of phosphoramidon (3.68×10^{-5} M), however, ANP evoked a marked attenuation in the responses evoked by methacholine ($-\log EC_{50}$; 4.12 ± 0.38, compared to control 5.85 ± 0.1, $P<0.05$) (Figure 2).

Phosphoramidon (3.68×10^{-5} M) in the absence of ANP had no effect either on reversing induced tone or protecting against methacholine-induced contraction (data not shown).

Effect of K^+-channel antagonists on peptide-induced bronchodilatation

Tolbutamide Preincubation of the bronchi with 10^{-7} M tolbutamide had no effect on relaxations evoked by urodilatin (10^{-9}–10^{-6} M) in the presence of phosphoramidon (3.68×10^{-5} M) (Figure 3). There was a marked reduction in the mean maximum inhibition evoked by urodilatin (plus phosphoramidon (3.68×10^{-5} M)) in the presence of 10^{-6} M (37.9 ± 3.7%) and 10^{-5} M (51.3% ± 5.2) tolbutamide compared with the control (81.7% ± 26.0, $n = 6$) ($P<0.01$ and $n = 5$ in each case, Figure 3). Tolbutamide alone did not alter the baseline tone of the bronchi.

Apamin Apamin 10^{-8} M did not significantly alter relaxations evoked by urodilatin (in the presence of phosphoramidon (3.68×10^{-5} M), Figure 4). In contrast, exposure to 10^{-7} M apamin resulted in a marked attenuation of the subsequent urodilatin-induced bronchodilator effects (mean

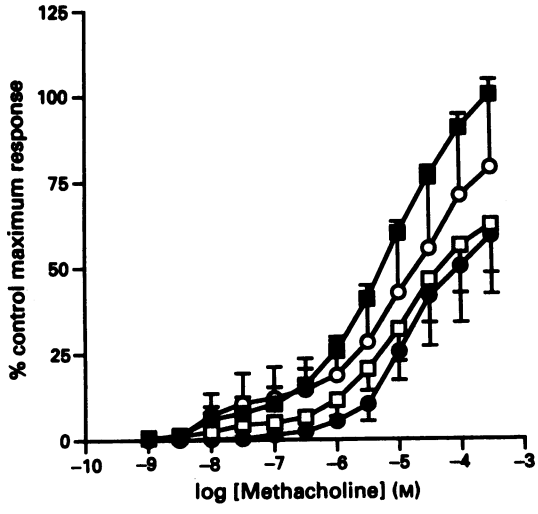

Figure 2 The effect on subsequent methacholine challenge (10^{-9}–3×10^{-4} M, ■) of preincubation with urodilatin 10^{-6} M, in the presence (□) and absence (○) of phosphoramidon (3.68×10^{-5} M) as well as the effect of atrial natriuretic peptide (ANP, ●, 10^{-6} M, in the presence of phosphoramidon), on such methacholine-challenge. Both urodilatin and ANP attenuated the methacholine contractions when phosphoramidon was present (n) = 6 in each case.

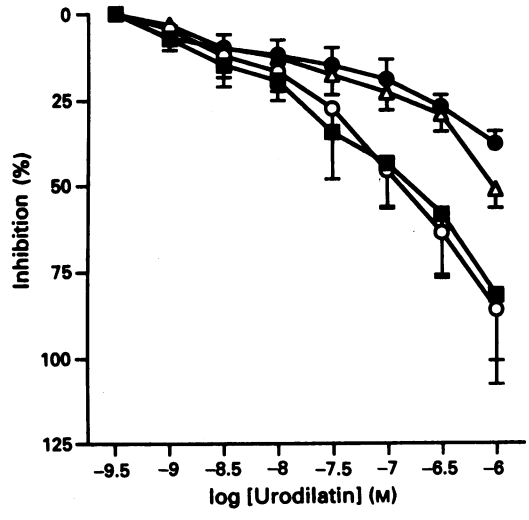

Figure 3 The effect of 10^{-7} M (○), 10^{-6} M (●) and 10^{-5} M (△) tolbutamide on (10^{-9}–10^{-6} M) urodilatin-induced relaxations (in the presence of phosphoramidon 3.68×10^{-5} M). Preincubation with 10^{-7} M tolbutamide had no significant effect on urodilatin responses compared with the control (■); however, a marked inhibition was seen in the presence of 10^{-6} M blocker. A similar degree of inhibition was seen with 10^{-5} M tolbutamide. Number of observations (n) = 6.

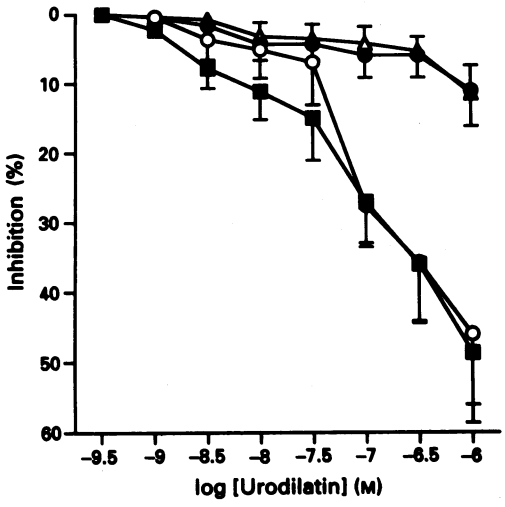

Figure 4 The effect of 10^{-8} M (○), 10^{-7} M (●) and 10^{-6} M (△) apamin on urodilatin-induced relaxations (in the presence of phosphoramidon 3.68×10^{-5} M). Apamin, 10^{-8} M, had no significant effect on relaxations evoked by urodilatin (10^{-9}–10^{-6} M) (■). In contrast, a marked attenuation of the responses occurred in the presence of 10^{-7} M and 10^{-6} M, with each of these concentrations evoking similar degrees of inhibition. Number of observations (n) = 6.

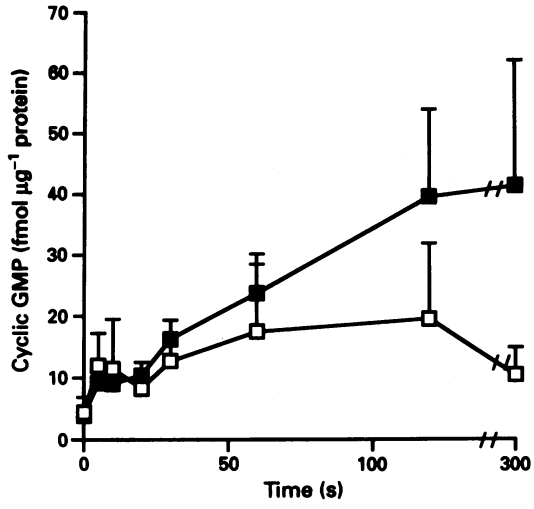

Figure 5 The effect of 10^{-6} M urodilatin, in the presence (■) and absence (□) of 3.68×10^{-5} M phosphoramidon, on the level of cyclic GMP (fmol μl^{-1} protein) within bovine bronchi. Urodilatin alone induced a small transient but insignificant elevation of cyclic GMP. In the presence of phosphoramidon, an initial significant elevation was apparent at 5 s whereupon levels reached a plateau. This was followed by a second rise apparent at 30 s, reaching a second plateau by 5 min. Number of observations (n) = 6 in each case.

maximum inhibition of methacholine-evoked contractions; 11.6 ± 4.07% at 10^{-6} M urodilatin, compared with control 48.6 ± 10.0%). There was no further inhibition of responses with 10^{-6} M compared to 10^{-7} M apamin ($P < 0.001$, $n = 6$ in each case) and the maximum urodilatin response was attenuated by similar amounts (37.5 ± 10% (10^{-7} M) and 37.0 ± 9.4% (10^{-6} M)) (Figure 4). Apamin alone did not alter the baseline tone of the bronchi.

Charybdotoxin The presence of 10^{-9}–10^{-7} M charybdotoxin had no significant influence on urodilatin-induced relaxations (in the presence of phosphoramidon, 3.68×10^{-5} M, data not shown, $n = 6$). Charybdotoxin, 10^{-7} M, alone evoked an increase in the resting tone of the bronchi prior to the methacholine challenge; however, neither 10^{-8} nor 10^{-9} M charybdotoxin had any effect on baseline tone (data not shown).

Effect of urodilatin on cyclic GMP levels

Mean basal cyclic GMP levels were 4.6 ± 1.6 fmol μg^{-1} protein. Stimulation of rings of bovine bronchi with urodilatin (10^{-6} M) alone did not significantly alter levels of cyclic GMP measured (Figure 5, $n = 6$).

In the presence of phosphoramidon (3.68×10^{-5} M), a significant increase in cyclic GMP levels was apparent 5 s after stimulation with 10^{-6} M urodilatin (140 ± 30%; $P < 0.05$), whereupon levels reached a plateau. This was followed by a second rise evident at 30 s ($P < 0.005$), reaching a plateau by 5 min whereupon cyclic GMP levels were 658 ± 99% of control ($P < 0.005$ at 5 min compared with control: $P < 0.005$ compared with 5 s level) (Figure 5, $n = 6$).

The rise in cyclic GMP preceded the time course for initiation of relaxations in response to urodilatin (which were apparent after approximately 1 min) and reached a maximum at a time in keeping with that required for development of maximum relaxations (5 min).

Phosphoramidon alone did not significantly alter cyclic GMP levels (data not shown) during the time course examined.

Discussion

Both ANP and urodilatin can reverse methacholine-induced tone in bovine bronchial tissue. Urodilatin evoked comparatively greater concentration-dependent relaxations than ANP when present alone. This appears to be the result of urodilatin being less sensitive than ANP to neutral endopeptidases, since, in the presence of phosphoramidon, ANP and urodilatin were equipotent in their ability to reverse methacholine-induced tone. This is in keeping with the findings of Kenny and co-workers (1993) who suggested that urodilatin was degraded at approximately half the rate of ANP, but in contrast to the findings of other studies where urodilatin was not significantly hydrolysed by neutral endopeptidases in (e.g. rat *in vivo*, Abassi *et al.*, 1992).

The equipotency between ANP and urodilatin, subsequent to protection against neutral endopeptidase activity, is likewise seen in the ability of these two peptides to provide protection against subsequent methacholine challenge. In this case, neither urodilatin nor ANP alone significantly altered subsequent contractions, but in the presence of phosphoramidon, these two peptides confer a similar degree of protection. The similarity between urodilatin and ANP suggests that both these peptides share the same receptors, as demonstrated in rat and human kidney (Valentin *et al.*, 1991; Heim *et al.*, 1989).

A role for K^+ channel activation in the actions of ANP has been suggested previously in bovine tracheal smooth muscle (Ishii & Murad, 1989). A number of potassium channel types have, however, been identified and little is known about the involvement of these in the responses evoked by natriuretic peptides. The present study examined the role of three subtypes of these channels in the response to urodilatin of bovine bronchi.

Neither small conductance calcium activated nor ATP-sensitive K^+ channels appeared to be open under resting conditions in this tissue since neither tolbutamide nor apamin altered baseline tension. This is in keeping with previous studies in human bronchi (Miura *et al.*, 1992). By contrast, we observed a rise in baseline tension in bovine bronchi on exposure to 10^{-7} M charbdotoxin, a selective large conductance Ca^{2+}-activated K^+ channel blocker. These results sug-

gest that charybdotoxin-sensitive channels are open in resting conditions in bovine airways. In contrast, in human airways (Miura *et al.*, 1992) charybdotoxin pretreatment did not alter baseline tone, possibly reflecting a species difference.

The opening of ATP-sensitive K^+ channels is at least partly involved in the mechanisms of action of urodilatin, as inferred from the significant attenuation of relaxations evoked by urodilatin observed after preincubation with tolbutamide. In addition, the opening of the small conductance Ca^{2+}-activated subtype of K^+ channel appears involved in urodilatin-induced bronchodilatations since apamin markedly inhibited responses. By contrast, although our results suggest that large conductance Ca^{2+}-activated K^+ channels may be open under resting conditions, they appear to have no involvement in the mechanism of action of urodilatin, since charybdotoxin did not attenuate the relaxations evoked by this peptide.

The involvement of cyclic GMP in the response evoked by urodilatin has not previously been studied. In bovine tracheal smooth muscle (Ishii & Murad, 1989), however, ANP-evoked relaxation was associated with generation of cyclic GMP. This has also been reported in guinea-pig trachea (Watanabe *et al.*, 1990) and in vascular smooth muscle preparations (for review see Winquist, 1985). It seemed likely that urodilatin might act in a similar manner. We found that the level of cyclic GMP in bovine bronchi was elevated in response to urodilatin, under the same conditions which evoked relaxations. Urodilatin, in the presence of phosphoramidon, evoked a significant increase in cyclic GMP levels at 5 s, followed by a second rise by 5 min. This increase was similar to that previously reported in bovine tracheal smooth muscle in response to ANP (Ishii & Murad, 1989).

A smaller effect on cyclic GMP accumulation was observed in response to urodilatin alone. There appeared to be a transient elevation of the second messenger, however, this was not significant. The rise in cyclic GMP in response to urodilatin (plus phosphoramidon) preceded the induction of dilatation, which required approximately 1 min, and this is in keeping with a receptor-operated event leading to smooth muscle relaxation. The time course of the peak increases in cyclic GMP coincides with the peak relaxant effect (attained in around 5 min), an observation in agreement with that of Ishii & Murad (1989). The mechanism by which cyclic GMP may induce relaxation remains to be elucidated, however, some reports suggest modulation of transmembrane mobilisation of Ca^{2+}, possibly through activation of a calcium extrusion pump (Popescu *et al.*, 1985; Ishihera *et al.*, 1985; Fujii *et al.*, 1986).

In summary, this investigation suggests, for the first time, a role for both the elevation of cyclic GMP and the opening of ATP-sensitive and small conductance calcium-activated K^+ channels in response to urodilatin. It is not clear from the present study whether these two mechanisms occur independently, or are intrinsically linked. Opening of K^+ channels accompanied by cyclic GMP elevation has previously been seen (Winquist *et al.*, 1984; Ohlstein & Berkowitz, 1985) and in addition, White *et al.* (1993) showed that cyclic GMP stimulated large conductance Ca^{2+}-activated K^+ channels through stimulation of protein kinase G in rat pituitary tumour cells. This was an indirect activation through protein dephosphorylation. These authors also reported that ANP rapidly increased the steady state outward current predominantly of large conductance Ca^{2+}-activated K^+ channels, an action mimicked by 8-bromo-cyclic GMP. This is in contrast to the present results using urodilatin, where large conductance Ca^{2+}-activated K^+ channels were not involved in the response to this peptide. It remains to be seen whether this represents a difference between these two peptides, or simply a species and tissue type difference.

In conclusion, this study showed that urodilatin can reverse and protect against, methacholine challenge in bovine bronchi *in vitro*. Furthermore urodilatin appeared to be less susceptible to degradation by neutral endopeptidases than ANP. The relaxant effects of urodilatin in bovine bronchi are associated with opening of ATP-sensitive and small conductance Ca^{2+}-activated K^+ channels as well as a rise in levels of cyclic GMP.

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Increase of meningeal blood flow after electrical stimulation of rat dura mater encephali: mediation by calcitonin gene-related peptide

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1 The dura mater encephali of the rat was exposed and the blood flow around branches of the medial meningeal artery was monitored with a laser Doppler flowmeter. Changes in the meningeal blood flow (MBF) following electrical stimulation of the dura mater at a parasagittal site were registered. The effects of human calcitonin gene-related peptide (h-αCGRP) and the CGRP antagonist (h-αCGRP$_{8-37}$) on the MBF were tested.

2 Electrical stimulation with rectangular pulses of 0.5 ms, 10–20 V, 5–10 Hz and a duration of 30 s caused an increase of the MBF in 14 out of 16 rats tested. The increases were dependent on stimulus strength and frequency.

3 The increase in MBF was inhibited in a dose-dependent manner by topical application of 0.1 ml of h-αCGRP$_{8-37}$ at concentrations of 10^{-7}–10^{-5} M. The highest dose abolished the increase in MBF.

4 Topical administration of 0.1 ml of h-αCGRP at a concentration of 10^{-4} M increased the basal MBF by 15% on average.

5 It is suggested that the increase in MBF following electrical stimulation of the dura mater is mediated by the release of CGRP. The contribution of the dural afferent and sympathetic and parasympathetic efferent nerve fibres to this response are discussed.

Keywords: Neurogenic inflammation; dura mater encephali; meningeal afferents; neuropeptide release; CGRP antagonist; blood flowmetry

Introduction

Neurogenic inflammatory responses of meningeal tissues, e.g. vasodilatation and plasma extravasation in the dura mater, have been proposed as an essential mechanism for migraine pain and other headaches (Moskowitz, 1984; 1993; Hardebo, 1990). In the rat a neurogenic inflammation of the dura mater was provoked by stimulation of trigeminal afferents, indicated by symptoms like plasma extravasation (Markowitz *et al.*, 1987), aggregation and adhesion of platelets, and activation of mast cells (Dimitriadou *et al.*, 1991; 1992). These responses are likely to be mediated by neuropeptides released from the peripheral terminals of fine trigeminal afferent fibres.

Histochemical studies by von Düring *et al.* (1990) and Keller & Marfurt (1991) as well as our recent investigation (Messlinger *et al.*, 1993) revealed that the dura mater encephali of the rat is densely innervated by both substance P and calcitonin gene-related peptide (CGRP) immunoreactive nerve fibres of small diameter suggesting the presence of these neuropeptides within distinct classes of dural afferents. Whereas substance P (as well as neurokinin A) seems to mediate mainly plasma extravasation that can be inhibited by the NK_1 receptor antagonist, RP 67580 (Moussaoui *et al.*, 1993; Shepheard *et al.*, 1993), it is suggested that CGRP contributes little to this response (Markowitz *et al.*, 1987). On the other hand, CGRP is well-known as a powerful vasodilator in various tissues including cerebral cortex (Brain *et al.*, 1985; McCulloch *et al.*, 1986; Andersson, 1989; Edwards *et al.*, 1991). In the dura mater a vasodilator action of CGRP has not so far been examined. Many of the CGRP immunoreactive fibres follow the course of the medial meningeal artery (MMA) up to the superior sagittal sinus, ramify in the parietal area of the dura mater and terminate both at dural vessels and within dural connective tissue (Messlinger *et al.*, 1993). Release of CGRP from these afferent terminals would be expected to dilate dural vessels and increase meningeal blood flow.

The aim of this study was to examine whether activation of meningeal afferents by electrical stimulation of the dura mater increases the meningeal blood flow, and whether these changes can be modified by the CGRP antagonist, h-CGRP$_{8-37}$, indicating an involvement of CGRP. Briefly, our measurements provide convincing evidence for a vasodilator action of CGRP released in meningeal structures after electrical stimulation of the dura mater.

Methods

Anaesthesia and general preparation

The study was performed on 16 Wistar rats of either sex (190–480 g). They were anaesthetized by an initial dose of 100–120 mg kg^{-1} thiopentone, (Trapanal) i.p., followed by additional doses of 2–25 mg kg^{-1} thiopentone i.p. when required. Depth of anaesthesia was routinely judged by observing the immobility to noxious stimuli and by monitoring the arterial blood pressure of the animal. The trachea was cannulated, and spontaneous respiration was assisted by blowing a gentle jet of oxygen toward the opening of the tracheal cannula. A catheter was placed into the right femoral vein for the i.v. infusion of solutions when necessary. Arterial blood pressure was continuously recorded with a pressure transducer via a catheter in the right femoral artery and maintained above 100 mmHg (systolic) by i.v. infusion of 4% Ficoll 70 (Serva, Heidelberg) and 5% glucose solu-

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tions. The animal's body temperature was maintained at 37.0–37.5°C with a thermostatically regulated heating plate.

Preparation for stimulation and recording

The animal's head was fixed in a stereotaxic frame. The skin covering the dorsal side of the head was divided by a sagittal section and pulled aside. Periosteum and the dorsal part of the masseter muscle were removed. The skull was carefully opened by means of an electric drill while being cooled with Tyrode solution (4°C). Two openings were formed for exposing the dura mater (Figure 1): one parasagittal slit of about 2 x 6 mm for electrical stimulation, and one rectangular window of about 5 x 6 mm in the parietal bone exposing the distal branches of the medial meningeal artery (MMA). A needle type of probe of a laser Doppler flowmeter was placed over a branch of the MMA without touching it. The parietal opening was covered with pieces of gauze soaked with Tyrode solution. A pair of parallel stimulating electrodes (wire diameter 0.2 mm, distance 1 mm) with a length of 4 mm was lowered onto the dural surface of the parasagittal opening, so that the wires touched the dural surface along their whole length parallel to the sagittal suture. The opening was filled with a cotton pad soaked with liquid paraffin.

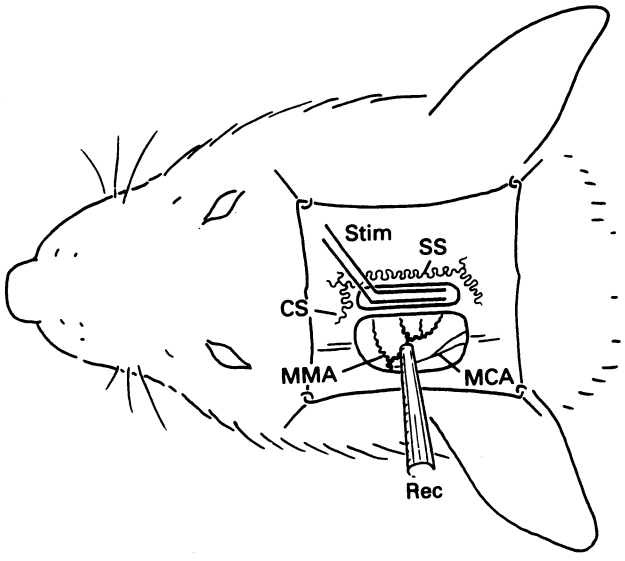

Figure 1 Experimental set-up for electrical stimulation of the rat dura mater by electrodes (Stim) oriented in parallel to the sagittal suture (SS) and for recording of the meningeal blood flow (Rec) around the middle meningeal artery (MMA) with a needle probe (tip diameter 1.0 mm) of a laser Doppler flowmeter. CS coronal suture, MCA branches of the medial cerebral artery visible through the dura.

Electrical stimulation and recording of blood flow

The dura was electrically stimulated by an isolated stimulator (HI-Med) with rectangular pulses of a constant length of 0.5 ms. Stimulus strength and frequency were varied from 5–20 V and 2–20 Hz, respectively, and were optimized at the beginning of each experiment to elicit maximal elevations of blood flow without changing the blood pressure (Figure 2). Blood flow was measured by a laser Doppler flowmeter (Moor Instruments, MBF3D; time constant 1.0 s) and recorded on-line by a penwriter (Rikadenki). Each test period was preceded by at least 4 control stimulations eliciting stable responses at intervals of 5 min. Then the test substance (on a gauze pad soaked with 0.1 ml solution of agents) was applied topically to the exposed dural surface. Two minutes later the first test response was elicited followed by further stimulations at intervals of 5 min.

Drug administration

Human calcitonin gene-related peptide (h-αCGRP, Bachem Chemicals) was administered at a concentration of 10^{-4} M, the CGRP antagonist h-$\alpha CGRP_{8-37}$ (Bachem) at concentrations of 10^{-7}, 10^{-6} and 10^{-5} M dissolved in Tyrode solution. The chemicals were kept frozen in a stock solution (10^{-4} M) and diluted immediately before use.

Evaluation of data

Changes of blood flow after electrical stimulation were assessed by measuring the area under the evoked response from the baseline before stimulation to the point where the curve returned to the baseline (see Figure 2) using morphometric software (HISTOL, List electronics). A constant

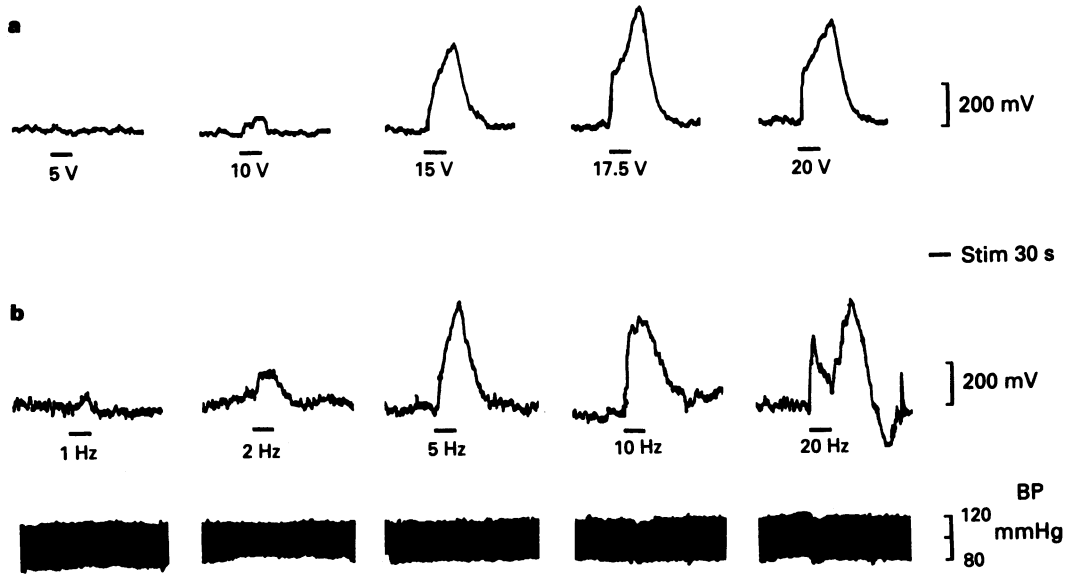

Figure 2 Increases in meningeal blood flow induced by electrical stimulation with pulses of 0.5 ms length for 30 s. (a) Variation of the stimulus strength (frequency kept constant at 10 Hz). The blood flow response was maximal at 17.5 V. (b) Variation of the stimulus frequency (strength constant at 15 V). The blood flow was optimal at 5–10 Hz. Higher frequencies caused big fluctuations of the blood flow and slightly lowered the systemic blood pressure (BP).

flow expressed by a horizontal baseline was a prerequisite for starting the test period. The increases in blood flow were expressed as relative values compared to the last control area (= 100%) immediately before the test sequence (see Figure 3). Comparison of the blood flow responses was made with one-way ANOVA and Fisher's least significance test.

Results

Increase of blood flow after electrical stimulation

In 14 of 16 experiments, electrical stimulation of the dura mater encephali via surface electrodes (see Figure 1) increased the blood flow that was measured at a parietal site over a branch of the medial meningeal artery. This increase was dependent on the stimulus strength and the frequency. Both these parameters were systematically varied in 6 experiments. Figure 2a shows an example where the stimulus strength was varied from 5 to 20 V. The blood flow increase was maximal at a stimulus strength of 17.5 V. As in this experiment, the maximum response was obtained between 10–20 V in all experiments. In Figure 2b the changes of blood flow are shown when the frequency of the stimulation was varied. A maximal effect was observed with stimulation frequencies of between 5 and 10 Hz. Higher frequencies caused a fluctuating response and changes in the systemic blood pressure. In view of these results, all further experiments were carried out with stimulus frequencies of 5 or 10 Hz. The duration of the blood flow response achieved by these stimulation parameters ranged from 1–2 min.

In two experiments the blood flow decreased during stimulation. This decrease could be abolished by local application of phentolamine at a concentration of 3 mM showing that it was due to an α-sympathetic activation. These two experiments were excluded from further analysis.

Inhibitory effect of h-CGRP$_{8-37}$ on blood flow increase

In 5 experiments Tyrode solution, topically administered on the dura mater as a control, did not change the evoked blood flow increase (Figure 3a). The effect of 100 µl of the CGRP antagonist, h-CGRP$_{8-37}$ at concentrations of 10^{-7} up to 10^{-5} M, administered in the same way, was examined in 10 experiments. In all these experiments h-CGRP$_{8-37}$ diminished the electrically evoked blood flow response measured around the MMA. In the experiment depicted in Figure 3 the response was reduced to about 30% of the control responses (at 2, 7 and 12 min) after the application of h-CGRP$_{8-37}$ (10^{-6} M), and it gradually returned toward the control response size.

The reduction of the electrically evoked blood flow response depended on the dose of h-CGRP$_{8-37}$ applied (Figure 4). H-CGRP$_{8-37}$ at a concentration of 10^{-7} M initially reduced the response to about 60% of its control size on average (n = 4 experiments). The inhibitory effect was short-lasting, i.e., it was significant only 2 min after administration of h-CGRP$_{8-37}$. The evoked increase of MBF recovered (without removing the antagonist) within 17 min. The higher concentration of 10^{-6} M caused an initial reduction of 75% on average (n = 4). The effect was long-lasting, and recovery was incomplete. The highest dose of h-CGRP$_{8-37}$ (10^{-5} M) totally abolished the blood flow response; the response showed no recovery within the observation time of 40 min.

Isolation of the dura mater

Since the blood flow measurement in this study may include pial and cortical flow in addition to the dural flow, at the end of 4 experiments the dura mater was incised and a thin sheet of black polyethylene was placed under the dura underlying the proble of the Doppler flowmeter to exclude the contribution of the blood flow beneath. In two experiments the electrically evoked increase of MBF did not significantly change after isolation of the dura (Figure 5). In one of these experiments the basal blood flow decreased after this manipulation, in the other experiment it increased accompanied by a bleeding from the lesioned dura mater. In two other experiments the evoked blood flow response decreased

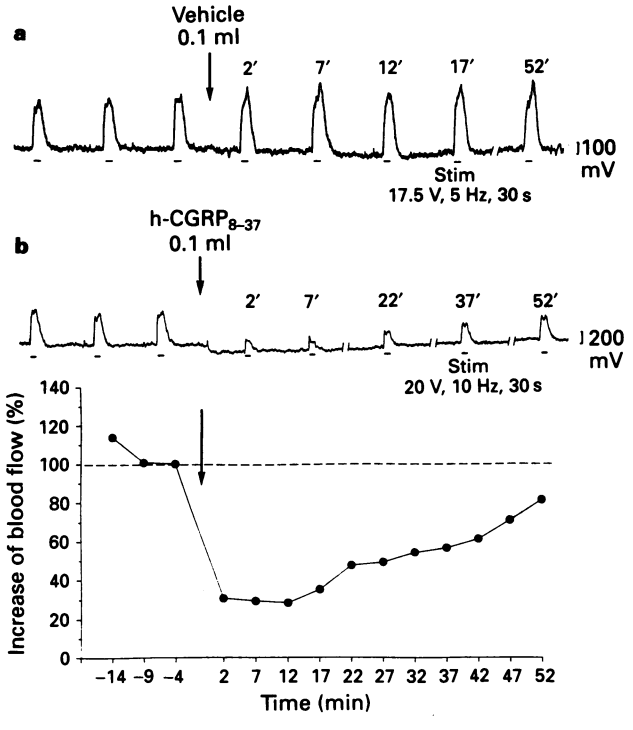

Figure 3 Specimen records of the blood flow responses to electrical stimuli before and at the indicated times after topical administration of Tyrode solution (vehicle) and h-CGRP$_{8-37}$. (a) Tyrode solution did not significantly change the evoked increases of blood flow. In this trial the responses slightly increased. (b) Inhibition of the electrically evoked blood flow responses by h-CGRP$_{8-37}$. In this trial also the basal blood flow was slightly decreased after application of the antagonist, as can be seen in the original records (upper diagram). The lower diagram shows the complete set of blood flow responses of the same trial, expressed as relative values to the control response recorded immediately before the h-CGRP$_{8-37}$ application.

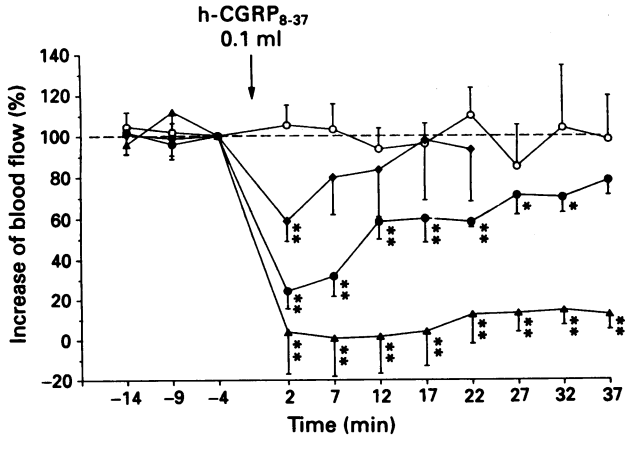

Figure 4 Effects of various concentrations of h-CGRP$_{8-37}$ (10^{-7} M, n = 4, ◆; 10^{-6} M, n = 4, ●; 10^{-5} M, n = 4, ▲) on the blood flow responses evoked by electrical stimulation of the dura mater, compared to the vehicle (Tyrode solution, n = 5, ○). Values are mean ± s.e.mean. Values after administration of h-CGRP$_{8-37}$ were tested for significant differences (*P < 0.05; **P < 0.01) versus values after Tyrode solution (one way ANOVA and Fisher's least significance test).

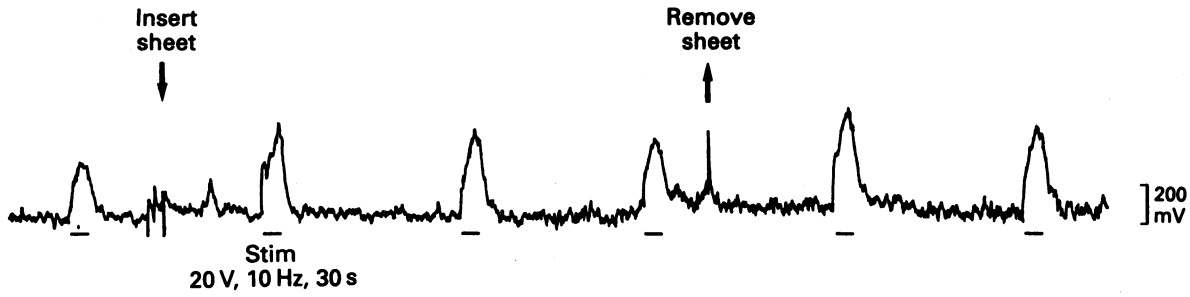

Figure 5 Evoked blood flow responses (bars below responses represent stimulation with 20 V and 10 Hz for 30 s) before and after isolation of the dura mater by inserting a black plastic sheet between dura and cortical surface.

to 36% and 49% respectively, of the previous average value, while the basal flow decreased in the first and increased in the latter case.

At the end of two experiments the MMA was selectively coagulated at a proximal site (at the lateral border of the recording window). The basal blood flow measured around the MMA decreased, in one case to nearly zero. In contrast, the basal cortical flow was not significantly changed by coagulation of the MMA when the probe of the Doppler flowmeter was positioned over a branch of the medial cerebral artery.

Increase of blood flow after h-CGRP

In 6 experiments human calcitonin gene-related peptide (100 µl) at a concentration of 10^{-4} M was administered topically on the dura mater (recording site). In all experiments it increased the basal blood flow (without electrical stimulation), an effect lasting for 4 to 16 min (Figure 6). The peak increase was 15% on average. Tyrode solution as a control did not change the blood flow.

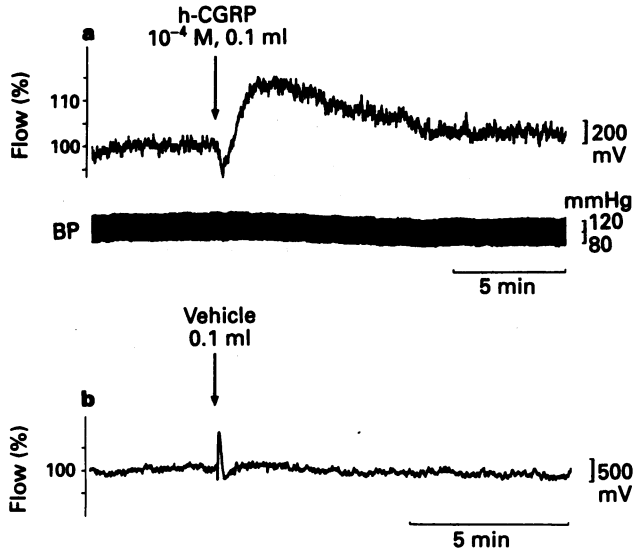

Figure 6 (a) Increase of meningeal blood flow (Flow) after topical administration of 100 µl h-CGRP at a concentration of 10^{-4} M. The brief decrease in blood flow immediately after the h-CGRP application was not seen in all experiments. The systemic blood pressure (BP) remained practically constant. (b) Administration of Tyrode solution (vehicle) in the same experiment (dripped onto the cotton pad) did not change the blood flow. The transient fluctuation of the blood flow is probably an artefact because the drop of vehicle briefly alters the amount of light reflected to the sensor.

Discussion

The present paper shows that electrical stimulation of the dura mater encephali with stimuli that are able to activate thin myelinated and unmyelinated nerve fibres increased the meningeal blood flow in the rat. This evoked increase was inhibited by the CGRP antagonist, h-CGRP$_{8-37}$, providing evidence that it was mediated by CGRP. Human CGRP itself caused an increase of the basal blood flow without electrical stimulation. Since the dura mater encephali is densely innervated by CGRP immunoreactive nerve fibres (Keller & Marfurt, 1991; Messlinger *et al.*, 1993), it is likely that CGRP is released from these nerve endings when they are activated by electrical stimulation. The same stimuli probably also release substance P (Edvinsson *et al.*, 1983) that is stored in a smaller proportion of dural nerve fibres (von Düring *et al.*, 1990; Messlinger *et al.*, 1993) where it may largely be co-localized with CGRP (O'Connor & van der Kooy, 1988), possibly in the same vesicles (Gulbenkian *et al.*, 1986). Substance P has been shown in different species (man, guinea-pig, cat) to act as a powerful vasodilator on isolated intracranial arteries including the middle meningeal artery *in vitro* (Jansen *et al.*, 1991; 1992) and on cerebral arterioles of the cat *in situ* (Jansen *et al.*, 1991). In our preparation, however, recent experiments have revealed that meningeal blood flow is not significantly changed by topical administration of substance P and the substance P analogue septide at concentrations of up to 10^{-3} M (unpublished results). These results may indicate different mechanisms of the two vasodilator neuropeptides: CGRP may act directly on the smooth muscle cells of arterial vessels, whereas the vasodilator effect of substance P may require the participation of the endothelium (Enokibori *et al.*, 1994) that is less accessible to topically applied peptides. Additionally, species-specific differences in the effectiveness of substance P may contribute to these discrepancies.

Sympathetic and parasympathetic contribution to blood flow response

It may be argued that electrical stimulation of the dural surface also activates other than afferent fibres, since the dura mater encephali of the rat is richly innervated by sympathetic nerve fibres (Keller *et al.*, 1989) and shows additionally some cholinergic fibres indicative of a parasympathetic innervation (Amenta *et al.*, 1980). Stimulation of the sympathetic nerves would probably decrease the dural blood flow by releasing catecholamines which would cause constriction of blood vessels. In fact, in some of our experiments a blood flow decrease was seen under stimulation. These phenomena may be interpreted by assuming opposite effects on the blood flow caused by vasoconstrictor catecholamines and vasodilator neuropeptides. It is likely that α-adrenoceptors were involved in the decreasing blood flow responses, since these effects could be abolished by phentolamine. It is further possible that in all other experiments

vasoconstrictor effects were present but hidden by the dominant blood flow increase. Assuming that h-CGRP$_{8-37}$ does not interfere with the catecholaminergic system, these effects probably did not alter our measurements that were based on the comparison before and after application of the antagonist.

Stimulation of the postganglionic parasympathetic fibres originating in the sphenopalatine ganglion has been shown to increase cortical blood flow (Suzuki *et al.*, 1990b). It was suggested that this increase was mediated by vasoactive intestinal polypeptide (VIP) which is co-localized in cholinergic nerve fibres. Since the dura mater also shows a sparse VIP-immunopositive innervation (Keller & Marfurt, 1991), it is possible that a small part of the increase of blood flow that persisted after the application of h-CGRP$_{8-37}$ was induced by VIP.

Selectivity of stimulation

Stimulation of the dural surface was used to activate selectively afferents of the dura mater. We know from immunohistochemical studies (Messlinger *et al.*, 1993) that many CGRP-immunoreactive nerve fibres which originate from dural nerves that accompany the MMA are likely to cross the stimulation site. On the other hand, it is unavoidable that at this site afferents are activated that innervate other than dural structures, e.g. the sagittal sinus, and these nerve fibres may have collaterals along the pial venous vessels extending down to the cortex. Electrical stimulation of the superior sagittal sinus of the cat has been shown to produce increases in cerebral blood flow (Lambert *et al.*, 1988). Thus activation of deeper structures, especially nerve fibres of pial vessels, cannot be excluded by our method. Other investigators stimulated the trigeminal ganglion to activate meningeal afferents antidromically and elicit neurogenically mediated plasma extravasation from dural blood vessels (Markowitz *et al.*, 1987; Dimitriadou *et al.*, 1991; Shepheard *et al.*, 1993). Since the trigeminal ganglion of different mammals gives rise to a dense CGRP peptidergic innervation of cerebral vessels (McCulloch *et al.*, 1986; Tsai *et al.*, 1988; Nozaki *et al.*, 1990) by pathways that do not project to the dura mater, e.g. through the nasociliary nerve (Suzuki *et al.*, 1989), stimulation of the trigeminal ganglion may be less appropriate for a selective activation of dural afferents than the method used in this study.

Selectivity of measurement

Suzuki *et al.* (1990a) showed that stimulation of the nasociliary nerve increases the cortical blood flow in the cat. In a recent study this increase could be inhibited by h-CGRP$_{8-37}$ demonstrating that CGRP contributes to this response (Goadsby, 1993). Our study suggests that a similar mechanism exists in the dura mater. Isolation of the dura mater as well as coagulation of the MMA showed that the blood flow registered in our experiments consisted mainly of dural blood flow.

Relevance of CGRP-dependent increase of meningeal blood flow during migraine attack

Several studies during the last decade using different methods have confirmed that migraine (migraine with aura and less consistently also migraine without aura) is accompanied by significant changes of the cerebral blood flow (see Friberg, 1991; Olesen, 1991 for recent overviews). Precise measurements with a sufficient time resolution showed that hypoperfusion is changed into hyperperfusion during the headache phase of migraine (Olesen *et al.*, 1990), probably due to the dilatation of large cerebral arteries (Friberg *et al.*, 1991). Thus both the migraine headache and the increased blood flow may depend on the same mechanisms. Results from several studies point to a neurogenic mechanism involved in migraine pain. A neurogenic inflammation of the meninges was proposed by Moskowitz and coworkers for the development of migraine headache (Moskowitz, 1984; 1993). This hypothesis was strengthened by several *in vitro* and *in vivo* experiments in which they measured plasma exudation and pathological changes of blood vessels and mast cells in the dura mater following antidromic stimulation of trigeminal afferents in the rat (Markowitz *et al.*, 1987; Dimitriadou *et al.*, 1991; 1992). Second, during migraine attacks increased levels of CGRP were found in the jugular vein of the affected side (Goadsby *et al.*, 1990). In analogy to this, an outflow of CGRP in the jugular vein of the cat (Goadsby *et al.*, 1988) and in the superior sagittal sinus of the rat (Buzzi *et al.*, 1991) could be measured after electrical stimulation of the trigeminal ganglion. These experiments suggest that the increase of cerebral blood flow during migraine attacks is at least partially due to a vasodilator effect of CGRP probably released from trigeminal fibres that innervate intracranial vessels. The results of the present study are consistent with this hypothesis. We conclude that the release and vasodilator action of CGRP following afferent stimulation take place in the dura mater as well as in pial structures increasing both cortical and meningeal blood flow. These processes may also occur in man during migraine headache.

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Assessment of renal dopaminergic system activity in the nitric oxide-deprived hypertensive rat model

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1 The present paper reports changes in the urinary excretion of dopamine, 5-hydroxytryptamine and amine metabolites in nitric oxide deprived hypertensive rats during long-term administration of N^G-nitro-L-arginine methyl ester (L-NAME). Aromatic L-amino acid decarboxylase (AAAD) activity in renal tissues and the ability of newly-formed dopamine to leave the cellular compartment where the synthesis of the amine has occurred were also determined.

2 Twenty four hours after exposure to L-NAME, both systolic (SBP) and diastolic (DBP) blood pressure were increased by 20 mmHg; heart rate was slightly decreased. During the next 13 days both SBP and DBP increased progressively reaching 170 ± 3 and 116 ± 3 mmHg, respectively.

3 Baseline urinary excretion of L-DOPA, dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT) and homovanillic acid (HVA) during the 4 day period of stabilization averaged 4.4 ± 0.5, 13.8 ± 0.3, 37.4 ± 0.8, 180.0 ± 2.7 and 206.1 ± 6.7 nmol day^{-1}, respectively. The urinary excretion of L-DOPA, dopamine and DOPAC, but not that of 3-MT and HVA, were increased from day 6-8 of L-NAME administration onwards (L-DOPA, up to 13.4 ± 2.1; dopamine, up to 23.0 ± 1.6; DOPAC, up to 62.8 ± 3.7 nmol day^{-1}). Baseline daily urinary excretion of 5-hydroxytryptamine and 5-hydroxyindolacetic acid (5-HIAA) averaged 73.5 ± 1.1 and 241.7 ± 5.4 nmol day^{-1}, respectively. During the first week of L-NAME administration, the urinary excretion of both 5-hydroxytryptamine and 5-HIAA did not change significantly; however, as was found with dopamine and DOPAC, changes in the urinary excretion of 5-hydroxytryptamine were evident during the second week of L-NAME administration.

4 In experiments performed on homogenates of isolated renal tubules, the decarboxylation of L-DOPA to dopamine was dependent on the concentration of L-DOPA used (10 to 5000 μM) and saturable at 1000 μM. AAAD activity as determined in homogenates (V_{max}, in nmol mg^{-1} protein h^{-1}; K_m in μM) was significantly ($P<0.01$) higher in rats given L-NAME for 14 days ($V_{max} = 25 \pm 2$; $K_m = 72 \pm 10$) than in control rats ($V_{max} = 14 \pm 1$; $K_m = 63 \pm 7$), rats given L-NAME for 7 days ($V_{max} = 15 \pm 1$; $K_m = 69 \pm 5$) and rats given L-NAME plus L-arginine ($V_{max} = 13 \pm 1$; $K_m = 60 \pm 3$) for 14 days.

5 A considerable amount of the total dopamine formed from added L-DOPA in kidney slices escaped into the incubation medium. The application of the Michaelis-Menten equation to the net transport of newly-formed dopamine allowed the identification of a saturable (carrier-mediated transfer) and a non-saturable component (diffusion). No significant differences in the diffusional rate of transfer (0.14 ± 0.02 μmol^{-1}) were observed between the four experimental groups. However, the saturable outward transfer of dopamine (V_{max}, in μmol mg^{-1} protein h^{-1}; K_m in μM) was higher in control animals ($V_{max} = 2.3 \pm 0.2$; $K_m = 568 \pm 67$) than that in rats treated with L-NAME for 14 days ($V_{max} = 0.8 \pm 0.02$; $K_m = 241 \pm 21$), but similar to that observed in rats receiving L-NAME plus L-arginine ($V_{max} = 2.4 \pm 0.2$; $K_m = 618 \pm 61$); the saturable dopamine outward rate of transfer in rats given L-NAME for 7 days ($V_{max} = 3.9 \pm 0.2$; $K_m = 1006 \pm 32$) was higher than in controls.

6 In conclusion, the present studies show that the hypertensive response resulting from the long-term administration of L-NAME is accompanied by an increased urinary excretion of dopamine and 5-hydroxytryptamine, which appears to follow an enhanced activity of renal AAAD. The observation that the increased AAAD activity can be reversed by the administration of L-arginine to L-NAME-treated rats favours the view that the adaptational response which results in an enhanced AAAD activity probably involves a decrease in the generation of nitric oxide.

Keywords: N^G-nitro-L-arginine methyl ester (L-NAME); hypertension; L-arginine; dopamine; L-DOPA; 5-hydroxytryptamine; kidney; aromatic L-amino acid decarboxylase

Introduction

The vascular endothelium is capable of modulating vascular smooth vascular tone, in part, by the generation of a diffusible and transferable substance, termed the endothelium-derived relaxing factor (EDRF), which activates soluble guanylate cyclase and increases levels of guanosine 3' : 5'-cyclic monophosphate (cyclic GMP) in the underlying smooth muscle cells to initiate vasorelaxation. Nitric oxide (NO) which is believed to account for the actions of the EDRF (Palmer *et al.*, 1987; Ignarro *et al.*, 1987) originates from the oxidation of one of the two equivalent guanidino nitrogen atoms of L-arginine by the action of a constitutive Ca^{2+}-dependent NO synthase (Palmer *et al.*, 1988; Sakuma *et al.*, 1988; Schmidt *et al.*, 1988). Analogues of L-arginine, such as N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-monomethyl-L-arginine (L-NMMA), produce competitive inhibition of the NO synthase and abolish NO-mediated events. Systemic administra-

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tion of L-arginine analogues, to inhibit the generation of NO, has been found to produce dose-dependent vasoconstriction of several vascular beds (including the mesenteric and renal beds) and an increase in systemic blood pressure (Aisaka *et al.*, 1989; Rees *et al.*, 1989; Vallance *et al.*, 1989; Whittle *et al.*, 1989). The hypertensive effect of systemic NO inhibition is accompanied by changes in renal function, namely decrements in renal blood flow, glomerular filtration rate and urine Na^+ excretion (Baylis *et al.*, 1990; Salazar *et al.*, 1992). However, dependent on the degree of NO synthase inhibition and magnitude of the hypertensive response, changes in renal perfusion pressure via a pressure natriuresis phenomenon can cause diverse effects on sodium excretion (Johnson & Freeman, 1992; Nakamura *et al.*, 1993). Because inhibition of NO formation appears to produce a sequence of events resembling the characteristics of essential volume-dependent hypertension (Romero *et al.*, 1992), it is possible that sustained inhibition of NO formation might provide additional information on the pathophysiology of this disorder.

There is evidence to suggest that some hypertensive subjects, namely in salt-sensitive forms of hypertension, might have a fault in renal dopamine production and this may also be associated with salt sensitivity of their blood pressure (for a review see Lee, 1993). In agreement with this view is also the fact that renal dopamine receptors might be upregulated in hypertensive patients (Lee, 1993). In the kidney, most of the dopamine has been demonstrated to have its origin in epithelial cells of renal tubules, namely those of the proximal portion of the nephron (Hayashi *et al.*, 1990). Because the dopamine produced in this area is in close proximity to renal cells which contain receptors for the amine, it has been hypothetized that the amine may act as a paracrine or autocrine substance (Siragy *et al.*, 1989). Early studies have demonstrated that the renal tubular transport of L-DOPA occurs through an energy-dependent and stereoselective carrier-mediated process and the synthesis of dopamine in tubular epithelial cells is regulated probably by the renal delivery of Na^+ (Soares-da-Silva & Fernandes, 1990). In both human and rat kidney preparations the production of dopamine is closely dependent on the extracellular Na^+, is related to the trans-tubular reabsorption of Na^+ (Soares-da-Silva *et al.*, 1993a,b) and dependent on the integrity of the tubular cytoskeleton and the functional integrity of the Na^+-K^+ATPase (Soares-da-Silva, 1992; 1993a). The rise of intracellular levels of cyclic GMP, as induced by the α-human atrial natriuretic peptide, by Zaprinast (MB 22,948) a relatively specific cyclic GMP phosphodiesterase inhibitor, or by 8-bromo cyclic GMP, have also been found to restrict the intracellular availability of L-DOPA and decrease the renal formation of dopamine (Soares-da-Silva & Fernandes, 1991; 1992). Despite the evidence for an association between the tubular reabsorption of Na^+, the levels of cyclic GMP and the uptake of L-DOPA, information is lacking upon the influence of NO on the renal formation of dopamine. However, inhibition of NO formation has been reported to enhance tubular reabsorption of Na^+ in the proximal nephron (Nakamura *et al.*, 1993).

The aim of the present work was to evaluate the activity of the renal dopaminergic system in the NO-deprived hypertensive rat model. This study describes the changes in the urinary excretion of dopamine and amine metabolites [3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT) and homovanillic acid (HVA)] in NO-deprived hypertensive rats during the oral consumption of L-NAME in the drinking water for 14 consecutive days; long-term oral administration of analogues of L-arginine has been shown to induce a dose- and time-dependent systemic hypertension (Baylis *et al.*, 1992; Arnal *et al.*, 1992; Ribeiro *et al.*, 1992; Dananberg *et al.*, 1993; Manning *et al.*, 1993). In the present work we have also determined the daily urinary excretion of 5-hydroxytryptamine and of its deaminated metabolite, 5-hydroxyindolacetic acid (5-HIAA), noradrenaline and adrenaline. In contrast to dopamine, 5-hydroxytyptamine has been found to increase the tubular reabsorption of Na^+ (Itskovitz *et al.*, 1988). However, both amines share a common synthetic pathway, the AAAD rich epithelial cells of the proximal convoluted tubules being also involved in the uptake of L-5-hydroxytryptophan and its decarboxylation into 5-hydroxytryptamine. Levels of noradrenaline and adrenaline in the urine are thought to reflect the activation of the sympathetic nervous system and the adrenal medulla, respectively. Aromatic L-amino acid decarboxylase (AAAD) activity in renal tissues and the ability of newly-formed dopamine to leave the cellular compartment where the synthesis of the amine has occurred were also determined. A preliminary account of these findings was presented by Soares-da-Silva *et al.* (1994).

Methods

In vivo studies

Normotensive male Wistar rats (Biotério do Instituto Gulbenkian de Ciência, Oeiras, Portugal) 30–45 days old and weighing 180–200 g were selected after a 7 day period of stabilization and following adaptation to blood pressure measurements. Animals ($n = 8$) were kept in metabolic cages under controlled environmental conditions (12 h light/dark cycle and room temperature 24°C); water intake and food consumption was monitored daily throughout the study. Twenty four hour urine was collected in vials containing 1 ml 6 M HCl to prevent spontaneous decomposition of monoamines and amine metabolites. Four rats were given L-NAME (50 μg ml^{-1}) in drinking water for 14 consecutive days (from LN1 to LN14) and blood pressure measurements performed every day. Control rats ($n = 4$) were given tap water and followed also during 14 days. In both groups of rats baseline measurements were performed in the last four days of the stabilization period. Blood pressure (systolic, SBP; diastolic, DBP) and heart rate were measured in conscious restrained animals, between 07 h 00 min to 10 h 00 min, with a photoelectric tail cuff pulse detector (LE 5000, Letica, Barcelona, Spain). On several occasions, blood pressure measurements were also performed between 18 h 00 min and 19 h 00 min; these blood pressure measurements were made late in the afternoon because L-NAME intake occurred mainly during the night. Three determinations were made each time and the means used for further calculations. After completion of these studies, L-NAME-treated and control rats were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.), blood from the renal vein was collected and the kidneys were rapidly removed through an abdominal midline incision. Samples of the renal cortex were dissected free with fine scissors, rinsed in cold saline (0.9% NaCl), weighed, placed in 2 ml perchloric acid (0.2 M) and stored frozen for a later determination of catecholamines. The blood was collected in iced tubes containing either heparin or EDTA for later determination of plasma catecholamines or plasma renin activity (PRA) and plasma aldosterone concentration (PAC), respectively.

In vitro *studies*

Renal tissues used in experiments carried out under *in vitro* conditions were obtained from three different experimental groups of rats: (1) animals given for 7 consecutive days L-NAME (50 μg ml^{-1} in drinking water; $n = 4$); (2) animals given for 14 consecutive days L-NAME (50 μg ml^{-1} in drinking water; $n = 6$) and (3) animals given for 14 consecutive days L-NAME (50 μg ml^{-1}) plus L-arginine (1.0 mg ml^{-1}) in drinking water ($n = 5$). Non-treated age-matched rats ($n = 6$) were used as controls. Rats in all groups had blood pressure and heart rate measurements performed every 2 days, the procedure being similar to that described below. Animals were kept two per cage under controlled environmental con-

ditions (12 h light/dark cycle and room temperature 24°C). Food and tap water were allowed *ad libitum* and experiments were all performed during daylight hours. Animals were killed by decapitation under ether anaesthesia and the kidneys removed through a midline abdominal incision, after which they were decapsulated and cut in half, placed in ice-cold saline (0.9% NaCl) and the renal medulla dissected free with fine scissors.

Homogenates of renal tubules were used in experiments designed to study the decarboxylation of L-DOPA. The preparation of renal tubules was based on the techniques described by Shah *et al.* (1979) and Bloch *et al.* (1992) with minor modifications. Kidneys were decapsulated, cut in half and placed in ice-cold Collins solution, containing (mM): KH_2PO_4 15, K_2HPO_4 50, KCl 15, $NaHCO_3$ 15, $MgSO_4$ 60 and glucose 140, PH = 7.4; this is an intracellular-like solution that does not contain calcium. Such solutions have been successfully used to preserve renal tissue for transplantation and for the preparation of proximal nephron segments. Thereafter, the outer cortex was cut out with fine scissors and minced with a scalpel into a fine paste. The cortical paste was then filtered sequentially through 180 μm and then 75 μm Nybolt nylon sieves. Unseparated cortex remained on the upper (180 μm) sieve, while the lower (75 μm) one retained predominantly cortical renal tubules. The sieves were continuously rinsed with cold Collins solution throughout the procedure. The viability of renal tubules used in this study was assessed by the trypan blue (0.2% wt/vol) exclusion method; more than 90% of the renal tubules excluded the dye. Most of the tissue fragments (>80%) were proximal convoluted tubules. The tubules were then washed off with cold Collins solution, collected into a pellet by centrifugation (200 *g*, 2 min, 4°C) and homogenized in a modified Krebs solution with Thomas Teflon homogenizers and kept continuously on ice. Aliquots of 250 μl of tubular homogenate plus 250 μl incubation medium were placed in glass test tubes and incubated for 30 min. Thereafter, L-DOPA (10 to 5,000 μM) was added to the medium for further 15 min; the final reaction volume was 1 ml. The composition of the incubation medium was as follows (mM): NaCl 50, choline chloride 70, KCl 4.7, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, EDTA 0.4, ascorbic acid 0.57 and glucose 11; 1-α-methyl-*p*-tyrosine (50 μM), pargyline (100 μM) tropolone (50 μM) were also added to the Hanks medium in order to inhibit the enzymes tyrosine hydroxylase, catechol-*O*-methyltransferase (COMT) and monoamine oxidase (MAO), respectively. The pH of the reaction medium was kept constant at 74. During incubation, homogenates of renal tubules were continuously shaken and gassed (95% O_2 and 5% CO_2) and maintained at 37°C. The reaction was stopped by the addition of 500 μl of 2 M perchloric acid and the preparations kept at 4°C for 60 min. The samples were then centrifuged (200 *g*, 2 min, 4°C) and 500 μl aliquots of the supernatant filtered on Millipore microfilters (MFl) were used for the assay of dopamine. K_m and V_{max} values for AAAD activity were calculated from linear regression analysis; the substrate decarboxylated (corresponding to the amount of dopamine formed) per mg of protein per hour of incubation was plotted against the ratio between the amount of the total dopamine formed and the substrate concentration (V against VS^{-1}).

The experiments performed with the aim of determining the kinetic parameters of an outward transport mechanism for dopamine were conducted in slices of renal cortex. Tissue slices approximately 0.5 mm thick and weighing 20 to 30 mg wet weight were prepared with a scalpel and preincubated for 30 min in 2 ml warm (37°C) and gassed (95% O_2 and 5% CO_2) Krebs solution. The Krebs solution had the following composition (mM): NaCl 120, KCl 4.7, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, EDTA 0.4, ascorbic acid 0.57 and glucose 11; 1-α-methyl-*p*-tyrosine 50 μM, pargyline (0.1 mM) and tropolone 50 μM, were also added to the Krebs solution in order to inhibit the enzymes tyrosine hydroxylase, monoamine oxidase and catechol-*O*-methyltransferase, respectively. After preincubation, renal slices were incubated for 15 min in Krebs solution with increasing concentrations (5 to 5000 μM) of L-DOPA for 15 min and the amount of newly-formed dopamine accumulated in the tissue and that which had escaped into the incubation medium determined; up to a concentration of 5000 μM L-DOPA, the pH of the incubation medium remained stable at 7.4. The preincubation and incubation were carried out in glass test tubes, continuously shaken throughout the experiment. After the incubation, renal slices were collected with fine forceps and immediately placed in 2 ml of 0.2 M perchloric acid at 4°C for the next 24 h. In order to allow the determination of the amount of dopamine which had leaked into the incubation medium, 250 μl of 2.0 M perchloric acid was added to the incubation medium after renal tissues had been collected; the samples of the incubation medium were also kept at 4°C for the next 24 h, until quantification of dopamine. In these experimental conditions, the movement of newly-formed dopamine is expected to be mainly from the intracellular to the extracellular medium; though it may be hypothesized that the net transfer of dopamine would involve a carrier-mediated transport and diffusion may also contribute to total amine transfer. The determination of carrier transport and diffusion in this experimental model was performed as defined by Neame & Richards (1976) with the use of the expression

$$\underset{\text{(carrier transport)}}{(V_{max}\cdot[S]/[S] + K_m)} + \underset{\text{(diffusion)}}{(K_d\cdot[S])}$$

where [S] is the concentration of the substrate to be transported and K_d the diffusion constant. K_m and V_{max} values for the outward transport of newly-formed dopamine were calculated from linear regression analysis; the substrate transported (nmol of dopamine present in the incubation medium) was plotted against the ratio between the substrate transported by the amount of the total dopamine formed (obtained from the algebraic sum of the amount accumulated in the tissue plus that present in the incubation medium). The fractional outflow of newly-formed dopamine was obtained by dividing the amount of the amine in the incubation fluid by the amount of the total dopamine formed.

The assay of L-DOPA, monoamines (dopamine, noradrenaline, adrenaline and 5-hydroxytryptamine) and amine metabolites (3,4,-dihydroxyphenylgylcol [DHPG], DOPAC, 3-MT, HVA and 5-HIAA) in urine and plasma samples, renal tissues and samples of the incubation medium and of kidney homogenates was performed by means of h.p.l.c. with electrochemical detection, as previously described (Soares-da-Silva *et al.*, 1993a). In brief, aliquots of 0.5 ml of acidified urine, 0.5 ml plasma, 1.5 ml perchloric acid in which tissues had been kept, 0.5 ml of acidified incubation medium or 1.5 ml acidified tubular homogenates were placed in 5 ml conical-based glass vials with 50 mg alumina and the pH of the samples adjusted to pH 8.6 by the addition of Tris buffer. The absorbed catecholamines were then eluted from the alumina with 200 μl of 0.2 M perchloric acid on Millipore microfilters (MF1); 50 μl of the eluate was injected into a high pressure liquid chromatograph (Gilson Medical Electronics, Villiers le Bel, France). For the quantification of 3-MT, HVA, 5-hydroxytryptamine and 5-HIAA in urine samples, 50 μl of urine was diluted to 500 μl with perchloric acid, the sample filtered on Millipore microfilters (MF1) and directly injected into the chromatograph. The lower limit for detection of L-DOPA, dopamine, adrenaline, noradrenaline, 5-hydroxytryptamine, DHPG, DOPAC, 3-MT, HVA and 5-HIAA ranged from 350 to 1000 fmol. Urinary Na^+ and K^+ were measured by flame photometry (model FML3) connected to a diluter (model A 6241) (Radiometer, Copenhagen, Denmark) and urine and plasma osmolality by means of an osmometer (Advanced Instruments, Inc., MA, U.S.A, model 3MO). Urinary phosphate and urinary and plasma creatinine were measured by a wave-length photometer

(Hitashi Automatic Analyzer, model 717, or a Beckman Analyzer II). The assay of PRA and PAC was performed by radioimmunoassay (RIA) kits of clinical assays RENTCK and ALDOCTK-2 (Sorin Biomedica, Saluggia, Italy), respectively. The protein content of the suspensions of renal tubules was determined by the method of Bradford (1976), with bovine serum albumin as a standard.

Results are means ± s.e.mean of values for the indicated number of determinations. Within-group analysis of data was performed by Friedman's test comparing all values to the baseline state or by the Student's t test for unpaired data comparing values of interest to the corresponding values in non-treated rats. Significance of difference between one control and several experimental groups was evaluated by the Tuckey-Kramer method. A P value less than 0.05 was assumed to denote a significant difference.

L-Arginine hydrochloride, L-dihydroxyphenylalanine, dopamine hydrochloride, adrenaline hydrochloride, homovanillic acid, 5-hydroxyindolacetic acid, 5-hydroxytryptamine creatinine sulphate, 3-methoxytyramine (3-MT), N^G-nitro-L-arginine methyl ester hydrochloride, noradrenaline bitartrate, pargyline hydrochloride and tropolone hydrochloride were obtained from the Sigma Chemical Company (St. Louis, Mo, U.S.A.).

Results

In vivo *studies*

Long-term inhibition of NO synthase by administration of L-NAME (~ 10 mg kg^{-1}) resulted in a persistent hyperten-

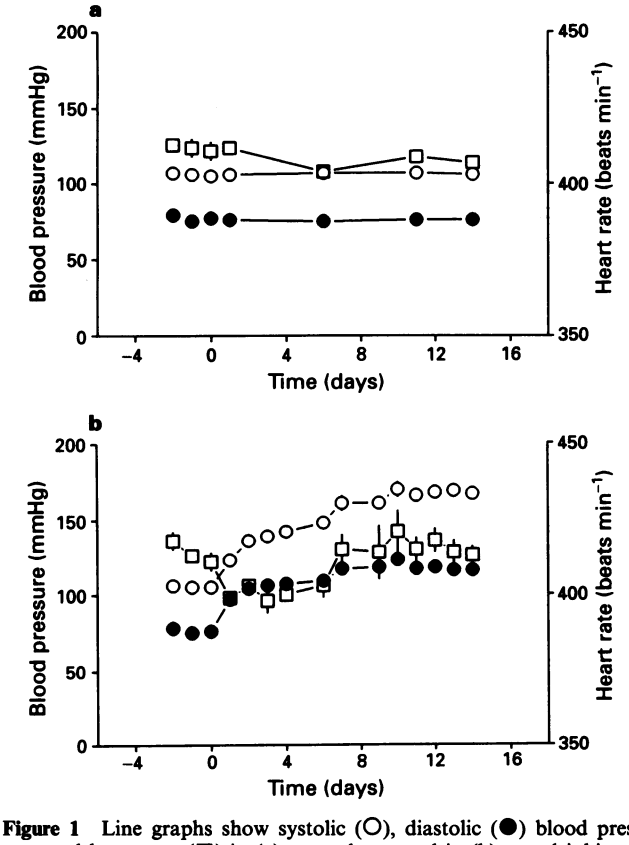

Figure 1 Line graphs show systolic (○), diastolic (●) blood pressure and heart rate (□) in (a) control rats and in (b) rats drinking a solution of N^G-nitro-L-arginine methyl ester (L-NAME; 50 μg ml^{-1}) during the baseline period (from day −4 till day 0) and during the administration of a solution of L-NAME (from day 1 till day 14). Each point represents the mean (with s.e.mean) of 4 determinations. All values corresponding to L-NAME treatment are significantly different from corresponding baseline values and from corresponding values in controls ($P<0.01$).

Table 1 Changes in water intake (ml day^{-1}), urine volume (ml day^{-1}), urinary sodium (mmol day^{-1}), potassium (mmol day^{-1}), phosphate (mg day^{-1}) and creatinine (mg day^{-1}) excretion and urine osmolality (mOsm kg^{-1} H_2O day^{-1}) in rats drinking water (Control; $n=4$) or a solution of N^G-nitro-L-arginine methyl ester (L-NAME; 50 μg ml^{-1}) ($n=4$)

	Water intake		*Urine volume*		*Urinary sodium*		*Urinary potassium*		*Urinary phosphate*		*Urinary creatinine*		*Urine osmolality*	
Day	*Control*	*L-NAME*	*Control*	*L-NAME*	*Control*	*L-NAME*	*Control*	*L-NAME*	*Control*	*L-NAME*	*Control*	*L-NAME*	*Control*	*L-NAME*
B1	42.8 ± 0.9	42.3 ± 0.8	21.4 ± 0.4	17.0 ± 1.3	1.1 ± 0.1	1.2 ± 0.1	4.1 ± 0.1	4.1 ± 0.6	4.0 ± 0.1	5.6 ± 0.5	7.9 ± 0.2	8.0 ± 0.1	1119 ± 28	1311 ± 87
B2	45.8 ± 2.0	37.5 ± 0.7	22.9 ± 1.0	17.0 ± 1.0	1.1 ± 0.1	1.2 ± 0.1	4.4 ± 0.2	3.7 ± 0.4	4.3 ± 0.2	5.7 ± 0.4	8.5 ± 0.2	8.5 ± 0.3	1091 ± 47	1276 ± 95
B3	46.0 ± 0.8	47.5 ± 2.4	23.0 ± 0.4	27.0 ± 2.2	1.2 ± 0.1	1.4 ± 0.1	4.4 ± 0.1	4.9 ± 0.6	4.3 ± 0.1	6.4 ± 1.1	8.5 ± 0.1	8.6 ± 0.1	1095 ± 18	979 ± 52
B4	45.7 ± 0.7	43.8 ± 3.0	22.8 ± 0.4	25.5 ± 2.4	1.1 ± 0.1	1.4 ± 0.1	4.3 ± 0.1	4.6 ± 0.4	4.3 ± 0.1	3.7 ± 0.4	8.9 ± 0.1	8.5 ± 0.1	1087 ± 17	1081 ± 88
LN1	46.2 ± 1.5	42.5 ± 3.9	23.1 ± 0.7	24.5 ± 3.2	1.2 ± 0.1	1.1 ± 0.1	4.4 ± 0.1	4.2 ± 0.2	4.3 ± 0.1	4.5 ± 0.4	8.6 ± 0.2	8.6 ± 0.2	1099 ± 36	1073 ± 129
LN2	47.5 ± 1.7	38.8 ± 2.8	23.8 ± 0.8	31.5 ± 3.1	1.1 ± 0.1	0.8 ± 0.1	4.5 ± 0.2	3.7 ± 0.4	4.5 ± 0.2	11.3 ± 0.9*	8.5 ± 0.3	8.8 ± 0.3	1131 ± 40	800 ± 51*
LN3	46.8 ± 1.4	52.5 ± 4.6	23.4 ± 0.7	28.5 ± 3.8	1.2 ± 0.1	1.1 ± 0.1	4.4 ± 0.1	6.0 ± 1.4	4.4 ± 0.1	4.6 ± 0.5	8.4 ± 0.3	8.7 ± 0.2	1115 ± 33	980 ± 121
LN4	46.8 ± 2.0	54.0 ± 2.9	23.4 ± 1.0	37.0 ± 2.5	1.2 ± 0.1	1.3 ± 0.2	4.4 ± 0.2	5.3 ± 0.7	4.4 ± 0.2	2.1 ± 0.4	8.8 ± 0.6	8.7 ± 0.3	1115 ± 47	762 ± 70*
LN5	46.2 ± 1.5	44.5 ± 3.4	23.1 ± 0.7	29.0 ± 3.0	1.2 ± 0.1	1.0 ± 0.2	4.4 ± 0.1	5.5 ± 1.6	4.3 ± 0.1	8.0 ± 1.4	8.4 ± 0.5	8.6 ± 0.2	1099 ± 36	938 ± 86*
LN6	48.3 ± 1.4	58.8 ± 4.5	24.2 ± 0.7	41.0 ± 4.6*	1.2 ± 0.1	1.2 ± 0.2	4.6 ± 0.1	5.3 ± 1.4	4.5 ± 0.1	6.7 ± 1.4	8.6 ± 0.2	8.0 ± 0.2	1150 ± 34	640 ± 64*
LN7	50.5 ± 2.0	73.0 ± 4.7*	25.3 ± 1.0	52.5 ± 4.1*	1.3 ± 0.1	1.8 ± 0.2	4.8 ± 0.2	4.8 ± 0.9	4.7 ± 0.2	2.8 ± 0.5	8.6 ± 0.3	8.3 ± 0.3	1102 ± 47	478 ± 41*
LN8	52.8 ± 1.5	86.3 ± 5.3*	26.4 ± 0.8	62.0 ± 5.6*	1.3 ± 0.1	1.7 ± 0.3	5.0 ± 0.1	3.8 ± 0.1	5.0 ± 0.1	4.8 ± 0.8	8.2 ± 0.3	8.7 ± 0.3	1157 ± 37	451 ± 70*
LN9	51.5 ± 1.9	75.0 ± 4.9*	25.8 ± 1.0	52.5 ± 4.3*	1.3 ± 0.1	1.5 ± 0.1	4.9 ± 0.2	8.7 ± 2.0	4.8 ± 0.2	8.6 ± 1.6	9.0 ± 0.3	8.5 ± 0.3	1125 ± 47	496 ± 57*
LN10	52.0 ± 0.9	77.5 ± 3.9*	26.0 ± 0.4	55.5 ± 3.7*	1.3 ± 0.1	1.6 ± 0.1	4.9 ± 0.1	4.0 ± 0.2	4.9 ± 0.1	2.1 ± 0.4*	8.4 ± 0.4	8.6 ± 0.1	1138 ± 21	410 ± 31*
LN11	51.5 ± 1.2	75.5 ± 4.8*	25.8 ± 0.6	56.3 ± 4.0*	1.3 ± 0.1	1.2 ± 0.1	4.9 ± 0.1	3.7 ± 0.4	4.8 ± 0.1	1.8 ± 0.5*	10.7 ± 0.8	8.5 ± 0.2	1127 ± 29	414 ± 44*
LN12	51.5 ± 1.8	77.5 ± 5.3*	25.8 ± 0.9	59.5 ± 5.1*	1.3 ± 0.1	1.7 ± 0.1	4.9 ± 0.2	3.5 ± 0.1	4.8 ± 0.2	2.7 ± 0.4*	7.9 ± 0.3	8.5 ± 0.3	1127 ± 43	424 ± 54*
LN13	51.2 ± 1.4	62.5 ± 6.3	25.6 ± 0.7	43.5 ± 4.6*	1.3 ± 0.1	1.5 ± 0.1	4.9 ± 0.1	3.7 ± 0.2	4.8 ± 0.1	1.4 ± 0.2*	8.4 ± 0.2	8.4 ± 0.2	1219 ± 53	623 ± 63*
LN14	51.2 ± 1.5	55.0 ± 7.6	25.6 ± 0.7	40.0 ± 6.1	1.1 ± 0.1	0.7 ± 0.1	4.9 ± 0.1	4.0 ± 0.1	4.8 ± 0.1	4.0 ± 0.9	7.5 ± 0.3	8.4 ± 0.2	1118 ± 35	717 ± 77*

Days B1 to B4 represent baseline period and days LN1 to LN14 represent the period during which L-NAME was given.
Values are means ± s.e.mean of the indicated number of animals.
Significantly different from corresponding control values (*$P<0.02$).

sion that reached a maximum level by day 10. Average baseline values of systolic (SBP) and diastolic (DBP) blood pressure and heart rate were 111 ± 2 mmHg, 84 ± 2 mmHg and 412 ± 1 beats min^{-1}, respectively. Twenty four hours after exposure to L-NAME, both SBP (134 ± 4 mmHg) and DBP (101 ± 3 mmHg) were increased by approximately 20 mmHg; heart rate was found to decrease from 412 ± 2 to 404 ± 2 beats min^{-1} ($P<0.02$). As shown in Figure 1b, both SBP and DBP during the first week of L-NAME administration were increased by 39% and 36%, respectively, which was even more marked by the end of the second week of treatment, reaching 173 ± 8 and 117 ± 7 mmHg, respectively. The initial decrease in heart rate observed during the first week of administration of L-NAME was no longer observed during the second week of the study; the average heart rate during the second week of L-NAME administration was 408 ± 1 beats min^{-1}. Blood pressure and heart rate measurements performed between 18 h 00 min and 19 h 00 min were similar to those obtained in the corresponding morning. As shown in Figure 1a, SBP (107 ± 2 mmHg), DBP (79 ± 2 mmHg) and heart rate (413 ± 2 beats min^{-1}) in control rats remained stable throughout the 18-day period of evaluation. Baseline values of blood pressure and heart rate of rats given L-NAME, corresponding to the first four days of the study, were similar to those of control animals.

At the beginning of the study average body weight (in g) was 212.3 ± 10.1 ($n = 4$) and 202.5 ± 5.7 ($n = 4$), respectively in the group of rats that was going to receive L-NAME and in control rats. At the end of the first week of L-NAME administration, body weight increased up to 254.6 ± 14.3 g and 238.9 ± 13.2 g in the L-NAME-treated group and the control group, respectively. By the end of the second week of L-NAME administration, average body weight in L-NAME-treated rats (312.3 ± 23.5 g) was not different from that in control animals (304.2 ± 11.4 g).

Daily average water intake in L-NAME-treated rats during the first week of treatment slightly increased when compared to baseline values (42.9 ± 2.5 vs 52.0 ± 4.4 ml day^{-1}), but no significant difference was observed when compared with the values of corresponding control rats (Table 1). During the second week of treatment with L-NAME the daily average water intake was significantly greater than that observed in control animals. The amount of L-NAME consumed during the first week of treatment averaged 10.6 ± 0.9 mg kg^{-1} and increased ($P<0.05$) up to 13.0 ± 0.7 mg kg^{-1} during the second week of treatment.

Urinary excretion of Na^+, K^+ and creatinine remained unchanged throughout the two week period of L-NAME administration (Table 1). The urinary excretion of phosphate in L-NAME-treated rats was similar to that observed in controls rats with the exception of a four day period during the second week of treatment (LN10 to LN13) during which there was a significant decrease in phosphate excretion in the urine. Baseline urine volume, which averaged 21.6 ± 2.7 ml day^{-1}, increased up to 34.9 ± 3.6 and 52.8 ± 3.1 ml day^{-1} in the two subsequent weeks of L-NAME administration, respectively; urine volume in control rats remained stable throughout the study. As also shown in this table, the increase in urine volume was accompanied by an increase in liquid consumption and a decrease in urine osmolality (baseline daily average being 1162 ± 79 mOsm kg^{-1} H_2O).

In the group of rats that was going to receive L-NAME, baseline daily urinary excretion of L-DOPA, dopamine, DOPAC, 3-MT and HVA were similar to those observed in control rats (Table 2). When compared with the corresponding controls, the urinary excretion of L-DOPA, dopamine and DOPAC, but not that of 3-MT and HVA, in L-NAME-treated rats was found to be increased (L-DOPA, from 73% to 157%; dopamine, from 29% to 40% increase; DOPAC, from 14% to 40% increase) between days LN8 and LN13. Daily urinary excretion of 5-hydroxytryptamine in L-NAME-treated rats significantly increased between day LN11 and LN14 when compared with corresponding controls (Table 3). In contrast to DOPAC, 5-HIAA levels in the urine did not change throughout the study (Table 3). As shown in Table 3, the urinary excretion of noradrenaline and adrenaline in L-NAME-treated rats was significantly increased between days LN8 and LN14 when compared with the corresponding control values.

In Table 4 are shown the concentrations in plasma of L-DOPA, noradrenaline, adrenaline, DPHG, aldosterone, Na^+, K^+, urea, creatinine and PRA in a terminal blood sample from the renal vein of L-NAME-treated rats and control animals. As indicated in this table, L-DOPA, noradrenaline, adrenaline and DPHG concentrations in plasma were found to be increased in L-NAME-treated rats, whereas PRA and PAC were significantly reduced in L-NAME-treated rats, when compared to control animals. No

Table 2 Changes in urinary excretion (in nmol day^{-1}) of L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT) and homovanillic acid (HVA) in rats drinking water (Control; $n = 4$) or a solution of N^G-nitro-L-arginine methyl ester (L-NAME; 50 μg ml^{-1}) ($n = 4$)

	L-DOPA		*Dopamine*		*DOPAC*		*3-MT*		*HVA*	
Day	*Control*	*L-NAME*	*Control*	*L-NAME*	*Control*	*L-NAME*	*Control*	*L-NAME*	*Control*	*L-NAME*
B1	4.4 ± 0.1	3.8 ± 0.6	13.6 ± 0.3	13.7 ± 0.6	36.9 ± 0.8	36.8 ± 1.3	164.7 ± 3.4	171.9 ± 5.9	171.3 ± 3.5	187.9 ± 1.4
B2	4.7 ± 0.2	3.3 ± 0.5	14.6 ± 0.6	13.1 ± 0.6	39.5 ± 1.7	35.5 ± 0.9	176.3 ± 7.7	181.8 ± 5.7	183.3 ± 8.0	207.1 ± 5.1
B3	4.7 ± 0.1	5.5 ± 0.4	14.6 ± 0.2	14.4 ± 0.5	39.7 ± 0.7	39.2 ± 0.6	176.9 ± 3.0	183.2 ± 9.1	184.0 ± 3.1	208.8 ± 6.0
B4	4.7 ± 0.1	5.0 ± 0.5	14.5 ± 0.2	14.1 ± 0.6	39.4 ± 0.6	38.0 ± 0.5	175.6 ± 2.7	182.9 ± 6.4	182.7 ± 2.9	220.4 ± 2.2
LN1	4.7 ± 0.2	3.7 ± 0.9	14.7 ± 0.5	13.2 ± 0.7	39.8 ± 1.3	35.1 ± 1.1	177.6 ± 5.7	154.1 ± 10.6	184.7 ± 6.0	205.1 ± 6.7
LN2	4.8 ± 0.2	2.9 ± 0.1*	15.1 ± 0.5	12.7 ± 0.6	40.9 ± 1.5	38.6 ± 1.5	182.7 ± 6.5	168.9 ± 6.0	190.0 ± 6.8	203.4 ± 2.8
LN3	4.8 ± 0.1	5.8 ± 1.8	14.9 ± 0.4	14.6 ± 0.9	40.4 ± 1.2	40.4 ± 0.8	180.1 ± 5.4	162.8 ± 4.5	187.3 ± 5.6	202.8 ± 6.9
LN4	4.8 ± 0.2	2.8 ± 0.4	14.9 ± 0.6	15.5 ± 0.2	40.4 ± 1.7	39.2 ± 0.4	180.1 ± 7.5	193.1 ± 8.8	187.3 ± 7.8	194.4 ± 3.1
LN5	4.7 ± 0.2	4.4 ± 0.4	14.7 ± 0.5	14.8 ± 0.5	39.8 ± 1.3	32.7 ± 1.3	177.6 ± 5.7	179.2 ± 9.0	184.7 ± 6.0	186.6 ± 3.4
LN6	4.9 ± 0.1	12.6 ± 3.7*	15.4 ± 0.5	17.8 ± 0.9	41.7 ± 1.2	36.9 ± 1.7	185.9 ± 5.5	162.0 ± 4.3	193.3 ± 5.7	191.6 ± 0.5
LN7	5.1 ± 0.2	7.9 ± 0.8	16.1 ± 0.6	16.2 ± 0.4	43.5 ± 1.7	41.5 ± 2.3	194.2 ± 7.6	145.9 ± 24.8	202.0 ± 7.9	214.5 ± 6.5
LN8	5.4 ± 0.2	12.3 ± 1.8*	16.8 ± 0.5	15.3 ± 0.6	45.5 ± 1.3	43.2 ± 2.8	203.2 ± 5.9	167.7 ± 10.2	211.3 ± 6.1	207.3 ± 9.8
LN9	5.2 ± 0.2	10.3 ± 1.8*	16.4 ± 0.6	23.0 ± 1.6*	44.4 ± 1.7	51.0 ± 3.3	198.1 ± 7.6	168.3 ± 10.9	206.0 ± 7.9	223.3 ± 4.5
LN10	5.3 ± 0.1	11.3 ± 2.0*	16.6 ± 0.3	19.9 ± 1.4	44.8 ± 0.8	62.8 ± 3.7*	200.0 ± 3.4	174.5 ± 3.6*	200.0 ± 3.4	198.7 ± 4.3
LN11	5.2 ± 0.1	13.4 ± 2.1*	16.4 ± 0.4	21.5 ± 1.4*	44.4 ± 1.1	60.7 ± 3.2*	198.1 ± 4.7	167.8 ± 15.2	198.1 ± 4.7	184.7 ± 7.8
LN12	5.2 ± 0.2	13.2 ± 1.5*	16.4 ± 0.6	22.5 ± 1.6*	44.4 ± 1.6	62.2 ± 3.7*	198.1 ± 6.9	160.8 ± 11.0	198.1 ± 6.9	166.4 ± 7.3*
LN13	5.2 ± 0.1	9.0 ± 1.9*	16.3 ± 0.4	21.0 ± 1.3*	44.1 ± 1.2	56.3 ± 3.5*	196.8 ± 5.4	175.4 ± 5.7	196.8 ± 5.4	169.3 ± 8.8
LN14	5.2 ± 0.1	9.9 ± 3.0	16.3 ± 0.5	18.9 ± 1.6	44.1 ± 1.3	55.2 ± 3.2*	196.8 ± 5.7	158.3 ± 2.3*	196.8 ± 5.7	187.1 ± 10.1

Days B1 to B4 represent baseline period and days LN1 to LN14 represent the period during which L-NAME was given.
Values are means ± s.e.mean of the indicated number of animals.
Significantly different from corresponding control values (*$P<0.02$).

Table 3 Changes in the urinary secretion (in nmol day^{-1}) of 5-hydroxytryptamine (5-HT), 5-hydroxyindolacetic acid (5-HIAA), noradrenaline (NA) and adrenaline (Ad) in rats drinking water ($n = 4$) or a solution of N^G-nitro-L-arginine methyl ester (L-NAME; 50 μg ml^{-1}) ($n = 4$)

	5-HT		*5-HIAA*		*NA*		*Ad*	
Day	*Control*	*L-NAME*	*Control*	*L-NAME*	*Control*	*L-NAME*	*Control*	*L-NAME*
B1	75.0 ± 2.0	70.8 ± 0.7	236.4 ± 6.4	234.0 ± 3.2	2.0 ± 0.1	1.7 ± 0.1	2.2 ± 0.1	2.1 ± 0.1
B2	75.9 ± 2.3	75.3 ± 0.9	239.1 ± 7.3	230.7 ± 2.4	2.1 ± 0.1	2.1 ± 0.3	2.3 ± 0.1	2.1 ± 0.1
B3	80.2 ± 2.2	75.2 ± 2.3	252.7 ± 7.0	252.1 ± 3.3	2.1 ± 0.1	1.8 ± 0.1	2.3 ± 0.1	2.5 ± 0.2
B4	73.9 ± 1.5	72.9 ± 1.5	232.8 ± 4.7	250.0 ± 3.6	2.1 ± 0.1	2.3 ± 0.2	2.3 ± 0.1	2.3 ± 0.1
LN1	79.0 ± 3.4	79.6 ± 1.0	249.1 ± 10.8	232.4 ± 2.8	2.1 ± 0.1	2.8 ± 0.3	2.4 ± 0.1	1.8 ± 0.1
LN2	79.3 ± 1.3	78.2 ± 1.4	250.0 ± 4.2	234.0 ± 3.4	2.2 ± 0.1	3.1 ± 0.4	2.4 ± 0.1	1.7 ± 0.1
LN3	78.7 ± 1.2	83.3 ± 2.4	248.2 ± 3.9	231.6 ± 2.9	2.2 ± 0.1	2.9 ± 0.4	2.4 ± 0.1	1.9 ± 0.1
LN4	79.6 ± 2.6	85.4 ± 3.7	250.9 ± 8.1	247.1 ± 1.2	2.2 ± 0.1	3.2 ± 0.5	2.4 ± 0.1	2.3 ± 0.1
LN5	81.9 ± 2.9	114.5 ± 2.3	258.2 ± 9.2	235.1 ± 2.5	2.1 ± 0.1	2.2 ± 0.3	2.4 ± 0.1	1.9 ± 0.2
LN6	80.7 ± 2.4	80.7 ± 1.9	254.5 ± 7.6	244.0 ± 2.8	2.2 ± 0.1	2.2 ± 0.3	2.5 ± 0.1	2.0 ± 0.2
LN7	80.7 ± 3.4	72.2 ± 2.0	254.5 ± 10.6	259.4 ± 2.6	2.3 ± 0.1	2.9 ± 0.2	2.6 ± 0.1	1.9 ± 0.1
LN8	79.6 ± 2.6	75.5 ± 4.5	250.9 ± 8.1	252.1 ± 4.0	2.4 ± 0.1	3.8 ± 0.4*	2.7 ± 0.1	2.0 ± 0.1
LN9	83.3 ± 2.5	72.6 ± 3.8	262.7 ± 7.8	233.8 ± 2.2	2.4 ± 0.1	4.0 ± 0.9*	2.6 ± 0.1	2.0 ± 0.3
LN10	87.1 ± 3.4	67.6 ± 4.1	274.5 ± 10.8	240.5 ± 1.2	2.4 ± 0.1	6.7 ± 0.8*	2.6 ± 0.1	6.1 ± 0.9*
LN11	91.1 ± 2.6	123.6 ± 4.0*	287.1 ± 8.4	233.7 ± 3.1	2.4 ± 0.1	7.4 ± 0.9*	2.6 ± 0.1	6.8 ± 0.8*
LN12	88.8 ± 3.4	151.1 ± 7.6*	279.9 ± 10.8	236.4 ± 2.5	2.4 ± 0.1	9.7 ± 1.0*	2.6 ± 0.1	7.3 ± 0.6*
LN13	89.7 ± 1.5	155.2 ± 3.2*	282.6 ± 4.9	237.5 ± 3.6	2.4 ± 0.1	7.5 ± 0.7*	2.6 ± 0.1	5.1 ± 0.5*
LN14	88.8 ± 2.1	140.7 ± 3.0*	279.9 ± 6.7	229.7 ± 2.8	2.4 ± 0.1	7.0 ± 0.9*	2.6 ± 0.1	4.3 ± 0.4*

Days B1 to B4 represent baseline period and days LN1 to LN14 represent the period during which L-NAME was given.
Values are means ± s.e.mean of the indicated number of animals.
Significantly different from corresponding control values (*$P < 0.02$).

Table 4 Plasma concentrations of L-3,4-dihydroxyphenylalanine (L-DOPA), noradrenaline (NA), adrenaline (Ad) and 3,4-dihydroxyphenylglycol (DHPG), aldosterone (PAC), Na^+, K^+, urea, creatinine and plasma renin activity (PRA) and plasma osmolality in control animals ($n = 4$) and rats given a solution of N^G-nitro-L-arginine methyl ester (L-NAME; 50 μg ml^{-1}) for 14 days ($n = 4$)

	Control	*L-NAME*
L-DOPA (pmol ml^{-1})	7.9 ± 1.7	16.1 ± 1.8*
NA (pmol ml^{-1})	2.4 ± 0.5	8.4 ± 1.3**
Ad (pmol ml^{-1})	1.9 ± 0.4	9.1 ± 0.7**
DHPG (pmol ml^{-1})	3.0 ± 0.3	7.9 ± 1.4*
PAC (pg ml^{-1})	310 ± 33	125 ± 13**
Na^+ (mmol ml^{-1})	139 ± 2	139 ± 1
K^+ (mmol ml^{-1})	5.3 ± 0.5	4.6 ± 0.1
Urea (mg dl^{-1})	18 ± 2	17 ± 2
Creatinine (mg dl^{-1})	0.4 ± 0.1	0.3 ± 0.1
PRA (ng ml^{-1} h^{-1})	29 ± 2	17 ± 2**
Osmolality (mOsm kg^{-1} H_2O)	289 ± 2	284 ± 1

Values are means ± s.e.mean of the indicated number of determinations.
Significantly different from corresponding control values (*$P < 0.05$; **$P < 0.01$) using Student's t test.

significant differences were observed in plasma concentrations of creatinine and urea and plasma osmolality between L-NAME-treated rats and controls. The levels of noradrenaline and dopamine in the renal cortex of L-NAME-treated animals (noradrenaline = 0.89 ± 0.03 nmol g^{-1}; dopamine = 0.16 ± 0.05 nmol g^{-1}) were found not to differ from those in control rats (noradrenaline = 0.78 ± 0.06 nmol g^{-1}; dopamine = 0.11 ± 0.01 nmol g^{-1}).

In vitro *studies*

Baseline blood pressure and heart rate values of rats ($n = 21$) used in *in vitro* experiments were similar to those described above (SBP = 108 ± 2 mmHg, DBP = 80 ± 4 mmHg, heart rate = 411 ± 3 beats min^{-1}). In Table 5, are shown the blood pressure and heart rate values, body weight, liquid consumption, dose of L-NAME and L-NAME plus L-arginine administered to rats used in this set of experiments: rats given L-NAME for 7 ($n = 4$) or 14 ($n = 6$) days, rats given for 14 days L-NAME plus L-arginine ($n = 5$) and vehicle-treated animals ($n = 6$). Rats treated for 7 or 14 days with L-NAME alone had significantly higher SBP and DBP than control rats. Animals given L-NAME plus L-arginine for 14 days had significantly lower blood pressure values than animals treated with L-NAME alone; the difference between the L-NAME plus arginine and the vehicle group did not achieve statistical significance (Table 5).

In experiments performed on homogenates of isolated renal tubules, the decarboxylation of L-DOPA to dopamine was found to be dependent on the concentration of L-DOPA used (10 to 5000 μM) and saturable at 1000 μM (Figure 2). AAAD activity as determined in tubular homogenates was found to be significantly ($P < 0.01$) higher in rats given L-NAME for 14 days than in controls, rats given L-NAME for 7 days and rats given L-NAME plus L-arginine for 14 days (see Table 6). No statistically significant differences were found between the K_m values in the four experimental groups.

The levels of total dopamine (Figure 3 a) and the amount of that dopamine which had escaped into the incubation fluid (Figure 3 b), in kidney slices incubated with increasing concentrations (5 to 5000 μM) of L-DOPA were dependent on the concentration of L-DOPA used and reached their maximum at 2500 μM L-DOPA. The renal tissues used in these experiments were obtained from rats given L-NAME alone for 7 or 14 days, rats given L-NAME plus L-arginine for 14 days and control animals. The major difference between the four experimental groups concerns the higher production of dopamine from added L-DOPA in rats given L-NAME for 14 days; this is particularly shown by a greater accumulation of dopamine in the tissue. At 2500 μM L-DOPA, a concentration at which decarboxylation in kidney slices appears to be saturated (the apparent K_m in control animals was 535 ± 37 μM), the fractional outflow of newly-formed dopamine in control animals was similar to that observed in rats given L-NAME for 7 days and rats receiving L-NAME plus L-arginine, but higher than that in rats treated with L-NAME for 14 days (Figure 3 c). No significant differences were observed between control animals and rats given L-NAME for 7 days and rats given L-NAME plus L-arginine.

As shown in Figure 3, a considerable amount of the total

Table 5 Systolic (SBP) and diastolic (DBP) blood pressure (mmHg), heart rate (beats min^{-1}), liquid consumption and body weight in control animals ($n = 6$), rats given a solution of N^G-nitro-L-arginine methyl ester (L-NAME; 50 $\mu g\ ml^{-1}$) for 14 days ($n = 6$) or 7 ($n = 4$) days and rats given L-NAME (50 $\mu g\ ml^{-1}$) plus L-arginine (1 $mg\ ml^{-1}$) for 14 days ($n = 5$)

	Control	*L-NAME* (7 days)	*L-NAME* (14 days)	*L-NAME + L-arginine* (14 days)
SBP	106 ± 1	150 ± 12*	171 ± 9*	111 ± 2#
DBP	75 ± 2	109 ± 10*	119 ± 6*	77 ± 5#
Heart rate	411 ± 4	409 ± 2	411 ± 5	343 ± 2#
Body weight (g)	215.4 ± 21.3	207.6 ± 14.7	224.6 ± 19.8	218.3 ± 14.2
Liquid intake ($ml\ day^{-1}$)	39.6 ± 2.5	49.8 ± 2.1*	62.5 ± 4.8*	40.8 ± 5.9#
Dose of L-NAME ($mg\ kg^{-1}\ day^{-1}$)	-	10.7 ± 0.6	11.8 ± 1.4	10.2 ± 0.9

Values are means ± s.e.mean of four to six experiments per group.
Significantly different from corresponding control values (*$P < 0.01$) or values for L-NAME (#$P < 0.01$) using the Tuckey-Kramer method.

Table 6 Kinetic parameters (V_{max} in $nmol\ mg^{-1}$ protein h^{-1}; K_m, in μM) of aromatic L-amino acid decarboxylase (AAAD) activity in homogenates of rat renal cortex in control animals, rats given a solution of N^G-nitro-L-arginine methyl ester (L-NAME; 50 $\mu g\ ml^{-1}$) for 14 days or 7 days and rats given L-NAME (50 $\mu g\ ml^{-1}$) plus L-arginine (1 mg ml^{-1}) for 14 days

	Control	*L-NAME* (7 days)	*L-NAME* (14 days)	*L-NAME + L-arginine* (14 days)
V_{max}	13.8 ± 0.6	15.2 ± 0.7	25.2 ± 2.4*	12.9 ± 0.9
K_m	62.6 ± 6.8	68.8 ± 5.1	72.0 ± 10.1	60.4 ± 2.5

Values are means ± s.e.mean of four to six experiments per group.
Significantly different from corresponding control values (*$P < 0.02$) using the Tuckey-Kramer method.

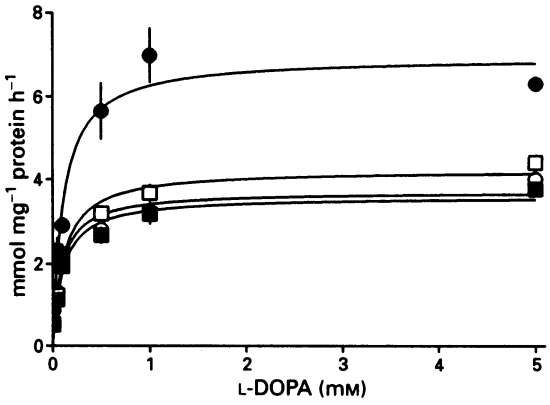

Figure 2 Saturation curves of aromatic L-amino acid decarboxylase (AAAD) activity in homogenates of renal cortical tubules obtained from control animals (○), rats given N^G-nitro-L-arginine methyl ester (L-NAME, 50 $\mu g\ ml^{-1}$) for 7 (□) or 14 days (●) and rats given L-NAME (50 $\mu g\ ml^{-1}$) plus L-arginine (1 $mg\ ml^{-1}$) for 14 days (■). AAAD activity is expressed as the rate of formation of dopamine (in $nmol\ mg^{-1}$ protein 15 min^{-1}) vs. concentrations of L-DOPA (μM). Each point is the mean of four to six experiments per group; vertical lines show s.e.mean.

dopamine formed from added L-DOPA in kidney slices was found to escape into the incubation medium. The application of the Michaelis-Menten equation to the net transport of newly-formed dopamine allowed the identification of a saturable (carrier-mediated transfer) and a non-saturable component (diffusion). In Figure 4 are shown both the saturable component of dopamine transport and the diffusional rate of transfer, as a plot of the amount of dopamine which has escaped into the incubation medium (rate of transport) against the total amount of dopamine formed (substrate concentration), for experiments carried out in renal tissues from rats in the four experimental groups. No significant differences in the diffusional rate of transfer were observed between the four experimental groups. However, the saturable outward transfer of dopamine in control animals was higher than in rats treated with L-NAME for 14 days, but similar to that observed in rats receiving L-NAME plus L-arginine; the saturable dopamine outward rate of transfer was found to be higher in rats given L-NAME for 7 days than that in controls. (Figure 4). The kinetic parameters (V_{max}, K_m and diffusion constant) for the saturable component of dopamine transfer for the control and the three experimental groups are listed in Table 7.

Discussion

The present studies show that long-term administration of L-NAME, an inhibitor of NO synthase, was associated with the development of sustained high blood pressure. The hypertensive response to L-NAME administration is accompanied by an increased urinary excretion of dopamine and 5-hydroxytryptamine, which appears to result from an enhanced activity of renal AAAD. Because the increased renal AAAD activity is reversed by the concomitant administration of L-arginine to L-NAME-treated animals it is suggested that this renal response may correspond to an adaptational response to sustained NO synthase inhibition.

Urinary dopamine is believed to derive mainly from the tubular decarboxylation of filtered or circulating L-DOPA (Lee, 1993); there is, however, lack of information on the amount of urinary 5-hydroxytryptamine which derives from the renal decarboxylation of L-5-hydroxytryptophan. Since the increase in 5-hydroxytryptamine levels in the urine of L-NAME-treated rats parallels that of dopamine, it is possible that considerable amounts of both amines might have originated in the same cellular compartment, the epithelial cells of renal proximal tubules. The same does not appear to apply to other amine metabolites. In fact, only changes in DOPAC levels in the urine paralleled those of dopamine and 5-hydroxytryptamine, whereas urinary 3-MT, HVA and 5-HIAA remained unchanged throughout the study. This would agree with the view that most of the 3-MT, HVA and 5-HIAA in the urine is of extrarenal origin, namely deriving from the metabolism of their parent amines in brain or other extrarenal tissues. The finding that a major component of dopamine metabolism in the kidney is represented by MAO fits well the suggestion that a substantial amount of DOPAC in the urine may derive from the deamination of renal dopamine (Fernandes & Soares-da-Silva, 1993).

The parallel increase in the urinary excretion of dopamine, DOPAC and 5-hydroxytryptamine during the second week of L-NAME administration probably results from an increased AAAD activity in renal tissues. This suggestion is supported by the findings that the specific activity of the enzyme, as measured under *in vitro* experimental conditions, is almost twice that occurring in renal tissues taken from non-treated rats. The question of whether this renal response is related to a deficit in NO production or may correspond to an adaptational response to high blood pressure cannot be answered at present. However, the finding that AAAD activity on day 7 of L-NAME treatment was similar to that in control rats, despite the increase in blood pressure, suggests that the enhanced ability of renal tissues to decarboxylate L-DOPA observed at day 14 may represent a slow developing adapta-

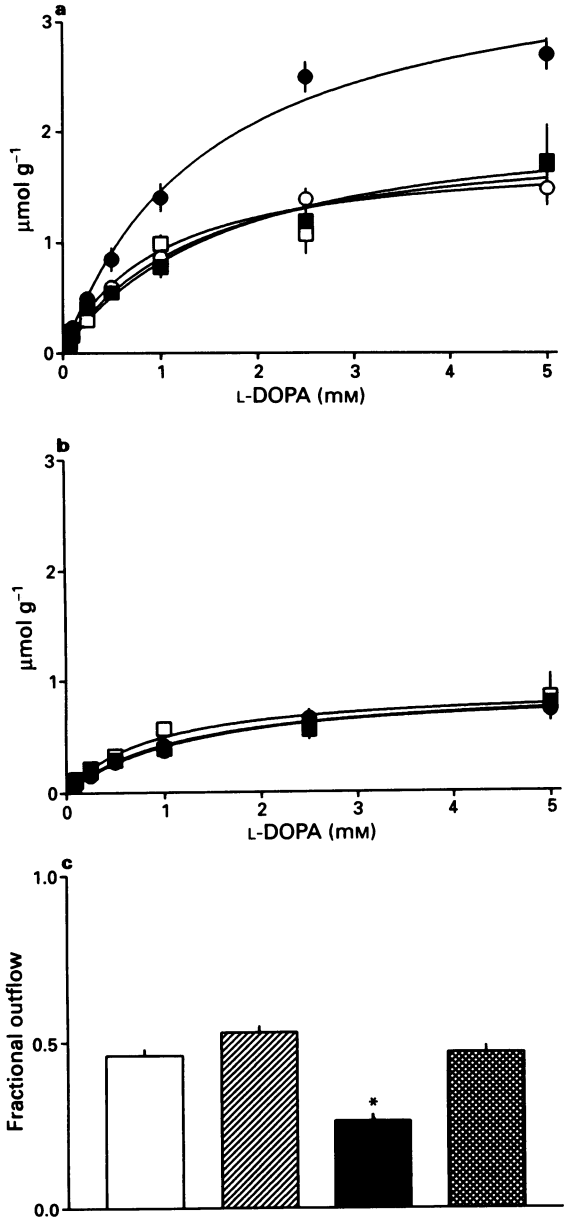

Figure 3 Levels of (a) total dopamine formed, obtained by the algebraic sum of the amine levels in the tissue and the incubation medium, and (b) levels of dopamine in the incubation medium, in slices of renal cortex of control animals (○), rats given N^G-nitro-L-arginine methyl ester (L-NAME, 50 μg ml^{-1}) for 7 (□) or 14 days (●) and rats given L-NAME (50 μg ml^{-1}) plus L-arginine (1 mg ml^{-1}) for 14 days (■). Graphs (c) show fractional outflow of dopamine from kidney slices of control animals (open columns), rats given L-NAME for 7 (hatched columns) or 14 days (solid columns) and rats given L-NAME (50 μg ml^{-1}) plus L-arginine (1 mg ml^{-1}) for 14 days (cross hatched columns) incubated with 2000 μM L-DOPA. Each point or column is the mean (with s.e.mean) of four to six experiments per group. Significantly different from corresponding control values (*$P<0.01$).

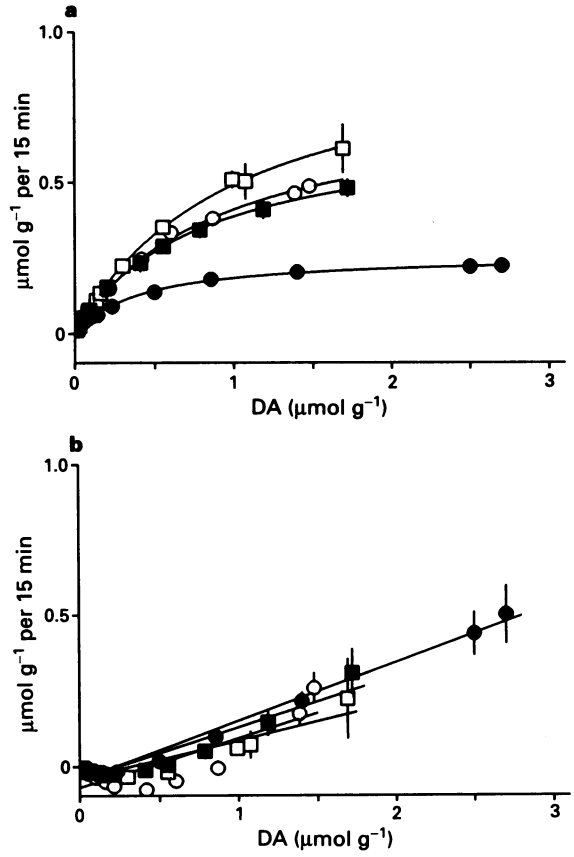

Figure 4 Outward transfer of dopamine in slices of renal cortex obtained from control animals (○), rats given N^G-nitro-L-arginine methyl ester (L-NAME, 50 μg ml^{-1}) for 7 (□) or 14 days (●) and rats given L-NAME (50 μg ml^{-1}) plus L-arginine (1 mg ml^{-1}) for 14 days (■) incubated with increasing concentrations of L-DOPA (5 to 5000 μM) for 15 min. The results are of the two components of dopamine transfer, (a) saturable and (b) non-saturable, as a plot of the amount of dopamine which has escaped into the incubation medium (rate of amine transport; [V], in μmol g^{-1} 15 min^{-1}) against the total amount of dopamine formed (amine concentration; [S], in μmol g^{-1}). Each point is the mean (with s.e. mean) of four to six experiments per group.

Table 7 Kinetic parameters (V_{max} in μmol g^{-1} h^{-1}; K_m, in μM) of the saturable component of newly-formed outward transfer of dopamine and diffusion constant (in μmol^{-1}) in kidney slices of control animals ($n=6$), rats given for 7 ($n=4$) or 14 days ($n=6$) a solution of N^G-nitro-L-arginine methyl ester (L-NAME; 50 μg ml^{-1}) and rats given for 14 days L-NAME (50 μg ml^{-1}) plus L-arginine (1 mg ml^{-1}) ($n=5$), incubated with increasing concentrations of L-DOPA (5 to 5000 μM)

	Control	*L-NAME* (7 days)	*L-NAME* (14 days)	*L-NAME* + *L-arginine* (14 days)
V_{max}	2.3 ± 0.2	3.9 ± 0.2*	0.8 ± 0.02*	2.4 ± 0.2
K_m	568 ± 67	1006 ± 26*	241 ± 21*	534 ± 36
Diffusion	0.14 ± 0.02	0.11 ± 0.04	0.19 ± 0.02	0.17 ± 0.02

Values are means ± s.e.mean of four to six experiments per group.
Significantly different from corresponding control values (*$P<0.05$) using the Tuckey-Kramer method.

tional response to NO synthase inhibition. In agreement with this view are the results of Yuasa *et al.* (1992) who showed that the acute increase in blood pressure produced no change in the urinary excretion of dopamine. Furthermore, the finding that the increased AAAD activity can be reversed by the administration of L-arginine to L-NAME-treated rats favours the view that the adaptive response leading to enhanced AAAD activity might have to do with generation of a signal which is dependent on chronic NO synthase inhibition.

The finding that the enhanced formation of dopamine was observed when both kidney slices or homogenates were used indicates that the enhanced production of dopamine is due to the enzyme itself and not differences in the intracellular availability of L-DOPA. As has been discussed elsewhere (Soares-da-Silva, 1994) the regulation of renal AAAD activity is far from being completely understood and these results appear to suggest that the reduced availability of NO, cyclic GMP production or both, may be of importance for the regulation of decarboxylase activity. It should be mentioned, however, that changes in AAAD activity were also accompanied by increases in the urinary excretion of L-DOPA, and that by the end of the study, plasma levels of L-DOPA in L-NAME treated rats were greater than those in non-treated rats. It is, therefore, possible that a larger delivery of L-DOPA to the kidney might have contributed to the increase in the urinary excretion of dopamine.

This suggestion would agree with the results that in both man and rats a substantial amount (up to 77%) of plasma L-DOPA is removed by the kidneys and about 80% of urinary dopamine derives from plasma L-DOPA (Grossman *et al.*, 1992; Wolfovitz *et al.*, 1993). Sympathetic neurones are believed to be a major source of plasma L-DOPA (Grossman *et al.*, 1992) and the rate of noradrenaline synthesis in sympathetic nerve terminals may even determine the rate at which L-DOPA appears in plasma (Eisenhofer *et al.*, 1989a, b). Assuming that plasma levels of noradrenaline and adrenaline may reflect the release of these catecholamines, respectively, from sympathetic neurones and the adrenal medulla, and since L-NAME-treated rats were found to have significantly higher levels of the two catecholamines, the presence of an increased sympathoadrenal tone in this experimental model of hypertension might be suggested. It is possible, therefore, that the increased sympathetic drive in L-NAME treated rats would be associated with an increased neuronal spillover rate of L-DOPA into the plasma. The increased plasma levels of noradrenaline and adrenaline fully agree with the data reported on the urinary excretion of the two amines, levels of which in the urine are thought to derive mainly from the circulating amines. The suggested increased sympathetic activity would also agree with the results reported by both Sakuma *et al.* (1992) and Harada *et al.* (1993) showing that inhibition of NO formation is accompanied by an increased sympathetic nerve activity; this appears to be a centrally mediated effect not depending on functioning baroreflex mechanisms.

Another result which is worthwhile mentioning is that concerning the outflow of dopamine in kidney slices. It should be stressed, however, that the results reported here would mainly reflect the outward transfer of dopamine at the basolateral cell border, because the apical cell border may not be in operation due to collapse of the tubular lumen (Wedeen & Weiner, 1973). The data reported here show that the outflow of newly-formed dopamine in kidney slices loaded with exogenous L-DOPA follows a Michaelis-Menten kinetics with a saturable component and a non-saturable one. It is interesting that the maximal rate of transfer of dopamine through the saturable component was lower in preparations with the highest ability to synthesize dopamine (rats given L-NAME for 14 days) than in preparations obtained from control animals or the two other experimental groups. This might suggest the occurrence of down regulation of the outward dopamine transporter, following exposure to increased amounts of the amine. The result of similar values for the dopamine diffusional rate of transfer in all experimental groups also agrees with this suggestion. The nature of the renal dopamine transporter has not been characterized in detail; however, the similarity of K_m values for the dopamine transporter in rat and canine renal tissues suggests that the renal outward transporter of dopamine in the two species may share some functional properties (Soares-da-Silva, 1993b). It should be mentioned that the activation of tubular α_1- and α_2- adrenoceptors has been demonstrated to enhance the outward transfer of dopamine in rat kidney slices; indirect evidence suggests that the underlying mechanism involves the stimulation of the Na^+-H^+ exchanger (Soares-da-Silva, 1993c). Neuronally released noradrenaline and circulating adrenaline have been suggested to be particularly active at tubular α_1- and α_2-adrenoceptors, respectively. Therefore, it could be speculated that the increased urinary excretion of dopamine observed during the second week of L-NAME was related to a facilitatory effect on the amine outward transfer, in addition to the increased renal decarboxylating activity and the enhanced renal delivery of L-DOPA.

Long-term L-NAME administration was associated with no change in the urinary excretion of Na^+, K^+ or creatinine or changes in plasma creatinine levels during the duration of the study. This suggests that in this model of hypertension, Na^+ retention may not be an important factor, as suggested by other authors (Dananberg *et al.*, 1993; Jover *et al.*, 1993), and that the high blood pressure associated with the long-term inhibition of NO synthase may have minimal effects on renal function (Jover *et al.*, 1993; Pollock *et al.*, 1993). This appears not to be the case in experimental models employing higher doses of L-NAME (Baylis *et al.*, 1990), when animals are acutely infused with NO synthase inhibitors (Romero *et al.*, 1992) or when experiments are performed under anaesthesia (Ribeiro *et al.*, 1992). In fact, dependent on the degree of NO synthase inhibition and magnitude of the hypertensive response, changes in renal perfusion pressure via a pressure natriuresis phenomenon can cause diverse effects on sodium excretion (Johnson & Freeman, 1992; Nakamura *et al.*, 1993). Although it has been suggested that both dopamine and 5-hydroxytryptamine may interfere with the tubular reabsorption of Na^+, namely that occurring in segments of the proximal nephron, it is interesting to observe that despite increases in the urinary excretion of both amines during the second week of L-NAME administration, no significant change in the urinary excretion of Na^+ occurred. The fact that dopamine and 5-hydroxytryptamine have opposite effects upon tubular reabsorption of Na^+ may explain this.

Another interesting observation concerns the increase in water consumption and excretion observed during L-NAME administration. Although we have no direct evidence on the nature of this phenomenon, the most probable explanation is that L-NAME interferes with the release of antidiuretic hormone in the pituitary or upon its renal actions; in fact, Na^+ and K^+ excretion were found not to change and plasma osmolality was similar to that in non-treated animals. Similar observations have been reported by Pollock *et al.* (1993). The finding that NO synthase is present both in the posterior pituitary and the hypothalamus (Bredt *et al.*, 1990; Föstermann *et al.*, 1990) gives support to this suggestion.

As has been reported before (Arnal *et al.*, 1992; Ribeiro *et al.*, 1992; Dananberg *et al.*, 1993; Manning *et al.*, 1993; Pollock *et al.*, 1993), we have observed that long-term L-NAME administration decreased PRA and PAC. Apparently, this decrease in PRA contrasts with the finding that both angiotensin converting enzyme inhibitors and angiotensin II receptor antagonists prevent the development of and reverse L-NAME-induced hypertension (Jover *et al.*, 1993; Pollock *et al.*, 1993).

In conclusion, the present studies show that the hypertensive response resulting from long-term administration of L-

NAME is accompanied by an increased urinary excretion of dopamine and DOPAC, which appear to result from an enhanced activity of renal AAAD and an increase in the delivery of L-DOPA to renal tissues. The result that the increased AAAD activity and hypertension can be reversed by the administration of L-arginine to L-NAME-treated rats favours the view that the enhanced AAAD activity probably involves a decrease in the generation of NO or has to do with the generation of a signal which depends on high blood pressure values.

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Sustained contraction produced by caffeine after ryanodine treatment in the circular muscle of the guinea-pig gastric antrum and rabbit portal vein

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1 Caffeine inhibited spontaneous mechanical activity at 0.3–1 mM, but produced a tonic contraction at concentrations higher than 3 mM in the circular muscle of the guinea-pig gastric antrum. In the circular muscle of the rabbit portal vein, caffeine at concentrations higher than 1 mM produced an early phasic contraction followed by a small tonic component. The caffeine-induced contraction was abolished by removal of the external Ca^{2+} more rapidly in the gastric antrum than the portal vein.

2 When the preparations were pretreated with ryanodine (1 μM) a sustained contraction developed on wash-out of caffeine (10 mM) both in the gastric antrum and portal vein. This contraction was not affected by nicardipine (3 μM) or verapamil (3 μM), but was readily abolished by removal of the external Ca^{2+} or by addition of cobalt (1 mM). Spontaneous electrical activity, the slow wave, in gastric muscles was blocked in the presence of 10 mM caffeine, but reappeared during the sustained contraction.

3 Both the contractions induced directly by caffeine and those produced following caffeine wash-out after ryanodine treatment were accompanied by a maintained increase in intracellular Ca^{2+} concentration measured with fura-2.

4 The presence or absence of Ca^{2+} during the application of ryanodine did not affect the ability of caffeine to initiate sustained contractions, provided Ca^{2+} was present during the exposure to caffeine.

5 It is concluded that caffeine can induce a sustained contraction after ryanodine treatment both in the guinea-pig gastric antrum and rabbit portal vein, by activating a Ca^{2+} influx pathway insensitive to organic Ca^{2+} channel blockers. No clear evidence was obtained for involvement of the Ca^{2+} influx pathway activated through depletion of intracellular Ca^{2+} stores. A hypothesis is proposed that the plasma membrane of these preparations is similar to the sarcoplasmic reticulum membrane in that Ca^{2+} permeability can be increased almost irreversibly by a combination of caffeine and ryanodine in the presence of the external Ca^{2+}.

Keywords: Caffeine; ryanodine; smooth muscle; Ca influx; guinea-pig gastric antrum; rabbit portal vein

Introduction

Caffeine is known to produce a contraction by releasing Ca^{2+} from intracellular stores in various vascular and intestinal smooth muscles (Itoh *et al.*, 1981; 1982; Iino, 1989). The effect of caffeine, however, may differ in different smooth muscles or experimental conditions. In myometrium, caffeine has only an inhibitory effect (Osa, 1973; Savineau & Mironneau, 1990) and in some intestinal smooth muscles, both contractile and relaxant action of caffeine have been reported (Osa, 1973; Ito *et al.*, 1974).

The circular muscle of the guinea-pig gastric antrum has spontaneous rhythmic activity, but the mechanism underlying this activity is not known. It is possible that intracellular Ca^{2+} release may be involved in some way in the generation of this activity. Since caffeine can release Ca^{2+} from the intracellular stores probably by potentiating the Ca^{2+}-induced Ca^{2+} release mechanism (Iino, 1989) and ryanodine is considered to unlock the Ca^{2+}-induced Ca^{2+} release channels, thereby resulting in depletion of the stores (Iino *et al.*, 1988; Ganitkevich & Isenberg, 1993), we examined whether caffeine and ryanodine modified the spontaneous activity. During these experiments, caffeine was found to produce a very prolonged contraction when preparations were pretreated with ryanodine. In the present experiments, some of the properties of this sustained contraction were analysed. Since caffeine-induced tonic contractions in gastric muscle were quickly abolished by removal of the external Ca^{2+}, we compared this muscle with rabbit portal vein in which caffeine induced phasic contractions which were relatively resistant to Ca^{2+} removal.

Methods

For gastric muscle, Hartley guinea-pigs (250–350 g) of either sex were killed by stunning and bleeding and the stomach was excised. After careful removal of the mucosa under a binocular microscope, circular muscle strips (approximately 1 × 5 mm) were separated from the longitudinal muscle layer, after dissecting out from the antral region. For portal vein, rabbits (2–2.5 kg) of either sex were killed by bleeding under anaesthesia with pentobarbitone (100 mg kg^{-1}). Circular muscle strips (approximately 1 × 5 mm) were obtained after carefully removing connective tissue. Mechanical activity was recorded simultaneously from the muscle strips of stomach and the portal vein with strain gauges in the same small chamber (0.3 ml) in which each preparation was mounted horizontally. In some experiments only gastric muscles were used. The chamber was perfused at a constant rate of 3 ml min^{-1} with physiological solution prewarmed to 35°C.

The normal solution contained (mM): NaCl 127, $KHCO_3$ 6, $CaCl_2$ 2.4, $MgCl_2$ 1.2, glucose 12, tris-hydroxymethyl-aminomethane (Tris) 10 (pH adjusted to 7.4 at 35°C with HCl). When caffeine was added, NaCl was substituted to maintain the osmolarity. In some experiments, atropine (1 μM), phenoxybenzamine (1 μM), N^G-nitro-L-arginine (50 μM), and

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indomethacin (1 μM) were added, to block contributions of acetylcholine, noradrenaline, nitric oxide, and prostaglandins, respectively, but no fundamental differences were found.

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was also measured from a small muscle strip (1 × 5 mm) which was mounted in a chamber (0.2 ml) fixed on a stage of an inverted microscope (IMT-2, Olympus Optical Co., Japan) and one end was connected to an isometric strain gauge to record tension development. The chamber was perfused at a rate of 3 ml min^{-1} with physiological solution prewarmed at 35°C, except for a period of fura-2 AM loading. The preparation was exposed to physiological solution containing 10 μM fura-2 AM for 1 h and the measurement of fura-2 signals was started about 20 min after restarting superfusion to remove external fura-2 AM. Fluorescence signals (510 nm) excited at 340 and 380 nm wavelength were collected at 3 s intervals with an image processor (ARGUS-50, Hamamatsu Photonics, Japan). The ratio of fluorescence intensities at 340 and 380 nm ($R_{340/380}$) was used to estimate changes in $[Ca^{2+}]_i$. The size of spot where the fluorescent signals were detected was about 60 μm square. In these experiments, physostigmine (10 μM), atropine (1 μM), and probenecid (20 μM) were added throughout to inhibit the esterase activity of the tissues and to improve the maintenance of intracellular fura-2 (Watanabe *et al.*, 1992).

The membrane potential was measured only in the gastric preparations, by the conventional microelectrode technique, as previously described (Katayama *et al.*, 1993). In these experiments, mechanical activities were also recorded simultaneously with a strain gauge.

The chemical agents used were obtained from Sigma (St. Louis, U.S.A.), except nicardipine (Wako Pure Chemical, Japan), fura-2-AM (Dojin, Japan) and phenoxybenzamine (Tokyo Chemical, Japan). Numerical data were expressed as means ± s.e.mean.

Results

Circular muscle strips of the guinea-pig gastric antrum have spontaneous activity, whereas those of the rabbit portal vein are usually quiescent, although some preparations produce irregular rhythmic activity at the beginning of experiments. As shown in Figure 1, caffeine inhibited the activity of gastric muscles at less than 1 mM, but produced a tonic contraction at concentrations higher than 3 mM. In the portal vein, caffeine produced a transient contraction followed by a small tonic contraction at 1 mM concentration or higher.

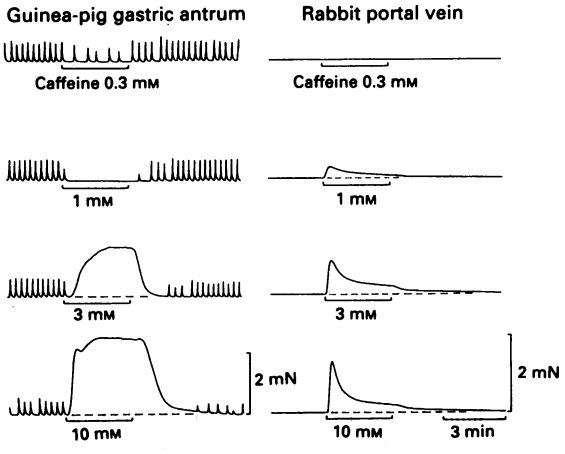

Figure 1 Simultaneous recording of tension responses to caffeine in the circular muscle strips of guinea-pig gastric antrum (left) and rabbit portal vein (right). Gastric muscle shows spontaneous phasic contractions which were inhibited by caffeine. Increasing concentration of caffeine was applied at 20 min intervals. Caffeine produced concentration-dependent contractions in both tissues.

Caffeine-induced contractions quickly disappeared in Ca^{2+}-free solution and after more than 3 min no contraction could be observed in the gastric muscle ($n = 6$) (Figure 2b), whereas in the portal vein, the decrease in contractions induced by Ca^{2+} removal was slow and was reduced to 37 ± 8% ($n = 6$) of the control after 5 min exposure to Ca^{2+}-free solution (Figure 2d). When 2.4 mM Ca^{2+} was reintroduced in the presence of 10 mM caffeine, a tonic contraction reappeared in gastric muscle (Figure 2b), but not in the portal vein (Figure 2d). Application of 1 mM cobalt had effects very similar to Ca^{2+} removal, but caffeine-induced contractions were not significantly affected by 3 μM verapamil or 3 μM nicardipine either in the gastric muscle or portal vein (not shown).

When ryanodine (1 μM) was applied for 2–5 min during caffeine-induced contractions, no effect was observed until caffeine was washed out, but a slow sustained contraction developed following removal of caffeine both in the gastric muscle and portal vein (Figure 2e,f). This contraction lasted for more than 1 h and the maximum tension developed was 237 ± 32% of the level of the caffeine-induced contraction after 3 min application in gastric muscle ($n = 16$) and 326 ± 45% in portal vein ($n = 14$). The sustained contraction was not affected by nicardipine (3 μM, Figure 2e,f) or verapamil (3 μM, not shown), but was reversibly abolished by removal of the external Ca^{2+} (Figure 2e,f) or by application of cobalt (1 mM, not shown), suggesting involvement of Ca^{2+} influx through a pathway insensitive to organic Ca^{2+} channel inhibitors. In the portal vein, relaxation caused by Ca^{2+} removal was slow and sometimes incomplete in 5 min (e.g. Figure 5f), but this was not studied further. Subsequent reapplication of caffeine (10 mM) during the sustained contraction reduced the tension almost to the level before caffeine wash-out (not shown).

In the stomach, some rhythmic activity could be observed during the sustained contraction, although there was no such activity in the presence of caffeine. Other xanthine derivatives, such as theophylline (1–10 mM) and isobutylmethylxanthine (1–100 μM) did not produce any contraction, but inhibited spontaneous rhythmic activity, before and also after treatment with ryanodine (1 μM).

Figure 3 shows changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) estimated by fura-2 fluorescence signal, recorded simultaneously with mechanical activity in the guinea-pig

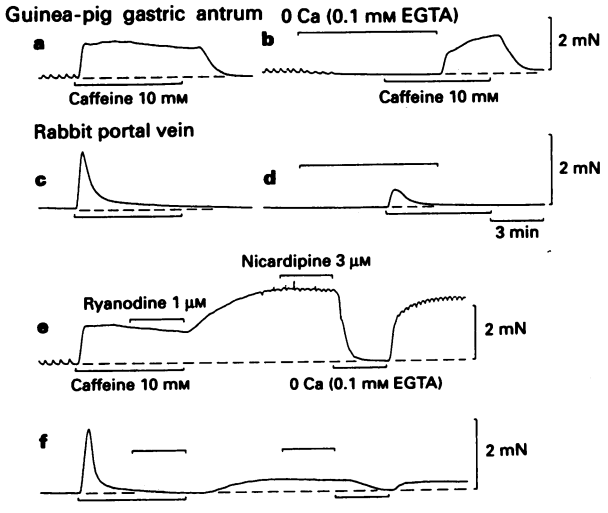

Figure 2 Simultaneous recording of mechanical responses to caffeine and ryanodine in muscle strips of guinea-pig stomach (a,b,e) and rabbit portal vein (c,d,f). (a and c) Control responses to caffeine (10 mM); (b and d) caffeine application 5 min after exposure to Ca^{2+}-free solution containing 0.1 mM EGTA and reapplication of 2.4 mM Ca^{2+} in the presence of caffeine; and (e and f) sustained contraction developed on wash-out of caffeine and ryanodine, and the lack of effect of nicardipine and reversible relaxation by removal of the external Ca^{2+}. Interval of caffeine application was 20 min.

gastric muscle. Caffeine (10 mM) produced a sustained increase in $[Ca^{2+}]_i$. In this experiment ryanodine (1 μM) was applied for 4 min and washed out for 2 min before the second caffeine application. Ryanodine did not produce any clear effect during the application, but a sustained contraction was produced following the second application of caffeine, as shown in Figure 3. There was a close correlation between changes in $[Ca^{2+}]_i$ and tension development, except that the rate of increase in $[Ca^{2+}]_i$ caused by caffeine was faster than that of tension development. When the external Ca^{2+} was removed during the sustained contraction, tension and $[Ca^{2+}]_i$ both decreased rapidly suggesting the sustained contraction is supported by continuous Ca^{2+} influx. Regular rhythmic $[Ca^{2+}]_i$ changes corresponding to rhythmic contractions could not be observed in this preparation due to a poor signal-noise ratio. Similar results showing a long-lasting increase in $[Ca^{2+}]_i$ during the sustained contraction following caffeine wash-out were obtained in three preparations of gastric antrum and two preparations of portal vein examined.

It is possible that an increase in $[Ca^{2+}]_i$, rather than caffeine itself, triggers a standard contraction after a ryanodine treatment. Another possibility is that an increase in $[Ca^{2+}]_i$ as well as adenosine 3':5'-cyclic monophosphate (cyclic AMP) is a prerequisite for the sustained contraction, since caffeine is expected to increase cyclic AMP due to inhibition of phosphodiesterase. Therefore, contractions produced by carbachol alone or together with forskolin, an adenylase cyclase activator, were compared with caffeine-induced contractions in gastric muscle, as shown in Figure 4. The experiment was carried out in the continuous presence of verapamil and indomethacin (1 μM) to inhibit phasic contractions produced by carbachol (CCh) and a possible involvement of endogenous prostaglandins, respectively. Both CCh (1 μM) alone and CCh plus forskolin (1 μM) failed to produce a sustained contraction after treatment with ryanodine (1 μM) for 5 min (Figure 4c,d). Caffeine (10 μM) applied 34 min after ryanodine treatment was still able to initiate a sustained contraction which was partially reversed by forskolin (1 μM) (Figure 4e).

Caffeine was still effective in initiating the sustained contraction even when ryanodine was applied in Ca^{2+}-free solution and also when caffeine was applied more than 1 h after removal of ryanodine. In the experiment shown in Figure 5, the preparations were treated with ryanodine for 5 min in

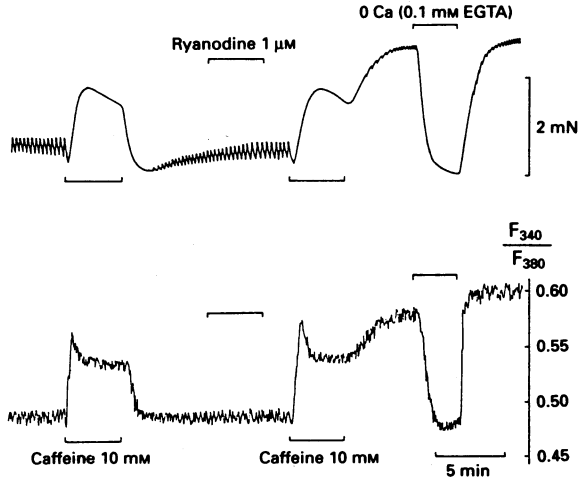

Figure 3 Simultaneous recording of fura-2 signals (lower) and mechanical response (upper trace) obtained from a gastric muscle strip. Fura-2 signal was expressed by the ratio of fluorescence intensities activated by 340 and 380 nm wavelengths (F_{340}/F_{380}). Caffeine application is indicated by horizontal bars underneath, and ryanodine application and removal of Ca^{2+} are shown by bars above the records.

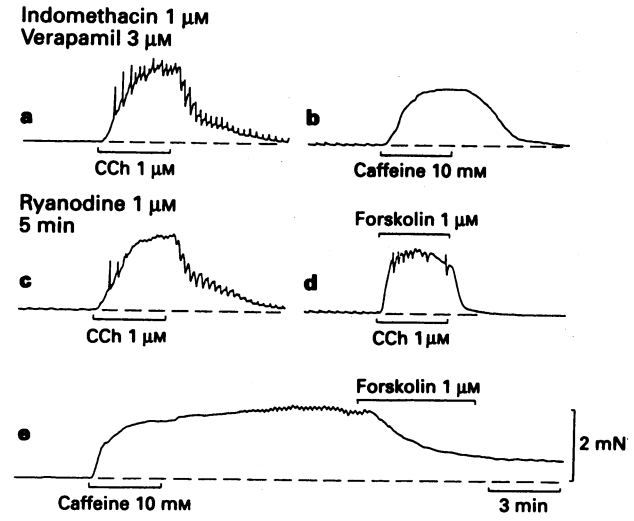

Figure 4 Contractions produced by carbachol (CCh) and caffeine before (a,b) and after ryanodine treatment (c,d,e) in gastric muscle. Indomethacin (1 μM) and verapamil (3 μM) were present throughout the experiment to inhibit phasic contractions. Ryanodine (1 μM) was applied between (b) and (c) for 5 min and CCh (1 μM) was applied 5 min after ryanodine wash-out (c). In (d) CCh was applied together with forskolin (1 μM). Note that caffeine applied 35 min after ryanodine wash-out initiated a sustained contraction (e), but not by CCh (c) or CCh plus forskolin (d). Forskolin partially inhibited the sustained contraction (e). CCh or caffeine was applied at 12 min intervals except for (c) in which CCh was applied at 15 min interval.

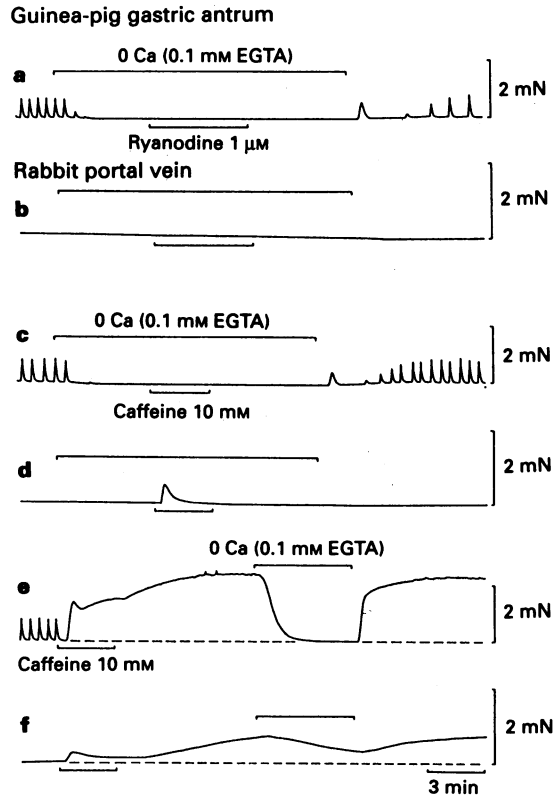

Figure 5 Simultaneous record of mechanical response in muscle strips of stomach (a,c,e) and portal vein (b,d,f). (a and b) Ryanodine application in Ca^{2+}-free solution as indicated; (c and d) caffeine application in Ca^{2+}-free solution. Note that ryanodine pretreatment in Ca^{2+}-free solution was still effective in producing the sustained contraction on caffeine wash-out in the presence (e,f), but not in the absence of Ca^{2+} (c,d); (e) and (f) also show effect of Ca^{2+} removal during the sustained contraction.

Ca^{2+}-free solution. On reapplication of 2.4 mM Ca^{2+}, a transient contraction was produced in gastric muscle (Figure 5a), but no clear response was observed in the portal vein (Figure 5b). The gastric preparation shown in this figure produced a transient contraction on Ca^{2+} reapplication, but in 2 out of 4 preparations no such contraction was observed. When caffeine was applied, 30 min after ryanodine wash-out, in the absence of Ca^{2+}, no contraction was produced in gastric muscle (Figure 5c) and a transient contraction in portal vein (Figure 5d), as observed without ryanodine-pretreatment (Figure 2b,d). The second application of caffeine, 58 min after ryanodine wash-out, in the presence of Ca^{2+} produced a typical sustained Ca^{2+}-sensitive contraction (Figure 5e,f).

It appears that the presence of external Ca^{2+} is necessary for caffeine to evoke a sustained contraction after ryanodine treatment. The ability of intracellular Ca^{2+} to evoke the sustained contraction was examined by use of caffeine (10 mM) and CCh (100 μM) in the absence of the external Ca^{2+} (Figure 6). CCh (100 μM) is considered to produce a contraction by releasing Ca^{2+} from intracellular stores in this muscle (Parekh & Brading, 1991). As shown in Figure 6b, caffeine applied together with CCh produced a transient contraction in Ca^{2+}-free solution, but on reapplication of 2.4 mM Ca^{2+} only large phasic contractions, but no sustained contraction, could be observed. Caffeine applied 10 min after Ca^{2+} reapplication produced a typical sustained contraction (Figure 6c). These results suggest that the presence of extracellular Ca^{2+} (or Ca^{2+} influx) is necessary for the caffeine-induced sustained contraction following exposure to ryanodine.

In muscle strips of the gastric antrum, ryanodine did not affect either the resting tension or the frequency of spontaneous activity at 1 μM, but when the concentration was increased to more than 3 μM a tonic contraction slowly developed with a small increase in frequency. The increase in frequency of spontaneous activity was $17 \pm 3\%$ ($n = 6$) 10 min after 5 min treatment with 10 μM ryanodine. When the basal tone was increased the size of rhythmic contractions became reduced.

Figures 7 and 8 show simultaneous recordings from gastric strips of mechanical and electrical activity recorded with intracellular microelectrodes. Caffeine (10 mM) produced a tonic contraction and blocked rhythmic mechanical and electrical activity, the slow wave, with membrane depolarization (Figure 7a). Ryanodine (1 μM) had no noticeable effects on mechanical activity and slow wave on its own, but a sustained contraction was produced after a subsequent application of caffeine. In the preparation shown in Figure 7, in which caffeine produced a relatively large depolarization (9.5 mV), the membrane stayed depolarized, the amplitude of the slow wave was reduced, and its frequency was slightly increased during the sustained contraction on which small ripples were superimposed (Figure 7b). Although successful recordings were difficult, similar results were obtained in four other preparations, a mean value of depolarization being 8.6 ± 2.2 mV ($n = 5$) and the resting potential (the most negative value between slow waves) -66 ± 4 mV. In two additional preparations, however, membrane depolarization produced by caffeine (10 mM) was relatively small (3–4 mV) and no clear changed in membrane potential and slow wave were observed during the sustained contraction, except that the frequency during the recovery from the caffeine effect was slightly reduced (Figure 8b). The reason for the difference was not clear, but inhomogeneity of electrical properties within the preparation is unlikely, because the mechanical activity recorded from the whole preparation correlated well with the pattern of electrical response recorded with a microeletrode.

Discussion

There is a clear difference in caffeine-induced contractions between guinea-pig gastric muscle and rabbit portal vein, the former being of a tonic and the latter of a phasic type. This may be due to the fact that the contraction in portal vein depends more on intracellular Ca^{2+} release and that in gast-

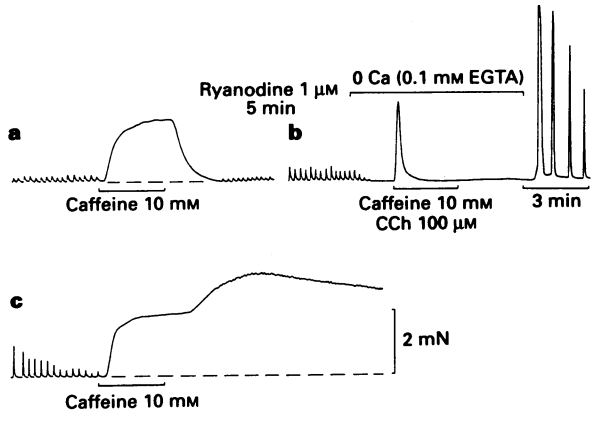

Figure 6 Contractions produced by simultaneous application of carbachol (CCh) and caffeine in the absence of Ca^{2+} (b) and by caffeine in the presence of Ca^{2+} (c) after ryanodine treatment in gastric muscle. (a) Control response to 10 mM caffeine; (b) CCh (100 μM) and caffeine (10 mM) were applied 7 min after ryanodine wash-out and 2 min after exposure to Ca^{2+}-free solution containing 0.1 mM EGTA. (c) Caffeine was applied 25 min after ryanodine treatment.

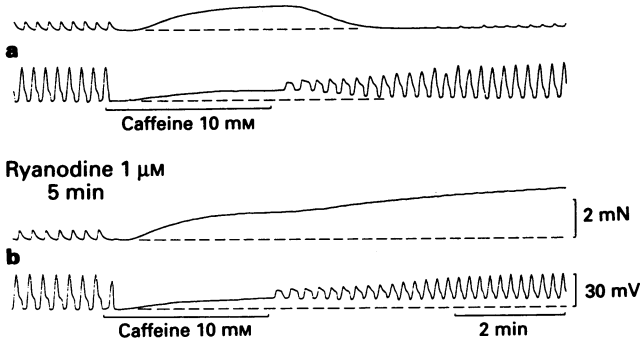

Figure 7 Simultaneous recording of mechanical activity (upper) and intracellular membrane potential (lower trace) in a gastric muscle strip. Electrical activity was obtained from the same cell and interval between records (a) and (b) is 12 min. Ryanodine (1 μM) was applied for 5 min, 10 min after wash-out of caffeine shown in (a).

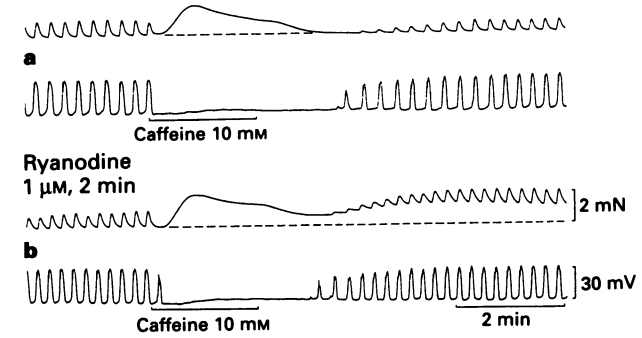

Figure 8 Similar records to Figure 7 in a gastric muscle strip obtained from a different guinea-pig. In this experiment, ryanodine was applied for only 2 min. Note that in this preparation, the sustained contraction, produced by caffeine wash-out after ryanodine pretreatment, was not accompanied by any clear change in membrane potential (the most negative value between slow waves).

ric muscle more on Ca^{2+} influx. This idea is supported by the observations that caffeine-induced contraction is more resistant to removal of Ca^{2+} from the external medium in the portal vein than in gastric muscle and that in gastric muscle the contraction can be readily restored by reapplication of Ca^{2+} in the presence of caffeine.

It has been reported in guinea-pig portal vein, pulmonary artery, and taenia caeci (Iino *et al.*, 1988) and coronary myocytes (Ganitkevich & Isenberg, 1993), that pretreatment with 1–30 μM ryanodine for a few minutes abolishes the caffeine-induced increase in $[Ca^{2+}]_i$, probably by depleting Ca^{2+} in the stores releasable by caffeine. Similarly, caffeine-induced contractions in rat aorta and mesenteric artery are inhibited by ryanodine pretreatment (Shima & Blaustein, 1992). In the present experiments, however, pretreatment with 1–10 μM ryanodine for 1–5 min did not essentially affect the caffeine-induced contraction, but resulted in a sustained contraction accompanied by a maintained high $[Ca^{2+}]_i$ level subsequent to caffeine removal both in gastric muscle and portal vein. The difference could be due either to the failure to deplete the store in the guinea-pig gastric antrum and rabbit portal vein or to a different action of caffeine and ryanodine in these preparations.

The sustained contraction initiated by removal of caffeine, following exposure to ryanodine is dependent on the presence of the external Ca^{2+}, suggesting that continuous Ca^{2+} influx is necessary. The Ca^{2+} influx pathway responsible for the sustained contraction was not clarified in the present experiments, but voltage-gated L-type channels do not seem to be involved because verapamil or nicardipine had no effect on the contraction. Although involvement of other types of voltage-gated Ca^{2+} channels insensitive to organic Ca^{2+} channel blockers cannot be completely disregarded, this is rather unlikely because the sustained contraction can be observed with little change in membrane potential and slow wave activity (Figure 8). It is known that some Ca^{2+} influx pathway in the plasma membrane can be activated when intracellular Ca^{2+} stores are depleted (Putney, 1990). In the present experiments, however, no clear evidence for such a pathway was obtained. Caffeine application in Ca^{2+}-free medium is expected to deplete Ca^{2+} stores, but on Ca^{2+} reapplication no contraction or only a very small transient contraction can be evoked with or without ryanodine pretreatment (Figure 5). Furthermore, since the caffeine-induced contraction was readily abolished by removal of the external Ca^{2+}, there was no clear evidence for Ca^{2+} release with caffeine in the gastric muscle. The Ca^{2+} influx pathway responsible for the sustained contraction is likely to be blocked by intracellular cyclic AMP, because the contraction is inhibited by caffeine and forskolin, which are known to increase cyclic AMP.

The presence or absence of external Ca^{2+} during exposure to ryanodine does not appear to affect the ability of caffeine to initiate a sustained contraction. Caffeine (in the presence of Ca^{2+}) applied about 1 h after ryanodine treatment in the absence of the external Ca^{2+} could still produce a typical sustained contraction on wash-out. On the other hand, caffeine applied in Ca^{2+}-free solution after pretreatment with ryanodine failed to produce a sustained contraction when Ca^{2+} was reapplied (Figure 5). Furthermore, this is also true even if a transient contraction is evoked by simultaneous application of CCh in Ca^{2+}-free solution. The idea that ryanodine acts on the Ca^{2+}-induced Ca^{2+} release channels of intracellular stores only when they are open (Iino *et al.*, 1988) is not sustained by the results in the present experiments. The present results suggest that ryanodine is nearly irreversibly bound at some sites controlling Ca^{2+} influx through the plasma membrane which can be activated by caffeine only when Ca^{2+} is present in the external medium. High $[Ca^{2+}]_i$ is not a sufficient condition to produce the sustained contraction, because carbachol which produced contractions of a similar size to caffeine-induced contractions could not produce a sustained contraction after ryanodine pretreatment. Excess K^+ also failed to evoke a sustained contraction (unpublished observations). In conclusion the plasma membrane of the circular muscles of the guinea-pig gastric antrum and rabbit portal vein appear to have properties similar to the sarcoplasmic reticulum, as previously considered (Ito & Kuriyama, 1971), so that Ca^{2+} permeability of the plasma membrane may be increased directly by caffeine and following an exposure to ryanodine this pathway may be kept open, once activated by caffeine, in the presence of Ca^{2+}.

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Study of the *in vivo* and *in vitro* cardiovascular effects of (+)-glaucine and N-carbethoxysecoglaucine in rats

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1 The cardiovascular and vasorelaxant effects of (+)-glaucine and of a semisynthetic derivative (N-carbethoxysecoglaucine) were studied in rats.

2 N-carbethoxysecoglaucine did not modify either systolic arterial pressure or heart rate values in conscious (25 mg kg^{-1}, p.o.) and anaesthetized normotensive rats (5 mg kg^{-1}, i.v.). Furthermore, this compound showed no activity in the experiments carried out on rat isolated aorta [contractility and $^{45}Ca^{2+}$ influx assays (5 μM)] and did not modify the rate and force of contraction in rat isolated atria (5 μM).

3 In conscious normotensive rats, oral administration of (+)-glaucine (25 mg kg^{-1}) did not modify either systolic arterial pressure or heart rate.

4 In anaesthetized normotensive rats, (+)-glaucine (5 mg kg^{-1}, i.v.) produced a remarkable fall in mean arterial pressure (MAP) accompanied by a significant decrease in heart rate. In the same preparation, (+)-glaucine (5 mg kg^{-1}, i.v.) did not modify the cardiovascular effects induced by noradrenaline (NA) (5 μg kg^{-1}) and 5-hydroxytryptamine (5-HT) (300 μg kg^{-1}) but markedly inhibited those induced by nicotine (200 μg kg^{-1}).

5 In isolated intact aorta of rat, (+)-glaucine (0.15–5 μM) competitively inhibited the contractions induced by NA (with a pA_2 value of 7.14) and non-competitively those induced by 5-HT (in normal Krebs solution) and Ca^{2+} (in depolarizing Ca^{2+}-free high-K^+ 50 mM solution), with depression of the maximal response and with pD_2' values of 5.56 and 5.26, respectively.

6 In experiments in Ca^{2+}-free medium, (+)-glaucine (3 μM) inhibited the contractions induced by NA and had no effect on either 5-HT- or caffeine-induced contractions.

7 Furthermore, in the experiments with radioactive Ca^{2+}, (+)-glaucine (3 μM) did not modify the basal uptake of $^{45}Ca^{2+}$ but strongly inhibited the influx of $^{45}Ca^{2+}$ induced by NA, 5-HT and K^+.

8 (+)-Glaucine (5 μM) had no effect on rate and force of contraction in rat isolated atria.

9 These results indicate that: (a) the cardiovascular effects (hypotension and bradycardia) of (+)-glaucine in anaesthetized normotensive rats (5 mg kg^{-1}) may be due, at least in part, to a ganglioplexic effect; (b) the vasorelaxant action of (+)-glaucine (0.15–5 μM) in rat isolated aorta can be attributed to an α_1-adrenoceptor blocking property (which may explain its inhibition of noradrenaline-induced $^{45}Ca^{2+}$ influx and contractions in normal Krebs solution and noradrenaline-induced contractions in Ca^{2+}-free medium) and to a Ca^{2+}-antagonist activity (which may be responsible, at least in part, for the inhibition of $^{45}Ca^{2+}$ uptake induced by NA, 5-HT and K^+ and the contractions induced by both NA and 5-HT in normal Krebs solution and by Ca^{2+} in Ca^{2+}-free high-K^+ medium) and (c) there is no correlation between the mechanisms of action observed for (+)-glaucine *in vivo* and *in vitro*, which suggests that the vasorelaxant activity of this alkaloid does not contribute to its hypotensive activity.

Keywords: Conscious and anaesthetized normotensive rats; rat aorta; rat atria; (+)-glaucine; N-carbethyoxysecoglaucine; hypotensive activity

Introduction

Glaucium flavum Crantz (Papaveraceae) has been used for years as a remedy for cough and other illnesses. (+)-Glaucine [(S)-1,2,9,10-tetramethoxyaporphine], an alkaloid isolated by Ivanov & Ivanova (1958) from the above-ground parts of this plant and present in other different genera (Guinaudeau *et al.*, 1988), attracts the attention of pharmacologists nowadays due to its antitussive potency, which is similar to that of codeine but with fewer side effects (physical dependence, constipation, respiratory depressant effects, etc.) (Kasé *et al.*, 1983) (Figure 1). Hypotension is the characteristic result of the action of (+)-glaucine on the cardiovascular system (CVS). In fact, in anaesthetized normotensive rats, this alkaloid showed a hypotensive activity accompanied by a significant decrease in heart rate (Fdez. Alzueta *et al.*, 1992a) the mechanism of which, though not extensively studied and clarified as yet, may be basically related to its *in vitro* vasodilator effect on the vascular smooth muscle, as has been previously described by Loza *et al.* (1993).

Like papaverine, (+)-glaucine has been observed to relax directly vascular and non-vascular smooth muscle. The mechanism of this activity is unknown and seems to be different from that of papaverine (Cortés *et al.*, 1990). Several hypotheses have been postulated, including an inhibition of adenosine 3':5'-cyclic monophosphate (cyclic AMP) phosphodiesterase in intestinal smooth muscle (Petkov & Stancheva, 1980) and bovine aorta (Ivorra *et al.*, 1992). In the rat uterus, (+)-glaucine shows a relaxant activity, possibly related to inhibition of Ca^{2+} entry through potential-operated Ca^{2+} channels (Anselmi *et al.*, 1992). Similar Ca^{2+}-antagonist properties have been described in rat cerebral cortex (where (+)-glaucine inhibits [^{3}H]-(+)-*cis*-diltiazem binding, Ivorra *et al.*, 1992). In rat vas deferens, (+)-glaucine has been characterized as an agent with weak

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relaxant activity, possibly due to its Ca^{2+}-antagonist and non-selective α-adrenoceptor blocking properties (Orallo *et al.*, 1993). The vasorelaxant action of (+)-glaucine in rat aorta seems to be related to α_1-adrenoceptor blocking properties because it relaxes the contractile response induced by noradrenaline (NA) more strongly than that induced by K^+ (Ivorra *et al.*, 1992; Loza *et al.*, 1993). Furthermore, similar results have been obtained in rat cerebral cortex, where (+)-glaucine blocks [^{3}H]-prazosin binding (Ivorra *et al.*, 1992).

In view of these reports and with the aim of further elucidating the mechanism(s) of the hypotensive action of (+)-glaucine, the effects of this alkaloid on the CVS *in vivo* (conscious and anaesthetized normotensive rats) and *in vitro* (rat isolated aorta and atria) were studied. We have also studied, for the first time, the potential cardiovascular activity of a semisynthesized derivative, the N-carbethoxysecoglaucine (Figure 1).

A preliminary account of this study has been published in abstract form by the Sociedad Española de Farmacología (Fdez. Alzueta *et al.*, 1992a).

Methods

Conscious normotensive rats

A group of five normotensive male Sprague–Dawley rats weighing 250–300 g received water (control) p.o. (10 ml kg^{-1}) and another similar group (treated) received (+)-glaucine and N-carbethoxysecoglaucine solutions p.o. (25 mg kg^{-1}, 10 ml kg^{-1}). The systolic arterial pressure and heart rate were measured by an indirect tail-cuff method, as described by Orallo *et al.* (1992a).

Anaesthetized normotensive rats

Normotensive male Sprague–Dawley rats weighing 250–300 g were anaesthetized by intraperitoneal injection of urethane following the method described in Orallo *et al.* (1992b).

In the first set of experiments, after blood pressure and heart rate had stabilized, saline (1 ml kg^{-1}, for control group) and (+)-glaucine or N-carbethoxysecoglaucine solutions (5 mg kg^{-1}, 1 ml kg^{-1}, for treated groups) were injected intravenously via the left femoral vein, in order to observe the effects on blood pressure and heart rate.

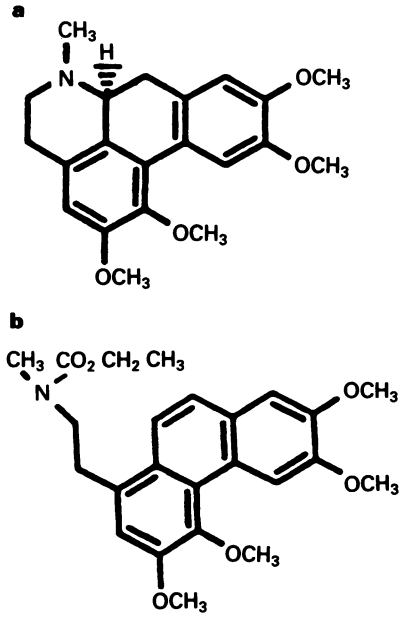

Figure 1 Chemical structures of (a) (+)-glaucine and (b) N-carbethoxysecoglaucine.

In the second set of experiments, NA, 5-hydroxytryptamine (5-HT) and nicotine were injected via the left femoral vein. After observing the cardiovascular responses produced by these drugs and that the haemodynamic parameters (blood pressure and heart rate) returned to basal values (15–20 min), a dose of saline or (+)-glaucine was injected. After 10–15 min, the same dose of the pressor agent was administered to test (a) the reproductibility of the cardiovascular effects induced by successive administrations of NA, 5-HT and nicotine in the control group or (b) whether (+)-glaucine could modify the cardiovascular responses induced by the pressor agents in the treated preparations.

Contraction studies in rat isolated thoracic aorta rings

Male Sprague–Dawley rats weighing 250–300 g were killed by stunning and exsanguination. The thoracic aorta was rapidly removed and placed in a Petri dish with Krebs bicarbonate solution oxygenated with carbogen (95% O_2 + 5% CO_2) at room temperature (20 ± 2°C) of the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2 H_2O 2.5, $MgSO_4$ 7 H_2O 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, EDTA 0.03, ascorbic acid 0.6 and glucose 11. Aortic rings were transferred to an organ bath according to the general procedure of Gil-Longo *et al.* (1992).

NA and 5-HT concentration-response curves Cumulative concentration-response curves were obtained by the method of Van Rossum (1963). In control rings, four consecutive concentration-response curves were obtained at 60-min intervals to allow washout and to minimize the possibility of receptor desensitization; the first curve differed from the last three, which were reproducible. In treated rings, after obtaining two similar curves with the agonist, tissues were incubated with (+)-glaucine/N-carbethoxysecoglaucine for 25 min (a sufficient exposure time to achieve equilibrium) and a fourth was constructed.

Calcium concentration-response curves After an equilibrium period of 1 h in Krebs bicarbonate solution, tissues were incubated for 30 min in Ca^{2+}-free depolarizing Krebs bicarbonate solution (containing 50 mM KCl instead of the equivalent amount of NaCl in order to maintain osmolarity). Calcium chloride (10 μM–30 mM) was then added to the bath in stepwise fashion. As above, after two reproducible control concentration-response curves had been obtained, tissues were incubated with (+)-glaucine/N-carbethoxysecoglaucine for 25 min and a fourth curve was then obtained.

Studies in calcium-free medium were carried out as described by Gil-Longo *et al.* (1992) but with a normal Krebs solution and a Ca^{2+}-free Krebs solution (containing 0.5 mM EGTA). Contraction was elicited with NA (10 μM); 5-HT (300 μM) and caffeine (10 mM) and the drugs under study were (+)-glaucine/N-carbethoxysecoglaucine.

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

The experimental protocol followed was the one described in Orallo *et al.* (1993) using Krebs bicarbonate solution (containing 1.5 mM instead of 2.5 mM $CaCl_2.2H_2O$). The vasoconstrictor agents used were NA (10 μM), 5-HT (100 μM) and K^+ (50 mM). To investigate the actions of (+)-glaucine/N-carbethoxysecoglaucine on basal and induced $^{45}Ca^{2+}$ uptake, the compounds were added to the bath 20 min before and for the incubation period after the addition of $^{45}Ca^{2+}$.

Rat isolated atria

Male Sprague–Dawley rats (250–300 g) were killed by a blow on the head and exsanguinated. The atria were set up in an organ-bath under 1 g resting tension as described by

Orallo et al. (1992a). After a 60 min stabilization time period, de-ionized water (for control group) or (+)-glaucine/N-carbethoxysecoglaucine solution (for treated group) was added and measurements of force and rate of contraction registered each minute for a period of 15 min, in order to observe whether the inotropic and/or chronotropic properties were modified by these compounds.

Data presentation and statistical analysis

Unless otherwise specified, results shown in the text and figures are expressed as means ± s.e.mean or 95% confidence interval. Significant differences between two means ($P<0.05$ or $P<0.01$) were estimated by Student's two-tailed t test for paired or unpaired data.

Systolic blood pressure and heart rate values are expressed in mmHg and beats min^{-1}, respectively. Mean arterial pressure was calculated according to the formula: (2 diastolic pressure + systolic pressure)/3. Unless otherwise specified, increases or decreases of mean blood pressure and heart rate values are expresssed as percentages with respect to basal values.

In functional postsynaptic studies, contractile responses to vasoconstrictor agents (in the presence or absence of (+)-glaucine) are expressed as a percentage of the maximal contraction reached in the concentration-response curves obtained before incubation with (+)-glaucine.

Vasoconstrictor agent pD_2 values (negative $\log_{10}$ of the molar concentration of agonist required to elicit 50% of maximal response) and antagonist pD_2' values (negative $\log_{10}$ of the molar concentration of antagonist required to cause a 50% depression of the maximal contractile effect of the agonist) were calculated (Van Rossum, 1963). The (+)-glaucine pA_2 value was obtained according to Arunlakshana & Schild (1959), by linear regression of Schild plots (Tallarida & Murray, 1987) derived from mean concentration-response curves. The value of the slope was used to assess whether antagonism was competitive or not.

$^{45}Ca^{2+}$ tissue uptake was calculated from the formula: $^{45}Ca^{2+}$ uptake [nmol (kg wet tissue)$^{-1}$] = [c.p.m. in tissue (wet tissue weight, kg)$^{-1}$] × [nmol $^{45}Ca^{2+}$ in 1 litre solution (c.p.m. in 1 litre of solution)$^{-1}$]. Note that the numerator of the second factor in this expression is the concentration of $^{45}Ca^{2+}$, not the total Ca^{2+} concentration.

Drugs, chemicals and radioisotopes

The following drugs were used: (+)-glaucine hydrochloride [isolated from *Glaucium flavum* Crantz var. *glabratrum* Wilk. (Papaveraceae) in the Departamento de Química Orgánica de la Universidad de Santiago de Compostela], N-carbethoxysecoglaucine hydrochloride (a (+)-glaucine derivative synthetized in the above-mentioned laboratory). (−)-Noradrenaline bitartrate, 5-hydroxytryptamine creatinine sulphate complex, 5-hydroxytryptamine hydrochloride, (−)-nicotine di-(+)-tartrate, caffeine, EGTA [ethylene glycol-bis (B-aminoethylether) N,N,N',N'-tetraacetic acid], EDTA (disodium salt of ethylenediaminetetraacetic acid) and urethane were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The radioisotope used was $^{45}Ca^{2+}$ (New England Nuclear).

The (+)-glaucine and N-carbethoxysecoglaucine hydrochlorides were dissolved in saline (for i.v. administration) or de-ionized water immediately before use.

The following solutions were prepared daily: (−)-NA bitartrate from 500 μg ml^{-1} or 100 mM stock solutions in saline or de-ionized water, respectively (sodium bisulphite (0.2%) was added to the NA stock solutions to prevent oxidation); 5-HT creatinine sulphate complex and 5-HT hydrochloride from 3 mg ml^{-1} or 100 mM stock solutions in saline or de-ionized water, respectively, and (−)-nicotine di-(+)-tartrate from a stock solution (2 mg ml^{-1}) in saline. All the stock solutions were kept at −20°C. Caffeine was prepared daily in Ca^{2+}-free medium. Urethane was dissolved in saline to make several solutions of 25 g (100 ml)$^{-1}$ and 12000 i.u. (100 ml)$^{-1}$, respectively and kept at 4°C. All reagents used in the preparation of physiological solutions were of analytical grade.

Results

Conscious normotensive rats

The mean basal values of systolic blood pressure and heart rate (measured in control and treated groups before administration) were 116 ± 5 mmHg and 413 ± 9 beats min^{-1} ($n = 15$).

The mean values of systolic blood pressure and heart rate, measured 7 times in control animals for a 24 h period after administration of saline, were not significantly different from the basal ones ($P>0.05$, $n = 5$).

(+)-Glaucine and N-carbethoxysecoglaucine (25 mg kg^{-1}, p.o.) did not significantly modify either systolic blood pressure or heart rate mean values when compared against basal and control values, after 0.5, 1, 3, 5, 7 and 24 h of initiating the treatment ($P>0.05$, $n = 5$) (Figure 2a,b).

Anaesthetized normotensive rats

The mean basal values for mean arterial pressure (MAP) and heart rate in anaesthetized normotensive rats were 101 ± 4 mmHg and 351 ± 13 beats min^{-1} ($n = 45$). In control

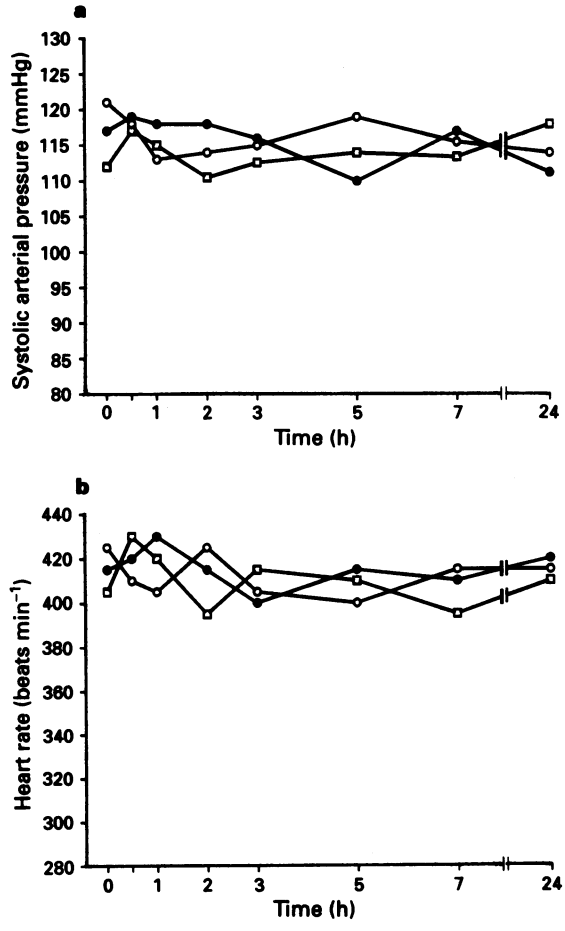

Figure 2 Effects of (+)-glaucine (25 mg kg^{-1}) (●) and N-carbethoxysecoglaucine (25 mg kg^{-1}) (□) on (a) systolic arterial pressure and (b) heart rate in conscious normotensive rats. Each point is the mean of five experiments. S.e.means have been omitted for clarity. Control is represented by (○).

animals, these values were not significantly different throughout the period in which the measurements were carried out ($P > 0.05$, $n = 5$).

Administration of (+)-glaucine (5 mg kg^{-1}, i.v.) caused a pronounced and prolonged fall in MAP accompanied by a significant decrease in heart rate. The maximal effect of the alkaloid was reached in the first minute of administration and persisted for over 30 min (Table 1) (Figure 3a,b; Figure 4a).

N-carbethoxysecoglaucine (5 mg kg^{-1}, i.v.) had no significant effects on either MAP or heart rate 1 and 15 min after administration. The values obtained did not differ with respect to basal values ($P > 0.05$; $n = 5$) (Figure 3a,b; Figure 4b).

NA (5 μg kg^{-1}, i.v.) produced an increase of MAP and heart rate. (+)-Glaucine (5 mg kg^{-1}, i.v.) was injected and the values of blood pressure and heart rate, after 15 min, were 69 ± 5 and 298 ± 11 ($n = 5$). Hence, the same dosage of NA produced a maximal increase of these two haemodynamic parameters, whose increments in mmHg and beats min^{-1} were not significantly different from those obtained in the absence of (+)-glaucine ($P > 0.05$) (Table 2) (Figure 5a).

5-HT (300 μg kg^{-1}, i.v.) significantly modified MAP and heart rate with a characteristic triphasic response: (1) an

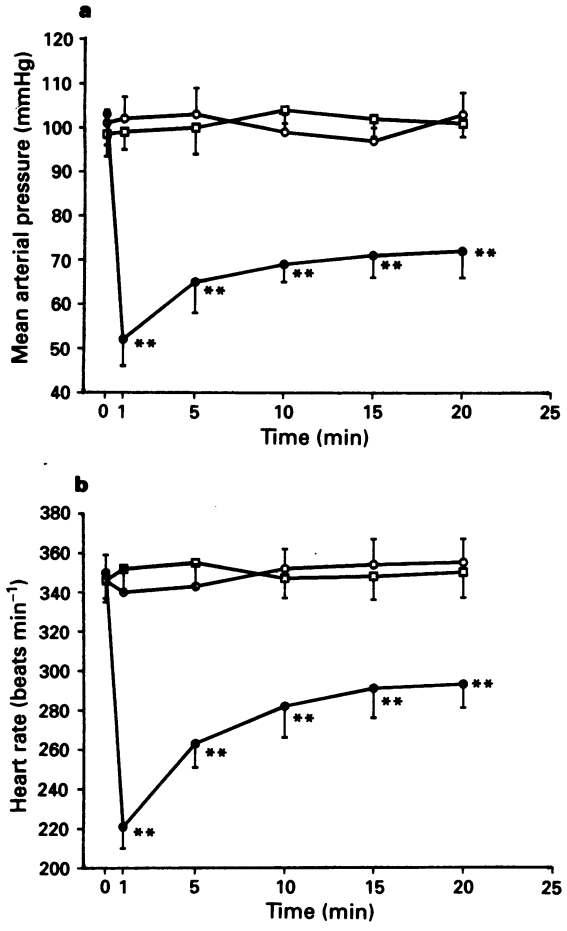

Figure 3 Effects of (+)-glaucine (5 mg kg^{-1}) (●) and N-carbethoxysecoglaucine (5 mg kg^{-1}) (□) on (a) mean arterial pressure and (b) heart rate in anaesthetized normotensive rats. Each point represents the mean value ± s.e.mean from five experiments. Level of statistical significance with respect to control values (○): $^{**}P < 0.01$.

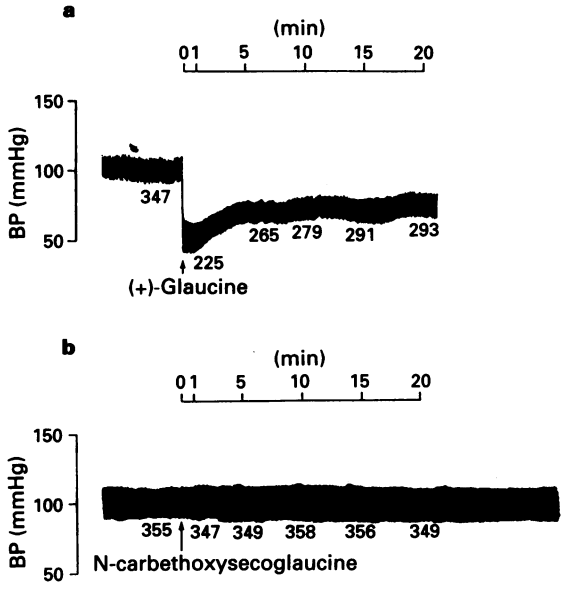

Figure 4 Typical tracings of the effects of (a) (+)-glaucine (5 mg kg^{-1}, i.v.) and (b) N-carbethoxysecoglaucine (5 mg kg^{-1}, i.v.) on blood pressure in anaesthetized normotensive rats. Numbers along the x-axis represent heart rate values.

Table 1 Effects of (+)-glaucine (5 mg kg^{-1}, i.v.) on mean arterial pressure (MAP) and heart rate (HR) in normotensive anaesthetized rats

	n	*MAP* (mmHg)		*HR* (beats min^{-1})	
Basal values	45	101 ± 4	Decrease	351 ± 13	Decrease
Values 1 min after administration	5	52 ± 6*	49 ± 4	221 ± 11*	130 ± 7
Values 15 min after administration	5	71 ± 5*	30 ± 3	291 ± 15*	60 ± 6

Data are expressed as means ± s.e.mean of n experiments. ($^{*}P < 0.01$ with respect to basal values).

Table 2 Effects of different pressor agents on mean arterial pressure (MAP) and heart rate (HR) values in anaesthetized normotensive rats before and after administration of (+)-glaucine (5 mg kg^{-1}, i.v.)

	NA (5 μg kg^{-1}, i.v.)		*5-HT* (300 μg kg^{-1}, i.v.)		*Nicotine* (200 μg kg^{-1}, i.v.)	
Before administration		Increments		Increments		Increments
Maximum value of MAP (mmHg)	159 ± 6*	58 ± 5	154 ± 6*	53 ± 4	178 ± 7*	79 ± 6
Maximum value of HR (beats min^{-1})	419 ± 13*	68 ± 9	385 ± 13*	32 ± 2	398 ± 6*	48 ± 4
Fifteen min after administration						
Maximum value of MAP (mmHg)	124 ± 7**	55 ± 4	126 ± 5**	51 ± 4	99 ± 4**	26 ± 2
Maximum value of HR (beats min^{-1})	372 ± 10**	73 ± 3	322 ± 12**	33 ± 6	317 ± 12**	21 ± 1.5

Each value is the mean ± s.e.mean of 5 experiments. $^{*}P < 0.01$ with respect to basal values. $^{**}P < 0.01$ with respect to values obtained in the presence of (+)-glaucine.

initial decrease, (2) a fast increase, (the values for which are shown in Tables 2 and 3) a long-lasting hypotensive effect. The values of MAP and heart rate were 74 ± 6 mmHg and 289 ± 9 beats min^{-1} ($n = 5$) after 15 min of administering (+)-glaucine. Again, a new dose of 5-HT was administered and, as above, a characteristic triphasic response was produced. There were no statistically significant differences between the values of the increments induced by 5-HT obtained before and after administration of (+)-glaucine ($P > 0.05$) (Table 2) (Figure 5b).

Nicotine (200 μg kg^{-1}, i.v.) increased MAP and heart rate. (+)-Glaucine was administered and, after 15 min, MAP and heart rate values were 73 ± 6 mmHg and 296 ± 11 beats min^{-1} ($n = 5$). Afterwards, the same dosage of the pressor agent produced a maximal increase in MAP and heart rate. In this case, the values of the increments were significantly less than those obtained without the alkaloid ($P < 0.01$) (Table 2) (Figure 5c).

Contraction studies in rat isolated thoracic aortic rings

The preparation lacked spontaneous activity. Resting tone was unaffected by (+)-glaucine (0.15–5 μM) ($n = 5$) or N-carbethoxysecoglaucine (5 μM) ($n = 5$).

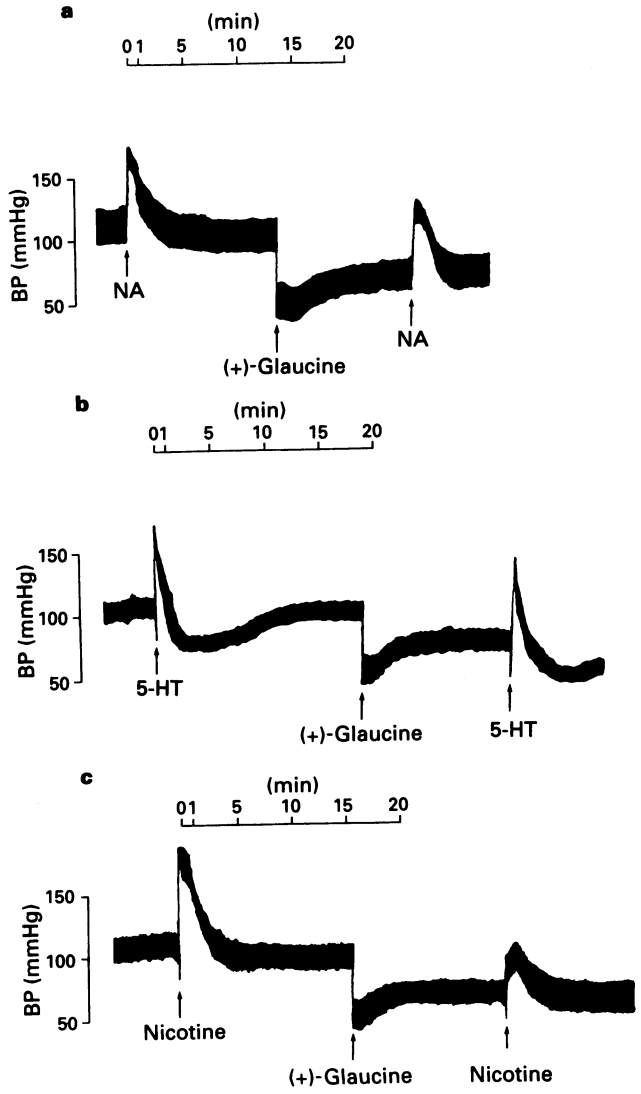

Figure 5 Cardiovascular effects of (a) noradenaline (NA, 5 μg kg^{-1}), (b) 5-hydroxytryptamine (5-HT, 300 μg kg^{-1}) and (c) nicotine (200 μg kg^{-1}) before and after administration of (+)-glaucine (5 mg kg^{-1}) in normotensive anaesthetized rats. Heart rate values omitted for clarity.

NA elicited dose-related contractions in intact aorta rings. The pD_2 and the maximal tension (mg) values were 8.27 ± 0.54 and 6434 ± 420, respectively ($n = 5$). (+)-Glaucine (0.5–5 μM) shifted the concentration-response curve for NA to the right without depression of the maximal response (Figure 6a). The pA_2 value was 7.14 (6.40 to 7.88) and the slope of the Schild plot [−0.98 (−1.52 to −0.46)] did not differ significantly from unity ($P > 0.05$, $n = 5$).

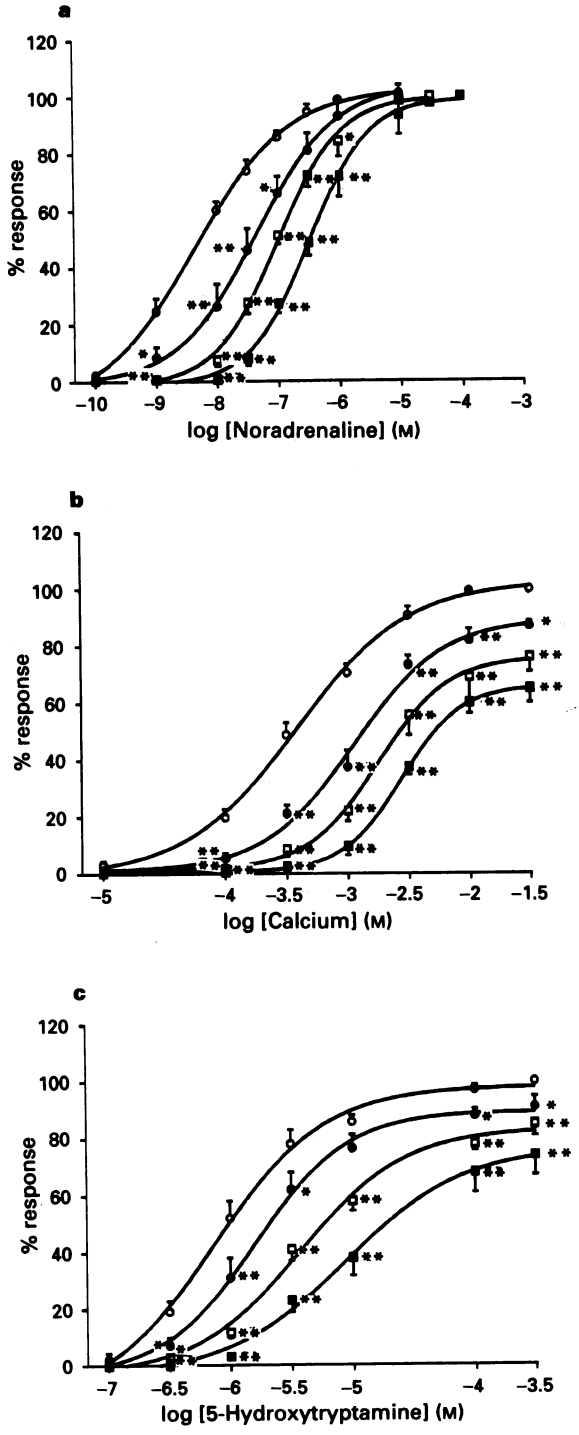

Figure 6 Cumulative concentration-response curves for (a) noradrenaline (○) in the absence or presence of (+)-glaucine (● 0.5 μM, □ 1.5 μM and ■ 5 μM); (b) Ca^{2+} in Ca^{2+}-free high-K^+ solution (○) in the absence or presence of (+)-glaucine (● 0.5 μM, □ 1.5 μM and ■ 5 μM); (c) 5-hydroxytryptamine (○) in the absence or presence of (+)-glaucine (● 0.15 μM, □ 0.5 μM and ■ 1.5 μM). Each point represents the mean value ± s.e.mean from 5 experiments. Level of statistical significance with respect to control curves: $^{**}P < 0.01$ or $^{*}P < 0.05$.

Table 3 Effects of N-carbethoxysecoglaucine and (+)-glaucine on the fast and slow components of the contractions induced by different agonists in rat isolated aorta in Ca^{2+}-free medium

Agonist	*Fast component* (mg)			*Slow component* (mg)		
		N-carbethoxysecoglaucine	*(+)-glaucine*		*N-carbethoxysecoglaucine*	*(+)-glaucine*
NA (10 μM)	2359 ± 170	2180 ± 159*	1250 ± 113**	1471 ± 127	1394 ± 145*	740 ± 68**
5-HT (300 μM)	1048 ± 97	934 ± 74*	1005 ± 93*	523 ± 48	492 ± 41*	517 ± 36*
Caffeine (10 mM)	510 ± 31	502 ± 27*	493 ± 40*	–	–	–

Data are expressed as means ± s.e.mean from 5 experiments. Stastistical significance with respect to basal values: *$P>0.05$, **$P<0.01$.

In Ca^{2+}-free high-K^+ (50 mM) medium, addition of Ca^{2+} to the bath induced a gradual increase in tension. The maximal tension reached was 4450 ± 490 mg and the EC_{50} value (50% effective concentration) was 371 ± 27 μM ($n = 5$). (+)-Glaucine (0.5–5 μM) antagonized non-competitively the Ca^{2+}-induced contractions with depression of the maximal response (Figure 6b). The pD_2' value was 5.26 ± 0.13 ($n = 5$).

(+)-Glaucine (0.15–1.5 μM) significantly inhibited 5-HT-induced contractions in the intact aortic rings and caused a shift to the right of the concentration-response curves for this vasoconstrictor agent without recovery of the maximal tension (Figure 6c). The pD_2 and the maximal tension (mg) reached were 6.03 ± 0.49 and 3471 ± 230 ($n = 5$). The pD_2' value was 5.56 ± 0.12 ($n = 5$).

N-carbethoxysecoglaucine (5 μM) did not modify the concentration-response curves elicited by NA, Ca^{2+} and 5-HT in intact aorta rings ($P>0.05$, $n = 5$; data not shown).

In Ca^{2+}-free medium, NA (10 μM) and 5-HT (300 μM) produced a characteristic contraction with two distinct components: an initial transient contraction (fast component) that relaxed to a sustained tension (slow component). Caffeine (10 mM) produced only an initial transient contraction. These contractions were unaffected by the addition of N-carbethoxysecoglaucine (5 μM).

(+)-Glaucine (3 μM) modified neither the constrictor effect induced by caffeine nor the one induced by 5-HT but did modify that induced by NA (Table 3).

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

Basal uptake of $^{45}Ca^{2+}$ by the segments of rat aorta was 11.8 ± 1.1 nmol kg^{-1} ($n = 5$). (+)-Glaucine (3 μM) and N-carbethoxysecoglaucine (5 μM) did not significantly modify this value ($^{45}Ca^{2+}$ tissue content: 10.8 ± 0.6 nmol kg^{-1}, $P>0.05$, $n = 10$).

NA (10 μM), 5-HT (100 μM) and high K^+ (50 mM) significantly increased $^{45}Ca^{2+}$ uptake (evoked uptake) ($^{45}Ca^{2+}$ tissue content: 16.7 ± 0.7 nmol kg^{-1} (NA), $P<0.05$ with respect to basal value, $n = 5$; 19.2 ± 1.1 nmol kg^{-1} (5-HT), $P<0.05$ with respect to basal value, $n = 5$; 22.0 ± 0.6 nmol kg^{-1} (K^+); $P<0.01$ with respect to basal value, $n = 5$). (+)-Glaucine (3 μM) considerably reduced this value: $^{45}Ca^{2+}$ tissue content in the presence of (+)-glaucine: 11.7 ± 1.3 nmol kg^{-1} (NA), $P<0.01$ with respect to evoked value, $n = 5$; 13.9 ± 0.6 nmol kg^{-1} (5-HT), $P<0.05$ with respect to evoked value, $n = 5$; 17.7 ± 0.9 nmol kg^{-1} (K^+); $P<0.01$ with respect to evoked value, $n = 5$.

N-carbethoxysecoglaucine (5 μM) had no significant inhibitory action on induced ^{45}Ca uptake ($^{45}Ca^{2+}$ tissue content: 16.4 ± 0.9 nmol kg^{-1} (NA), $P>0.05$ with respect to evoked value, $n = 5$; 18.9 ± 0.7 nmol kg^{-1} (5-HT), $P>0.05$ with respect to evoked value, $n = 5$; 22.3 ± 0.9 nmol kg^{-1} (K^+); $P>0.05$ with respect to evoked value, $n = 5$).

Rat isolated atria

The mean basal values of force and rate of contraction, measured in control and treated groups before administration, were 52 ± 3 mg and 303 ± 5 beats min^{-1} ($n = 15$). In the control preparations, the mean values obtained in the 15 min period were not significantly different from the basal ones ($P>0.05$, $n = 5$).

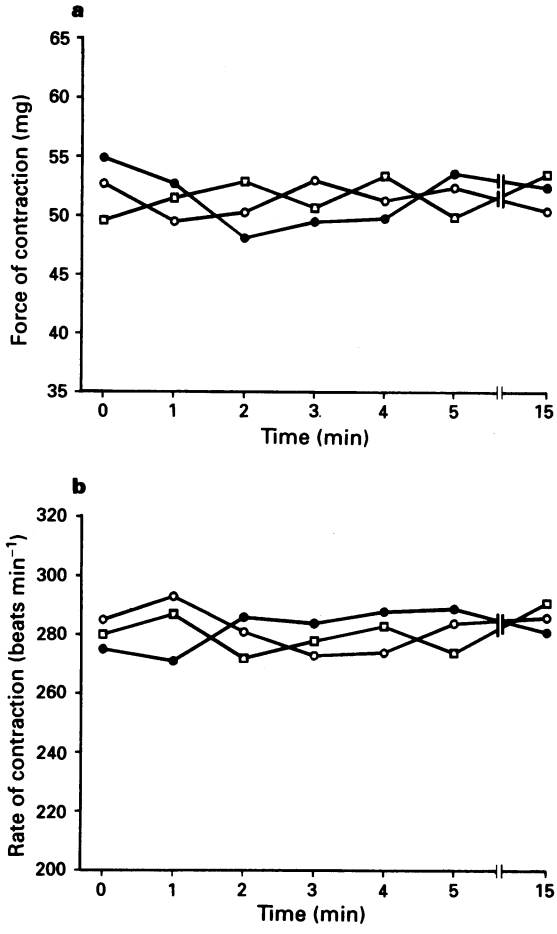

Figure 7 Effects of (+)-glaucine (5 μM) (●) and N-carbethoxysecoglaucine (5 μM) (□) on (a) force of contraction and (b) rate of contraction in rat isolated atria. Each point is the mean of five experiments. S.e.means omitted for clarity. Control is represented by (○).

(+)-Glaucine (5 μM) and N-carbethoxysecoglaucine (5 μM) did not significantly modify the mean atrial force and rate values, for the period of 15 min, compared against basal and control values ($P>0.05$, $n = 5$) (Figure 7a,b).

Discussion

The objective of this work was to examine the potential activity on the CVS of the alkaloid (+)-glaucine and the derivative N-carbethoxysecoglaucine.

In vivo, N-carbethoxysecoglaucine failed to modify the

blood pressure and heart rate values in both conscious (25 mg kg^{-1}, p.o.) and anaesthetized normotensive rats (5 mg kg^{-1}, i.v.). In the experiments carried out on rat isolated aorta, this derivative (5 μM) showed no activity in the assays of contractility (concentration-response curves induced by NA, 5-HT and Ca^{2+}; studies of contraction in Ca^{2+}-free medium using NA, 5-HT and caffeine as agonists) and did not modify the basal values of $^{45}Ca^{2+}$ influx. In rat isolated atria (5 μM), this compound again did not change the basal values of rate and force of contraction.

On the other hand, intravenous bolus injection of (+)-glaucine produced a strong hypotensive effect accompanied by an intense decrease in heart rate. However, in conscious normotensive rats (25 mg kg^{-1}) no hypotensive effect was observed for this alkaloid, which may be due to the fact that the dose was unable to reach the site of action (receptors) involved in the cardiovascular response in the anaesthetized rat. The possibility of a poor bioavailability for (+)-glaucine is ruled out because of the significant alterations in rat central nervous system activity (analgesic and sedative effects) seen when administered p.o. (data not shown), as previously described (Zetler, 1988).

Therefore, with the objective of elucidating the mechanism of action of (+)-glaucine *in vivo*, a series of experiments in anaesthetized normotensive rats was designed.

In this preparation, intravenous injection of NA increased systolic and diastolic pressures due to increases in peripheral vascular resistance (which involves vasoconstriction, through stimulation of postsynaptic α_1- and α_2-adrenoceptors) and heart rate (mediated by activation of β_1-adrenoceptors) (Jie *et al.*, 1985). The results presented here show that (+)-glaucine did not antagonize either the hypertensive or positive chronotropic effects induced by NA, which indicates that: (i) the alkaloid-induced vasodilatation cannot be attributed to a blockade of the postsynaptic α_1- and/or α_2-adrenoceptors of smooth muscle cells and (ii) the bradycardia is not due to a blockade of the β_1-adrenoceptors.

In the same preparation, the blood pressure response to intravenous 5-HT administration was triphasic (Meller *et al.*, 1990): (1) a brief depressor phase immediately following the injection, initiated with the coronary chemoreflex (Bezold-Jarisch reflex) and due to interaction with cardiac 5-HT$_3$ receptors, which led to profound bradycardia and hypotension (Saxena & Villalón, 1991); (2) a pressor phase, as a consequence of vasoconstriction mediated through 5-HT$_2$ receptors (recently renamed 5-HT$_{2A}$ receptors) and due mainly to the direct effects of 5-HT in increasing total peripheral resistance, heart rate and cardiac output (Cohen *et al.*, 1993), and finally, within 1 or 2 min after the injection, a prolonged hypotensive phase due to the activation of 5-HT$_1$ receptors of endothelial cells and release of endothelial vasodilator factors (Bodelsson *et al.*, 1993). (+)-Glaucine did not modify the 5-HT pharmacological response on blood pressure or heart rate, which suggests that the alkaloid does not interact with any of the 5-HT receptors and, therefore, the hypotensive response of the compound cannot be attributed to a stimulation of 5-HT$_1$/5-HT$_3$ receptors or to a blockade of 5-HT$_2$ receptors.

Some drugs produce a hypotensive effect by blocking neurotransmission at a ganglionic level, which was demonstrated with the assays carried out with nicotine. Intravenous administration of nicotine in anaesthetized normotensive rats caused vasoconstriction, tachycardia and, subsequently, an increase of blood pressure values. This is due to stimulation of nicotinic receptors in the sympathetic ganglia and the adrenal medulla, which involves the discharge of catecholamines, in which the activation of chemoreceptors of the aortic and carotid bodies also plays a part (Shimada *et al.*, 1984). In this respect, the cardiovascular response of nicotine was significantly antagonized by intravenous injection of (+)-glaucine, which suggests that the cardiovascular effects of this alkaloid in anaesthetized normotensive rat are due to a ganglioplexic activity.

On the other hand, it is also interesting to note that the (+)-glaucine-induced bradycardia that accompanies the hypotensive effect cannot be attributed to a direct action of the compound on the heart because this alkaloid did not affect: (i) the noradrenaline-induced heart rate increase in anaesthetized normotensive rats, which also indicates that it does not block β_1-adrenoceptors and (ii) the basal values of inotropic and chronotropic properties in rat isolated atria.

We have also investigated the vasorelaxant effect of (+)-glaucine in rat isolated intact aorta by evaluating its action on the contractile responses of rat aorta induced by NA, 5-HT and Ca^{2+} (in Ca^{2+}-free high-K^+ 50 mM solution) in order to ascertain whether the action of this alkaloid was selective or not.

It has been reported that high K^+ concentrations cause contractions in smooth muscle by depolarizing cell membranes and by increasing the influx of Ca^{2+} through L and T voltage-dependent channels (Godfraind & Govoni, 1989). It has also been shown that the activation of several postsynaptic α_1-adrenoceptors by NA (Muramatsu *et al.*, 1990) and 5-HT$_{2A}$ receptors by 5-HT (Sufka *et al.*, 1990) in rat aorta induces a biphasic contraction: an initial transient contraction produced by IP_3-mediated release of Ca^{2+} from intracellular stores (via activation of IP_3 specific receptors), and a slow, sustained contraction caused by Ca^{2+} influx through receptor-operated Ca^{2+} channels (Karaki & Weiss, 1988; Van Breeman & Saida, 1989), although the existence of these channels is now considered to be doubtful (Nelson *et al.*, 1990; Gil Longo *et al.*, 1992).

The results of the present experiments show that the inhibition of the NA-induced contractions may be due to a competitive antagonism of the vascular postjunctional α_1-adrenoceptors, owing to the fact that: (i) (+)-glaucine causes a shift to the right of the NA concentration-response curve without a depression of the maximal response and (ii) the slope value in the Schild plot is not significantly different from unity. However, (+)-glaucine antagonizes non-competitively with almost equal effectiveness (similar pD_2' values) the contractions induced by 5-HT (in normal solution) and Ca^{2+} (in Ca^{2+}-free high-K^+ medium), with depression of the maximal response.

The absence of an endothelial system does not significantly modify the vasorelaxant effects of (+)-glaucine (see Fdez. Alzueta *et al.*, 1992b), which suggests that the vasorelaxant activity is not due to an effect on the endothelial system, increasing the release of endogenous relaxant factors, such as EDRF, EDHF and PGI_2, from endothelial cells (for review see Moncada, 1982; Komori & Vanhoutte, 1990; Radomski & Moncada, 1993).

The selectivity shown by (+)-glaucine on NA-induced contractions in normal Krebs solution was checked in the experiments carried out in a Ca^{2+}-free medium. Under these conditions, NA, 5-HT and caffeine induce a contractile response in the vascular smooth muscle which is due only to the release of intracellular Ca^{2+} from the internal stores sensitive to the agonists. The mechanism whereby NA, 5-HT and caffeine cause Ca^{2+}-release is not entirely known but it has been suggested that the two first vasoconstrictor agents (via activation of postsynaptic α_1-adrenoceptor subtypes and 5-HT$_{2A}$ receptors, respectively) induce a fast contractile response (attributed to IP_3-mediated release of Ca^{2+} by interacting with a specific receptor of intracellular stores, Chiu *et al.*, 1987; Karaki & Weiss, 1988) and a subsequent sustained contraction (which is thought to involve the breakdown of phosphoinositide to diacylglycerol followed by the activation of protein kinase C and induction of contraction in the presence of a low concentration of Ca^{2+}, Koch *et al.*, 1990). However, the caffeine-induced Ca^{2+}-release is due to a different mechanism (Sato *et al.*, 1988; Noguera *et al.*, 1993), no type of membrane receptor being involved, but probably ryanodine specific receptors located in a sensitive intracellular store (for review see Missiaen *et al.*, 1992; Sorrentino & Volpe, 1993) which could partially overlap with the sensitive

pool of NA (Noguera & D'Ocon, 1992) and 5-HT (Noguera & D'Ocon, 1993).

In Ca^{2+}-free medium, (+)-glaucine strongly inhibited the NA-induced contractile response and had no effect on 5-HT and caffeine-induced contractions, which suggests that the alkaloid may selectively inhibit Ca^{2+}-release mediated by α_1-adrenoceptor activation in rat aorta. This effect, which would exclude a possible intracellular action (see below), may be due to an antagonism of postsynaptic α_1-adrenoceptors, previously described in rat vas deferens by Orallo *et al.* (1993) and in rat cerebral cortex by Ivorra *et al.* (1992).

Otherwise, the fact that pD_2' values for (+)-glaucine were similar for contractions induced by 5-HT (in normal Krebs) and Ca^{2+} (in depolarizing calcium-free solution), would also tend to suggest: (1) that the alkaloid may display an intracellular activity and (2) the existence of a non-selective mechanism of (+)-glaucine action on the cell membrane, involving the blockade of Ca^{2+} influx through both voltage-dependent and receptor-operated Ca^{2+} channels and/or the opening of K^+ channels. The effect of (+)-glaucine on the smooth muscle cell membrane of rat aorta does not seem to be due to the opening of K^+ channels, since cromakalim and other K^+ channel openers do not inhibit contractions induced by Ca^{2+} in Ca^{2+}-free high-K^+ medium at concentrations greater than about 30 mM in vascular smooth muscle. This last effect is due to the fact that, with high extracellular KCl concentrations, the cell membrane is depolarized to a level far from the K^+ equilibrium potential and, therefore, the K^+ channel openers do not hyperpolarize the smooth muscle cells. As a consequence, the vasorelaxant effects of these openers are negligible (Lebrun *et al.*, 1990; Pérez-Vizcaíno *et al.*, 1993; for review see Cook & Quast, 1990).

The action of (+)-glaucine on Ca^{2+} channels is illustrated by the experiments involving $^{45}Ca^{2+}$ uptake. Basal $^{45}Ca^{2+}$ uptake, i.e. the amount of Ca^{2+} entering by means of leak channels (Van Breemen & Saida, 1989), is unchanged by the addition of (+)-glaucine (3 μM), whereas the same concentration of the alkaloid strongly inhibited the uptake of $^{45}Ca^{2+}$ induced by K^+, NA and 5-HT. These results suggest that (+)-glaucine may inhibit, at least in part, the contractions induced by Ca^{2+} (in Ca^{2+}-free high-K^+ medium), NA and 5-HT by blocking transmembrane Ca^{2+} influx through voltage- and receptor-dependent Ca^{2+} channels directly or indirectly (through inhibition of various phosphodiesterase subtypes, previously described in vascular smooth muscle by Ivorra *et al.* (1992), which involves increased intracellular cyclic nucleotide levels and phosphorylation of transmembrane Ca^{2+} channels, Bean, 1989). However, the inhibitory effect on $^{45}Ca^{2+}$ influx induced by noradrenaline may also be due, at least in part, to the α_1-adrenoceptor blocking activity of (+)-glaucine.

An intracellular action direct or indirect (mediated by cyclic-nucleotides) which could cause a reduction of actin-myosin system sensitivity and/or a decrease in free intracellular Ca^{2+} concentration via: (i) an activation of Ca^{2+} uptake by intracellular stores (via phosphorylation of phospholamban and activation of the corresponding Ca^{2+}-ATP-ase); (ii) an increase of Ca^{2+} efflux, stimulating the Ca^{2+}-ATP-ases of the plasmalemma and the Na^+-Ca^{2+} exchange and (iii) an inhibition of intracellular Ca^{2+}-release from intracellular stores, is unlikely owing to the fact that (+)-glaucine does not inhibit contractions induced by either caffeine or 5-HT in Ca^{2+}-free medium (for review of all these mechanisms see Van Breemen & Saida, 1989; Missiaen *et al.*, 1992).

To summarize, (+)-glaucine has been characterized as an agent with a long-lasting hypotensive activity in the anaesthetized normotensive rat (possibly related to its ganglioplexic activity) and clear vasorelaxant effects on rat aortic smooth muscle (probably related to its α_1-adrenoceptor blocking and Ca^{2+} antagonist properties). Furthermore, a correlation between the mechanism of action of (+)-glaucine *in vivo* and *in vitro* was not observed, which indicates that the direct vasorelaxant activity of this alkaloid cannot play an important role in the onset of hypotension in the anaesthetized normotensive rat.

In view of these hypotensive properties, (+)-glaucine, an agent isolated from a plant source, is likely to have an interesting therapeutic potential as a model for the design of more efficient semisynthetic antihypertensive drugs, in order to improve the pharmacological treatment of hypertension.

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5-HT_2-mediated bronchial responses after syngeneic lung transplantation in the rat

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1 The effect of lung transplantation was assessed on the subsequent bronchial responses to 5-hydroxytryptamine (5-HT) in the rat *in vitro*.

2 Bronchial ring segments from syngeneic lung transplant recipients, from sham-operated and from non-operated control animals were studied in organ baths one month after unilateral left lung transplantation.

3 Responses of the bronchial smooth muscle to 5-HT (10^{-8} to 10^{-4}M) significantly increased after lung transplantation (80.6 ± 7.6% of the maximal contractile response to acetylcholine) compared with the responses of all other controls (ca. 40% of the maximal contractile response to acetylcholine, $P<0.01$). pD_2 values were similar for the left transplanted (5.70 ± 0.11) and all other control bronchi (ranging from 5.53 to 5.82).

4 Contraction to 5-HT was blocked by the selective 5-HT_2 antagonist, ketanserin. Responses to the selective 5-HT_2 agonist, α-methyl-5-HT, were similar to those to 5-HT; pD_2 values were similar in all groups (ranging from 5.41 to 5.74).

5 The selective 5-HT_3 agonist, 1-*m*-chlorophenyl biguanide, had no contractile effect on rat bronchus, whether transplanted or not.

6 Atropine, a muscarinic cholinoceptor blocking agent, had no effect on 5-HT-induced bronchial contraction. Hence, acetylcholine released from nerve terminals does not play any role in the observed bronchial hyperresponsiveness to 5-HT.

7 These results show bronchial hyperresponsiveness to 5-HT *in vitro* one month after lung transplantation in the rat, mediated through 5-HT_2 receptor activation. This might be caused by upregulation of 5-HT_2 receptors in the lung after transplantation.

Keywords: Lung; transplantation; bronchial hyperresponsiveness; 5-hydroxytryptamine; contraction; bronchus; 5-HT_2 receptors

Introduction

Bronchial hyperresponsiveness to both the cholinoceptor agonist, methacholine and the mast cell mediator, histamine, has been reported after lung transplantation in man *in vivo* (Glanville *et al.*, 1987; Banner *et al.*, 1988; Maurer *et al.*, 1989; Higenbottam *et al.*, 1989). In addition, in an experimental model of syngeneic lung transplantation in the rat, enhanced bronchial contraction to endogenous acetylcholine released by electrical field stimulation was also present *in vitro* one month after transplantation (Tavakoli *et al.*, 1992). Enhancement was not a result of medication, inflammation or rejection, absent from our syngeneic transplant model, or of modified contractile properties of the bronchial smooth muscle since responses to exogenous acetylcholine were unmodified (Tavakoli *et al.*, 1992).

We report here the effect of syngeneic lung transplantation on subsequent responses of the bronchial smooth muscle *in vitro* to exogenous 5-hydroxytryptamine (5-HT), the bronchoconstrictor derived from the mast cell in the rat. Since 5-HT may activate different receptor types, we have assessed the effect of the selective 5-HT_2 receptor antagonist, ketanserin on 5-HT-induced contraction and have compared the reactivity of the bronchi to 5-HT, to the 5-HT_2 receptor agonist, α-methyl-5-HT and to the 5-HT_3 receptor agonist, 1-*m*-chlorophenyl biguanide. Since hyperresponsiveness to 5-HT was observed after lung transplantation, the question of whether it might be related to the activation of a cholinergic pathway, i.e. to release of endogenous acetylcholine, was clarified by use of the muscarinic cholinoceptor blocking agent, atropine.

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Methods

Animals

Male inbred Lewis rats weighing 280–350g (Janvier, Le Jennest, France) maintained in similar conditions were used. They were separated into three groups: (1) 21 rats underwent orthotopic transplantation of the left lung from 21 syngeneic donor rats; (2) 5 sham-operated rats underwent left thoracotomy via the fifth intercostal space, the hilar structures being left intact; (3) 21 rats were not operated and served as controls.

All animals received humane care in compliance with the Principles of Laboratory Animal Care (NIH Publication No. 86-23, revised 1985).

Unilateral left lung transplantation was carried out as previously described (Tavakoli et al., 1992). Bronchi from our syngeneic transplantation were devoid of rejection phenomena, and no medication was used, either anti-inflammatory, immunomodulatory or antibiotic. The animals were maintained under similar conditions for one month. Histological examination of the airways showed no evidence of inflammation or of smooth muscle alteration.

Tissue preparation

One month after transplantation, each animal was killed by a blow to the head. The thorax was opened and the heart and lungs removed en bloc. On the left side, two adjacent bronchial ring segments were excised from the left main stem bronchus. In transplanted animals, the rings were taken distal to the suture line. On the right side, two adjacent bronchial ring segments were excised above and below the upper lobe bronchus take-off from the right bronchus.

Measurement of tissue contraction

Bronchial ring segments from animals of each group were placed in 10 ml organ baths containing modified Krebs buffer bubbled with 95%O_2: 5%CO_2, pH 7.4, at 37°C. The tissue was attached to isometric force-displacement transducers (Narco F 60, Houston, TX) connected to amplifiers and recorders (Physiograph MKIV, Narco) under 200 mg tension. During a 30 min period, the tissue was washed three times. An initial tension of 400 mg was then applied. The tissue was stabilized for 30–45 min before the experiment began. Four sets of experiments were carried out: (a) On bronchi from five transplanted, five sham-operated and five control animals, 5-HT (10^{-8} to 10^{-4} M) was added to the organ bath following a cumulative schedule. (b) On adjacent bronchial ring segments from six transplanted and six control rats, the effect of cumulative concentrations of either α-methyl-5-HT or 1-*m*-chlorophenyl biguanide (10^{-8} to 10^{4} M) was evaluated. (c) Adjacent bronchial ring segments from five transplant recipients and five control rats were preincubated with either ketanserin (1 μM) or solvent (distilled water) for 20 min prior to the addition of 5-HT (10^{-8} to 10^{-4} M). (d) Adjacent bronchial ring segments from five transplant recipients and five control rats were preincubated with either atropine (1 μM) or solvent (distilled water) for 20 min prior to the addition of 5-HT (10^{-8} to 10^{-4} M).

At the end of each experiment, the tissue was washed three times during a 30 min period. Cumulative concentrations of acetylcholine (ACh, 10^{-7} to 3×10^{-2} M) were added to the bath followed by the addition of KCl (100 mM) to assess the maximal contractile response of each bronchial ring segment. As previously reported (Tavakoli *et al.*, 1992), concentration-response curves to ACh were similar in all preparations. ACh gave the maximal response in each bronchus except in some atropine-pretreated bronchi. Bronchi with maximal contractions of less than 50 mg were discarded from the analysis of results.

Chemicals and buffer

The modified Krebs buffer had the following composition (in mM): NaCl 120, KCl 5.9, $CaCl_2$ 2.5, $MgSO_4$ 1.2, glucose 5.6, KH_2PO_4 1.2, and $NaHCO_3$ 25.5, 5-HT chloride, ACh chloride and atropine sulphate (Sigma, St. Louis, MO), α-methyl-5-HT and 1-*m*-chlorophenyl biguanide (Research Biochemical Inc., Bioblock, Strasbourg, France) were used. Ketanserin was a gift from Janssen Pharmaceutica (Beerse, Belgium).

Expression of results and statistical analysis

Contractile response was expressed as a percentage of the maximal response of each bronchus to ACh for the first 3 sets of experiments and to KCl for the last one. Comparisons of the curves and of pD_2 values within and between groups were made by analysis of variance followed by a Scheffe's *F* test (Statview II, Abacus Concepts, USA). Results were expressed as means ± standard error of the mean (s.e.). A *P* value of less than 0.05 was considered significant.

Results

Contraction to 5-HT

Concentration-response curves to 5-HT are shown in Figure 1. Contraction of the left transplanted bronchus was significantly enhanced compared to that of the right non-transplanted bronchus. The latter was identical to contractions of the left and right bronchi from control and from sham-operated animals. Maximal contraction of the left transplanted bronchus (80.6 ± 7.6% of the maximal contraction to ACh) was significantly greater than that of the right non transplanted bronchus (38.2 ± 11.6%, $P<0.01$) and than those of left and right bronchi from controls (38.8 ± 3.2 and 36.4 ± 5.2%, respectively; $P<0.01$ for both) and from sham-operated animals (42.2 ± 10.4%, respectively; $P<0.01$ for both).

There was no change in the mean effective concentration (expressed as pD_2 values) in the left transplanted bronchus (5.70 ± 0.11) compared with the other bronchi (5.82 ± 0.10 in right non-transplanted, 5.72 ± 0.05 in left control, 5.64 ± 0.11 in right control, 5.82 ± 0.09 in left sham-operated and 5.53 ± 0.11 in right sham-operated, NS). The curves were superimposed for right non-transplanted and left and right bronchi from control and sham-operated animals.

*Responses to α-methyl-5-HT and 1-*m*-chlorophenyl biguanide*

Concentration-response curves to the selective 5-HT_2 agonist α-methyl-5-HT are shown in Figure 2. Like the response to 5-HT, the maximal contraction of the left transplanted bronchus to α-methyl-5-HT (73.3 ± 10.5%) was significantly greater than that of the right non-transplanted bronchus (30.2 ± 4.7%, $P<0.01$) and than that of left and right bronchi from control animals (38.8 ± 32.1 and 31.8 ± 3.8%, respectively, $P<0.02$ for both).

pD_2 values were not significantly different for left transplanted bronchi (5.74 ± 0.12 μM) and for right non-transplanted bronchi (5.57 ± 0.10) and left and right bronchi from control groups (5.45 ± 0.06 and 5.41 ± 0.09, respectively, NS). The curves were similar for right non-transplanted and control bronchi on both sides.

The selective 5-HT_3 agonist, 1-*m*-chlorophenyl biguanide

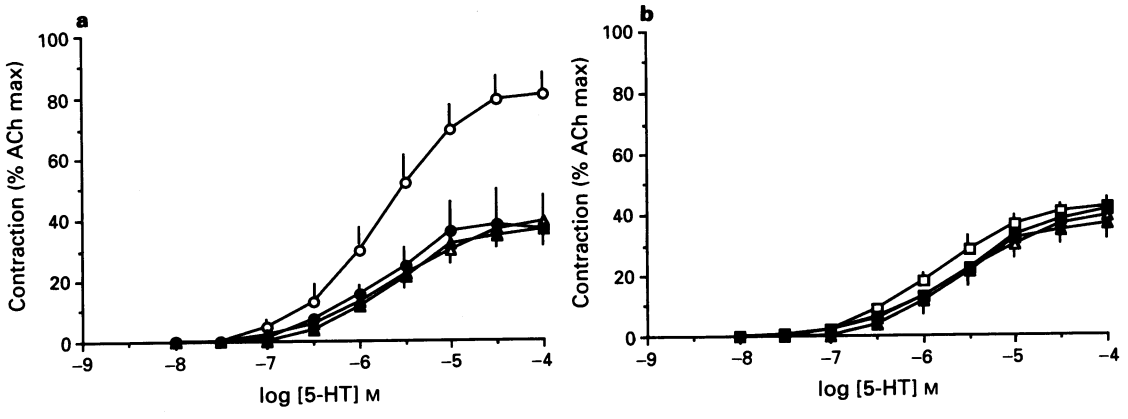

Figure 1 Concentration-response curves to 5-HT in ring segments from left transplanted (○) and right non-transplanted (●) bronchi, from left lung transplant recipient, left (△) and right (▲) bronchi from control animals and left (□) and right (■) bronchi from sham-operated animals. (a) Transplant *vs* control; (b) sham-operated *vs* control. Each point represents the mean ± s.e. of 5 experiments.

produced no contraction in bronchial ring segments in any group of animals.

Effect of ketanserin

The effect of ketanserin (1 μM) on concentration-response curves to 5-HT are shown in Figure 3. Bronchial contractions were significantly reduced by preincubation with ketanserin in transplanted as well as in other groups of animals. In the transplanted bronchus, contraction occuring at 0.1 mM of 5-HT was reduced from 75.4 ± 5.2 to 12.8 ± 8.8%. In the presence of ketanserin, the curves were identical for right non-transplanted and control bronchi on both sides. Contraction to 5-HT (0.1 mM) in right non-transplanted bronchus was reduced from 38.2 ± 8.8 to 4.2 ± 1.7% and in left and right control bronchi from 38.8 ± 2.4 and 37.2 ± 5.7% to 2.3 ± 2.3 and 1.0 ± 1.0%, respectively, suggesting antagonism of the 5-HT-induced contraction by the 5-HT_2 receptor antagonist ketanserin in the rat bronchus, whether transplanted or not. However, even in the presence of ketanserin, bronchial contractions to 5-HT remained significantly enhanced in left transplanted as compared to all other groups ($P<0.01$).

Effect of atropine

The effect of atropine on concentration-response curves to 5-HT is shown in Figure 4. Preincubation of the tissue with atropine did not reduce the 5-HT-induced contraction of the left transplanted bronchus. Maximal contractions reached 77.8 ± 4.6 in the absence and 84.4 ± 2.2% in the presence of atropine (NS). These values were significantly greater than those obtained in right non-transplanted bronchus and left and right control bronchi, both in the absence and presence of atropine: 39.1 ± 6.8 and 30.7 ± 3.0% for right non-transplanted, 45.3 ± 5.3% for left control, 38.8 ± 7.6 and 29.1 ± 6.2% for right control respectively. Although there was a trend towards inhibition by atropine in the 3 control groups, differences did not reach significance.

pD_2 values were not modified by atropine in left transplanted bronchi (5.68 ± 0.08 and 5.69 ± 0.10 in the absence and presence of atropine, respectively). Similarly, atropine did not modify pD_2 values in right non-transplanted bronchus (5.68 ± 0.11 and 5.62 ± 0.10, respectively) and in control bronchi (5.60 ± 0.05 and 5.55 ± 0.10 for left and 5.53 ± 0.11 and 5.51 ± 0.06 for right bronchi, respectively). The curves were identical for right non-transplanted and for left and right bronchi from control and sham-operated animals.

Discussion

We have shown that the contraction of the left transplanted bronchus to 5-HT was significantly enhanced one month after lung transplantation in the rat compared with that of the contralateral non-transplanted bronchus and with that of bronchi from control and sham-operated animals. This in-

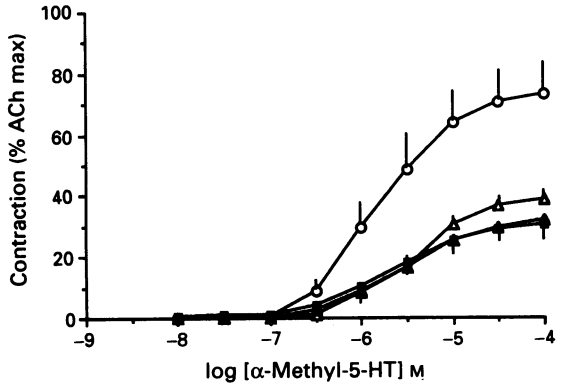

Figure 2 Concentration-response curves to α-methyl-5-HT in ring segments from left transplanted (○) and right non-transplanted (●) bronchi from left lung transplant recipient and left (△) and right (▲) bronchi from control animals. Each point represents the mean ± s.e. (bar) of 6 experiments.

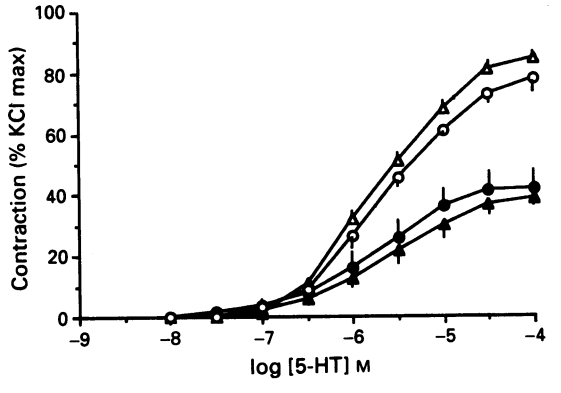

Figure 4 Concentration-response curves to 5-HT in the presence (triangles) and absence (circles) of atropine in ring segments from left transplanted bronchi from left lung transplant recipient (○, △) and left bronchi from control animals (●, ▲). Each point represents the mean ± s.e. of 5 experiments.

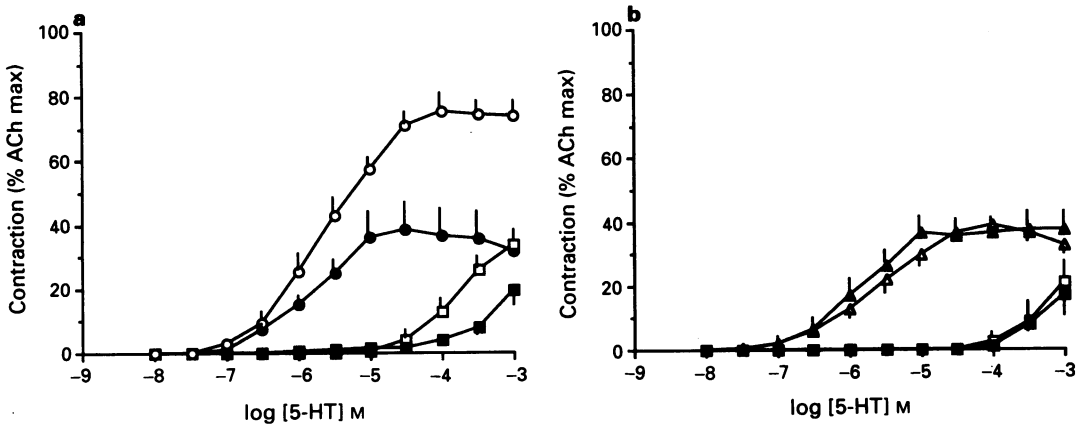

Figure 3 Concentration-response curves to 5-HT in the presence (squares) and absence (circles and triangles) of ketanserin. (a) Left transplanted (○, □) and right non-transplanted (●, ■) bronchi from left lung transplant recipient; (b) left (△, □) and right (▲, ■) bronchi from control animals. Each point represents the mean ± s.e. of 5 experiments.

creased bronchial responsiveness of the transplanted bronchus *in vitro* was also produced by the selective 5-HT_2 receptor agonist, α-methyl-5-HT, whereas the 5-HT_3 agonist, 1-*m*-chlorophenyl biguanide, produced no contraction in any preparation. The potency of α-methyl-5-HT for inducing contraction of the bronchial smooth muscle after lung transplantation was similar to that of controls as shown by the pD_2 values. This agonist potency fits with that reported for a 5-HT_2 receptor and is in accordance with studies in the guinea-pig showing that contraction of airway smooth muscle is mediated through 5-HT_2 receptor activation (Cohen *et al.*, 1985; Watts & Cohen 1993). Moreover, the 5-HT_2 antagonist, ketanserin, abolished the contraction, also suggesting 5-HT_2 receptor activation mediates contraction of the rat bronchus both in control and in transplanted animals. This suggests the increased responsiveness is mediated through 5-HT_2 receptor activation.

The muscarinic cholinoceptor antagonist, atropine, did not affect the increased bronchial responsiveness to 5-HT. 5-HT was previously shown to facilitate cholinergic neurotransmission, produced by electrical field stimulation, via activation of 5-HT_2 receptors in the rat (Szarek *et al.*, 1993). However, our results show that cholinergic neurotransmission does not play any role in 5-HT-mediated bronchial contraction and, furthermore, does not account for the increased responsiveness to 5-HT observed after lung transplantation.

These results were obtained in our syngeneic transplant model where no inflammation or rejection phenomenon occur and no treatment is necessary (Tavakoli *et al.*, 1992). Results were also not affected by the operative procedure itself since bronchi from sham-operated animals developed similar concentration-response curves to 5-HT to those from controls. Therefore, the increased bronchial responsiveness to exogenous 5-HT was present in the absence of confounding factors. This is of importance since inflammation caused by exogenous stimuli is associated with bronchial hyperresponsiveness in experimental models (Holtzman *et al.*, 1983; O'Byrne *et al.*, 1984; Boushey & Holtzman 1985). Our results rule out the essential association between airway inflammation and bronchial hyperresponsiveness (Tavakoli *et al.*, 1992; and this study). This is in accordance with the lack of association between bronchial hyperresponsiveness and lung rejection or airway inflammation in human lung transplant recipients (Maurer *et al.*, 1989; Higenbottam *et al.*, 1989). In these patients, bronchial hyperresponsiveness to both inhaled methacholine and the mast cell mediator, histamine, is described after a single lung, double lung and heart-lung transplantation. Our study is the first evidence of the existence of increased bronchial responsiveness to the mast cell bronchoconstrictor mediator, 5-HT, in the rat *in vitro* after lung transplantation.

Lung transplantation results in interruption of vascularization and innervation of the transplanted lung. This probably induces phenotypic changes of the cells and structures within the lung tissue which could account for the observed increased bronchial responsiveness. Accordingly, 5-HT-induced increased responsiveness might be related to modified 5-HT containing cells, i.e. the mast cells in rat lung, and/or to modified expression of 5-HT receptors. An increase in mast cell number is generally associated with tissue repair (Galli 1990; 1993) and this also occurs after lung transplantation in the rat (Buvry *et al.*, 1993). An increase in the lung content of 5-HT, secondary to an increase in the mast cell number, might be associated with a modified expression of 5-HT receptors, either in affinity or in density. Since there was no change in the contractile potency to both 5-HT and 5-HT_2 selective agonist in bronchi from transplanted lungs compared to controls, the hypothesis of increased 5-HT receptor affinity can be ruled out. The hypothesis of an increased density, i.e. an upregulation of 5-HT receptors in the transplanted lung, would better explain the post-transplant increase in bronchial responsiveness to 5-HT. The regulation of the expression of 5-HT receptors is, to date, not well established, but seems to follow an unusual pattern (Leysen & Pauwels 1990; Leysen & Schotte 1992). In fact, administration of either 5-HT_2 agonist or antagonist in rats leads to a similar desensitization and down regulation of 5-HT_2 receptors in the front cortex (Leysen *et al.*, 1986; Leysen & Pauwels, 1990; Leysen & Schotte, 1992). In the periphery, there is no report on the regulation of the 5-HT_2 receptor. Our results suggest an upregulation of 5-HT_2 receptors in transplanted bronchi after lung transplantation. Therefore, determination of 5-HT_2 receptor density together with the quantification of mast cells in the lung will bring important insight into the understanding of 5-HT_2 receptor regulation.

We conclude that one month after lung transplantation in rats, bronchial responsiveness to exogenous 5-HT is markedly enhanced and that the observed increased responsiveness is mainly mediated through 5-HT_2 receptors. This could be accounted for by upregulation of 5-HT_2 receptor population and/or phenotypical changes in 5-HT-containing cells.

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Effects of acute and chronic ethanol on cyclic AMP accumulation in NG108-15 cells: differential dependence of changes on extracellular adenosine

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1 This study investigated the effects of acute and chronic ethanol on basal, agonist- and forskolin-stimulated cyclic AMP formation in NG108-15 mouse neuroblastoma x rat glioma hybrid cells, and examined the role of changes in extracellular adenosine concentrations on the effects observed.

2 NG108-15 cells incubated acutely with ethanol (1-200 mM) displayed concentration-dependent increases in basal and iloprost-stimulated (300 nM; a prostanoid IP receptor agonist) cyclic AMP accumulation but a concentration-dependent decrease in forskolin-stimulated (10 μM) accumulation.

3 Cells treated chronically with ethanol (200 mM) for 48 h displayed increases over control in basal, iloprost- (0.001-10 μM) and forskolin (0.01-100 μM)-stimulated cyclic AMP formation. However, chronic ethanol did not affect [^{3}H]-iloprost binding to cell membranes.

4 Inclusion of adenosine deaminase (ADA; 1 unit ml^{-1}) during the incubation period to measure cyclic AMP accumulation completely abolished the increase in basal accumulation following chronic ethanol, but did not affect the increase in iloprost stimulation. On the other hand ADA partially reversed the increase in forskolin stimulation following chronic ethanol, but even in the presence of high concentrations of ADA (5 units ml^{-1}) the forskolin stimulation remained elevated above control.

5 Cells treated chronically with the adenosine receptor agonist 5'-(N-ethylcarboxamido)-adenosine (NECA; 10 μM for 48 h) displayed a reduction in subsequent NECA- and forskolin-stimulated cyclic AMP accumulation, but iloprost stimulation was not affected. ADA included acutely during the incubation period to measure cyclic AMP accumulation abolished the reduction in forskolin but not NECA stimulation produced by the chronic NECA pretreatment.

6 We have previously noted that ethanol inhibits NG108-15 cell proliferation and alters cell morphology. To mimic this, cells were incubated in the absence of foetal calf serum for 48 h. Following this time, basal, iloprost- and forskolin-stimulated cyclic AMP formation was enhanced over that in cells grown in the presence of serum.

7 These results indicate that chronic ethanol enhances cyclic AMP formation in intact NG108-15 cells by more than one mechanism: one involves increased extracellular adenosine concentrations and the other a change in the transduction system beyond the receptor, possibly involving the adenylyl cyclase enzyme. Furthermore the ethanol-induced changes in cyclic AMP accumulation may relate to alterations in NG108-15 cell growth and development.

Keywords: Ethanol; cyclic AMP; adenosine; NG108-15 cells

Introduction

Chronic ethanol treatment of neuronal tissue is reported to produce a number of adaptational changes in cell signalling, such that the effect produced by acute ethanol is often reversed following chronic ethanol exposure (eg. Richelson *et al.*, 1986). It is thought that these effects may have relevance to the pathophysiological changes that occur in alcoholism (Charness *et al.*, 1989). For example, ethanol acutely inhibits Ca^{2+} uptake and voltage-sensitive Ca^{2+} currents in neuronal cells, whereas chronic ethanol enhances these parameters (Messing *et al.*, 1986; Grant *et al.*, 1993). The NG108-15 mouse neuroblastoma x rat glioma hybrid cell line has been widely used as a model of neuronal function. Acute ethanol treatment of these cells can elevate basal and agonist-stimulated adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation whereas chronic ethanol treatment has been reported to induce heterologous desensitization of agonist-stimulated cyclic AMP formation (Gordon *et al.*, 1986). In further studies chronic ethanol was reported to reduce selectively membrane $G_s\alpha$ expression in NG108-15 cells by a reduction in $G_s\alpha$ mRNA levels (Mochly-Rosen *et al.*, 1988), thus accounting for the heterologous desensitization. It was proposed that this desensitization was secondary to a chronic ethanol-induced inhibition of adenosine uptake by the cells, which thus accumulates extracellularly and in some ways leads to a reduction in $G_s\alpha$ mRNA (Nagy *et al.*, 1989; 1990).

On the other hand in our own studies we found that chronic ethanol affects the expression of multiple G-protein α subunits in NG108-15 cells, and that none of these changes are dependent upon extracellular adenosine (Williams *et al.*, 1993; Williams & Kelly, 1993). Furthermore, we found that unlike previous studies, chronic ethanol leads to a novel *increase* in agonist-stimulated cyclic AMP formation in NG108-15 cells. In this paper we characterize these changes in cyclic AMP formation, employing both receptor-dependent and independent means of increasing cyclic AMP formation to investigate the effects of acute and chronic ethanol in NG108-15 cells. We also investigate the role of extracellular adenosine in the changes observed.

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Methods

Cell culture

NG108-15 neuroblastoma x glioma hybrid cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 6% (v/v) foetal calf serum and supplemented with 1 μM aminopterin, 100 μM hypoxanthine and 16 μM thymidine. In some experiments foetal calf serum was omitted from the medium. Cells (passages 21–35) were grown in the presence or absence of various concentrations of ethanol or NECA (5'-(N-ethylcarboxamido)-adenosine) in culture flasks (25 cm^2) maintained at 37°C in a humidified atmosphere of 6% CO_2/94% air. Growth medium, ethanol or NECA were renewed daily. Subconfluent cells were harvested in 10 ml of phosphate buffered saline (PBS) and pelleted by centrifugation at 200 *g* for 1 min. The resultant pellets were washed twice in 10 ml of PBS at 37°C and either used immediately or frozen at −80°C until required. Cell counts were performed with a haemocytometer. In all experiments, control and drug-treated cells were grown in parallel.

Cyclic AMP measurements

Cyclic AMP levels in NG108-15 cells were measured by a protein binding assay (Brown *et al.*, 1971). Control, ethanol or NECA pretreated cells were washed twice in PBS at 37°C, and the pellets resuspended in ice-cold Krebs-Ringer-HEPES buffer (composition, mM: NaCl 110, KCl 5 $MgCl_2$ 1, $CaCl_2$ 1.8, glucose 25, HEPES 10 and Ro20 1724 (4-(3-butoxy-4-methoxybenzyl)imidazolidin-2-one) 100 μM, as phosphodiesterase inhibitor, pH 7.4); 100 μl of buffer containing relevant drug concentrations was then pipetted into tubes on ice, to which was then added 100 μl aliquots (containing about 250–500 mg protein) of the cell suspension, and the tubes were then placed in a shaking water bath at 37°C for 8 min (accumulation of cyclic AMP over this time period is linear). At the end of this time 20 μl of ice cold 100% trichloroacetic acid was added to each tube and placed on ice for 20 min. Precipitated protein was pelleted by centrifugation at 2,900 *g* for 20 min at 4°C, and 100 μl of the supernatant added to 100 μl 1 M NaOH and 1 ml of 50 mM Tris, 4 mM EDTA, pH 7.4 (TE buffer); 100 μl of this solution was then added to fresh tubes containing 50 μl TE buffer, 100 μl [^{3}H]-cyclic AMP in TE buffer (about 30,000 c.p.m.) and 100 μl of binding protein in TE buffer (to give final concentration of ~750 mg protein ml^{-1}; prepared from bovine adrenal cortex). Tubes containing 100 μl of standard concentrations of cyclic AMP (0.125–10 pmol) were used to construct a standard curve. After 2–3 h incubation, 200 μl of TE buffer containing charcoal (Norit GSX; 50 mg ml^{-1} concentration) and bovine serum albumin (2 mg ml^{-1} final concentration) were added and 15 min later the tubes were centrifuged at 2,900 *g* for 20 min at 4°C. The resulting supernatant was poured into vials for liquid scintillation counting. Standard curve data were fitted to a logistic expression (Graphpad) and the unknowns read off. Protein content of cell suspensions was determined (Bradford, 1976) and the results expressed as pmol cyclic AMP min^{-1} mg^{-1} protein.

[^{3}H]-iloprost binding

Frozen cells were thawed and homogenized in ice-cold 20 mM HEPES, 10 mM EDTA, 0.3 M sucrose, pH 7.4 in a Teflon/glass homogenizer. The lysates were centrifuged at 1,500 *g* for 10 min at 4°C and the resultant supernatant centrifuged at 16,000 *g* for 30 min at 4°C. The membrane pellets were then washed in 2 ml of 20 mM HEPES, 1 mM $MgCl_2$, pH 7.4. The final washed pellets were resuspended in 20 mM HEPES, 1 mM $MgCl_2$, pH 7.4, to a concentration of 1–2 mg ml^{-1} and stored at −80°C until required. The membranes (0.1–0.2 mg protein per sample) were incubated in a final volume of 250 μl of 20 mM HEPES, 1 mM $MgCl_2$, pH 7.4, containing 10 nM [^{3}H]-iloprost in the absence or presence of 10 μM unlabelled iloprost to determine non-specific binding, and where appropriate, 100 μM 5'-guanylylimidodiphosphate (GppNHp). Samples were incubated for 90 min at 4°C, then filtered through Whatman GF/B glass fibre filters and washed with 3 × 3.5 ml ice-cold assay buffer, using a Brandel Cell Harvester. Scintillation fluid was added to the filters and they were left overnight before counting.

Drugs

[8-^{3}H]-cyclic AMP (26 Ci $mmol^{-1}$) and [^{3}H]-iloprost (15.5 Ci $mmol^{-1}$) were obtained from Amersham International plc (U.K.), cell growth medium from GIBCO BRL (Paisley, Scotland), ethanol from Hayman Limited (U.K.), whilst iloprost was a generous gift from Schering AG (Berlin, Germany). The protein assay reagent was from Pierce and Warriner (U.K.) Ltd. All other reagents and drugs were obtained from the Sigma Chemical Co. Ltd.

Data analysis and statistics

Where appropriate, concentration-effect curves were analysed by the iterative fitting programme GraphPad InPlot (GraphPad Software, San Diego, U.S.A.). Due to the variability in absolute cyclic AMP levels between experiments, some of the data were normalised to show % changes; data from these were analysed statistically by the Mann-Whitney test. Untransformed data from other experiments was analysed by a paired *t* test.

Results

Effect of acute ethanol on basal, iloprost- and forskolin-stimulated cyclic AMP accumulation

Ethanol included acutely in the cyclic AMP incubation experiment concentration-dependently increased basal and iloprost-stimulated (300 nM) cyclic AMP formation, but inhibited forskolin-stimulated (10 μM) cyclic AMP formation in NG108-15 cells (Figure 1). Acute ethanol also enhanced NECA-stimulated (10 μM) cyclic AMP formation (data not shown). The dependence of these changes on extracellular adenosine was then investigated. Inclusion of adenosine deaminase (ADA; 1 unit ml^{-1}) reduced basal cyclic AMP accumulation and blocked the increase in basal cyclic AMP formation due to acute ethanol (200 mM), but did not affect the increase in iloprost-stimulated formation (Figure 2). Furthermore, although ADA reduced control forskolin stimulation of cyclic AMP formation by almost 40%, acute ethanol was still able to inhibit forskolin-stimulated cyclic AMP formation in the presence of ADA (Figure 2).

Effect of chronic ethanol on basal, iloprost- and forskolin-stimulated cyclic AMP accumulation

In chronic studies, treatment of NG108-15 cells for 48 h with ethanol (200 mM) increased basal, iloprost- and forskolin-stimulated cyclic AMP formation (Figure 3). The increase in stimulated levels appeared to represent increases in maximal stimulation rather than changes in EC_{50}s. Thus the EC_{50} for iloprost was 0.12 ± 0.06 μM in control cells and 0.062 ± 0.034 μM in chronic ethanol-treated cells, but the maximum response was increased from 62.5 ± 13.2 in controls to 106.7 ± 15.5 pmol cyclic AMP min^{-1} mg^{-1} protein in ethanol-treated cells ($P<0.05$, paired *t* test; $n=4$). For forskolin the EC_{50} was 3.6 ± 1.4 μM in control cells and 4.1 ± 1.8 μM in chronic ethanol-treated, and the maximum response was increased from 295.4 ± 59.3 in control cells to 551.9 ± 97.5 pmol cyclic AMP min^{-1} mg^{-1} protein in ethanol-treated cells ($P<0.05$, paired *t* test; $n=8$). Chronic ethanol concentration-dependently increased basal, iloprost- and

forskolin-stimulated cyclic AMP formation, with significant increases being observed at 100 and 200 mM ethanol for the latter two agents (Figure 4).

Following chronic ethanol treatment, cells were washed before exposure to agonists. To investigate whether the increases in cyclic AMP accumulation we observe are due to the sudden withdrawal of ethanol, we included 200 mM ethanol in the cyclic AMP incubation buffer. However, this did not abolish the chronic ethanol-induced increases in basal, iloprost- and forskolin-stimulated cyclic AMP formation, but the forskolin-stimulated response was reduced (Figure 5). To investigate whether osmotic changes in the medium might account for the increases in cyclic AMP accumulation, cells were incubated with 200 mM sucrose for

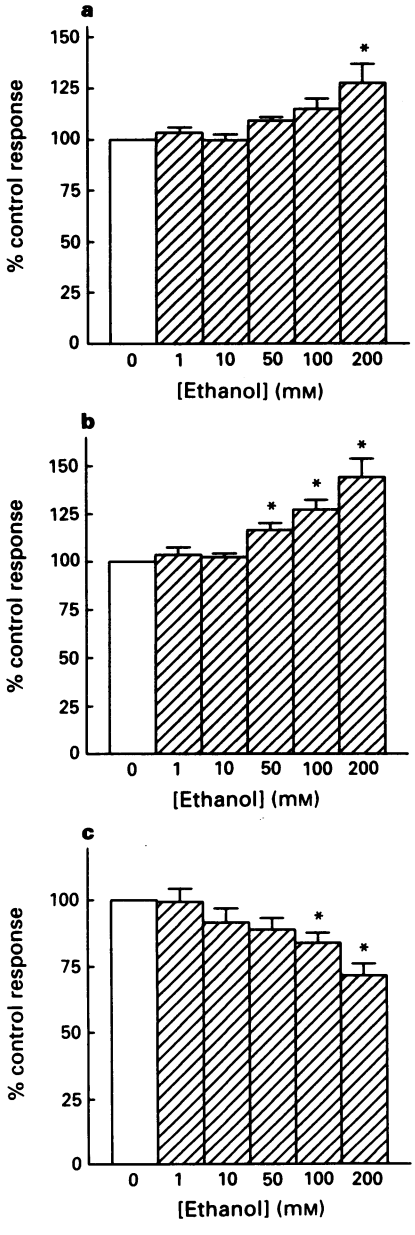

Figure 1 Concentration-dependent effects of acute ethanol on (**a**) basal, (**b**) 300 nM iloprost stimulated and (**c**) 10 μM forskolin stimulated cyclic AMP formation in NG108-15 cells. Cells were incubated at 37°C for 8 min in buffer containing 100 μM Ro 20-1724. In each case, control values (open columns) are normalised to 100%. Values are means ± s.e. mean from 5-7 separate experiments. $*P<0.05$, vs. no ethanol, Mann-Whitney test. Basal cyclic AMP accumulation in the absence of ethanol was 23.7 ± 4.6, iloprost-stimulated accumulation in the absence of ethanol was 67.5 ± 15.5, and forskolin-stimulated accumulation in the absence of ethanol was 207.1 ± 54.4 pmol cyclic AMP $min^{-1} mg^{-1}$ protein.

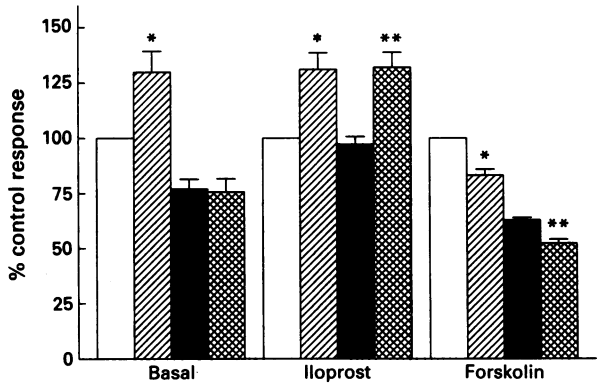

Figure 2 Effect of adenosine deaminase (ADA; 1 unit ml^{-1}) on the changes in cyclic AMP accumulation induced by acute addition of ethanol (200 mM) to NG108-15 cells. Cells were incubated under the following conditions: basal, iloprost- (1 μM) or forskolin-stimulated (10 μM; open columns; basal, iloprost or forskolin in the presence of ethanol (hatched columns); basal, iloprost or forskolin in the presence of ADA (solid columns); basal, iloprost or forskolin in the presence of both ADA and ethanol (cross hatched columns). In each case, control values (basal, iloprost or forskolin alone) are normalised to 100%. Values are means ± s.e. mean from 5 separate experiments. $*P<0.05$, vs. no ethanol in absence of ADA; $**P<0.05$, vs. no ethanol in presence of ADA, Mann-Whitney test.

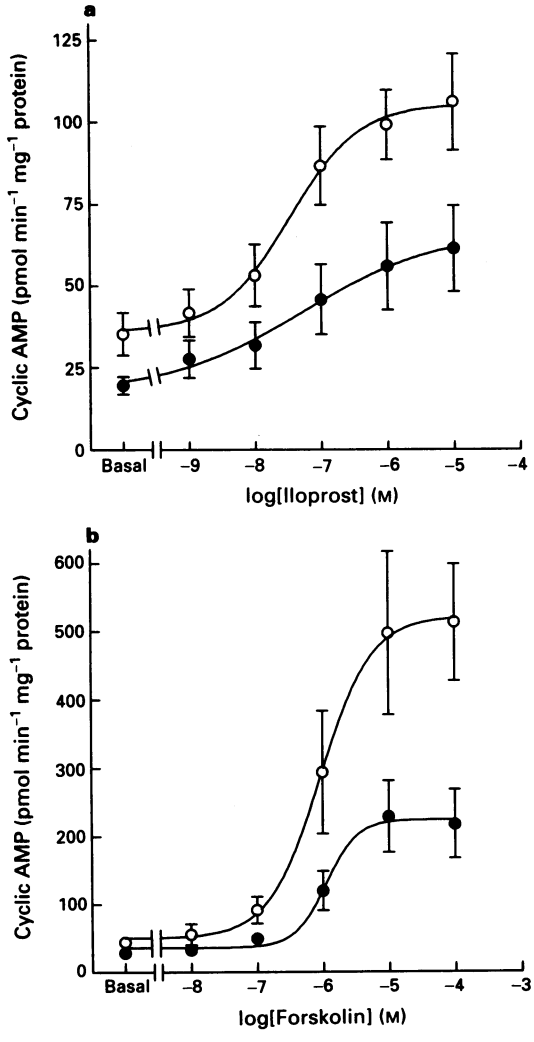

Figure 3 Effect of chronic ethanol treatment (200 mM; 48 h) on the iloprost and forskolin concentration-effect curves for cyclic AMP accumulation in NG108-15 cells. Control (●) or ethanol pretreated cells (○) were washed and incubated with different concentrations of (**a**) iloprost or (**b**) forskolin. Values are means ± s.e. mean from 4-8 separate experiments.

48 h; however, the cells did not survive this treatment. On the other hand incubation of the cells with 200 mM methanol for 48 h did not produce the increases in cyclic AMP accumulation as observed with ethanol (the levels of cyclic AMP achieved in the presence of 10 μM forskolin were 200.5 ± 18.2, 430.9 ± 89.8 and 279.3 ± 58.4 pmol cyclic AMP min^{-1} mg^{-1} protein for control, chronic 200 mM ethanol and chronic 200 mM methanol, respectively). Only the increase following ethanol was significantly greater than control ($P<0.05$, paired t test). This indicates that osmotic effects cannot account for the effects of ethanol, otherwise methanol would have produced similar changes.

To investigate whether the increase in iloprost-stimulated cyclic AMP formation may be secondary to an increase in IP receptor number, binding of 10 nM [^{3}H]-iloprost to membranes from control and chronic ethanol (200 mM; 48 h) treated cells was assessed. However, chronic ethanol did not affect [^{3}H]-iloprost binding in the presence or absence of 100 μM GppNHp (control binding in the absence of GppNHp 33.9 ± 3.2 and in the presence of GppNHp 15.9 ± 1.0 fmol mg^{-1} protein; binding following chronic ethanol 32.9 ± 4.7 in the absence of GppNHp and 16.2 ± 1.2 fmol mg^{-1} protein in the presence of GppNHp; means ± s.e. mean, $n = 3$).

Involvement of adenosine in chronic ethanol-induced changes in cyclic AMP accumulation

To assess the contribution of extracellular adenosine to the chronic ethanol-induced increases in cyclic AMP formation. ADA (1 unit ml^{-1}) was included in the cyclic AMP incubation buffer. This concentration of adenosine deaminase completely abolished the increase in basal cyclic AMP, but did not affect the increase in iloprost-stimulated cyclic AMP formation following chronic ethanol (Figure 6a). On the other hand, the increase in forskolin-stimulated cyclic AMP formation was partially inhibited by ADA, due partly to a decrease in forskolin's potency following removal of extracellular adenosine (Figure 6b). In fact, ADA concentration-dependently inhibited forskolin-stimulated cyclic AMP formation in the presence or absence of chronic ethanol pretreatment (Figure 7), but the forskolin-stimulated cyclic AMP level in chronic ethanol pretreated cells was still greater than controls (cyclic AMP accumulation in presence of 10 μM forskolin and 5 units ml^{-1} ADA was 74.2 ± 11.0 and 137.7 ± 22.1 pmol cyclic AMP min^{-1} mg^{-1} protein in control and chronic ethanol-treated cells, respectively). The adenosine receptor antagonist xanthine amine congener (1 μM), also inhibited the increase in basal and forskolin stimulated cyclic AMP accumulation produced by chronic ethanol to the same extent as ADA (data not shown).

Since changes in adenosine receptor activation have previously been implicated in the action of chronic ethanol on these cells (Nagy *et al.*, 1989), we investigated the effects of chronic treatment with an adenosine receptor agonist, NECA, on subsequent stimulation of cyclic AMP accumulation by NECA, iloprost and forskolin (Figure 8). A 48 h treatment with 10 μM NECA did not affect basal or iloprost-

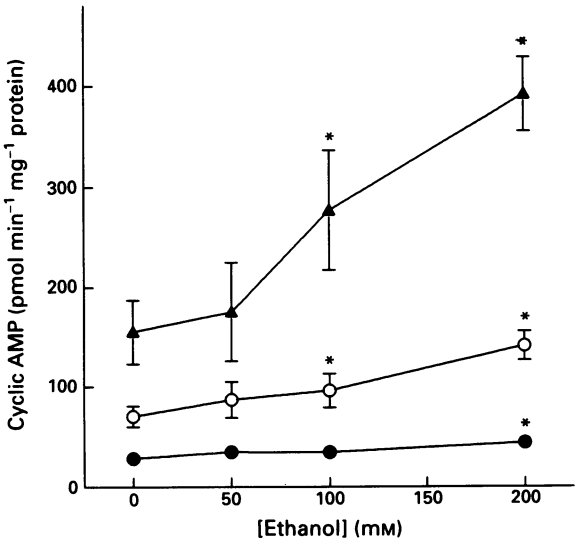

Figure 4 Concentration-dependent effects of chronic ethanol on basal (●), 1 μM iloprost-stimulated (○) and 10 μM forskolin-stimulated (▲) cyclic AMP formation in NG108-15 cells. Values are mean ± s.e. mean from 9–10 separate experiments. *$P<0.05$, vs. no ethanol, paired t test.

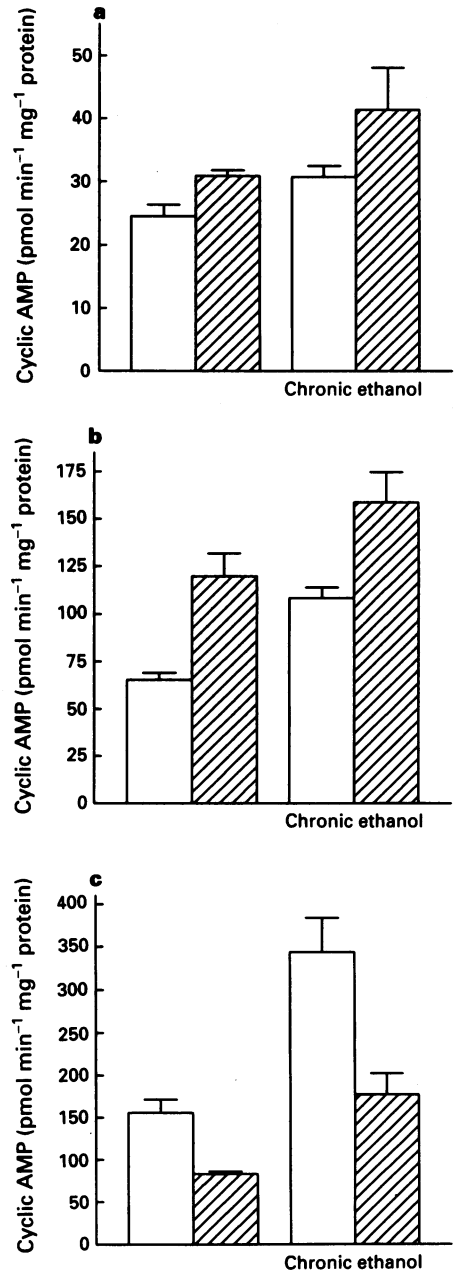

Figure 5 Effect of acute ethanol on the chronic ethanol induced increases in (**a**) basal, (**b**) iloprost-stimulated (1 μM) and (**c**) forskolin-stimulated (10 μM) cyclic AMP accumulation in NG108-15 cells. Cells were incubated in the absence (columns to left of x-axis) or presence (columns to right of x-axis) of 200 mM ethanol for 48 h. They were then washed and resuspended in buffer containing no ethanol (open columns) or 200 mM ethanol (hatched columns). Values are means ± s.e. mean from a single experiment performed in triplicate; similar results were obtained in 1 further experiment.

stimulated cyclic AMP formation, but abolished subsequent NECA-stimulated cyclic AMP formation, whilst that due to forskolin was partially reduced (Figure 8a). In the presence of ADA during the cyclic AMP incubation, the NECA stimulation was still abolished by chronic NECA, but the reduction in forskolin-stimulated cyclic AMP formation was no longer evident (Figure 8b).

Effect of neuronal differentiation on basal, iloprost and forskolin stimulated cyclic AMP accumulation

Incubation of NG108-15 cells with ethanol concentration-dependently reduced cell proliferation and increased protein content per cell (Figure 9). The effects of ethanol on these parameters occurred at broadly similar concentrations to those which acutely modified cyclic AMP formation (compare Figure 1 with Figure 9), and those which enhanced cyclic AMP formation following chronic treatment (compare Figure 4 with Figure 9).

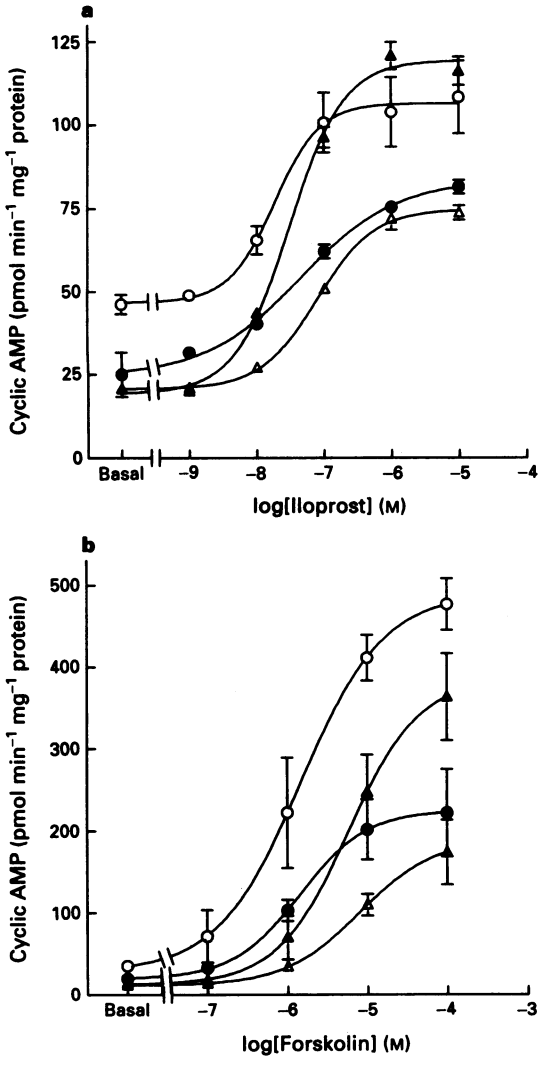

Figure 6 Effect of adenosine deaminase (ADA 1 unit ml^{-1}) on (**a**) iloprost and (**b**) forskolin-stimulated cyclic AMP formation with or without chronic ethanol pretreatment (200 mM; 48 h). Control cells were incubated in the presence (Δ) or absence (●) of ADA during the 8 min cyclic AMP accumulation, whilst chronic pretreated cells were also incubated in the presence (▲) or absence (○) of ADA. Values in (**a**) are means ± s.e. mean from a single experiment performed in triplicate, whilst (**b**) includes data from 3 separate experiments. From the fitted curves, the EC_{50} for forskolin was 1.4 and 7.9 μM for control cells in the absence and presence of ADA respectively, and 1.5 and 5.8 μM for chronic ethanol-treated cells in the absence and presence of ADA.

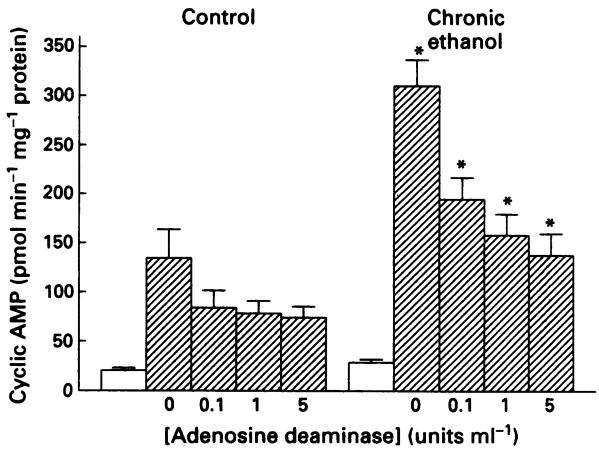

Figure 7 Effect of increasing concentrations of adenosine deaminase (ADA) on forskolin-stimulated (10 μM) cyclic AMP formation. Control or chronic ethanol-treated (200 mM; 48 h) cells were washed and resuspended in buffer for 8 min in the absence (open columns) or presence (hatched columns) of forskolin and ADA (0.1–5 units ml^{-1}). Values show means ± s.e. mean from 5 separate experiments. $*P<0.05$, vs. corresponding control cells, paired t test.

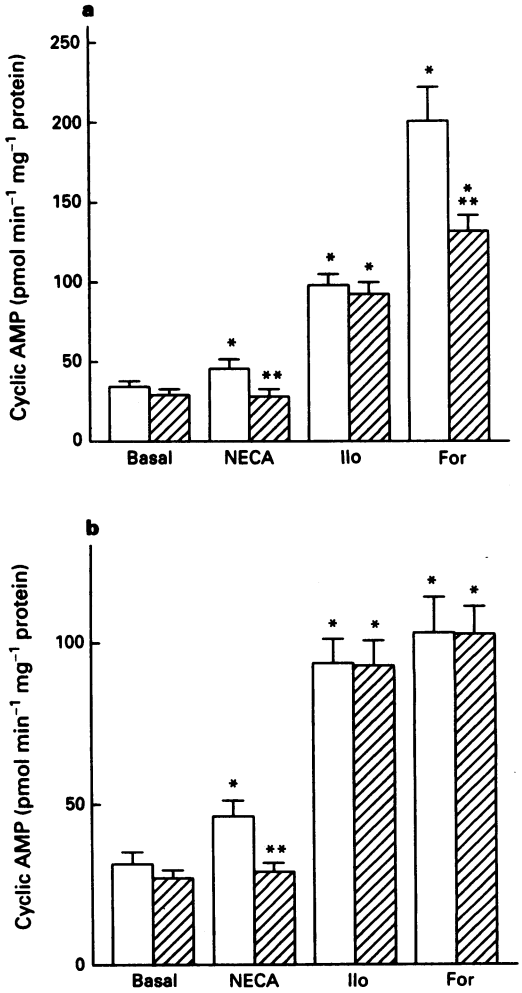

Figure 8 Effect of chronic treatment of NG108-15 cells with 5′-(N-ethyl)-carboxamidoadenosine (NECA) on cyclic AMP accumulation. Cells were incubated for 48 h in the presence (hatched columns) or absence (open columns) of NECA (10 μM), following which they were washed and incubated for 8 min without agonist (basal), or with NECA (10 μM), iloprost (1 μM) or forskolin (10 μM). (**a**) Incubation performed with agonists in the absence of ADA; (**b**) incubation performed with agonists in the presence of ADA (1 unit ml^{-1}). Values show means ± s.e. mean from 8 separate experiments. $*P<0.05$, greater vs. control basal value; $**P<0.05$, lower vs. respective value from control cells, t test.

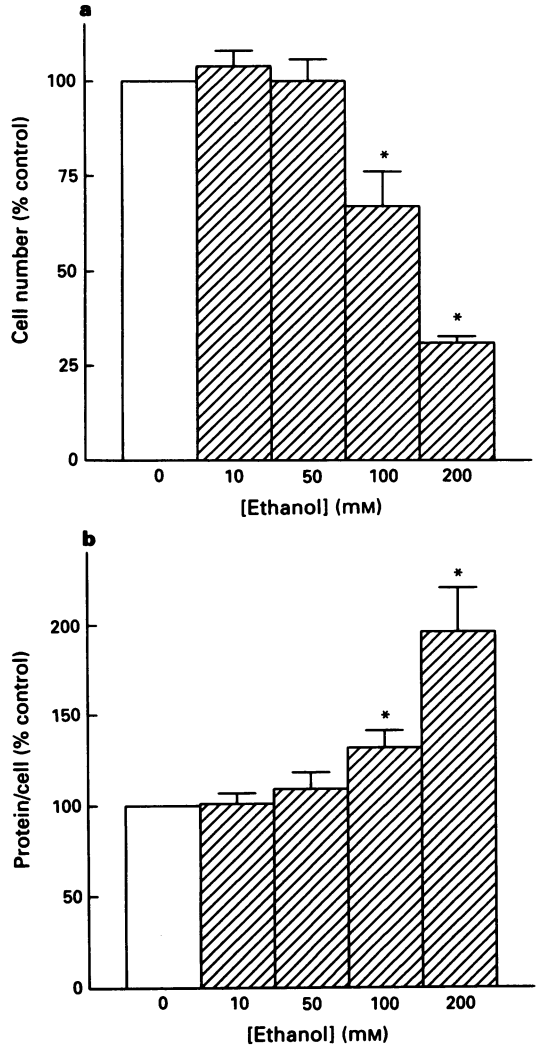

Figure 9 Concentration-dependent effects of chronic ethanol on (**a**) NG108-15 cell number and (**b**) cell protein content. Cells were incubated with 0–200 mM ethanol for 48 h prior to these determinations. In each case, control values (no ethanol) are normalised to 100%. Control values for cell number were 2.03 ± 0.31 million cells per 25 cm^2 flask and for protein content $8.70 \times 10^{-6} \pm 0.69 \times 10^{-6}$ mg protein per cell. Values are means ± s.e. mean from 5 separate experiments. $^*P<0.05$, vs. no ethanol, Mann-Whitney test.

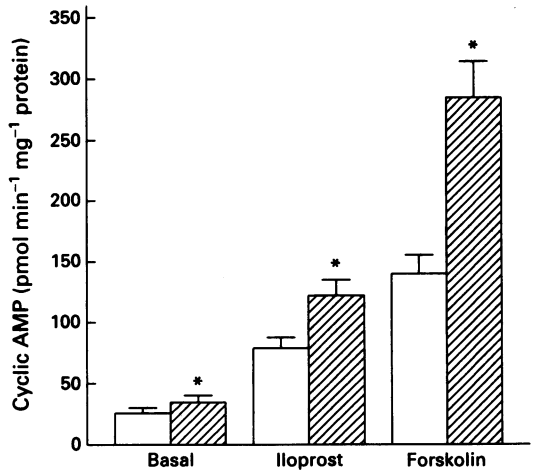

Figure 10 Effect of incubation of NG108-15 cells without foetal calf serum on cyclic AMP levels. Following splitting, cells were grown for 48 h in the presence of 6% foetal calf serum, following which they were grown for a further 48 h either in the presence (open columns) or absence (hatched columns) of serum. Cells were then washed and cyclic AMP accumulation assessed under basal conditions, or in the presence of iloprost (1 μM) or forskolin (10 μM). Values are means ± s.e. mean from 11 separate experiments. $^*P<0.05$, vs. control, paired *t* test.

Incubation of cells in the absence of foetal calf serum for 48 h inhibited cell division and enhanced neurite outgrowth, a result similar to that for ethanol treatment (data not shown). This treatment enhanced basal, iloprost- and forskolin-stimulated cyclic AMP formation (Figure 10).

Discussion

Acute and chronic ethanol treatments are reported to have a number of different effects on cyclic AMP signal transduction (reviewed in Hoffman & Tabakoff, 1990). In rodent brain, acute ethanol enhances hormone- and guanine nucleotide-stimulated adenylyl cyclase activity (eg Rabin & Molinoff, 1981), whereas chronic ethanol pretreatment of rats is generally reported to reduce subsequent activation of brain adenylyl cyclase by the same agents (Saito *et al.*, 1987; Wand *et al.*, 1993). Due to the heterogeneity of brain tissue, a number of studies have employed transformed neuronal cell lines, since the effects obtained in a homogeneous cell population are often easier to interpret. In NG108-15 cells, heterologous desensitization of agonist-stimulated cyclic AMP formation (Gordon *et al.*, 1986) and a selective reduction in $G_s\alpha$ mRNA and membrane $G_s\alpha$ protein expression following chronic ethanol have been reported (Mochly-Rosen *et al.*, 1988). It should be noted however that we (Williams *et al.*, 1993) and others (Hu *et al.*, 1993) have not been able to detect a chronic ethanol-induced reduction in $G_s\alpha$ mRNA in this cell line. It has been proposed that the changes in cell signalling in NG108-15 cells described above are subsequent to ethanol-induced increases in extracellular adenosine (Nagy *et al.*, 1989), since ethanol can inhibit cellular adenosine uptake (Nagy *et al.*, 1990). However, the role of extracellular adenosine in ethanol-induced changes in cyclic AMP signal transduction has been questioned by us (Williams *et al.*, 1993) and by others (Rabin *et al.*, 1993).

In our own studies we find that similar concentrations of chronic ethanol do not selectively reduce $G_s\alpha$ expression in NG108-15 cells, since we observe changes in the expression of multiple G-protein α-subunits including reductions in the expression of $G_s\alpha$, $G_i\alpha 2$ and $G_q\alpha/_{11}\alpha$, but a marked increase in the expression of $G_o\alpha$ (Williams *et al.*, 1993; Williams & Kelly, 1993). Furthermore, unlike previous findings we did not observe heterologous desensitization of adenylyl cyclase in these cells following chronic ethanol, but rather an increase in basal and agonist-stimulated cyclic AMP accumulation (Williams *et al.*, 1993). In the light of these findings we decided to investigate in more detail the effects of acute and chronic ethanol on cyclic AMP signal transduction in NG108-15 cells. For comparative reasons, the concentrations of ethanol we used were in the same range as those used in previous studies (eg. Gordon *et al.*, 1986).

In the present experiments, acute ethanol enhanced basal cyclic AMP levels, in line with previous findings in NG108-15 cells (Nagy *et al.*, 1989). This increase was due solely to an increase in extracellular adenosine, which would activate the adenosine A_2 receptor present on these cells (Keen *et al.*, 1989), since the increase was abolished by co-inclusion of ADA with ethanol. Acute ethanol also concentration-dependently enhanced iloprost-stimulated cyclic AMP formation in NG108-15 cells, again in line with findings for adenosine agonist-stimulated cyclic AMP formation in these cells (Gordon *et al.*, 1986). This increase was unaffected by removal of extracellular adenosine, and it has been suggested that ethanol acutely increases agonist-stimulated cyclic AMP

formation by an interaction with G_S, or the ability of $G_S\alpha$ to interact with the adenylyl cyclase enzyme (Hoffman & Tabakoff, 1990). On the other hand, we found acute ethanol to inhibit forskolin-stimulated cyclic AMP formation in NG108-15 cells, similar to findings in N1E-115 cells (Stenstrom *et al.*, 1985). Inclusion of ADA also reduced forskolin-stimulated cyclic AMP by around 40%, indicating that part of the forskolin response is dependent upon extracellular adenosine. Hormonal activation of G_S-coupled receptors is known to activate synergistically adenylyl cyclase with forskolin (Seamon & Daly, 1986), thus explaining the large effect produced by extracellular adenosine in the presence of forskolin. However, the present results demonstrate that the ethanol-dependent inhibition of forskolin-stimulated cyclic AMP formation is not dependent upon extracellular adenosine, since it remains in the presence of ADA. Taken together, these results indicate that the acute effects of ethanol on cyclic AMP accumulation in NG108-15 cells are differentially dependent upon extracellular adenosine.

In the present experiments, chronic ethanol enhanced basal, iloprost- and forskolin-stimulated cyclic AMP formation over that in control NG108-15 cells. It is possible that the increase in iloprost stimulation is due to a chronic ethanol-induced increase in IP receptor number, since chronic ethanol has recently been reported to increase expression of δ-opioid receptor, α_2-adrenoceptor and muscarinic acetylcholine receptor expression in NG108-15 cells (Charness *et al.*, 1993; Hu *et al.*, 1993). However, the limited membrane binding study performed here with a single concentration of [^{3}H]-iloprost suggested no change in receptor number and no apparent change in coupling to G_S since GppNHp decreased binding in control and ethanol pretreated cells to the same extent. It therefore seems likely that the change in iloprost efficacy following chronic ethanol is due to changes beyond the level of the IP receptor. Interestingly two recent studies also report chronic ethanol to enhance receptor- and forskolin-stimulated cyclic AMP accumulation in other cell types, including rat hepatocytes (Nagy & DeSilva, 1992) and human placental trophoblasts (Karl *et al.*, 1994). Thus the chronic ethanol-induced increase in cyclic AMP accumulation is a characteristic of different cell types.

The increases in basal, iloprost- and forskolin-stimulated cyclic AMP formation were dependent upon the concentration of chronic ethanol used (Figure 4). The concentrations of chronic ethanol that produced increases were similar to those that we found to alter G-protein expression (Williams *et al.*, 1993), and which others have found to increase δ-opioid expression in NG108-15 cells (Charness *et al.*, 1993). Although the ethanol concentrations used in these studies are high, it is possible that incubation of cells with lower concentrations for longer periods may also alter cyclic AMP formation, as has been demonstrated for PC12 cells incubated with 25 mM ethanol for 7 days (Rabin, 1993).

It is possible that the changes in cyclic AMP accumulation observed in the present study are due to the sudden withdrawal of ethanol from the cells, since the cells are thoroughly washed following the 48 h incubation with ethanol. To examine this, cells were pretreated with or without ethanol for 48 h, then washed and exposed to agonists in the presence or absence of ethanol. If a rebound increase in cyclic AMP following chronic ethanol is occurring, then the readdition of ethanol should blunt this effect, however, no such reversal was observed (Figure 5). Indeed not only did the replacement of ethanol not reverse the increases in cyclic AMP formation following chronic ethanol, but basal and iloprost-stimulated levels were further increased. The ability of acute ethanol to inhibit forskolin-stimulated cyclic AMP levels was likewise left intact following chronic ethanol. Thus although chronic ethanol markedly alters cyclic AMP accumulation in these cells, they remain normally responsive to the acute effects of ethanol, which may suggest that the chronic effects are not a direct adaptation to the acute effects.

Since adenosine plays an important part in the changes in cyclic AMP accumulation due to acute ethanol, we decided to examine the involvement of extracellular adenosine in the changes following chronic ethanol using ADA, which deaminates adenosine to inosine. Following chronic ethanol, acute ADA abolished the increase in basal cyclic AMP levels, but did not affect the maximum increase in iloprost-stimulated cyclic AMP levels (Figure 6a). Thus, as we have previously shown (Williams *et al.*, 1993), increased extracellular adenosine is responsible for the increase in basal cyclic AMP, but plays no part in the increased agonist-stimulated cyclic AMP formation. Recent work confirms that the increase in basal cyclic AMP is due to increased extracellular adenosine, and occurs in a number of different neuronal cell lines (Charness *et al.*, 1993). Furthermore the finding that an adenosine receptor antagonist, xanthine amine congener, also inhibited the increase in basal and forskolin-stimulated cyclic AMP accumulation following chronic ethanol indicates that adenosine is exerting its effect via an adenosine A_2 receptor (Rivkees & Reppert, 1992).

Acute ADA reduced forskolin-stimulated cyclic AMP formation both before and after chronic ethanol, shifting the forskolin concentration-effect curve to the right. These data are consistent with the synergistic effect of hormone receptor stimulation on forskolin-activated adenylyl cyclase (Seamon & Daly, 1986), such that when extracellular adenosine is removed there is a reduction in the potency of forskolin. Higher concentrations of ADA did not further inhibit the response to forskolin in control and chronic ethanol-treated cells (Figure 7), demonstrating that the increase in forskolin-stimulated cyclic AMP formation is due to increases in both the adenosine-dependent and -independent components of the response. The enhancement of the adenosine-independent forskolin response may reflect an increase in the function of the adenylyl cyclase enzyme itself, and could explain the increase in agonist-stimulated cyclic AMP formation we see following chronic ethanol.

To investigate further the involvement of adenosine A_2 receptors in altered cyclic AMP formation, NG108-15 cells were chronically incubated with the adenosine receptor agonist NECA. If extracellular adenosine plays a role in the changes in cyclic AMP observed, then prolonged activation of adenosine A_2 receptors should mimic to some extent the effects of ethanol on cyclic AMP formation. However, the effects of chronic NECA we observed were unlike those of ethanol, but were consistent with simple homologous desensitization of adenosine A_2 receptors (Keen *et al.*, 1989). In the present experiments, NECA pretreatment also reduced forskolin-stimulated cyclic AMP formation, but this difference was no longer observed when ADA was present (Figure 8b), indicating that the reduction of the forskolin response was due solely to loss of the adenosine-dependent component of forskolin-activated cyclic AMP formation. Thus these results suggest that prolonged adenosine receptor stimulation is not involved in the chronic ethanol-induced increase in receptor-stimulated cyclic AMP accumulation in NG108-15 cells that we observe, and is in agreement with recent work in PC12 cells (Rabin *et al.*, 1993). If, as seems likely, chronic ethanol increases extracellular adenosine concentrations in an NG108-15 cell culture, then it is unclear why this does not lead to desensitization of adenosine A_2 receptors, but actually enhances NECA responsiveness (Williams *et al.*, 1993). One possible explanation could be that although there is some adenosine A_2 receptor desensitization due to extracellular adenosine following chronic ethanol, this is masked by the enhancement of receptor-stimulated cyclic AMP accumulation observed with NECA.

We have previously noted that 200 mM chronic ethanol pretreatment inhibits NG108-15 cell proliferation and enhances neuronal differentiation (Williams *et al.*, 1993). In the present study chronic ethanol was found to inhibit cell proliferation and increase cell protein content concentration-dependently, and interestingly the concentrations at which

these effects were observed were similar to those which produced both acute and chronic changes in cyclic AMP responsiveness. It is therefore possible that the changes in cyclic AMP accumulation following chronic ethanol might be due to the morphological changes that occur under these conditions. In an attempt to answer this question, cells were depleted of foetal calf serum for 48 h, which greatly reduced NG108-15 cell division and tended to promote neurite outgrowth, not unlike that seen following chronic ethanol. Under these conditions, basal, iloprost- and forskolin-stimulated cyclic AMP accumulation were all increased compared to control, suggesting that at least a component of the altered cyclic AMP formation following chronic ethanol is due to the change in cell morphology.

Some mention should be made of the differences in the effects of chronic ethanol on cyclic AMP signal transduction in this cell line reported in the literature. Although it is clear that ethanol reduces membrane $G_s\alpha$ expression in NG108-15 cells (Mochly-Rosen *et al.*, 1988; Williams *et al.*, 1993), it is less clear what happens to cyclic AMP levels. Heterologous desensitization of agonist-stimulated cyclic AMP formation was originally reported (Gordon *et al.*, 1986), but we have observed enhanced cyclic AMP responsiveness (Williams *et al.*, 1993 and the present study). In line with our findings other studies have reported chronic ethanol to increase basal (Charness *et al.*, 1993) and adenosine receptor-stimulated cyclic AMP formation (Charness *et al.*, 1986). These differences cannot be explained by the use of different ethanol concentrations; incubation of cells with 200 mM ethanol for 48 h has been employed by ourselves and Gordon *et al.* (1986) with opposite results. On the other hand differences could arise because of the culture conditions employed (we used complete medium whereas Gordon *et al.*, 1986 used defined medium). Under defined medium conditions the latter group reported little or no effect of chronic ethanol on cell number but with complete medium we observed a marked inhibitory effect of ethanol on cell division. Thus ethanol may exert differential effects on receptor-stimulated cyclic AMP accumulation depending upon whether the cells are actively dividing or not, a possibility that we are currently examining. Another potential difference may relate to differences in the cells used by different groups; in a recent study acute ethanol was reported to exert opposite effects on cyclic AMP accumulation in two subclones of PC12 cells obtained from different sources (Rabe *et al.*, 1990). These differences underline the likelihood that ethanol not only has different effects in different cell types, but also that the conditions used in a particular cell type may be crucial.

In conclusion, we describe a chronic ethanol-induced enhancement of cyclic AMP formation in NG108-15 cells. Increased basal cyclic AMP was due exclusively to extracellular adenosine, that due to iloprost occurred independently of extracellular adenosine, and that due to forskolin was partially adenosine-dependent. The increase in iloprost responsiveness and the adenosine independent part of the increased forskolin responsiveness may reflect an increase in the function of the adenylyl cyclase enzyme. Furthermore, ethanol-induced morphological changes in the cells may in part underlie the observed changes in the cyclic AMP signal transduction system. These studies may be of relevance to the pathological effects of ethanol in the developing nervous system, as occurs with excessive alcohol intake during pregnancy (Charness *et al.*, 1989).

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Inhibition of serum-induced proliferation of bovine tracheal smooth muscle cells in culture by heparin and related glycosaminoglycans

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1 The effect of heparin and related glycosaminoglycans on bovine airway smooth muscle proliferation has been investigated.

2 Foetal bovine serum stimulated division of bovine trachealis smooth muscle cells in a concentration-dependent fashion at concentrations between 1 and 30%.

3 Heparin ($0.1-100\,\mu g\,ml^{-1}$), heparan sulphate ($0.1-100\,\mu g\,ml^{-1}$) and fragmin ($0.1-100\,\mu g\,ml^{-1}$) inhibited smooth muscle division in a concentration-dependent fashion between $0.1-100\,\mu g\,ml^{-1}$. A heparin disaccharide did not exhibit inhibition of division at $100\,\mu g\,ml^{-1}$.

4 Dextran sulphate at molecular weights of 5×10^3 and 5×10^5 concentration-dependently inhibited division between $0.1-100\,\mu g\,ml^{-1}$. Dextran without sulphation did not exhibit inhibition of division at $100\,\mu g\,ml^{-1}$.

5 The magnitude of inhibition of proliferation did not reach 100% for any compounds examined at concentrations up to $100\,\mu g\,ml^{-1}$ during incubations for 5 and 14 days. IC_{50} values for inhibition of proliferation ranged between $1-5\,\mu g\,ml^{-1}$.

6 These findings suggest that heparin and related glycosaminoglycans inhibit bovine airway smooth muscle cell division.

Keywords: Heparin; glycosaminoglycans; airway; smooth muscle; division

Introduction

The smooth muscle component of airways has largely been investigated for its contribution to airway smooth muscle tone in several diseases such as asthma and bronchitis. Changes in structure of the airway wall including increases in airway smooth muscle mass have also been observed in asthmatics (Dunnill, 1969; Ebina *et al.*, 1990; 1993) and there is evidence supporting a relationship between changes in airway smooth muscle mass and the bronchial hyperresponsiveness characteristic of asthma (James *et al.*, 1989). However, the mechanisms controlling airway smooth muscle cell (ASMC) division and size are poorly defined. The range of potential mitogenic stimuli for ASMC is extensive, but the number of endogenous factors with an established antiproliferative action on ASMC is very limited.

The inhibitory action of glycosaminoglycans including heparin and heparan sulphate on vascular smooth muscle cell (VSMC) (Karnovsky & Edelman, 1994) and fibroblast division (Jackson *et al.*, 1991) is well established. Heparin containing mast cells are abundant in the airways and cell surface heparan sulphate glycosaminoglycans are ubiquitous. Furthermore, it has recently been demonstrated that human lung parenchyma can release heparin at antiproliferative concentrations following allergen exposure (Green *et al.*, 1993). Glycosaminoglycans therefore provide potential regulators of ASMC behaviour. The absence of a proliferative action of heparan sulphate on ASMC division has been demonstrated (Panettieri *et al.*, 1990), but the potential antiproliferative effects of glycosaminoglycans on ASMC division have not been examined.

Heparins are polymers composed of alternating glucuronic and iduronic saccharides with O- and N- sulphation and carboxylation. The antiproliferative action of heparin in VSMC is independent of anticoagulant activity, indicating that the antithrombin III binding site is not a requirement (Guyton *et al.*, 1980). Certain characteristics of the glycosaminoglycans are required for antiproliferative activity, including a degree of charge density and minimum molecular size (Castellot *et al.*, 1984; Wright *et al.*, 1989). Thus, desulphated heparin or heparin fragments of less than hexasaccharide units are not antiproliferative in VSMC (Karnovsky & Edelman, 1994).

In the present investigation we have examined the possible influence of glycosaminoglycans on ASMC. In addition we have examined requirements of minimum molecular size and sulphation by examining antiproliferative activity of dextrans and low molecular weight heparin fragments on ASMC division. Preliminary findings of this work have been presented to the European Respiratory Society (Kilfeather *et al.*, 1993).

Methods

Smooth muscle cell culture

Airway smooth muscle cells were obtained from bovine trachealis muscle. Trachealis muscle was obtained from adult cattle killed by captive bolt and was dissected free of connective tissue under aseptic conditions under a dissection microscope. The muscle was washed in calcium- and magnesium-free phosphate buffered saline (PBS) and cut into $1-2\,mm^3$ pieces for explanting. The explants were washed in medium 199 (M199) supplemented with gentamicin ($50\,\mu g\,ml^{-1}$) and plated in 100 mm tissue culture-treated Petri dishes in M199 at pH 7.3 supplemented with foetal bovine serum (FBS, 10%), glutamine (2 mM), gentamicin ($50\,\mu g\,ml^{-1}$) and amphoterecin B ($2.5\,\mu g\,ml^{-1}$), and maintained at 95% air/5% CO_2 in a humidified atmosphere at 37°C. Cells migrated from the explants after one week of plating. The explants

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were removed when cells migrating from the explant had reached a density of 10 per cent. The cells were then allowed to reach confluence before replating. For replating, cells were washed three times in PBS and removed from the plate as previously described (San Antonio *et al.*, 1992) by incubation with PBS and EDTA (1 mM). The cells were replated for further population growth or directly onto a 96 well tissue culture plated at 10 or 20% confluence for examination of cell division at 5 days or plated at 100 cells/well for examination of division at 14 days. A lower cell density was adopted for plating in experiments conducted over 14 days to maintain exponential growth characteristics during the incubation period. Cells were refed every 3 days. Cells were examined up to passage 4. Cells from separate animals were used for each experiment.

Smooth muscle cell identification

Smooth muscle cells were identified by immunofluorescence using an antibody against alpha smooth muscle actin (anti-α-SM-1) as previously described (Skalli *et al.*, 1986).

Smooth muscle cell division

Following plating for 24 h in 10% FBS, cells were growth arrested by maintenance in M199 supplemented with 0.5% FBS for 72 h. This concentration of FBS was insufficient to stimulate division (Figure 1). Cell division was reinitiated by incubation with FBS (10%) or varying concentrations for examination of concentration-dependent stimulation of division by FBS. Compounds under examination were introduced with 10% FBS. Cell division was monitored after 5 or 14 days by cell counting following nuclear staining with propidium iodide and giemsa. Cells were washed in phosphate buffered saline with calcium and magnesium three times before fixing in methanol and air drying. Fixed cells were then stained with propidium iodide (0.05 mg ml^{-1}, 30 min) or giemsa (GS-500, Sigma 2 min). Stained cells were then washed in deionised water. Staining with propidium iodide and giemsa gave identical results, although giemsa staining was found to be preferable due to stability of the stain and lack of requirement for fluorescence microscopy. Cell location while counting was assisted by creation of a grid on the underside of the wells with a scalpel blade.

Compounds

All compounds including tissue culture components and antibodies for immunofluorescence were obtained from Sigma (Poole, UK). Fragmin was obtained from Pharmacia (Milton Keynes, UK). Tissue culture plastics were obtained from Bibby Sterilin (UK).

Data analysis

Comparison of cell densities within experiments was by Student's *t* test. Concentration-response curves were analysed from Hill coefficients. Statistical significance was accepted with $P<0.05$.

Results

Smooth muscle cell culture

After four days following explant plating, cells were observed migrating from explants. These cells and cells at subsequent passages stained positive for α-smooth muscle actin. Maintenance of cells at confluence in the presence of FBS (10%) resulted in the formation of multilayered cells characteristic of smooth muscle cells and often referred to as 'Hill and valley'.

Effect of heparin and related compounds on smooth muscle cell division

FBS induced a concentration-related increase in cell division between 1 and 30% (Figure 1). A submaximal concentration of 10% serum was chosen in subsequent experiments to examine potential inhibitory effects of glycosaminoglycans. In initial experiments, effects of glycosaminoglycans were examined at 100 μg ml^{-1}, since the findings of previous investigations have demonstrated maximal antiproliferative effect on VSMC at this concentration (Wright *et al.*, 1989). Heparin, heparan sulphate, fragmin and dextran sulphate all inhibited cell division at 5 days (50% at 100 μg ml^{-1}, $P<0.05$) (Figure 2). The antiproliferative action of heparin in VSMC is dependent upon a minimum molecular weight. We have examined molecular weight dependency in the antiproliferative action of heparin against ASMC. The disaccharide of heparin (a-DUA-[1-4]-GlcNS-6S, derived from heparinase I and II-treated heparin) did not influence cell division in experiments where heparin exhibited significant inhibition of division (Figure 2). Previous studies in VSMC and fibroblasts have revealed a requirement for sulphation in the antiproliferative action of heparin. We have investigated a requirement for sulphation in the antiproliferative action of dextran sulphate by examining a dextran devoid of sulphation, but with high molecular weight (5×10^5) to avoid

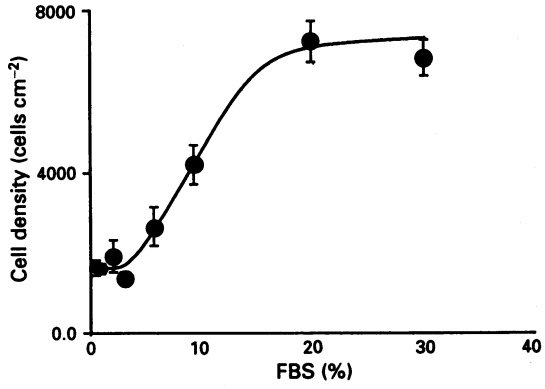

Figure 1 The effect of foetal bovine serum (FBS) on bovine trachealis smooth muscle division. Cells were incubated with FBS for 5 days and cell density measured as described in methods. The data points are mean with s.e.mean derived from one of two experiments conducted with eight replicates for each mean.

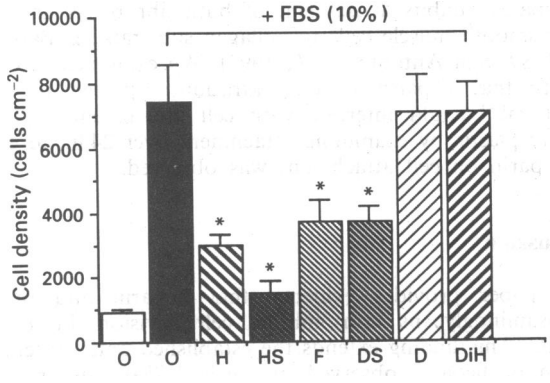

Figure 2 The effect of heparin, and related glycosaminoglycans (100 μg ml^{-1}) on FBS (10%)-induced ASMC division. Cells were incubated with FBS for 5 days and cell density measured as described in methods: (H) heparin, (HS) heparan sulphate, (F) fragmin, (DS) dextran sulphate, (D) dextran, (DiH) heparin disaccharide. Columns are means with s.e.mean derived from one of three experiments conducted with eight replicates for each mean. *$P<0.05$ compared to FBS alone.

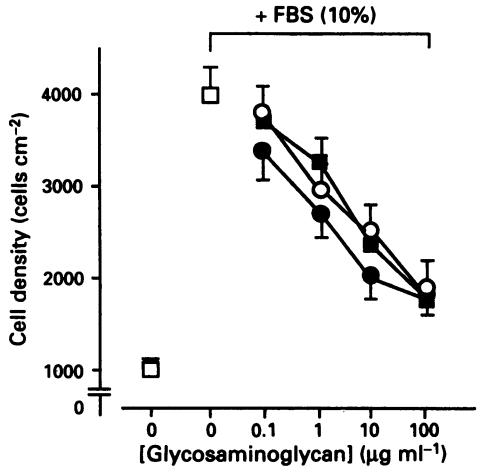

Figure 3 The concentration-dependency of inhibition of ASMC division by glycosaminoglycans. Cells were incubated with and without FBS and glycosaminoglycans (GAG) for 5 days and cell density measured as described in methods. Cells incubated without glycosaminoglycan (□), with heparin (●), heparan sulphate (○) and fragmin (■). The data points are mean with s.e.mean derived from one of three experiments conducted with eight replicates for each mean.

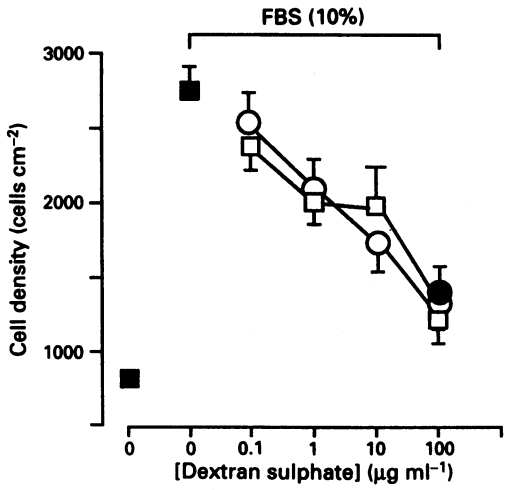

Figure 4 The concentration-dependent inhibition of ASMC division by dextran sulphate. Cells were incubated with and without FBS for 5 days and cell density measured as described in methods. Cells incubated without dextran sulphate (■), with dextran sulphate (molecular weight 5,000, ○; molecular weight 500,000 □) for 5 days and cell density measured as described in methods. The effect of heparin (100 μg ml^{-1}, ●) on cell division is also included. The data points are mean with s.e.mean derived from one of two experiments conducted with eight replicates for each mean.

potential limitation of molecular size. Division in response to 10% serum was inhibited in a concentration-dependent fashion by heparin (0.1–100 μg ml^{-1}) (Figure 3). Heparan sulphate and fragmin, a low molecular weight heparin also induced concentration-dependent inhibition of cell division (0.1–100 μg ml^{-1}) (Figure 3). Dextran sulphate at molecular weights of 5×10^3, and 5×10^5 (0.1–100 μg ml^{-1}) also exhibited concentration-dependent inhibition of cell division (Figure 4). Heparin included as a positive control inhibited division to a similar extent as observed in other experiments. An intermediate size dextran sulphate (molecular weight 4.5×10^4) also inhibited division at 100 μg ml^{-1} by 50% (54.4 ± 3.3%). We have not observed significant differences in potency between heparin-related compounds and dextran sulphates exhibiting inhibition of ASMC division.

The possibility that proliferation could be restored in the presence of heparin over a period of 14 days was also examined. In these experiments cells were incubated with heparin for 14 days before monitoring effects of cell division. Heparin inhibited division over 14 days by approximately 75% (75.1 ± 2.3%) (Figure 5), providing support for sustained inhibition of ASMC division by heparin.

Heparin inhibits attachment of both fibroblasts and vascular smooth muscle cells to collagen substrata (LeBaron *et al.*, 1989; San Antonio *et al.*, 1992). We examined the possibility that heparin at concentrations between 0.1 and 100 μg ml^{-1} could interfere with cell attachment to tissue culture plastic by examining attachment over 24 h; no effect of heparin on cell attachment was observed.

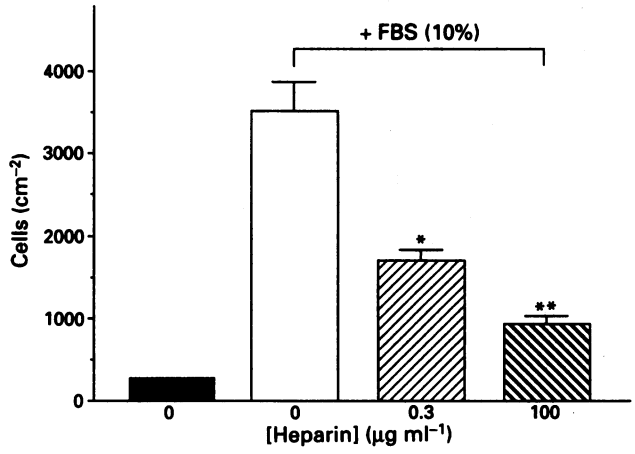

Figure 5 Inhibition of ASMC division by heparin during a 14 day incubation. Cells were maintained for 14 days in the presence and absence of FBS. Cell density was measured as described in Methods. The data points are means with s.e.mean derived from one of three experiments conducted with 12 replicates for each mean. *$P < 0.01$; **$P < 0.001$ compared to FBS without heparin.

Discussion

This paper provides evidence that heparin and related glycosaminoglycans can inhibit cell division in bovine ASMC. This finding extends the established antiproliferative action of heparin observed in aortic VSMC and recently observed in pulmonary vascular smooth muscle (Benitz *et al.*, 1986; 1990). The present findings in ASMC with respect to potency and efficacy of heparin for antiproliferative effects also reflect findings in VSMC (Castellot *et al.*, 1984).

The antiproliferative properties of heparin were first recognised following investigations of the interdependence of anticoagulant and antiproliferative activity in VSMC *in vivo* (Guyton *et al.*, 1980). The findings of these and subsequent investigations *in vitro* demonstrated a direct inhibitory effect on smooth muscle division independent of anticoagulant activity (Hoover *et al.*, 1980). Further examination of antiproliferative actions of heparin on VSMC *in vitro* has revealed a direct relationship between antiproliferative effect and both molecular size and charge density (Castellot *et al.*, 1984). Fragmin, used in the present investigation, contains fragments of molecular weight in the region of 4,000, while the heparin disaccharide has a molecular weight of 665. The difference in inhibitory activity on ASMC between these compounds is indicative of a minimum molecular size requirement in the inhibitory action of glycosaminoglycans on ASMC division. These findings suggest similarity in molecular size requirements of glycosaminoglycan actions in ASMC and VSMC, since disaccharides of heparin are not antiproliferative in VSMC (Wright *et al.*, 1989).

The range of dextran sulphate molecular weights examined in this investigation (5,000–500,000) are above the molecular weight threshold observed between fragmin and the heparin disaccharide and this could account for the absence of a relationship between molecular size and antiproliferative activity in this range. Although dextran sulphate inhibits VSMC and Swiss 3T3 fibroblast division (San Antonio *et al.*, 1992), a relationship between molecular size and antiproliferative effect has not been demonstrated for dextran sulphates.

The present findings demonstrate an antiproliferative effect of dextran sulphate against ASMC that appears to be dependent on sulphation, since dextran without sulphation at the highest molecular weight (500,000) was without effect on ASMC division. This finding also reflects inhibition by dextran sulphate and absence of inhibition by dextran in fibroblasts (San Antonio *et al.*, 1992). The nature of sulphation could be important, since O-sulphation is required in antiproliferative activity in VSMC (Wright *et al.*, 1989) and antiproliferative activity is not observed in dematan sulphate and the condroitins 4 and 6 sulphate in either VSMC or 3T3 fibroblasts (Castellot *et al.*, 1981; San Antonio *et al.*, 1992).

Interruption of ASMC attachment by heparin to tissue culture-treated plastic was not observed under conditions in which significant inhibition of division occurred. This finding does not exclude the possibility that interference by glycosaminoglycans in ASMC attachment and migration could be a contributory factor to potential antiproliferative activity *in vivo* or a collagen substratum *in vitro*. Interference with cell attachment through competition with glycosaminoglycan binding sites in attachment-dependent cells remains a potential mode of inhibition of division or migration.

Taken together our findings demonstrate that certain classes of glycosaminoglycans exhibit antiproliferative activity in ASMC. The requirements for this action that have been identified by the present investigation include a certain molecular size within heparins and sulphation within dextrans. In these aspects and in terms of efficacy and potency, the profile of glycosaminoglycan activity is consistent with the actions of glycosaminoglycans in VSMC. Further investigation is required to establish whether sulphation alone or the nature of the sulphation is important in defining antiproliferative activity in ASMC.

Airways are embryologically derived from the intestine and we cannot assume that the actions and underlying mechanisms of glycosaminoglycans will be the same in ASMC and VSMC. The mechanism of action of glycosaminoglycans in VSMC has not been completely defined and speculation concerning the mechanism of antiproliferative action of glycosaminoglycans in ASMC is not possible without extensive biochemical examination. It is improbable, however, that the incomplete inhibition of ASMC division by glycosaminoglycans reflects development of a glycosaminoglycan-resistant cell population during the course of these experiments. Heparin also inhibits thymidine labelling conducted over 1 h, 24 h after mitogenic stimulation by approximately 50%. It is more probable therefore that incomplete inhibition reflects the glycosaminoglycan insensitivity of certain intracellular mitogenic signalling pathways. Heparin-resistant cell populations can be developed following prolonged division in the presence of heparin (Pukac *et al.*, 1990), but this process is not achieved over the time courses used in the present investigation. Experiments conducted over 14 days in the present investigation demonstrate that a significant antiproliferative action is maintained over this period as opposed to a possible resumption of normal division rate after inhibition for 5 days. Division occurred in the presence of heparin during this period, indicated by incomplete inhibition of division by heparin. This implies that over this time period adaption to the inhibition by heparin is not observed. Division is not, therefore, delayed by heparin, but subject to prolonged inhibition for at least 14 days. The extent of inhibition is apparently greater at 14 days than at 5 days. This is a cumulative effect reflecting divergence in population size of two cell populations exhibiting different exponential growth rates.

These findings have relevance to broader issues of inflammation, structure and hyperresponsiveness of airways. Several changes in structural cell behaviour occur in asthma including increased smooth muscle mass that appear to be related to airway hyperresponsiveness (James *et al.*, 1989). Heparin is released from human parenchyma upon exposure to allergen or calcium ionophores at concentrations that would influence ASMC division *in vitro* (Green *et al.*, 1993). Glycosaminoglycans modulate vascular smooth muscle phenotypic expression towards a nondividing contractile phenotype (Chamley-Campbell *et al.*, 1981; Campbell *et al.*, 1992); an action that could contribute to antiproliferative effects on ASMC division observed in the present investigation. Inflammatory cells are major sources of heparanase, which disrupt glycosaminoglycan linkages and thereby release fragments from cellular heparan sulphate. Released glycosaminoglycans could influence lymphocyte (Sy *et al.*, 1983; Brenan & Parish, 1986; Lider *et al.*, 1990) and eosinophil trafficking (Seeds *et al.*, 1993), while disrupted cell surface heparan sulphate could affect glycosaminoglycan antiproliferative activity in the airway, as previously suggested for the vasculature (Campbell *et al.*, 1992). Persistent or recurring inflammation in the airways could thereby influence structural changes observed in airways of asthmatics. Glycosaminoglycans, either free or incorporated as proteoglycans could be involved in this process.

In conclusion, these findings suggest a role for endogenous glycosaminoglycans in regulation of airway smooth muscle division and airway structure. Manipulation of airway glycosaminoglycan levels would therefore be of relevance to airway remodelling and consequent influence on airway responsiveness.

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Inhibitory effects of SR 58611A on canine colonic motility: evidence for a role of β_3-adrenoceptors

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1 In order to clarify whether atypical or β_3-adrenoceptors can modulate canine colonic motility *in vivo*, we studied the effects of SR 58611A (a selective agonist for atypical β-adrenoceptors) alone and after pretreatment with β-adrenoceptor antagonists on colonic motility in the conscious dog. The gastrocolonic response (postprandial increase in motility) was monitored by means of electrodes and strain-gauge force transducers chronically implanted along the distal colon. In some experiments, heart rate was also measured. The possible role of β_3-adrenoceptors in mediating the effects of SR 58611A was also tested *in vitro* in circular muscle strips taken from the canine distal colon.

2 Intravenous infusion of SR 58611A, ritodrine or isoprenaline at doses inducing the same degree of tachycardia inhibited the gastrocolonic response to a different extent, with SR 58611A and ritodrine being more effective than isoprenaline.

3 In a dose-response study, SR 58611A was more potent in inhibiting colonic motility than in inducing tachycardia: the ED_{35} values for inhibition of colonic motility and induction of tachycardia were 23 and 156 $\mu g\,kg^{-1}$, i.v., respectively.

4 The inhibitory effect of SR 58611A 100 $\mu g\,kg^{-1}$, i.v., on the gastrocolonic response was reversed by alprenolol (non-selective β-adrenoceptor antagonist), but resistant to CGP 20712A (β_1-adrenoceptor antagonist) or ICI 118551 (β_2-adrenoceptor antagonist).

5 *In vitro*, SR 58611A concentration-dependently relaxed circular muscle strips, an effect that was competitively antagonized by alprenolol with a pA_2 value of 7.1, but resistant to CGP 20712A (100 nM), ICI 118551 (100 nM) or tetrodotoxin (1 μM).

6 The present study provides strong functional evidence for a role of atypical or β_3-adrenoceptors in the modulation of canine colonic motility both *in vivo* and *in vitro* by an inhibitory effect most likely at the smooth muscle level.

Keywords: β_3-Adrenoceptors; colonic motility; gastrocolonic response; smooth muscle

Introduction

β-Adrenoceptors were originally divided into β_1- and β_2-subtypes on the basis of agonist potency (Lands *et al.*, 1967a,b). More recently, pharmacological studies have suggested the existence of atypical or β_3-adrenoceptors, which are characterized by resistance to classical β-adrenoceptor antagonists and are stimulated by novel agonists such as SR 58611A (for review, see Arch & Kaumann, 1993). Several lines of evidence suggest the presence of atypical or β_3-adrenoceptors mediating relaxation at different gut levels in the rat, guinea-pig and rabbit (Bond & Clarke, 1987; 1988; Bianchetti & Manara, 1990; Blue *et al.*, 1990; McLaughlin & MacDonald, 1990; Norman & Leathard, 1990; Croci *et al.*, 1991; Taneja & Clarke, 1992; Growcott *et al.*, 1993a,b; de Boer *et al.*, 1993; MacDonald & Lamont, 1993). However, most of these studies were performed *in vitro* and no data are available on the possible role of β_3-adrenoceptors in the modulation of canine colonic motility *in vivo*.

In dogs, as in man, meal ingestion induces the so-called 'gastrocolonic response', i.e. an increase in colonic motility starting within a few minutes and lasting several hours postprandially (Sarna, 1991). This excitatory response is well reproducible (Sarna & Lang, 1989) and therefore particularly suitable when one wants to evaluate the effects of drugs, such as β-adrenoceptor agonists, which inhibit colonic motility. A study carried out in the dog isolated colon showed a low apparent pA_2 value for antagonism of isoprenaline by propranolol (Grivegnée *et al.*, 1984). Because this is now an established hallmark of atypical, β_3-type adrenoceptors (Arch & Kaumann, 1993; Emorine *et al.*, 1994), we decided to study the effects of SR 58611A (a selective agonist for β_3-adrenoceptors, Bianchetti & Manara, 1990) on the gastrocolonic response in the conscious dog (monitoring heart rate at the same time) and compare its effects with those of isoprenaline (non-selective β-adrenoceptor agonist) and ritodrine (β_2-adrenoceptor agonist). The possible role of β_3-adrenoceptors in mediating the effect of SR58611A was also tested by using selective antagonists *in vivo* and in isolated colonic circular muscle strips.

A preliminary account of these data was presented at the V[th] Joint Meeting between the British and Italian Societies of Pharmacology (De Ponti *et al.*, 1994b).

Methods

Experimental model

All experiments were carried out according to the guidelines set forth by EEC Directive 86/609 on the care and use of experimental animals. Five female mixed breed dogs (18–22 kg body weight), purchased from an authorized local breeder (BMG Allevamento, Cividate al Piano, Bergamo, Italy) were used. The animals were operated upon under general pentobarbitone anaesthesia (pentobarbitone sodium, 30 $mg\,kg^{-1}$, i.v.) with aseptic techniques and assisted respiration (respiration pump model 16/24, Palmer Ltd, London, England). Through a midline laparotomy, we implanted five

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bipolar electrodes (nichrome wire, 0.12 mm in diameter, Driver Harris, Rho, Milan, Italy) along the distal colon. Atropine (100 $\mu g\,kg^{-1}$, i.v.) was administered after abdominal incision to minimize shortening of the bowel due to handling. All wires had been inserted previously into a silastic tube (inside diameter, 1.47 mm; outside diameter, 1.96 mm, Medical Grade Tubing, Dow Corning Corporation, Midland, MI, U.S.A.) and soldered to the terminal of a multi-pin connector. Electrodes were placed as follows: the fifth electrode (E_5) was implanted 8–12 cm from the anal verge, the fourth electrode (E_4) was placed 3 cm proximal to E_5, the third electrode (E_3) 5 cm proximal to E_4 and finally the second and first electrodes (E_2 and E_1) 3 and 6 cm proximal to E_3, respectively. A strain-gauge force transducer (type EA-06-090DG-120, Micro-Measurements, Raleigh, NC, U.S.A.) was implanted between E_3 and E_4.

The wires connected to the strain-gauge and the tube containing the electrodes were exteriorized in the midscapular region through a subcutaneous tunnel. To avoid infection, the point of exit of the wires and the tube was cleansed daily. Antibiotic prophylaxis consisted of i.m. Vettrimicina (Vetem, Milan, Italy: 10,000 iu, kg^{-1} day^{-1} of penicillin and 10 mg kg^{-1} day^{-1} of dihydrostreptomycin) for three days after surgery.

Experimental procedure

After allowing a period of 2 weeks for recovery from surgery, the fasted, conscious animals were studied while comfortably standing in a sling. Intestinal myoelectrical and mechanical activities were recorded by a multi-channel recorder (R711, Sensormedics, Anaheim, CA, U.S.A.). Myoelectrical activity was recorded with a time constant of 0.03 s and stored simultaneously by a computer system (Compaq Deskpro 386s computer, provided with a 110 Mbyte hard disk) for later analysis via software. After monitoring colonic motility for at least 2 h, treatments were administered intravenously as detailed below (see Experimental Design). The standard meal was administered during a quiescent phase of motility. It consisted of 3 cans (405 g each) of Pedigree Pal (Unisabi, St. Denis de l'Hôtel, France), corresponding to ~1,000 Kcal, and was invariably consumed within 5 min. Colonic motility was then monitored for at least 3 h after meal administration.

In some experiments, in order to monitor heart rate, electrocardiographic recordings were obtained at the following times: −5, 0 (control, immediately before β-adrenoceptor agonist administration), 5, 10, 15, 20, 30, 40, 50, 60, 70, 80 and 90 min after the start of the i.v. infusion or after i.v. bolus injection of the β-adrenoceptor agonist being tested. At the same times, in some experiments with SR 58611A, systolic blood pressure was measured by the tail-cuff method (Blood Pressure Recorder, model 8006, Basile, Comerio, Italy).

Experimental design and data analysis

Four sets of experiments were carried out *in vivo* on each dog, allowing an interval of at least 3 days between two experimental sessions with the same dog.

In the first set of experiments we assessed the gastrocolonic response in each dog by administering intravenously, in random order to each animal, 10 ml of 154 mM NaCl (control) or 300 $\mu g\,kg^{-1}$ atropine 15 min before the standard meal. Atropine was used as a reference drug, for its ability to inhibit the colonic motor response to a meal (Sarna, 1991) and for its well-known positive chronotropic effect. The dose of atropine chosen had previously been found to be a supramaximal dose in increasing heart rate in the dog (De Ponti *et al.*, 1993).

In the second set of experiments, we evaluated the colonic and chronotropic effects of SR 58611A, isoprenaline and ritodrine. SR 58611A (10 $\mu g\,kg^{-1}$ min^{-1}), isoprenaline (0.1 $\mu g\,kg^{-1}\,min^{-1}$) or ritodrine (10 $\mu g\,kg^{-1}\,min^{-1}$) were randomly administered to each dog via a 30 min i.v. infusion (0.5 ml min^{-1}), starting 15 min before the standard meal. These doses (having approximately the same positive chronotropic effect) were chosen on the basis of pilot experiments.

In the third set of experiments, we carried out dose-response studies with SR 58611A (10, 30, 100 and 300 $\mu g\,kg^{-1}$). The treatments were randomly administered to each dog as an i.v. bolus 15 min before the standard meal.

In the fourth set of experiments, we assessed the effects of β-adrenoceptor antagonists on colonic motility and on the inhibitory effect of SR 58611A. In each dog, alprenolol, CGP 20712A (β_1-adrenoceptor antagonist, Dooley *et al.*, 1986) or ICI 118551 (β_2-adrenoceptor antagonist, Bilski *et al.*, 1983) were randomly infused intravenously for 1 h, starting 45 min before the meal. Each antagonist was administered at the dose of 300 $\mu g\,kg^{-1}$, i.v. bolus immediately followed by 300 $\mu g\,kg^{-1}\,h^{-1}$ infusion (infusion rate: 10 ml h^{-1}). The doses of alprenolol and CGP 20712A were chosen on the basis of pilot experiments in conscious dogs, where they antagonized the positive chronotropic effect of isoprenaline (1.5 $\mu g\,kg^{-1}$, i.v.). The dose of ICI 118551 was selected from a review of the literature (Bilski *et al.*, 1983) and for its ability to antagonize the fall in diastolic blood pressure induced by salbutamol (30 $\mu g\,kg^{-1}$, i.v.) in the anaesthetized dog (unpublished data). In those experiments in which SR 58611A was combined with β-adrenoceptor antagonists, SR 58611A (100 $\mu g\,kg^{-1}$) was injected as an i.v. bolus 15 min before the meal (i.e. 30 min after starting the i.v. infusion of the antagonist).

Colonic spike activity was evaluated with the aid of a computer system which has already proved reliable in several studies (De Ponti *et al.*, 1988; 1994a). Briefly, the number of spikes per unit of time (10 min or 1 h in the present experiments) was calculated automatically by original software identifying spikes on the basis of a discriminant function. The hardware and software specifications of the system have been detailed elsewhere (De Ponti *et al.*, 1988; 1994a).

According to Sarna & Lang (1989), the colonic motor response to the meal was divided into three phases: the immediate response (10 min period immediately following the end of the meal), the early response (first 2 h after meal ingestion) and the late response (which begins ~2 h after meal ingestion). Since the late response is believed to depend on entry of chyme into the colon (Sarna & Lang, 1989; Flourie *et al.*, 1989), it cannot be considered a true gastrocolonic response and therefore it will not be considered further. From now on, the immediate and early response will be referred to as the first and second phase of the gastrocolonic response, respectively.

Mechanical tracings were analysed visually. According to Sarna & Lang (1989) and Flourie *et al.* (1989), a contractile state was defined as a burst of contractions that lasted for at least 1 min. Contractile activity was then expressed as total duration of contractions per hour.

In the dose-response study with SR 58611A, the effect of the compound on the first phase of the gastrocolonic response was assessed by calculating the difference, for each animal, between the number of spikes recorded in the 10 min period following the end of the meal in the control experiments (meal with i.v. injection of 154 mM NaCl) and in the experiments with SR 58611A. The value obtained was then expressed as a percentage of the variation observed with 300 $\mu g\,kg^{-1}$, i.v., atropine. The effects of SR 58611A on heart rate (difference between the peak post-treatment value and the control value) were scored as a percentage of the variation observed after 300 $\mu g\,kg^{-1}$, i.v., atropine. Dose-response relationships were evaluated by linear regression analysis according to Tallarida & Murray (1987). The best linear fitting of the dose-response curves was obtained by the log transfor-

mation and the least-squares method. ED_{35} values (i.e. the doses having an effect that was 35% of that of a supramaximal dose of atropine) with 95% confidence limits were derived from log dose-response curves.

In vitro *experiments*

Circular muscle strips (10–15 mm long) were obtained from specimens of distal colon removed from male and female beagle dogs (10–15 kg body weight) euthanized with thiopentone sodium (25 mg kg^{-1}, i.v.) followed by 0.3 ml kg^{-1} Tanax (Hoechst, Milan, Italy). The preparations were deprived of the mucosa by sharp dissection, connected to isotonic transducers (load: 1.5 g) and incubated in 20 ml organ baths containing a modified Krebs solution (composition, mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.1, $NaHCO_3$ 25, ascorbic acid 0.11), continuously bubbled with a mixture of 95% O_2 and 5% CO_2 and maintained at a constant temperature of 37°C. After a tissue-equilibration time of 60 min, we carried out cumulative-concentration-response studies with β-adrenoceptor agonists alone and after preincubation (30 min) with β-adrenoceptor antagonists or tetrodotoxin. All experiments were carried out in the presence of 30 μM hydrocortisone (to block extraneuronal uptake of isoprenaline) and 1 μM phentolamine (to antagonize possible α-mediated effects of high concentrations of isoprenaline: see Smith *et al.*, 1993).

In order to avoid desensitization, only one concentration-response curve to an agonist was carried out on each preparation. Concentration-response curves in the presence and absence of antagonists were performed on preparations obtained from the same animal.

The relaxations observed with β-adrenoceptor agonists were expressed as percentages of the response to a supramaximal dose of isoprenaline. EC_{50} values (i.e. the concentrations producing a half-maximal relaxation) with 95% confidence limits were derived from log concentration-response curves. The pA_2 value for alprenolol, as defined by Arunlakshana & Schild (1959), was obtained from a constrained plot (best straight line with a slope of unity) according to Tallarida & Murray (1987) after verifying that the experimental data fitted a theoretical Schild plot (slope not significantly different from unity).

Drugs

The following drugs were used: SR 58611A {N-[(2S)-7-ethoxycarbonyl-methoxyl-1,2,3,4-tetrahydro-naphth-2-yl](2R)-2-(3-chloro-phenyl)-2-hydroxyethanamine hydrochloride} from Sanofy-Midy (Milan, Italy); (±)-isoprenaline hydrochloride, ritodrine hydrochloride, (±)-alprenolol hydrochloride (non-selective β-adrenoceptor antagonist), atropine sulphate, phentolamine hydrochloride and tetrodotoxin from Sigma (St. Louis, MO); hydrocortisone hemisuccinate (Flebocortid ampoules) from Lepetit (Milan, Italy). CGP 20712A (1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethyl amino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl) phenoxy]-2-propanol methanesulphonate) and ICI 118551 (erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) were kindly donated by Ciba-Geigy (Basel, Switzerland) and ICI (Milan, Italy), respectively.

Drugs were dissolved in 154 mM NaCl for *in vivo* experiments and in distilled water for *in vitro* experiments.

Statistical analysis

Statistical comparisons between two groups were made by Student's *t* test for paired data (two tailed). For multiple comparisons, repeated measures analysis of variance followed by the Dunnett's test (for comparisons of values obtained before and after a given treatment) or the Student-Newman-Keuls test (for comparisons between different treatments and controls) was used. A P value < 0.05 was considered significant. Values are given as mean ± s.e.mean.

Results

In vivo *experiments*

Colonic motor response to the standard meal and effect of atropine In control experiments, colonic myoelectrical and mechanical activities were stimulated by meal ingestion. The mean numbers of spikes were 730 ± 69 ($n = 5$) in the 10 min period immediately following the end of the meal (first phase of the gastrocolonic response) and 298 ± 55 ($n = 5$) in the 10 min period preceding meal ingestion ($P < 0.01$). The numbers of spikes during the second phase of the gastrocolonic response were 3,455 ± 416 and 3,737 ± 431 ($n = 5$) spikes h^{-1} in the first and second hour, respectively as compared to 2315 ± 431 spikes h^{-1} ($n = 5$) in the 1 h period preceding meal ingestion ($P < 0.01$). The increase in spike activity was associated with an increase in mechanical activity: the total durations per hour of contractile activity in the first and second postprandial hour were 21 ± 3 and 22 ± 2 min ($n = 5$), respectively, as compared to 13 ± 2 min ($n = 5$) in the 1 h period preceding meal ingestion ($P < 0.01$).

The reference drug atropine (300 μg kg^{-1}, i.v.) significantly inhibited both the first and the second phase of the gastrocolonic response (Figure 1).

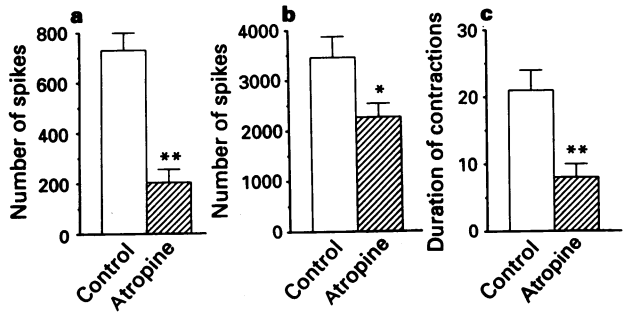

Figure 1 Inhibition by atropine (300 μg kg^{-1}, i.v.) of the gastrocolonic response in the dog. (a) Shows the number of spikes in the 10 min period after the end of the meal (first phase of the gastrocolonic response) in the control experiments and after pretreatment with atropine. (b) Shows the number of spikes h^{-1} in the second phase of the gastrocolonic response in the control experiments and after pretreatment with atropine. (c) Shows the total duration of contractions (min h^{-1}) in the second phase of the gastrocolonic response in the control experiments and after pretreatment with atropine. Values are means ± s.e.mean ($n = 5$ animals); $^*P < 0.05$; $^{**}P < 0.01$ vs control.

Effect of β-adrenoceptor agonists Infusion of SR 58611A, ritodrine (both at 10 μg kg^{-1} min^{-1}) or isoprenaline (0.1 μg kg^{-1} min^{-1}) for 30 min induced significant tachycardia (Figure 2). The degree of tachycardia observed 15–30 min after starting the intravenous infusion was similar for the three β-adrenoceptor agonists. Heart rate quickly returned to control values after isoprenaline, while it remained significantly higher than control levels for at least 40–60 min after stopping the SR 58611A or ritodrine infusion.

SR 58611A (10 μg kg^{-1} min^{-1}), ritodrine (10 μg kg^{-1} min^{-1}) and isoprenaline (0.1 μg kg^{-1} min^{-1}) inhibited the first phase of the gastrocolonic response with this dose of isoprenaline being significantly ($P < 0.05$) less effective than SR 58611A or ritodrine (Figure 3).

SR 58611A and ritodrine significantly reduced spike activity also in the second phase of the gastrocolonic response, while the small decrease in motility observed with isoprenaline did not reach statistical significance (Figure 4). Inhibition of spike activity by SR 58611A or ritodrine in the

second phase of the gastrocolonic response was associated with inhibition of mechanical events, as assessed by calculating the duration of contractions recorded via the strain-gauge force transducer (Figure 4).

Dose-response study with SR 58611A Injection of SR 58611A as an i.v. bolus 15 min before the standard meal, dose-dependently inhibited the gastrocolonic response (Figure 5). Its ED_{35} value for inhibition of the gastrocolonic response was 23 (11–46) $\mu g\,kg^{-1}$. SR 58611A also dose-dependently increased heart rate, with an ED_{35} value of 156 (78–309) $\mu g\,kg^{-1}$.

Systolic blood pressure was dose-dependently reduced by SR 58611A. This hypotensive effect reached statistical significance ($P<0.05$) only at the two highest doses (100 and 300 $\mu g\,kg^{-1}$): Δ at peak = -18 ± 5 and -26 ± 4 mmHg ($n=5$), respectively.

Effect of β-adrenoceptor antagonists alone and combined with SR 58611A None of the β-adrenoceptor antagonists tested when given alone, affected the gastrocolonic response: the mean number of spikes in the first phase of the gastrocolonic response during β-adrenoceptor antagonist infusion did not significantly differ from control experiments [744 ± 83, 722 ± 66 and 673 ± 71 ($n=5$) during alprenolol, CGP 20712A or ICI 118551 infusion, respectively].

The inhibitory effect of 100 $\mu g\,kg^{-1}$ SR 58611A on the gastrocolonic response was reversed by alprenolol but resistant to CGP 20712A or ICI 118551 (Figure 6).

In vitro *experiments*

SR 58611A concentration-dependently relaxed circular muscle strips: this effect was competitively antagonized by alprenolol, but resistant to 100 nM CGP 20712A, 100 nM ICI 118551 (Figure 7, Table 1) and the tetrodotoxin (% relaxation with 6 μM SR 58611A = 42 ± 4 and 44 ± 2% in the absence and in the presence of 1 μM tetrodotoxin, respectively). After blockade of β_1- and β_2-adrenoceptors with combined CGP 20712A and ICI 118551 (both at 100 nM), the pA_2 value calculated for alprenolol against SR 58611A-induced relaxations was 7.1 (6.9–7.3).

Isoprenaline and ritodrine concentration-dependently relaxed circular muscle strips. Ritodrine-induced relaxations

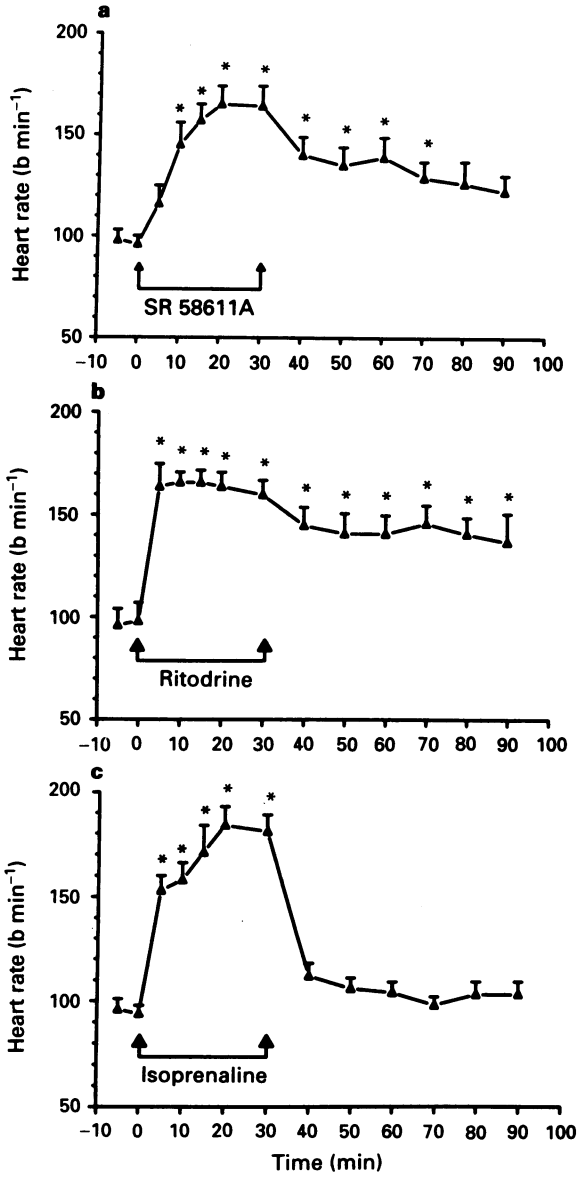

Figure 2 Effect of SR 58611A (10 $\mu g\,kg^{-1}\,min^{-1}$; a) ritodrine (10 $\mu g\,kg^{-1}\,min^{-1}$; b) and isoprenaline (0.1 $\mu g\,kg^{-1}\,min^{-1}$; c) on heart rate in the dog. Values are means ± s.e.mean ($n=5$ animals); $^*P<0.05$ vs time 0.

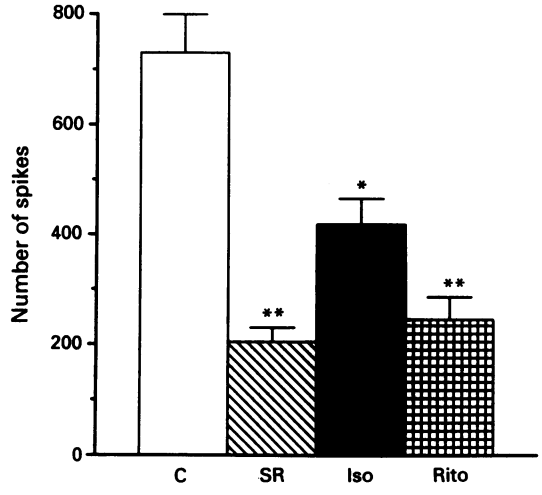

Figure 3 Inhibition of the first phase of the gastrocolonic response by SR 58611A (10 $\mu g\,kg^{-1}\,min^{-1}$), isoprenaline (0.1 $\mu g\,kg^{-1}\,min^{-1}$) or ritodrine (10 $\mu g\,kg^{-1}\,min^{-1}$): on the y-axis, number of spikes in the 10 min period after the end of the meal in control experiments (C) and during i.v. infusion of SR 58611A (SR), isoprenaline (Iso) or ritodrine (Rito). Values are means ± s.e.mean ($n=5$ animals); $^*P<0.01$ vs control; $^{**}P<0.01$ vs control and $P<0.05$ vs isoprenaline.

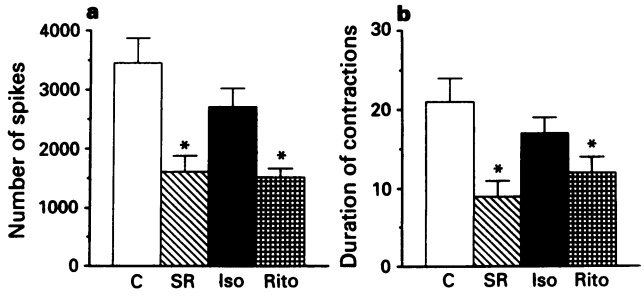

Figure 4 Inhibition of the second phase of the gastrocolonic response by β-adrenoceptor agonists. (a) On the y-axis, number of spikes h^{-1} during the second phase of the gastrocolonic response in the control experiments (C) and after i.v. pretreatment with 10 $\mu g\,kg^{-1}\,min^{-1}$ SR 58611A (SR), 0.1 $\mu g\,kg^{-1}\,min^{-1}$ isoprenaline (Iso) or 10 $\mu g\,kg^{-1}\,min^{-1}$ ritodrine (Rito). (b) On the y-axis, total duration (min h^{-1}) of contractions during the second phase of the gastrocolonic response in the control experiments (C) and after i.v. pretreatment with the same doses of SR 58611A (SR), isoprenaline (Iso) or ritodrine (Rito). Values are means ± s.e.mean ($n=5$ animals); $^*P<0.01$ vs control.

were antagonized by alprenolol and ICI 118551, but not by CGP 20712A, while isoprenaline-induced relaxations were antagonized by any of the three antagonists. EC_{50} values are shown in Table 1.

Discussion

The present study has provided strong functional evidence that atypical or β_3-adrenoceptors can modulate canine colonic motility both *in vivo* and *in vitro*. Furthermore, the *in vivo* experiments have shown that β-adrenoceptor agonists, at doses producing the same degree of tachycardia, inhibit the gastrocolonic response to a different extent. SR 58611A and ritodrine virtually abolished the first phase of the gastrocolonic response, while isoprenaline, administered at a dose that has been shown to induce maximal tachycardia in the dog (Tavernier *et al.*, 1992), was significantly less effective.

A few pharmacokinetic considerations need to be taken into account when comparing drug effects *in vivo*, since differences may simply be due to different pharmacokinetics of the compounds to be tested. In this respect, it should be noted that the first phase of the gastrocolonic response was assessed by calculating the number of spikes in the 10 min period after the end of the meal, i.e. in a time period between 15 and 30 min after starting β-adrenoceptor agonist infusion, when the chronotropic effect was at its plateau for all the three agonists tested (the meal was administered 15 min after starting the intravenous infusion and was consumed within 5 min, see Methods). Thus, we can conclude that, at doses inducing the same chronotropic effect, the ability of the three β-adrenoceptor agonists to inhibit colonic motility *in vivo* differed. The inhibitory effect of β-adrenoceptor agonists on

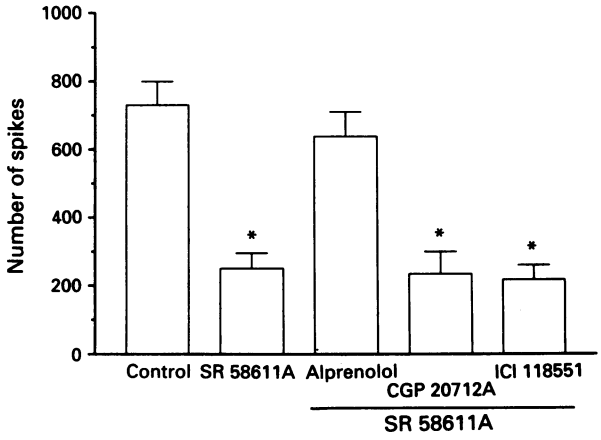

Figure 6 Effect of β-adrenoceptor antagonists (each at a dose of 300 $\mu g\ kg^{-1}$, i.v. bolus followed by 300 $\mu g\ kg^{-1}\ h^{-1}$ for 1 h) on the response to 100 $\mu g\ kg^{-1}$ SR 58611A. On the y-axis, number of spikes in the 10 min period after the end of the meal. Note inhibition of the gastrocolonic response by SR 58611A and antagonism by alprenolol, but not by CGP 20712A or ICI 118551. Values are means ± s.e.mean ($n = 5$ animals); * $P < 0.01$ vs control.

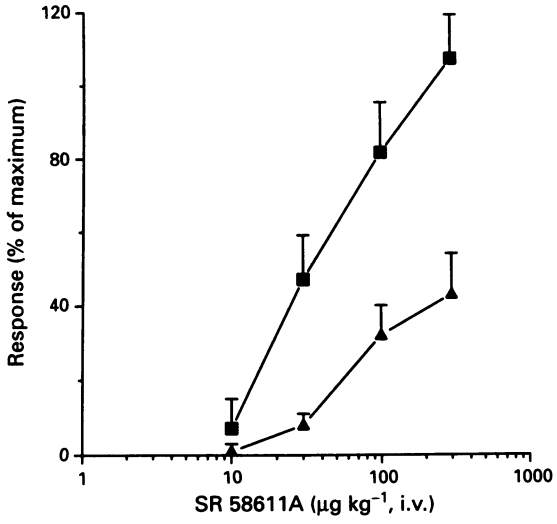

Figure 5 Dose-response relationships for inhibition of spike activity in the first phase of the gastrocolonic response (■) and induction of tachycardia (▲); on the x-axis, log dose of SR 58611A; on the y-axis, values are expressed as percentages with respect to the response to a supramaximal dose of atropine. Note the dissociation between the colonic inhibitory effect and the positive chronotropic effect. Values are means ± s.e.mean ($n = 5$ animals).

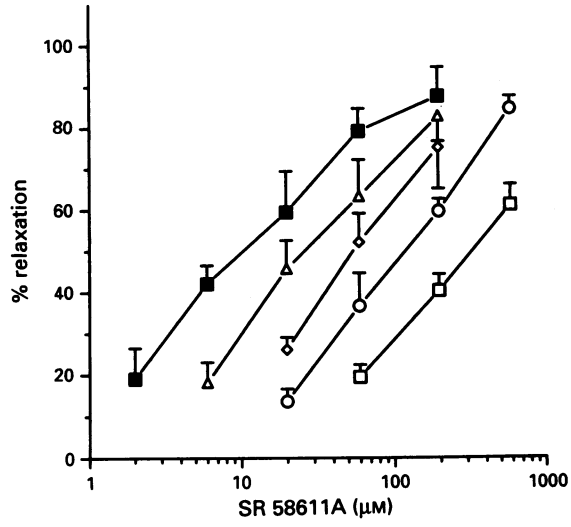

Figure 7 Concentration-response relationships for relaxation of colonic circular muscle strips by SR 58611A in the absence (■) and in the presence (open symbols) of 0.1 (△), 0.3 (◇), 1 (○) and 3 μM (□) alprenolol. Values are expressed as % relaxation with respect to the relaxation obtained with a supramaximal concentration of isoprenaline and are means ± s.e.mean ($n = 4$ preparations).

Table 1 EC_{50} values (μM) for relaxation of canine colonic circular smooth muscle by β-adrenoceptor agonists in the absence and in the presence of β-adrenoceptor antagonists

	Control	*Alprenolol* (0.1 μM)	*Alprenolol* (1 μM)	*CGP 20712A* (100 nM)	*ICI 118551* (100 nM)
SR 58611A	7.64 (4.57–12.77)	20.89 (13.06–33.33)	86.35 (75.91–98.24)	7.58 (3.88–14.75)	7.92 (6.14–10.22)
Ritodrine	0.33 (0.21–0.53)	1.59 (0.76–3.29)	8.59 (4.58–16.13)	0.31 (0.17–0.58)	4.89 (3.58–6.67)
Isoprenaline	0.16 (0.09–0.28)	0.61 (0.29–1.31)	5.17 (4.54–5.89)	0.51 (0.34–0.75)	0.56 (0.38–0.85)

Values in parentheses indicate 95% confidence limits.

the second phase of the gastrocolonic response was similar to that observed in the first phase of the response, except in the case of isoprenaline, which caused only a minor, non-significant inhibition. However, since the second phase of the response involved evaluation of spike activity after the end of drug infusion, this lack of effect was probably due to the very short half-life of isoprenaline (Conolly *et al.*, 1972), as indicated by the sudden drop in heart rate after the end of the isoprenaline infusion.

In the dose-response study with SR 58611A, we found that its ED_{35} value for induction of tachycardia was 6.7 time as high as its ED_{35} value for inhibition of colonic motility. This indicates that SR 58611A, after i.v. bolus administration (which should favour drug delivery to the cardiovascular system), still maintains preferential activity on the colon, as already described in the rat for other phenylethanolaminotetralines (Giudice *et al.*, 1989). This finding has important implications in the search for new therapeutic agents inhibiting intestinal motility, since currently available β-adrenoceptor agonists cannot be used for the treatment of intestinal motility disorders because of their significant cardiovascular effects (Lyrenäs *et al.*, 1985).

Although extrapolation to man of data generated in animal experiments is not without caveats, our model certainly appears a useful tool for the preclinical screening of compounds of potential application in the treatment of intestinal motor disorders such as the irritable bowel syndrome, a condition in which the gastrocolonic response is exaggerated and is associated with occurrence of symptoms (McKee & Quigley, 1993). Indeed, the gastrocolonic response in dogs is well reproducible and similar to that observed in man (Sarna & Lang, 1989; McKee & Quigley, 1993). Furthermore, our experimental model allows us to compare the colonic vs cardiovascular effects of β-adrenoceptor agonists *in vivo*, i.e. in a condition where the responses may be affected by several pharmacokinetic and pharmacodynamic variables, which can modify the selectivity profile observed *in vitro*. Our *in vivo* study has substantially confirmed the selectivity profile of SR 58611A reported *in vitro* by Bianchetti & Manara (1990). Concerning the positive chronotropic response observed after i.v. injection of β_3-adrenoceptor agonists, the mechanism underlying this effect seems to involve a baroreceptor-mediated reflex secondary to hypotension (Tavernier *et al.*, 1992), as no evidence for the presence of β_3-adrenoceptors in dog heart has been obtained so far (Tavernier *et al.*, 1992; Takayama *et al.*, 1993).

Since β-adrenoceptor agonists have significant cardiovascular effects, the question arises as to whether inhibition of colonic motility observed *in vivo* is secondary to variations in blood flow. A previous study by Esser *et al.* (1987) showed that β-adrenoceptor agonists inhibited colonic motility while increasing blood flow to the inferior mesenteric artery with no correlation between the two parameters. Moreover, SR 58611A was shown to inhibit colonic motility at doses with minor or no effect on heart rate or blood pressure. Thus, we believe that the inhibition of the gastrocolonic response by β-adrenoceptor agonists, namely SR 58611A, was due mainly to a direct effect on colonic smooth muscle, as confirmed by our *in vitro* experiments, where a concentration-dependent, tetrodotoxin-resistant relaxation was observed with SR 58611A. The existence of β-adrenoceptors on intestinal smooth muscle cells is indeed well documented (Bülbring & Tomita, 1987; Smith *et al.*, 1993).

Concerning the subtype of β-adrenoceptor involved in the inhibition of colonic motility, our experiments have provided evidence in favour of the hypothesis that β_3-adrenoceptors mediate the effect of SR 58611A both *in vivo* and *in vitro*, since the effect of the compound was resistant to β_1 and β_2-selective adrenoceptor antagonists and antagonized competitively *in vitro* by alprenolol with a pA_2 value of 7.1, which is similar to that reported in the guinea-pig ileum, rat ileum and rat colon (Bianchetti & Manara, 1990; Blue *et al.*, 1990; Growcott *et al.*, 1993a,b; Landi *et al.*, 1993). However, our experiments suggest that other β-adrenoceptor subtypes may contribute to the modulation of canine colonic motility, as indicated by the ability of CGP 20712A and ICI 118551 to antagonize, at least in part, isoprenaline-induced relaxations. Inhibition of ritodrine-induced responses by ICI 118551 is also in favour of an involvement of β_2-adrenoceptors, in line with a binding study carried out in smooth muscle preparations obtained from the canine colon (Smith *et al.*, 1993).

In conclusion, the present study has shown that SR 58611A, most likely by an action on β_3-adrenoceptors, inhibits canine colonic motility both *in vivo* and *in vitro*. However, our results should not be interpreted as showing a physiological role of β_3-adrenoceptors in mediating the gastrocolonic response: they simply provide evidence in favour of the existence of these receptors in the canine colon and that β_3-adrenoceptors can modulate a physiological response.

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Increase by N^G-nitro-L-arginine methyl ester (L-NAME) of resistance to venous return in rats

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1 The effects of the nitric oxide (NO) synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), on mean circulatory filling pressure (MCFP), total peripheral resistance (TPR), cardiac output (CO) and resistance to venous return (R_v) were studied in rats.

2 In conscious, unrestrained rats, L-NAME (0.5–16 mg kg^{-1}) dose-dependently increased mean arterial pressure (MAP) but not MCFP, an inverse index of venous compliance, either in the absence or presence of the ganglionic blocker mecamylamine (10 mg kg^{-1}).

3 In pentobarbitone-anaesthetized rats, L-NAME (2, 4, 8 mg kg^{-1}) increased MAP and reduced CO in a dose-related manner but did not change MCFP. TPR (+84, +140 and +192%) as well as R_v (+62, +72, +110%) were dose-dependently increased by L-NAME.

4 Our results show that L-NAME reduces CO by increasing arterial as well as venous resistances. L-NAME does not affect MCFP.

Keywords: N^G-nitro-L-arginine methyl ester (L-NAME); nitric oxide; mean circulatory filling pressure (MCFP); cardiac output, total peripheral resistance, venous resistance, capacitance vessels

Introduction

N^G-substituted arginine (Arg) analogues, which include N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-Arg (L-NOARG) and its methyl ester (L-NAME), are nitric oxide (NO) synthase inhibitors which suppress endothelium-dependent relaxation of arterial preparations *in vitro* and raise blood pressure *in vivo* (Aisaka *et al.*, 1989; Rees *et al.*, 1989; 1990; Wang & Pang, 1991; Wang *et al.*, 1993a,b,c; see Moncada *et al.*, 1991). The N^G-substituted Arg analogues also inhibit endothelium-dependent relaxations or cause contractions in venous smooth muscles which include the bovine pulmonary vein (Gold *et al.*, 1990), canine and rat femoral veins (Miller, 1991; Nagao & Vanhoutte, 1991), canine renal vein (Pawloski & Chapnick, 1991), rabbit external jugular vein (Martin *et al.*, 1992) and canine saphenous vein (Elmore *et al.*, 1992) *in vitro*.

There are few studies which investigate the effect of NO synthase inhibitors in the control of venomotor tone. The i.v. bolus injection of a single dose (10 mg kg^{-1}) of L-NAME into anaesthetized rabbits increased arterial pressure and reduced the cross-sectional area of the inferior vena cava suggesting venoconstriction (Schwarzacher *et al.*, 1992). However, it is unlear if the constriction was active or passive, due to a reduction in cardiac output. Intravenous bolus injection of L-NAME (37 μmol kg^{-1}) into ganglion-blocked, artificially-ventilated and anaesthetized cats receiving a continuous infusion of noradrenaline to maintain vascular tone, caused large increases in arterial and venous resistances but only a small rise in mean circulatory filling pressure (MCFP), an index of body venous tone (Bower & Law, 1993). In conscious, partially-restrained rats, i.v. injection of L-NMMA (25 mg kg^{-1}) caused a large increase in arterial pressure but only a small rise in MCFP (Glick *et al.*, 1993).

There is, as yet, no published study which concurrently investigates the dose-response effects of NO synthase inhibitors on MCFP and venous resistance in the intact animal. The aim of this study was to examine the dose-response effects of L-NAME on MCFP and resistance to venous return in rats. Since anaesthesia and surgical stress may affect the equilibration of central venous pressure and portal venous pressure, thereby altering the contribution of venous pressure from the splanchnic capacitance vessels (see Tabrizchi & Pang, 1992; Tabrizchi *et al.*, 1993; Pang, 1994), MCFP measurements were made in anaesthetized as well as conscious rats.

Methods

Surgery

Male Sprague-Dawley rats (320–500 g) were anaesthetized with pentobarbitone (60 mg kg^{-1}) or halothane (2% in air). Body temperature was maintained at 37°C via a rectal thermometer and a heating pad connected to a Thermistemp Temperature Controller (Model 71; Yellow Spring Instrument Co Inc., OH, U.S.A.). A polyethylene cannula (PE5O) was inserted into the left iliac artery, for the recordings of mean arterial pressure (MAP), by a pressure transducer (P23DB, Gould Statham, CA, U.S.A.), and heart rate (HR), which was derived electronically from the upstroke of the arterial pulse pressure by a tachograph (Grass, Model 7P4G). PE50 cannulae were also inserted into the right iliac vein, for the administration of vehicle or drugs, and the inferior vena cava *via* the left iliac vein, for the measurement of central venous pressure (CVP) by another pressure transducer (P23DB, Gould Statham). A saline-filled, balloon-tipped catheter was inserted into the right atrium *via* the right external jugular vein. The proper location of the atrial balloon was tested by injecting saline into the balloon and obtaining a simultaneous decrease of MAP to 20–25 mmHg and an increase of CVP within 5 s of circulatory arrest. MAP, HR and CVP were continuously monitored and recorded by a Grass Polygraph (Model RPS 7C8).

In pentobarbitone-anaesthetized rats, an additional cannula was inserted via the right carotid artery into the left ventricle for the injection of radioactively-labelled microspheres, and into the left iliac artery for blood withdrawal, as described in detail elsewhere (Pang, 1983). These rats were given 30 min to stabilize before MAP, MCFP and cardiac output (CO) measurements were made.

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In halothane-anaesthetized rats, all cannulae were tunneled s.c. along the back, exteriorized at the back of the neck and secured. Bupivacaine (local anaesthetic, 0.25% solution) and cicatrin (antibiotic) were applied topically to the surgical wounds to alleviate pain and prevent infection, respectively. Halothane was withdrawn after the completion of surgery which took about 30 min. The rats were in the upright posture within 10 min of removing halothane and were used 6–8 h later. In our experience, 6 h is sufficient time for the recovery of the effects of brief halothane anaesthesia and minor surgery on haemodynamic measurements. Readings of MAP, HR and MCFP obtained in rats recovered from anaesthesia and surgery for 6 h were not different from those recovered from anaesthesia and surgery for 24 h. The conscious rats were allowed to wander freely in a small cage. The method for measuring MCFP in rats has been described in detail (see Tabrizchi & Pang, 1992). Briefly, steady-state readings of MAP and CVP were noted at 4 to 5 s after circulatory arrest via inflation of the implanted balloon. To avoid rapid equilibration of arterial and venous pressures during circulatory arrest, the arterial pressure contributed by the small amount of trapped arterial blood was corrected by the following equation: MCFP = VPP + 1/60 (FAP-VPP), where FAP and VPP represents the final arterial pressure and venous plateau pressure, respectively, obtained within 5 s of circulatory arrest, and 1/60 represents the ratio of arterial to venous compliance.

Microspheres studies

A well-stirred suspension (200 μl) containing 20,000–25,000 microspheres (15 μm diameter), labelled with ^{57}Co (Du Pont Canada Inc., Ontario, Canada), was injected and flushed over 10 s into the left ventricle in the control period and 20 min after the i.v. bolus injection of a drug or vehicle. At 10 s before the injection of each set of microspheres, blood was withdrawn (Harvard infusion/withdrawal pump) from the iliac arterial cannula into a heparinized syringe at 0.35 ml min^{-1} for 45 s. The blood removed was slowly injected back to the rats immediately after the counting of radioactivity at 80–160 kev using a 1185 Series Dual Channel Automatic Gamma Counter (Nuclear-Chicago, IL, U.S.A.) with a 3 inch NaI crystal.

Experimental protocol

Pentobarbitone-anaesthetized rats Rats were randomly divided into 2 groups ($n = 6$ each). At 20 min after a baseline measurement of CO followed by MCFP, one group received i.v. bolus injections of cumulative doses of L-NAME (2, 4, 8 mg kg^{-1} or 7, 15, 30 μmol kg^{-1}) whereas in the other group, injections of equal volumes of the vehicle (0.9% NaCl, 0.2 to 0.5 ml kg^{-1}) were made, at dose-intervals of 20 min. CO followed by MCFP measurements were made after the injection of a drug or vehicle, at the plateau phase of the response to L-NAME. A blood sample was removed at the end of the stabilization period and after the completion of the study to monitor any changes in haematocrit.

Conscious rats Rats were randomly divided into four groups ($n = 6$ each). In two groups of intact rats a cumulative dose-response curve was obtained to i.v. bolus injections of L-NAME (0.5–16 mg kg^{-1} or 1.9–59.0 μmol kg^{-1}) or to equal volumes (0.06–1 mg kg^{-1}) of normal saline. MCFP measurements were made at 10 min after the injection of a drug or vehicle. Mecamylamine (10 mg kg^{-1}) was given as an i.v. bolus injection to another two groups of rats. This dose of mecamylamine was found to block ganglionic transmission effectively for more than 2 h (Wang & Pang, 1991). Ten minutes later, i.v. bolus injections of L-NAME or vehicle were also given to each group.

Drugs

L-NAME hydrochloride and mecamylamine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and dissolved in normal saline (0.9% NaCl solution). Cicatrin was from Burroughs Wellcome Inc., (Que., Canada) and bupivacaine HCl was from Sanofi Winthrop (Ont, Canada).

Calculations and statistics analysis

CO (ml min^{-1}), total peripheral resistance (TPR, mmHg min ml^{-1}) and venous resistance (R_v; mmHg min ml^{-1}) were calculated as follows:

$$CO = \frac{\text{rate of withdrawal of blood} \times \text{total injected c.p.m.}}{\text{c.p.m. in withdrawn blood}}$$

$$TPR = \frac{BP}{CO}$$

$$R_v = \frac{MCFP - CVP}{CO}$$

Due to the technical difficulty of measuring right atrial pressure and MCFP concurrently in small animals, CVP rather than right atrial pressure was used to estimate pressure gradient to venous return (MCFP-right atrial pressure). This is legitimate since mean CVP is nearly identical to mean right atrial pressure (see Rothe, 1993).

All results were expressed as mean ± s.e.mean and analysed by the analysis of variance/covariance followed by Duncan's multiple range test, with $P < 0.05$ selected as the criterion for statistical significance. Profile/trend analysis was used to compare dose-dependency of responses using the statistical package, SYSTAT v. 5.03 (SYSTAT Inc., IL, U.S.A.).

Results

Anaesthetized rats

The vehicle did not alter significantly MAP, HR, R_v, TPR or MCFP (Figure 1) but slightly increased CO, which, at the highest dose, was significantly different from baseline CO (Figure 1e). There was slight blood loss during surgery, as haematocrit was insignificantly reduced from 55 ± 3 to 50 ± 2% from the beginning to the end of the study ($P > 0.05$). L-NAME significantly and dose-dependently ($P < 0.05$) increased MAP (Figure 1a), R_v and TPR (Figure 1c,d) and reduced CO (Figure 1e) but did not alter MCFP (Figure 1f), when the values were compared with the corresponding readings in the vehicle group. HR was insignificantly decreased (Figure 1b). The haematocrit was also insignificantly decreased ($P > 0.05$) in L-NAME-treated rats, from a baseline value of 57 ± 1 to 51 ± 1% from the beginning to the end of the study.

Conscious rats

Circulatory arrest for <6 s did not cause any distress or visible behavioural disturbances in the rats. The four groups of rats had similar baseline readings of MAP, HR and MCFP (Table 1). In two groups of rats given mecamylamine, MAP, HR and MCFP were decreased by 35%, 7% and 23% of control readings (pooled values), respectively (Table 1).

In intact rats, there were slight decreases in MCFP but not MAP or HR with the passage of time (Figure 2a,b,c). L-NAME significantly and dose-dependently ($P < 0.05$) increased MAP and decreased HR but did not alter MCFP, when the values were compared with the corresponding time-control readings in rats given the vehicle (Figure 2a,b,c).

Table 1 Baseline values of mean arterial pressure (MAP), heart rate (HR) and mean circulatory filling pressure (MCFP) in four groups ($n = 6$ each) of conscious, unrestrained rats to be given i.v. bolus injections of N^G-nitro-L-arginine methyl ester (L-NAME) (I and III) or normal saline (II and IV)

	MAP (mmHg)		*HR* (beats min^{-1})		*MCFP* (mmHg)	
	Before	*After*	*Before*	*After*	*Before*	*After*
I	102 ± 3	–	407 ± 8	–	6.6 ± 0.3	–
II	100 ± 2	–	400 ± 7	–	6.8 ± 0.2	–
Pooled	101 ± 2	–	404 ± 6	–	6.7 ± 0.2	–
III	104 ± 4	72 ± 4[a]	378 ± 9	355 ± 11[a]	6.5 ± 0.3	5.1 ± 0.3[a]
IV	105 ± 2	65 ± 2[a]	398 ± 7	367 ± 14[a]	6.4 ± 0.2	4.9 ± 0.2[a]
Pooled	105 ± 2	68 ± 3[a]	390 ± 6	362 ± 10[a]	6.4 ± 0.2	5.0 ± 0.2[a]

Values are mean ± s.e.mean. [a]denotes significant difference from saline-treated control group ($P < 0.05$). Baseline readings of MAP, HR and MCFP in Groups III and IV were obtained before as well as 10 min after pretreatment with mecamylamine.

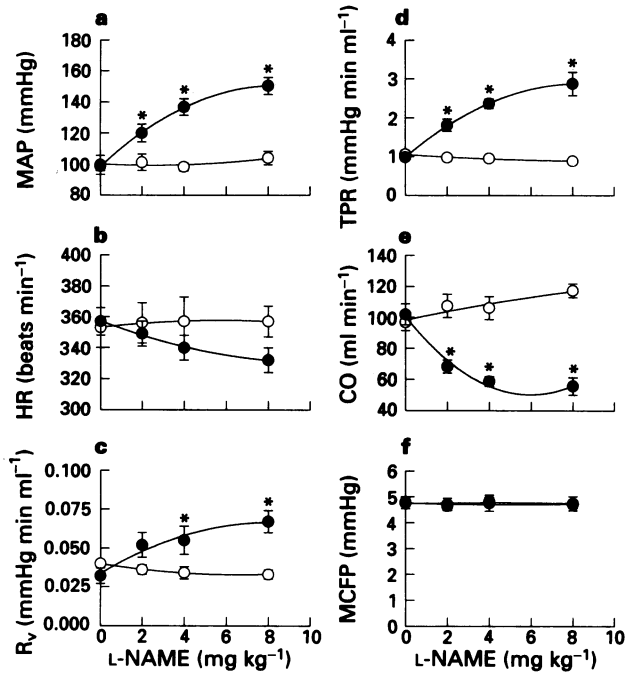

Figure 1 Effects (mean ± s.e.mean) of i.v. bolus injections of N^G-nitro-L-arginine methyl ester (L-NAME, ●) and equal volumes of vehicle (0.9% NaCl, ○) on mean arterial pressure (MAP, a), heart rate (HR, b), venous resistance (R_v, c), total peripheral resistance (TPR, d), cardiac output (CO, e) and mean circulatory filling pressure (MCFP, f) in pentobarbitone-anaesthetized rats ($n = 6$ each group). *Denotes significant difference from the corresponding reading in vehicle-treated rats ($P < 0.05$).

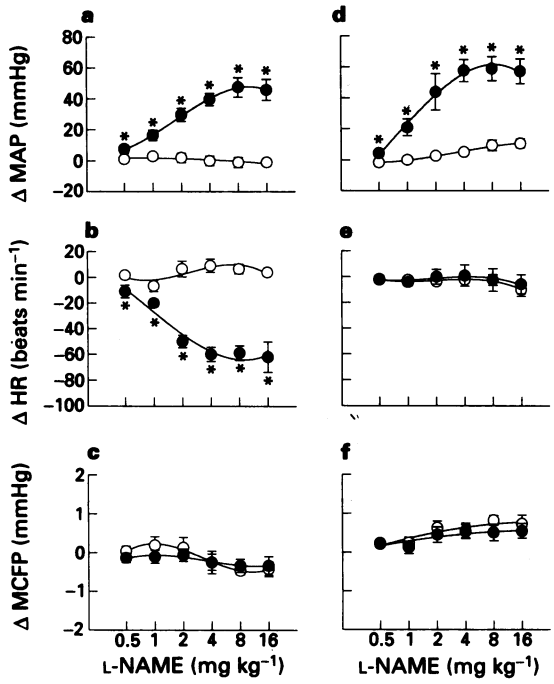

Figure 2 Effects (means ± s.e.mean) of i.v. bolus injections of N^G-nitro-L-arginine methyl ester (L-NAME, ●) and equal volumes of vehicle (0.9% NaCl, ○) on mean arterial pressure (MAP), heart rate (HR) and mean circulatory filling pressure (MCFP) in conscious, unrestrained rats ($n = 6$ each group) in the absence (a, b, c) and presence (d, e, f) of mecamylamine (10 mg kg^{-1}, i.v. bolus injection).

In mecamylamine-pretreated rats, the vehicle caused slight increases in MAP and MCFP but not HR (Figure 2d,e,f). L-NAME caused marked and dose-dependent ($P < 0.05$) increases in MAP but did not alter HR or MCFP when the readings were compared with the corresponding time-controls in rats given the vehicle (Figure 2d,e,f).

Discussion

Our results show that L-NAME raises MAP but not MCFP in anaesthetized rats. Since a combination of anaesthesia and surgical stress may interfere with the equilibration of CVP and portal venous pressure thereby reducing pressure contribution from the splanchnic venous bed (Tabrizchi & Pang, 1992; Tabrizchi *et al.*, 1993; Pang, 1994), MCFP measurements were also made in conscious, freely-moving rats. The results show that L-NAME also did not alter MCFP in either intact or ganglion-blocked conscious rats. Unlike L-NAME, noradrenaline, angiotensin II as well as angiotensin III (Pang & Tabrizchi, 1986; Tabrizchi & Pang, 1987; Tabrizchi *et al.*, 1992) were found in similar studies in our laboratory to raise MAP and MCFP dose-dependently. Therefore, the absence of a rise in MCFP in the present study is not due to a lack of sensitivity of the technique to detect changes. Our results are somewhat different from those of Bower & Law (1993) which show that a large dose of L-NAME caused a significant though small increase in MCFP in reflex-blocked but noradrenaline-infused, artificially-ventilated and anaesthetized cats. In another study using partially-restrained rats, a single dose of L-NMMA caused a large elevation of MAP but a small increase in MCFP in only one of the two groups of intact rats (Glick *et al.*, 1993). Since MCFP is inversely proportional to venous compliance in the absence of a change in stressed blood volume (see Tabrizchi & Pang, 1992; Pang, 1994), the small effect of L-NAME on MCFP in various studies suggests that L-NAME is unimportant in the control of venous compliance. It should be noted that MCFP measurements do not reveal whether or not unstressed blood volume is altered, since only stressed blood volume con-

tributes to pressure development (see Pang, 1994). MCFP measurements alone also do not reveal if venous resistance is altered since MCFP readings are obtained during circulatory arrest (hence, an absence of flow resistance).

L-NAME significantly reduced HR in conscious rats but not anaesthetized rats. Attenuated reflex changes in HR are characteristic of anaesthetized rats. The bradycardic effect of L-NAME in intact rats was abolished by mecamylamine indicating the completeness of ganglionic blockade. The effectiveness of mecamylamine in suppressing the reflex bradycardic but not the pressor effect of L-NAME in conscious rats is consistent with our previous findings (Wang & Pang, 1991).

Our present and previous (Wang & Pang, 1991) results of the preservation of pressor responses to L-NOARG following ganglionic blockade are in accordance with those of others using pentolinium or chlorisondamine as the ganglionic blocker (Chyu *et al.*, 1992; Pucci *et al.*, 1992; Pegoraro *et al.*, 1992; Bower & Law, 1993). However, there are reports that ganglionic blockers (chlorisondamine or hexamethonium) abolish or attenuate pressor responses to L-NMMA or L-NOARG in anaesthetized rats or dogs (Vargas *et al.*, 1990; Lacolley *et al.*, 1991; Toda *et al.*, 1993), which suggest the existence of nitrergic, vasodilator nerves innervating the arterial wall (Toda *et al.*, 1993). To resolve these discrepancies, a systematic study of the pressor effects of N^G-nitro-L-Arg analogues using various ganglion blockers in conscious and anaesthetized animals is needed.

Our results also show that L-NAME increased MAP by elevating TPR rather than CO. In fact, CO was dose-dependently decreased by L-NAME. Similar observations of the effects of L-NAME and L-NOARG on CO and TPR have been reported (Gardiner *et al.*, 1990; Bower & Law, 1993; Wang *et al.*, 1993a; Herity *et al.*, 1994). L-NOARG was found to cause generalized peripheral vasoconstriction, as vascular conductances in all beds, which include the lungs, heart, liver, stomach, intestine, colon, kidneys, spleen, muscle skin, testes and brain, were reduced (Wang *et al.*, 1993a). The greatest vasoconstrictor influence was in the bronchial bed whereas the least was in the hepatic arterial bed. In addition to increasing arteriolar resistance (+84, +140, +192%), L-NAME also dose-dependently increased resistance to venous return (+62, +72 and +110%) at 7, 15, 30 μmol kg^{-1}, respectively). The L-NAME-induced increases in R_v are similar to, though larger in magnitude than, those in areflex, noradrenaline-infused cats, in which L-NAME (37 μmol kg^{-1}) increased R_v by 58%. These results show that the reduction in CO by L-NAME was a consequence of increased arteriolar and venous resistances.

Our results are supportive of an important role for N^G-substituted Arg analogues in the maintenance of venomotor tone. The prominent venous effect of L-NAME and related compounds is in elevating venous resistance, thereby reducing venous return. In contrast to VPP at circulatory arrest, CVP was not elevated by L-NAME in either conscious or anaesthetized rats (results not shown). The absence of a change in CVP by L-NAME is to be expected since CVP is downstream venous pressure (the resultant pressure after pressure drops at arterial and venous resistance sites) and is therefore a poor indicator of venomotor tone.

The pressor responses of N^G-substituted-L-Arg analogues are generally believed to be secondary to the inhibition of endogenous NO biosynthesis (Aisaka *et al.*, 1989; Rees *et al.*, 1989; see Moncada *et al.*, 1991). On the basis of this hypothesis, the marked effect of L-NAME on R_v suggests that endogenously synthesized NO lowers R_v.

To summarize, L-NAME dose-dependently increased MAP, TPR and R_v and reduced CO but did not alter MCFP in pentobarbitone-anaesthetized rats. L-NAME also dose-dependently raised MAP but not MCFP in conscious rats, both in the absence and presence of the ganglion blocker, mecamylamine. Our results show that L-NAME reduces CO by increasing resistance in arterioles and capacitance vessels.

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Characterization of the PGE receptor subtype mediating inhibition of superoxide production in human neutrophils

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1 The aims of this study were to characterize the EP receptor subtype mediating the inhibition of superoxide anion generation by formyl methionyl leucine phenylalanine (FMLP)-stimulated human neutrophils, and to test the hypothesis that adenosine 3':5'-cyclic monophosphate (cyclic AMP) is the second messenger mediating the inhibition of the neutrophil by prostaglandin (PG)E_2.

2 PGE_2 (0.001–10 μM) inhibited FMLP (100 nM)-induced O_2-generation from human peripheral blood neutrophils in a concentration-dependent manner, with an EC_{50} of 0.15 ± 0.03 μM, and a maximum effect ranging from 36–84% (mean inhibition of 68.7 ± 2.5%, $n = 32$).

3 The EP_2-receptor agonists, misoprostol, 11-deoxy PGE_1, AH13205 and butaprost, all at 10 μM, inhibited O_2^- generation, causing 95.5 ± 2.9%, 56.8 ± 5.2%, 37.1 ± 6.6% and 18.9 ± 4.4% inhibition respectively, the latter two being much less effective than PGE_2. Similarly, the EP_1-receptor agonist, 17-phenyl PGE_2 (10 μM), and the EP_3/EP_1-receptor agonist, sulprostone (10 μM), also inhibited O_2^- generation, causing 32.2 ± 7.0% and 15.3 ± 3.4% inhibition respectively.

4 The non-selective phosphodiesterase inhibitor, isobutyl methylxanthine (IBMX, 0.25 mM) inhibited the FMLP response by 54.5 ± 5.0%. In addition, IBMX shifted concentration-effect curves for PGE_2, misoprostol, 11-deoxy PGE_1, butaprost, and AH 13205 to the left, to give EC_{50}s of 0.04 ± 0.03 ($n = 13$), 0.07 ± 0.03 ($n = 4$), 0.08 ± 0.03 ($n = 4$), 0.33 ± 0.13 ($n = 4$) and 0.41 ± 0.2 μM ($n = 3$) respectively, allowing equieffective concentration-ratios (EECs, $PGE_2 = 1$) of 11.5, 5.3, 50.7 and 12.7 to be calculated. This agrees well with the relative potencies of these agonists at EP_2 receptors.

5 By contrast, even in the presence of IBMX (0.25 mM), sulprostone and 17-phenyl PGE_2 were only effective at the highest concentration (10 μM), and gave EECs of > 700 and 486 respectively, suggesting that EP_1 or EP_3 receptors are not involved.

6 The selective type IV phosphodiesterase inhibitor, rolipram at 2 and 10 nM did not inhibit the FMLP response, but at the higher concentration of 50 nM, it decreased the FMLP response by 46.6 ± 7.3%. However, rolipram shifted concentration-effect curves for PGE_2 to the left to give EC_{50}s of 0.06 ± 0.022, 0.015 ± 0.0, 0.012 ± 0.006 μM at 2, 10 and 50 nM respectively, compared to the control EC_{50} of 0.27 ± 0.09 μM for PGE_2.

7 The EP_4/TP receptor blocking drug, AH 23848B (10 μM, 10 min) did not inhibit O_2^- generation by PGE_2, but was found to potentiate significantly the effect of PGE_2 at the lower concentrations of PGE_2 tested (0.001–0.1 μM).

8 The adenylate cyclase inhibitor, SQ 22,536 (0.1 mM, 2 min) reduced PGE_2-induced inhibition of O_2^- production, giving an EC_{50} in the absence of SQ 22,536 of 0.24 ± 0.1, and 1.9 ± 1.1 μM in its presence.

9 These results suggest that inhibition of superoxide generation by PGE_2 is mediated by stimulation of EP_2 receptors and activation of adenylate cyclase, leading to the elevation of intracellular levels of cyclic AMP.

Keywords: EP receptors; neutrophils; cyclic AMP; superoxide anion generation

Introduction

The main function of neutrophils is in host-defence. Neutrophils ingest microorganisms by phagocytosis and release toxic substances such as superoxide (O_2^-) and lysosomal enzymes, which enable the neutrophil to kill any invading pathogen.

It has been shown that prostaglandin E_2, through activation of EP receptors, inhibits the generation of O_2^- by activated human neutrophils (Wheeldon & Vardey, 1993). The main aim of the present study was to determine the subtype of EP receptor involved, the likely candidates being EP_2 and EP_4 subtypes, both of which have been linked to stimulation of adenylate cyclase (Coleman *et al.*, 1994; Milne *et al.*, 1994). Thus, we also set out to test the hypothesis that adenosine 3': 5'-cyclic monophosphate (cyclic AMP) is the second messenger mediating inhibition of neutrophil activation.

For these purposes, the inhibitory effects of selective EP agonists, and the antagonistic effect of selective EP receptor blocking drugs have been evaluated. In addition, the modulatory effects of the adenylate cyclase inhibitor, SQ 22,536 (Harris *et al.*, 1979), the non-selective phosphodiesterase inhibitor, isobutyl methylxanthine (IBMX), and the selective type IV phophodiesterase inhibitor, rolipram (Schudt *et al.*, 1991), have been assessed.

Furthermore, as it has previously been shown that adenosine released from neutrophils can inhibit O_2^- generation (Cronstein *et al.*, 1988), we have investigated the modulatory role of endogenous adenosine using adenosine deaminase (ADA) to break down adenosine (Cronstein *et al.*, 1984), and the non-selective A_1/A_2 antagonist, 8-phenyltheophylline (8-PT), to block the effects of released adenosine (see Collis & Hourani, 1993).

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Methods

Isolation of human neutrophils

Venous blood was taken from the forearm of healthy volunteers, and anti-coagulated with heparin 10 u ml^{-1} of blood, mixed with an equal volume of 3% dextran in 0.9% w/v saline and left to stand for 45 min. The leukocyte-rich plasma was then removed and centrifuged at 280 *g* for 10 min and the resultant pellet resuspended in 55% Percoll (Pharmacia) and layered on top of a discontinuous Percoll gradient (3 ml 70% Percoll layered on top of 5 ml 81% Percoll). The tubes were then centrifuged for 30 min at 650 *g* to separate neutrophils from mononuclear cells. The neutrophils (lower layer) were removed and washed twice. Red cells remaining in the neutrophil pellet were lysed by resuspending the pellet in 10 ml ice cold 0.2% w/v NaCl solution for 20 s, after which 10 ml of ice cold 1.6% w/v NaCl was added to return the cells to isotonic conditions. Cells were then counted in an improved Neubauer counting chamber, and their viability assessed by trypan blue exclusion. Finally the resultant pellet was resuspended at a concentration of 1.5×10^6 cells ml^{-1} in phosphate buffered saline (PBS containing Ca^{2+}/Mg^{2+}, Sigma), and kept at room temperature.

Superoxide anion production

Superoxide generation was assayed by spectrophotometric evaluation of the reduction of ferricytochrome C to ferrocytochrome C (A550nm).

Control tubes contained 5 μg per ml of cytochalasin B (used to enhance the amount of superoxide anion released during the reaction), 0.1 mg ml^{-1} cytochrome C and 90 u ml^{-1} superoxide dismutase (SOD). In sample tubes SOD was replaced with PBS. Cells (900 μl) were incubated with PBS or inhibitory agent in a gently shaking water bath for 10 min at 37°C prior to the addition of formyl methionyl leucine phenylalanine (FMLP) (100 nM). When antagonists were studied, they were incubated for 10 min prior to the addition of EP agonists or the adenosine mimetic, 5'-N-ethylcarboxamido-adenosine (NECA). Ten min after the addition of FMLP, the reaction was terminated by immersing the tubes in ice for 5 min and the samples centrifuged at 1000 r.p.m., 4°C, for 5 min, to sediment the cells.

The absorbance of the supernatants was measured with a Cary spectrophotometer.

Data analysis

Each incubation was carried out in duplicate and the values averaged. The amount of superoxide anion produced (nmol per 10^6 neutrophils per 10 min) was calculated with the following equation:

$$\frac{dE}{Q \times d} \times 10^6 \times \frac{1}{L}$$

$L = 1.5 \times 10^6$ cells per ml = concentration of human neutrophils; dE = (absorbance of samples without SOD)-(absorbance of samples with SOD); Q = coefficient of molar extinction $21.1 \times 10^3\ M^{-1}\ cm^{-1}$; d = thickness of cuvette = 1 cm.

Effects of the various drugs on O_2^- generation were expressed as the percentage inhibition of the response produced by a submaximally effective concentration of FMLP (100 nM).

EC_{50} values were calculated (concentration of agonist required to produce 50% of the maximal effect of PGE_2) for all the EP-receptor agonists, in the presence and absence of antagonists using the Apple Macintosh programme 'Kaleidagraph'. Kaleidagraph is not a graph-fitting but a graph-drawing programme and so uses the experimental maximum observed to determine the EC_{50} value. A control curve for PGE_2 was run in each neutrophil preparation, so that the EC_{50} values for other agonists could be determined relative to the maximum effect achieved with PGE_2. From EC_{50} values, equieffective concentration ratios (EECs) were calculated relative to the standard agonist, PGE_2 (EEC = 1).

Statistical analysis

Data are expressed as the mean ± standard error of the mean (s.e.mean), of the averaged result taken from a minimum of four separate experiments. Data were analysed using Student's paired or unpaired 2 tailed *t* test as appropriate.

Materials

The following compounds were gifts which we gratefully acknowledge: sulprostone and rolipram from Dr E Schillinger, Schering AG, Berlin; butaprost from Dr P. Gardiner, Bayer, U.K.; AH 13205 (*trans*-2[4-(1-hydroxyhexyl)pentyl) phenyl]-5-oxocyclo pentaneheptanoic acid), AH 6809 (6-isopropoxy-9-oxaxanthene-2-carboxylic acid) and AH 23848B 9[1α(Z), 2β, 5α]-(±)-7-[5-[[(1,1'-biphenyl)-4-yl]methoxyl]-2-(4-morpho-linyl) -3-oxocyclopentyl]-4-heptanoic acid) from Dr B. Bain, Glaxo, U.K.; 17-phenyl-ω-trinor PGE_2 and 11-deoxy PGE_1 were purchased from Cayman Chemicals, U.S.A.; SQ 22,536 ((9-tetrahydro-2-furyl)adenine) was synthesised in the department by Dr N.H. Wilson.

Isobutyl methylxanthine (IBMX), FMLP, ferricytochrome C and superoxide dismutase (SOD) were purchased from Sigma; Cytochalasin B from Aldrich; 5'-N-ethylcarboxamido-adenosine (NECA) from R.B.I. Ethanolic stock solutions of the prostanoids (10–30 mM) were stored at −20°C.

Results

Inhibition of superoxide generation by PGE_2

FMLP (100 nM) in the presence of cytochalasin B (5 μg per ml), induced a submaximal release of O_2^- of 6.7 ± 2.7 nmol per 10^6 cells per 10 min ($n = 6$). This concentration of FMLP achieved 91.9 ± 6.0% of the maximal effect induced by 300 nM FMLP, $n = 5$. PGE_2 (0.001–10 μM) produced a concentration-related inhibition of FMLP-induced O_2^- generation, achieving a maximal effect of 68.7 ± 2.5% inhibition at a concentration of 10 μM ($EC_{50} = 0.15 \pm 0.03$ μM, $n = 32$). The maximal degree of inhibition was variable ranging from 36–84%. A 10 min pre-incubation with PGE_2 prior to the addition of FMLP was selected as consistent with studies published by other workers. Pre-incubation times of 0, 15 and 30 min gave EC_{50}s for PGE_2 of 0.15 ± 0.07, 0.17 ± 0.07 and 0.11 ± 0.06 μM respectively ($n = 4$), none of which varied significantly ($P > 0.05$) from that at 10 min.

Inhibition of superoxide generation by selective EP agonists

The selective EP_2-receptor agonists, 11-deoxy PGE_1, AH13205 and butaprost, (all at 10 μM) were less potent and caused significantly less inhibition ($P < 0.05$) than PGE_2, causing 49.3 ± 5.4% ($n = 6$), 32.1 ± 3.4% ($n = 8$) and 13.9 ± 4.3% ($n = 16$) inhibition respectively at concentrations of 10 μM (Figure 1a). Taking the maximal effect of PGE_2 at 10 μM as 100% response, apparent EC_{50}s are 0.5 ± 0.2 ($n = 6$) and ⩾ 10.0 ($n = 8$) and > 10.0 μM ($n = 16$) respectively. Misoprostol, an EP_2/EP_3-receptor agonist, was slightly less potent than PGE_2 ($EC_{50} = 0.35 \pm 0.1$ μM ($n = 4$), with an EEC of 2.8), but more effective than PGE_2 at 10 μM, giving a maximal inhibition of 95.5 ± 2.9% (Figure 1a).

The cyclo-oxygenase inhibitor, indomethacin (3 μM) did not significantly effect inhibition of superoxide generation by PGE_2, butaprost or AH 13205 ($n = 4$) (Figure 1b).

The EP_1 receptor agonist, 17-phenyl-ω-trinor PGE_2 (0.1–10 μM) caused significantly ($P<0.05$) less inhibition than PGE_2 giving 32.2 ± 7.1% inhibition at a concentration of 10 μM (EC_{50} = 16.9 ± 8.4 μM, n = 4) (Figure 1c). Similarly, the EP_1/EP_3 receptor agonist sulprostone (0.1–10 μM) gave a maximal inhibition of only 15.3 ± 3.4% at 10 μM (EC_{25} = 17.7 ± 10.8 μM, n = 6), (Figure 1c).

Effect of IBMX on inhibitory effects of EP agonists

In the presence of IBMX (0.25 mM), concentration-effect curves for PGE_2 were shifted to the left (EC_{50} 0.04 ± 0.03 μM, n = 13), and the maximum inhibition at 10 μM increased to 83.9 ± 5.2%. IBMX (0.25 mM) itself reduced the FMLP response by 54.9 ± 5.0%, and % inhibitions in the presence of EP agonists were calculated from this reduced FMLP response.

The selective EP_2 receptor agonists, misoprostol, 11-deoxy PGE_1, AH 13205 and butaprost were also more effective inhibitors of O_2^- production by FMLP when the neutrophils were treated with IBMX (0.25 mM), causing 93.4 ± 3.5% inhibition at 10 μM (EC_{50} = 0.07 ± 0.03 μM, n = 4), 79.9 ± 9.7% (EC_{50} = 0.08 ± 0.03 μM, n = 4), 61.4 ± 6.0% at 10 μM (EC_{50} = 0.3 ± 0.1 μM, n = 4) and 57.1 ± 6.1% at 10 μM (EC_{50} = 0.4 ± 0.2, n = 4) respectively (Figure 2a). Thus, equieffective concentration-ratios (EECs) for misoprostol, 11-deoxy PGE_1, AH 13205 and butaprost of 1.75, 5.3, 12.7 and 50.7, respectively were determined.

In contrast, no significant difference ($P>0.05$) was observed with sulprostone in the presence of IBMX, whereas inhibition obtained with 17-phenyl PGE_2 was increased, but only at the highest concentration (10 μM, 56.8 ± 8.2% compared to 31.0 ± 6.0%) (Figure 2b).

Effect of selective prostanoid receptor antagonists

When neutrophils were pre-incubated with the EP_4 receptor antagonists, AH 23848B (10 μM, 10 min), there was no significant ($P>0.05$) antagonism of the inhibition produced by PGE_2. However, a potentiation of the inhibitory effect of PGE_2 at the lower concentrations of PGE_2 tested (0.001–0.1 μM) was observed (Figure 3a). This potentiation of inhibition by AH 23848B was also observed in the presence of IBMX (data not shown). An even greater potentiation of inhibition induced by butaprost (0.1–10 μM) was found (Figure 3b), although no inhibitory effect was observed with AH 23848B alone. Indeed, AH 23848B (10 μM, 10 min) increased the release of O_2^- stimulated by FMLP from

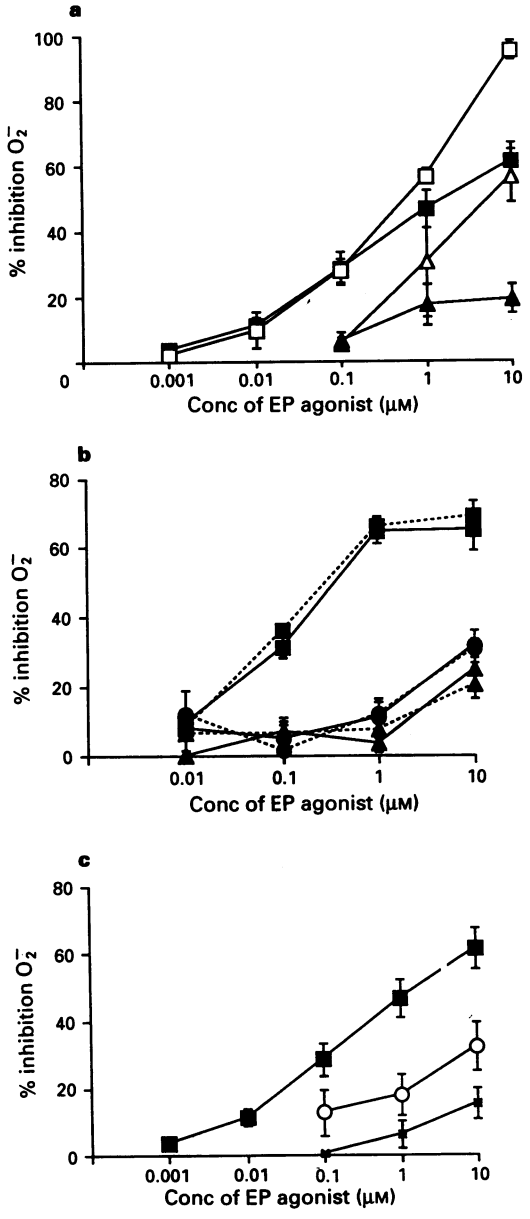

Figure 1 Log concentration-effect curves for EP-receptor agonist-inhibition of superoxide anion generation in human neutrophils induced by FMLP (100 nM). (a) PGE_2 (■), misoprostol (□), 11-deoxy-PGE_1 (△) and butaprost (▲); (b) PGE_2 (■), AH 13205 (●) and butaprost (▲). Continuous lines represent control neutrophils and dash lines, neutrohils treated with indomethacin (3 μM); (c) PGE_2 (■), 17-phenyl-ω-PGE_2 (○) and sulprostone (×). The values are the mean ± s.e.mean of 14 different donors for PGE_2, and 4 different donors for the other agonists. For abbreviations for this and all legends, see text.

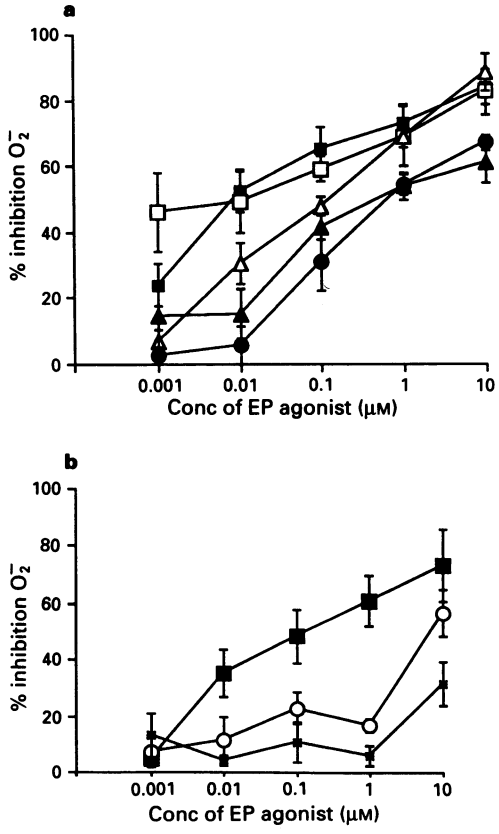

Figure 2 Log concentration-effect curves for EP-receptor agonist-inhibition of superoxide anion generation in neutrophils treated with IBMX (0.25 mM). (a) PGE_2 (■), misoprostol (□), 11-deoxy-PGE_1 (△), AH 13205 (●) and butaprost (▲); (b) PGE_2 (■), 17-phenyl-ω-PGE_2 (○) and sulprostone (×). The values are the mean ± s.e.mean of 4 different donors.

13.9 ± 1.4 to 16.0 ± 1.7 nmol O_2^- per 10 min per 10^6 cells, ($n = 5$). AH 23848B similarly potentiated inhibition induced by low concentrations of the adenosine-mimetic, 5'-N-ethylcarboxamido-adenosine (NECA) (Figure 3c).

The EP_1/DP-receptor antagonist, AH 6809 (10 μM, 10 min), significantly ($P < 0.05$) antagonized the inhibitory effects of 1.0 and 10, but not 0.1 μM PGE_2 (Figure 4a). From EC_{50} values, a pA_2 of 6.04 was calculated for AH 6809. AH 6809 on its own at 1 and 10 μM significantly ($P = 0.004$ and 0.02, respectively), increased O_2^- release induced by FMLP from 13.3 ± 2.4 to 15.9 ± 2.5 and 17.0 ± 2.0 nmol O_2^- per 10 min per 10^6 cells respectively ($n = 4$). AH 6809 also antagonized the inhibitory response induced by NECA (Figure 4b), with a pA_2 of 6.83.

Role of cyclic AMP in the inhibition of neutrophil activation

The adenylate cyclase inhibitor, SQ 22,536 (0.1 mM, 2 min), significantly ($P < 0.05$) reduced PGE_2-induced inhibition of O_2^- production, giving an EC_{50} in the absence and presence of SQ 22536 of 0.24 ± 0.1, and 1.9 ± 1.1 μM respectively (Figure 5). Conversely, non-selective inhibition of phosphodiesterase with IBMX (0.25 mM) potentiated the inhibitory effects of PGE_2 (Figure 2a). However, at this concentration, IBMX itself inhibited the FMLP response by 54.9 ± 5.0% ($n = 10$), a fact which could account for the increased effectiveness of PGE_2.

The specific type IV phosphodiesterase inhibitor, rolipram at concentrations of 2 and 10 nM, did not have any significant effect on FMLP response, reducing the FMLP effect by 8.8 ± 5.4% and 29.7 ± 15.1% respectively, whereas at the concentration of 50 nM, it significantly ($P < 0.05$) reduced the FMLP response, by 46.6 ± 8.4%. Yet rolipram, at all three concentrations (2, 10 and 50 nM) shifted the concentration-effect curve for PGE_2 leftwards, with EC_{50}s of 0.06 ± 0.02, 0.01 ± 0.0 and 0.012 ± 0.006 μM respectively (Figure 6). The cyclic AMP analogue, 8 bromo cyclic AMP (1.0–100 μM), did not give consistent results, inhibition varying from 0 to 14.5%.

Role of endogenously produced adenosine

Removal of endogenous adenosine with the enzyme, adenosine deaminase (ADA, 1.0 u ml^{-1}), had no significant effect upon concentration-effect curve for PGE_2 (EC_{50} of 0.42 ± 0.15 μM for PGE_2 and 0.1 ± 0.4 μM for PGE_2 + ADA) (Figure 7a), although the maximal inhibition was significantly increased from 55.3 ± 8.1 to 68.3 ± 6.8%, $n = 6$. ADA itself significantly ($P < 0.05$) increased the production of O_2^- by FMLP from $1.0 \times 10^{-8} \pm 0.2 \times 10^{-8}$ to $1.3 \times 10^{-8} \pm 0.1 \times 10^{-8}$ mol O_2^- per 10 min per 10^6 cells, $n = 6$. The non-selective adenosine receptor antagonist, 8-phenyl theophylline (8-PT, 10 μM), had no significant ($P > 0.05$) effect upon concentration-effect curves for PGE_2 (EC_{50}s of 0.4 ± 0.17 for

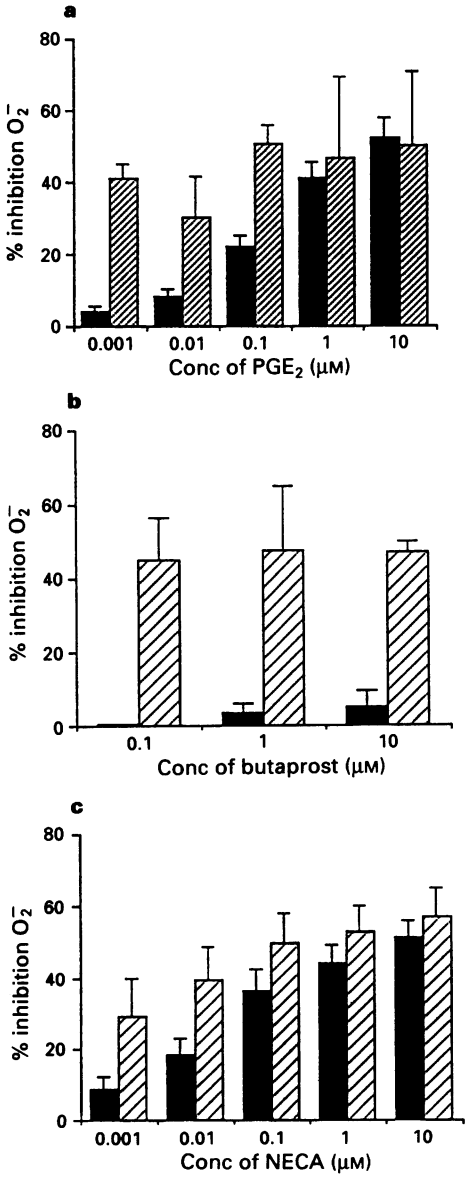

Figure 3 Effect of a 10 min pre-incubation with the EP_4 receptor anatagonist AH 23,848 (10 μM) on inhibition of FMLP-induced superoxide anion generation in human neutrophils by (a) PGE_2, (b) butaprost and (c) NECA. Solid columns represent control neutrophils and hatched columns, neutrophils treated with AH 23,848. The values are the mean ± s.e.mean of 4 different donors.

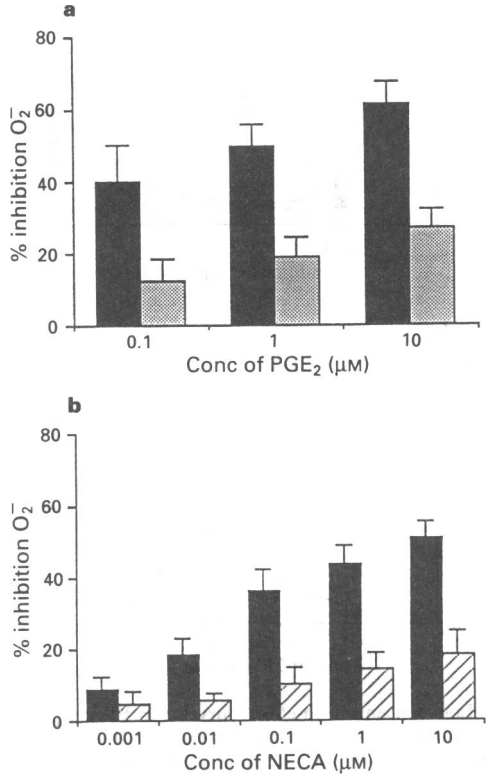

Figure 4 Effect of a 10 min pre-incubation with the EP_1 receptor anatagonist, AH 6809 (10 μM) on inhibition of FMLP-induced superoxide anion generation in human neutrophils by (a) PGE_2 and (b) NECA. Solid columns represent control neutrophils and hatched columns, neutrophils treated with AH 6809. The values are the mean ± s.e.mean of 4 different donors.

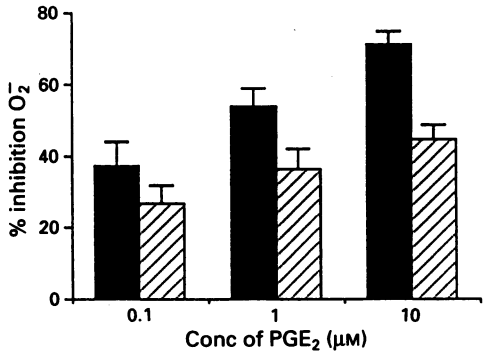

Figure 5 Effect of a 2 min pre-incubation with the adenylate cyclase inhibitor, SQ 22,536 (100 μM) on inhibition of FMLP-induced superoxide anion generation in human neutrophils by PGE_2. Solid columns represent control neutrophils and hatched columns, neutrophils treated with SQ 22,536. The values are the mean ± s.e.mean of 8 different donors.

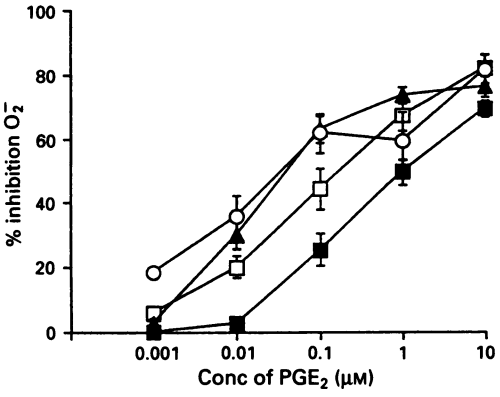

Figure 6 Log concentration-effect curves for inhibition of superoxide anion generation by PGE_2 in neutrophils treated with rolipram at (■) 0, (□) 2, (▲) 10 and (○) 50 nM. The values are the mean ± s.e.mean of 4 different donors.

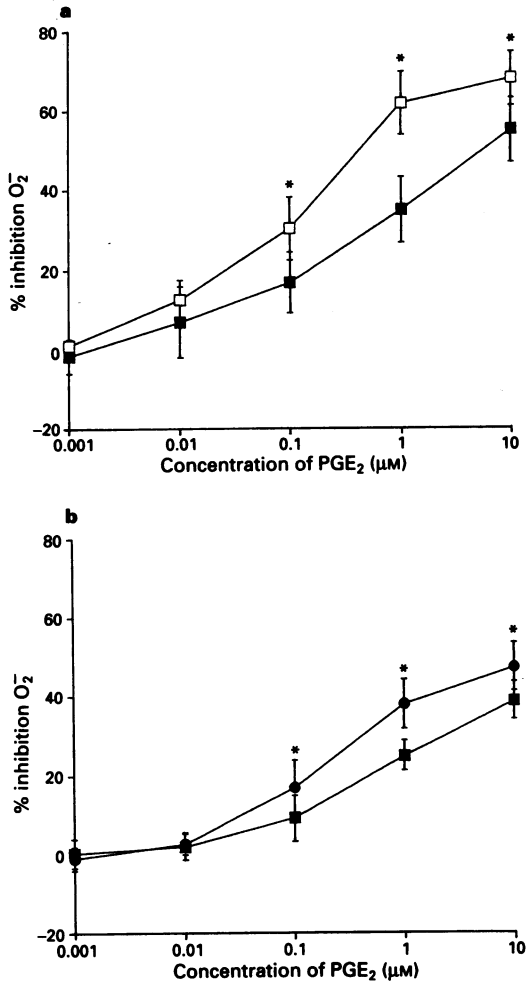

Figure 7 Log concentration-effect curves for inhibition of superoxide anion generation by PGE_2 in FMLP-stimulated human neutrophils. (a) PGE_2 in control neutrophils (■) and neutrophils treated with adenosine deaminase (1 u ml^{-1}) (□); (b) PGE_2 in control neutrophils (■) and neutrophils treated with 8-phenyl theophylline (10 μM) (●). * represents $P<0.05$. The values are the mean ± s.e.mean of a result from 6 and 5 donors respectively.

PGE_2 and 0.24 ± 0.1 μM for PGE_2 + 8-PT, $n = 5$) although the maximal response was increased slightly from 38.9 ± 4.9% to 47.7 ± 6.0% with 8-PT (Figure 7b).

Discussion

PGD_2 and PGE_2 are known to be potent inhibitors of O_2^- release in the human neutrophil (Gryglewski *et al.*, 1987; Wheeldon & Vardey, 1993). In the present study we have investigated the effects of selective EP receptor agonists and prostanoid receptor antagonists on FMLP-induced release of O_2^-, in order to elucidate the subtype of EP receptor occupied by PGE_2 in producing inhibition of O_2^- release.

The EP_2 receptor agonists, misoprostol, 11-deoxy PGE_1, butaprost (Gardiner, 1986; Lawrence & Jones, 1992) and AH 13205 (Nials *et al.*, 1993), while effective, were found to be less potent than PGE_2 itself, the latter two analogues producing a significant effect only at a concentration of 10 μM. However, one must question the selectivity of a compound at 10 μM, and the EP_1 agonist, 17-phenyl PGE_2 as well as the EP_1/EP_3 agonist, sulprostone, also showed weak inhibitory effects at this concentration, suggesting some crossover on to this inhibitory EP receptor at high concentrations. When the experiments were repeated with neutrophils treated with IBMX (0.25 mM), the concentration-effect curves for all the EP_2 agonists were significantly shifted to the left, allowing EECs of 11.5, 5.3, 50.7 and 12.7 to be calculated. This agrees well with the relative potency of these agonists on EP_2 receptor preparations (Nials *et al.*, 1991; 1993) where EECs of 1-4, 2-5, 6-30 and 30-100 have been reported.

In contrast, IBMX treatment did not significantly affect inhibition by sulprostone, and increased the activity of 17-phenyl PGE_2, only at the highest concentration 10 μM, allowing EECs of >700 and 486 to be calculated for sulprostone and 17-phenyl PGE_2 respectively. Sulprostone is a potent agonist at EP_3 receptors of the guinea-pig vas deferens, EEC = 0.15, PGE_2 = 1 (Coleman *et al.*, 1987) as well as at EP_1 receptors of the guinea-pig fundus, EEC = 2.5, PGE_2 = 1 (Coleman *et al.*, 1988) but shows weak activity at EP_2 receptors of the cat trachea, EEC > 7000 (Coleman *et al.*, 1988) or EP_4 receptors of the pig saphenous vein, EEC > 3000 (Coleman *et al.*, 1994). Similarly, 17-phenyl PGE_2 is a potent agonist at EP_1 receptors, but not at EP_2 receptors (Lawrence *et al.*, 1992). This apparent lack of activity by these agents, therefore, confirms that the EP receptor of major importance in mediating PGE_2-induced inhibition of O_2^--release is not of the EP_1 or EP_3 receptor subtypes.

The results so far imply that either EP_2 or EP_4 receptors are those involved in PGE_2-induced inhibition of O_2^- production. Investigation of the EP_4 receptor of the pig saphenous vein (Milne *et al.*, 1994) found 11-deoxy PGE_1, butaprost and AH 13205 to cause relaxation with EECs (PGE_2 = 1) of 2, 42 and 6600 respectively. With this in mind, the effect of

the EP_4 receptor antagonist AH 23848B was examined. AH 23848B (10 μM) did not block the inhibitory response to PGE_2, but surprisingly, a potentiation of the effects of the lower concentrations of PGE_2 (0.001–0.1 μM) was observed. In addition, AH 23848B was also found to potentiate the weak inhibitory action of butaprost. The action of AH 23848B on butaprost first led us to believe that AH 23848B may have been acting as a partial agonist and not as an antagonist as described by Coleman *et al.* (1994), but this is unlikely as AH 23848B showed no inhibitory effect when incubated with neutrophils on its own. It seems unlikely that AH 23848B is acting as a phosphodiesterase inhibitor as potentiation of inhibition was still observed when PGE_2 was examined in IBMX treated neutrophils. Furthermore, this effect appears to be unrelated to the EP_4 receptor blocking activity of AH 23848B as potentiation of the adenosine-mimetic, NECA was also observed. Since AH 23848B was originally synthesized as a thromboxane receptor antagonist (Coleman *et al.*, 1990), possible interactions with the TP receptor are under investigation.

The EP_1/DP-receptor antagonist AH 6809 (Keery & Lumley, 1988), was found to produce a significant inhibition of the effects of 1 and 10 μM PGE_2 ($pA_2 = 6.04$), which is unlikely to be due to an effect through EP_1-receptors as both sulprostone and 17-phenyl PGE_2 were such weak inhibitors of superoxide generation. This may relate to a nonspecific effect of this compound, as AH 6809 was also found to inhibit the effects of the adenosine agonist NECA ($pA_2 = 6.83$) suggesting that AH 6809 is not acting on either EP or DP receptors.

Prostaglandins of the E series are known to activate membrane-bound adenylate cyclase in human neutrophils, and to increase intracellular cyclic AMP levels (Bourne *et al.*, 1971). It is possible therefore, that the increase in cyclic AMP induced by PGE_2 may in turn inhibit the release of O_2^- from human neutrophils. Indeed, many groups have studied the effects of PGE series prostaglandins on cyclic AMP in human neutrophils with relation to superoxide and lysosomal enzyme release (Zurier *et al.*, 1974). PGE_2 can be shown to activate membrane adenylate cyclase, but the increase in cyclic AMP in the whole cell is small, and can only be seen in the presence of a phosphodiesterase inhibitor to prevent cyclic AMP breakdown (Lad *et al.*, 1984). Although it has been proposed that these small increases in cyclic AMP are sufficient to inhibit various specific functions in the neutrophil (Lad *et al.*, 1984), the fact that these observations could be seen only in the presence of a phosphodiesterase inhibitor, and that cyclic AMP levels slowly increased over a 10 min time scale still cast doubt on the hypothesis that cyclic AMP is the second messenger mediating inhibition of O_2^- generation by PGE_2.

The demonstration that prostaglandins can elicit an increase in cyclic AMP is not on its own sufficient proof that cyclic AMP is the second messenger involved. The present study set about trying to accommodate three more criteria thought to be essential for concluding that cyclic AMP is the second messenger:-

First, we have shown that the adenylate cyclase inhibitor, SQ 22,536 (Harris *et al.*, 1979), significantly antagonized the inhibition of O_2^- generation produced by PGE_2. This result suggests strongly a causal link between cyclic AMP and PGE_2, as it shows that inhibition of the enzyme responsible for cyclic AMP production (adenylate cyclase) significantly reduces the action of PGE_2 on O_2^- generation. Unfortunately SQ 22,536 is not a very effective inhibitor of adenylate cyclase, and it is not possible to block adenylate cyclase activity completely.

Secondly, the inhibitory effects of PGE_2 are significantly potentiated after phosphodiesterase block with IBMX and rolipram. The inhibition of phosphodiesterase and the resultant increase in neutrophil cyclic AMP levels was itself found to inhibit the FMLP response by about 50% with both IBMX (0.25 mM) and high concentrations of rolipram. This demonstrates that the production of O_2^- by FMLP is susceptible to inhibition by increased cyclic AMP levels. In addition, the finding that lower concentrations of rolipram, which do not inhibit the FMLP response, are still capable of potentiating inhibition by PGE_2 suggests that this action results from the phosphodiesterase block and not the reduction in FMLP stimulus.

Further evidence that the effect of IBMX is unlikely to result from adenosine receptor block, is provided by the observation that neither ADA nor 8-PT shift the concentration-effect curve for PGE_2 in a similar manner to IBMX.

Thirdly, there is an impressive correlation ($r = 0.92$) between the increase in cyclic AMP levels (Armstrong & Talpain, 1994) and the inhibition of O_2^- generation particularly when the phosphodiesterase inhibitor (IBMX) is used to evaluate the inhibition of the superoxide generation, as is the case when cyclic AMP levels are measured. As would be predicted, a threshold increase in cyclic AMP is required before any inhibitory effect can be detected.

However, results with 8 bromo cyclic AMP were poor, but inhibition of neutrophil activation has previously been shown by Wong *et al.* (1980) using the cyclic AMP analogue dibutyryl adenosine 3',5'-monophosphate.

Taken together, these results suggest that cyclic AMP acts as a second messenger to mediate the inhibition by PGE_2 and EP_2 agonists of FMLP-induced superoxide formation. This has also been suggested by Li *et al.* (1993). The exact mechanism by which cyclic AMP inhibits O_2^- generation still remains to be elucidated. However, it has been demonstrated (Hecker *et al.*, 1989; Ney & Schror, 1991) that the increase in intracellular cyclic AMP produced by PGE_2 in human neutrophils is paralleled by a decrease in extracellular Ca^{2+} influx which may lead to the attenuation of receptor-mediated neutrophil activation by FMLP. It is interesting to note that Sedgwick *et al.* (1985) found that although cyclic AMP levels increased in all neutrophils stimulated by the three different neutrophil activators, FMLP, phorbol myristate acetate and serum-treated zymosan, inhibition of O_2^- was seen only in the neutrophils treated with FMLP. This finding suggests that the influence of prostaglandins on O_2^- production depends on the nature of the neutrophil activation stimulus, and that an increase in cyclic AMP in the activated neutrophil alone is not always sufficient to limit O_2^- generation. As a result of this, any conclusions drawn in this study must be restricted to FMLP.

In conclusion, the results of the present study suggest that inhibition of FMLP-induced superoxide generation by prostanoids is mediated by the occupation of EP_2 receptors and activation of adenylate cyclase, leading to the elevation of intracellular levels of cyclic AMP.

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Blockade of mast cell histamine secretion in response to neurotensin by SR 48692, a nonpeptide antagonist of the neurotensin brain receptor

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1 Pretreatment of rat isolated mast cells with SR 48692, a nonpeptide antagonist of the neurotensin (NT) receptor, prevented histamine secretion in response to NT.

2 This inhibition was rapid in onset (~1 min) and dependent upon the concentration of SR 48692 (IC_{50} ~1-10 nM).

3 SR 48692 (1-1000 nM) did not inhibit histamine secretion elicited by substance P, bradykinin or compound 48/80, or by anti-IgE stimulation of sensitized mast cells.

4 When SR 48692 was injected intradermally (5 pmol in 50 µl) into anaesthetized rats, 15 min before the intradermal injection of NT, it reduced the effect of NT on vascular permeability.

5 When injected intravenously, SR 48692 attenuated the effects of NT on haematocrit and blood stasis.

6 These results demonstrate that SR 48692 selectively antagonizes the actions of NT on rat isolated mast cells as well as mast cells *in vivo*. Given the demonstrated specific interaction of SR 48692 with receptors for NT in brain, our results suggest the presence of specific NT receptors on mast cells.

Keywords: Neurotensin; mast cell; neurotensin receptor; antagonist; SR 48692; histamine secretion

Introduction

Neurotensin (NT, pZLYENKPRRPYIL) a tridecapeptide found in brain, gut and peripheral nerves, has a broad spectrum of biological activities. In the central nervous system, NT displays analgesic, neuromodulator, and neuroendocrine actions while in the gastrointestinal tract, it modulates smooth muscle contraction and endocrine function. When injected intravenously, NT produces several cardiovascular effects including a profound hypotension and increased vascular permeability (Carraway & Leeman, 1973; Kitabgi, *et al.*, 1985; Reinecke, 1985; Rioux *et al.*, 1985; Prange, 1992). These latter effects of NT are primarily attributable to its striking ability to stimulate secretion from mast cells as they are blocked by preventing mast cell secretion or by pretreatment with anti-histamines (Carraway *et al.*, 1982; 1991). The interaction between NT and mast cells has been suggested to be involved in the regulation of the microvasculature and in the response to inflammation (Carraway *et al.*, 1982; 1987; 1989; 1991; Cochrane, 1990; Cochrane *et al.*, 1991; 1992).

Specific receptors for NT have been demonstrated in membrane preparations from brain (Vincent, 1992) and gut (Seybold *et al.*, 1990) as well as in cell lines derived from a neuroblastoma (see Vincent, 1992). Circulating monocytes have also been shown to display specific NT receptors (Bar-Shavit *et al.*, 1982).

Recently, a nonpeptide antagonist (SR 48692) of brain NT-receptors was described (Gully *et al.*, 1993). This antagonist competitively and selectively inhibited the interaction of NT with high affinity binding sites in brain membranes and prevented a variety of NT-induced cellular responses (Gully *et al.*, 1993). SR 48692 had no inhibitory effect on a variety of other peptide receptor systems including those of bradykinin, neuropeptide Y, cholecystokinins A and B, neurokinin 1 and 2 ligands, and [Arg^8] vasopressin (Gully *et al.*, 1993). The aim of the present study was to examine the effects of SR 48692 on NT-induced histamine secretion from mast cells *in vitro* and on NT-induced, mast cell-dependent effects *in vivo*. A preliminary account of some of this work has been reported (Cochrane *et al.*, 1994).

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Methods

Mast cells were isolated from the peritoneal and pleural cavities of male Sprague-Dawley rats as previously described (Cochrane & Douglas, 1974). The cells were purified to >90% homogeneity by centrifugation through 22.5% metrizamide and counted in a haemocytometer as previously described (Carraway *et al.*, 1982). The composition of Locke solution has been given previously (Miller *et al.*, 1993). Histamine release from isolated mast cells was measured by fluorometric assay (Carraway *et al.*, 1982) and secretion expressed as histamine release (% total) = released histamine ÷ (cellular histamine + released histamine) × 100.

Changes in cutaneous vascular permeability were measured in anaesthetized (Nembutal) rats by use of Evans Blue dye as previously described (Cochrane *et al.*, 1991). Briefly, Locke (50 µl, without BSA) in the absence or presence of SR 48692 (5 pmol in 50 µl) was injected intradermally into the shaved abdominal skin of anaesthetized (Nembutal) rats pretreated 5 min before with Evans Blue dye (0.1 ml of 0.5% dye 100 g^{-1} body wt., i.v.). After 15 min, Locke (solution 50 µl, control sites) or NT (0.15, 1.5, 15 pmol in 50 µl) was injected into the preinjected sites. Five min later the animal was killed by decapitation, the skin site rapidly removed and analyzed for changes in vascular permeability. Dye extrusion at injected skin sites was quantified by densitometric scanning with a Hewlett-Packard Scanjet Plus digital scanner coupled to Image software with a Macintosh IIci computer (Cochrane *et al.*, 1991). Values for saline-injected sites were subtracted from treated skin sites. For experiments involving the intravenous injection of SR 48692, anaesthetized (Nembutal) rats (150-200 g) were pretreated by intravenous injection of

isotonic saline or antagonist in isotonic saline (~0.6 ml with indicated amount). Five min later, at time = 0, an intravenous injection of saline or NT in saline (4 nmol kg^{-1}) was given. Blood was collected from the vena cava into heparinized (10 u ml^{-1}) syringes containing EDTA (1 mg ml^{-1}) at time = 2 min for the measurement of histamine and at time = 15 min for determination of haematocrit, as previously described (Carraway *et al.*, 1991). Samples were stored on ice, the plasma removed and histamine levels measured by radioenzymatic assay as before (Carraway *et al.*, 1982; 1991). The resting (control) level of plasma histamine was < 0.1 ng in 10 µl of plasma. With the injection of NT the level increased to 5.6 ± 1.9 ng (mean ± s.e.mean) in the absence of antagonist. Pretreatment of animals with SR 48692 followed by the injection of saline 2 min later, had no effect on the level of plasma histamine or haematocrit (data not shown).

Neurotensin was purchased from Bachem. Substance P (SP), bradykinin (BK), somatostatin (SOM), and compound 48/80 were from Sigma Chemical Co. A23187 was from Cal Biochem. Anti-IgE serum was from ICN. Levocabastine was a gift from the Janssen Research Foundation. SR 48692 was generously provided by Dr Danielle Gully, Sanofi Recherche, 195 route d'Espagne, 31036 Toulouse Cedex, 34082, Montpellier Cedex, France. SR 48692 was dissolved in DMSO at 1 mM and stored at −20°C in a light-tight container. It was diluted to the appropriate concentration immediately before use. We did observe ~50% loss in the activity of SR 48692 when it was stored under the above conditions as a stock solution for more than nine months. All experiments reported here were done using stock solutions of SR 48692 stored for less than 2 months.

Results

Preincubation of mast cells (10^5–10^6 ml^{-1}) with SR 48692 resulted in an inhibition of histamine secretion in response to NT (0.1–20 µM) that depended upon the concentration of SR 48692 used (Figure 1a; IC_{50} ~1–10 nM). This inhibitory effect of SR 48692 was rapid in onset (within 1 min) and readily reversible at the 10 nM level by washing and resuspending the cells in Locke solution free of SR 48692 (Figure 2). If cells treated with SR 48692 were washed with Locke solution containing the antagonist, no recovery of NT stimulated secretion was seen (see Figure 2, legend). At higher concentrations of SR 48692 (100 and 1000 nM), the inhibitory effect was only partially (~30%) reversed by a 20 min wash period. Importantly, at the concentrations tested (1, 10, 100, and 1000 nM), SR 48692 was specific for NT (Figure 1). Thus, histamine release in response to various concentrations of SP, BK or compound 48/80 was unaffected (Figure 1, b, c, and d). Likewise, the secretory response elicited from IgE-sensitized mast cells stimulated by anti-IgE serum (1:10 dilution) for 20 min was not affected (without SR 48692 histamine release was 30 ± 2.0% and after a 10 min incubation with SR 48692 (10 nM), histamine release was 28 ± 1.7%, mean ± s.e.mean, $n = 3$). Higher concentrations (100 and 1000 nM) of the antagonist also had no effect (data not shown). Pretreatment of mast cells with levocabastine (10 µM), shown to block low affinity NT receptors in brain (see Gully *et al.*, 1993), had no effect on the inhibitory action of SR 48692. Thus, NT (10 µM) in the absence and presence of levocabastine elicited 18% and 17% histamine release (mean of 2, respectively). Preincubation for 10 min with SR 48692 (10, 25, 50, or 100 nM) with and without

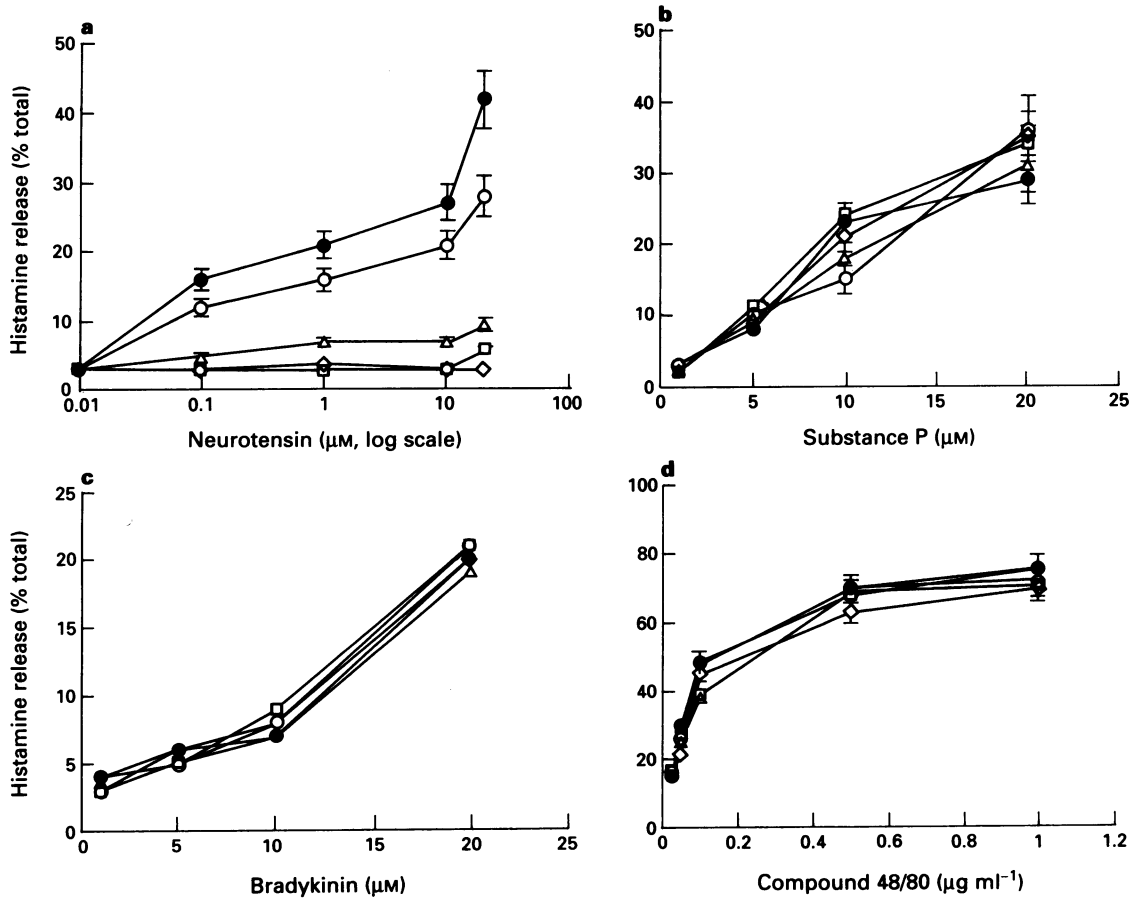

Figure 1 Effect of SR 48692 on the histamine releasing ability of neurotensin (NT) and other mast cell secretagogues. Mast cells were incubated for 10 min in the absence (●) or presence of SR 48692 (○ = 1 nM, △ = 10 nM, □ = 100 nM, ◇ = 1000 nM). NT, (a), substance P (b), bradykinin (c) or compound 48/80 (d), at the concentration indicated, were then added and histamine secretion determined after 5 min. Mean ± s.e.mean>, $n \geq 3$ for (a), (b) and (d). For (c) n = mean of 2.

levocabastine reduced NT stimulated histamine release to ≤ spontaneous release (3 ± 0.1%, mean ± s.e.mean).

Injected intradermally into anaesthetized rats, 10 min before the injection of NT, SR 48692 (5 pmol in 50 μl) inhibited by 90%, 88%, and 42% the increase in vascular permeability elicited by the injection of 0.15, 1.5 and 15 pmol NT respectively (Figure 3a). At a higher concentration (50 pmol in 50 μl), SR 48692 also reduced (35.8 ± 5.2% decrease, $n = 4$, mean ± s.e.mean) the increase in vascular permeability elicited by the intradermal injection of histamine (25 nmol in 50 μl). SR 48692 by itself did not increase cutaneous vascular permeability.

Given intravenously to anaesthetized rats 5 min before the intravenous injection of NT (4 nmol kg^{-1}), SR 48692 (0.25–2.0 mg kg^{-1}) reduced the subsequent elevation in haematocrit and plasma histamine (Figure 3b). At the 1.25 and 2.0 mg kg^{-1} doses, SR 48692 also blocked the cyanosis resulting from the blood stasis induced by NT.

Discussion

The results described here show that SR 48692, a non-peptide antagonist of NT-receptors (Gully *et al.*, 1993) inhibits the stimulatory action of NT on mast cells *in vitro* and on mast cell-dependent effects *in vivo*. On isolated mast cells, this inhibition was rapid in onset, concentration-dependent and was reversible at the 10 nM concentration (Figures 1 and 2). Moreover, SR 48692 was selective for NT; it did not inhibit histamine release in response to substance P, bradykinin, or compound 48/80 (Figure 1b–d) nor that elicited by anti-IgE stimulation of passively sensitized mast cells. The inhibition *in vivo* could be demonstrated for both intradermal and intravenous challenges with NT (Figure 3a and b). Gully *et al.* (1993) have shown that SR 48692 inhibits the specific binding of ^{125}I labelled-NT to high affinity sites on isolated brain membranes with IC_{50} values ranging from 0.3–50 nM depending on the species of animal used. They have also shown that SR 48692 is highly selective for NT as it had no antagonistic effects in other receptor systems. The results of our *in vitro* studies given here (Figure 1a) are in general agreement with these earlier findings (IC_{50}, ~1–10 nM). In order to inhibit NT-stimulated, mast cell-dependent effects *in vivo*, higher levels of SR 48692 were necessary (Figure 3b).

NT and several other peptides have been shown to stimulate a rapid, exocytotic secretory response from isolated mast cells that depends upon a cellular calcium pool (Carraway *et al.*, 1982; Shanahan *et al.*, 1985; Church *et al.*, 1989; Pearce *et al.*, 1989; Cochrane, 1990). However, the mechanisms by which these peptides elicit mast cell secretion remain ill-defined and no mast cell receptor protein for these peptides has as yet been identified. Some workers have suggested that some basic peptides like SP, BK and mastoparan as well as compound 48/80 stimulate mast cell secretion by directly activating guanine nucleotide binding (G) proteins (Bueb *et al.*, 1990; Aridor *et al.*, 1990; 1993) and thus act by a receptor-independent mechanism (Mousli *et al.*, 1990a,b). According to this hypothesis the positively charged portion of the molecule interacts with the C-terminal region of the α subunit of the G protein thereby activating secretion (Mousli *et al.*, 1990a).

Studies of the action of NT on isolated mast cells have shown that its stimulatory activity is evident at a 10–20 fold lower concentration than the stimulatory action of peptides such as SP or BK (Jasani *et al.*, 1979; Mazurek *et al.*, 1981; Carraway *et al.*, 1982; 1984 and Figure 1a, b, c) and that its biological activity resides in the C-terminal hexapeptide, NT^{8-13} (this is also true for other NT-activated systems, see Kitabgi *et al.*, 1985). Also, while the amount of positive charge is generally related to its histamine-releasing activity ($NT^{6-13} > NT^{8-13}$) there are also essential structural requirements for the C-terminal residues (Sagi-Eisenberg *et al.*, 1983; Carraway *et al.*, 1984; Kitabgi *et al.*, 1985). For example, replacing either Lue^{13} or Ile^{12} of NT^{8-13} with the

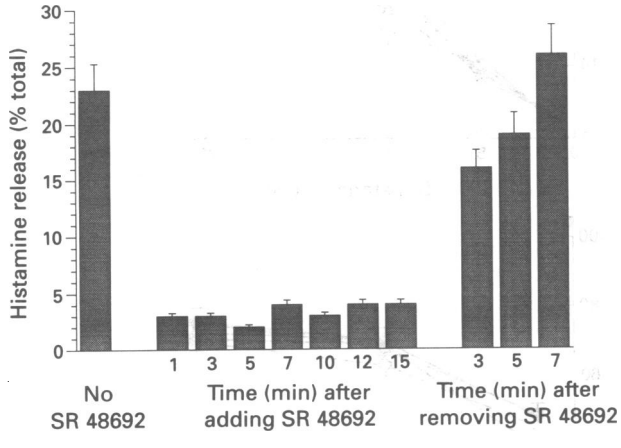

Figure 2 Time course of inhibition of neurotensin (NT) stimulated histamine release by SR 48692 and its reversibility. Mast cells were incubated with SR 48692 (10 nM) for the indicated times, and NT (10 μM) then added. Histamine release was determined 5 min later. For reversibility measurements, mast cells were incubated for 10 min with SR 48692 (10 nM), washed and resuspended in Locke solution. At the indicated times, NT (10 μM) was added and histamine secretion determined as before. The far lefthand column shows histamine release in response to NT (10 μM) in the absence of SR 48692. Mast cells that were pretreated for 10 min with SR 48692 (10 nM), washed with and resuspended in Locke solution containing SR 48692 (10 nM) and then stimulated with NT (10 μM) did not respond (histamine release < 4.1 ± 0.1%). Mean ± s.e.mean, $n \geq 3$.

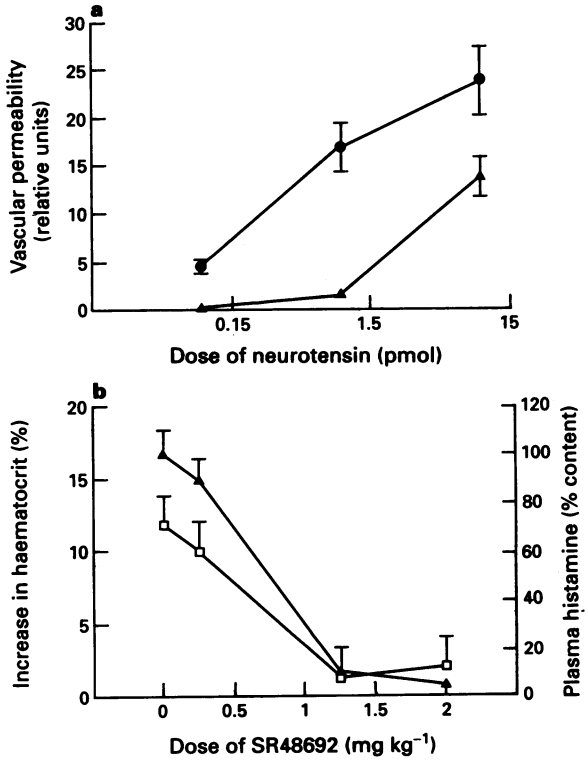

Figure 3 Antagonistic effects of SR 48692 *in vivo*. (a) Changes in cutaneous vascular permeability induced by the intradermal injection of neurotensin (NT) in the presence (▲) or absence (●) of SR 48692. Plotted is the increment above control (Locke solution or Locke + SR 48692) determined for each condition. Mean ± s.e.mean, $n = 3$. (b) Effect of SR 48692 on the increases in haematocrit (□) and plasma histamine (▲) produced by NT. Plasma histamine is expressed as a percentage of the NT value measured in the absence of SR 48692 (100%). Mean ± s.e.mean, $n \geq 3$.

charged amino acid, Asp, renders the resulting analogues, $Asp^{13}NT^{8-13}$ and $Asp^{12}NT^{8-13}$, ineffective in stimulating mast cell secretion (Miller *et al.*, 1993). Likewise, removal of the C-terminal Leu from NT to give NT^{1-12} significantly reduces its histamine-releasing activity (Cochrane *et al.*, 1991) as well as its ability to bind to NT-brain receptors (Kitabgi *et al.*, 1985). The subsequent removal of Ile^{12} produces a peptide (NT^{1-11}) with no biological activity on the mast cell (Cochrane *et al.*, 1991) or on other NT-activated systems (Kitabgi *et al.*, 1985).

The importance of C-terminal structure in stimulating mast cell secretion has also been shown for SP (Fewtrell *et al.*, 1982). Removal of two or three amino acids from the C-terminal of SP to give SP^{1-9} or SP^{1-8} respectively, reduced their histamine releasing activity by about two-thirds. Removing an additional C-terminal amino acid to give SP^{1-7} reduced histamine releasing activity by an order of magnitude.

The results described here, showing a selective inhibition of NT-evoked histamine secretion by a specific antagonist of the NT-receptor together with the results from earlier studies with NT, suggest to us that the stimulating action of NT on the connective tissue mast cell involves a selective NT-receptor mechanism.

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Characterization of kinin receptors modulating neurogenic contractions of the mouse isolated vas deferens

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1 This study analyses the receptors mediating the effects of bradykinin (BK) and analogues on neurogenic twitch contractions of the mouse isolated vas deferens evoked, in the presence of captopril (3 μM), by electrical field stimulation with trains of 4 rectangular 0.5 ms pulses of supramaximal strength, delivered at a frequency of 10 Hz every 20 s.

2 BK (0.1–300 nM) induced a graded potentiation of twitches, with an EC_{50} (geometric mean and 95% confidence limits) of 4.5 nM (1.7–11.6) and an E_{max} of 315 ± 19 mg per 10 mg of wet tissue ($n = 6$). Similar results were obtained in tissues challenged with Lys-BK, [Hyp^3]-BK, Met,Lys-BK and the selective B_2 receptor agonist [Tyr(Me)^8]-BK (0.1–300 nM).

3 The selective B_2 receptor antagonists, Hoe 140 (1-10 nM) and NPC 17731 (3–30 nM), caused graded rightward shifts of the curve to BK-induced twitch potentiation, yielding apparent pA_2 values of 9.65 ± 0.09 and 9.08 ± 0.13, respectively, and Schild plot slopes not different from 1. Both antagonists (100 nM) failed to modify similar twitch potentiations induced by substance P (3 nM) or endothelin-1 (1 nM). Preincubation with the selective B_1 receptor antagonist, [Leu^8,des-Arg^9]-BK (1 μM), increased the potentiating effect of BK on twitches at 30–300 nM.

4 In contrast to BK, the selective B_1 receptor agonist, [des-Arg^9]-BK (0.3–1000 nM) reduced the amplitude of twitches in a graded fashion, with an IC_{50} of 13.7 nM (10.4–16.1) and an I_{max} of 175 ± 11 mg ($n = 4$). The twitch depression induced by [des-Arg^9]-BK (300 nM) was not affected by Hoe 140 (30 nM) or NPC 17731 (100 nM), but was abolished by the selective B_1 receptor antagonist, [Leu^8,des-Arg^9]-BK (1 μM), which did not modify the twitch inhibitory effect of clonidine (1 nM) or morphine (300 nM).

5 In non-stimulated preparations, BK (100 nM) also potentiated, in a Hoe 140-sensitive (10 nM) manner, the contractions induced by ATP (100 μM), but not by noradrenaline (10 μM), whereas [des-Arg^9]-BK (300 nM) did not modify the contractions induced by either agonist.

6 It is concluded that the mouse vas deferens expresses both B_1 and B_2 receptors, which modulate sympathetic neurotransmission in opposing ways. Neurogenic contractions are inhibited by stimulation of possibly prejunctional B_1 receptors, whereas activation of B_2 receptors increases twitch contractions, in part by amplifying the responsiveness of the smooth muscle cells to the sympathetic co-transmitter ATP.

Keywords: Bradykinin; B_1 receptors; B_2 receptors; mouse vas deferens; neurotransmission; bradykinin antagonist; Hoe 140; NPC 17731

Introduction

In addition to its widespread biological effects (for reviews see Bhoola *et al.*, 1992; Hall, 1992), bradykinin (BK) can modify neurotransmission at certain autonomic neuroeffector junctions of the urogenital tract. Thus, BK potentiates neurogenic contractions of the rat isolated urinary bladder, possibly by increasing the responsiveness of smooth muscle cells to the sympathetic co-transmitter ATP (Acevedo *et al.*, 1990). Similarly, the peptide increases responses to electrical field stimulation of the vas deferens isolated from guinea-pigs (Zetler & Kampmann, 1979) or rats (Huidobro-Toro *et al.*, 1986; Tousignant *et al.*, 1987). In the rat vas deferens, BK also augments the release of [^{3}H]-noradrenaline, potentiates the contraction to ATP, but not noradrenaline, and causes smooth muscle contraction (Llona *et al.*, 1987; 1991; Donoso *et al.*, 1989). These effects appear to be mediated via stimulation of B_2 receptors (Llona *et al.*, 1987; Rifo *et al.*, 1987). Moreover, BK also triggers the release of adrenaline from adrenochromaffin cells and of noradrenaline from sympathetic ganglia (Collier, 1970).

Llona *et al.* (1991) reported that BK also enhances noradrenaline release from sympathetic nerves of the mouse vas deferens, but the effects of BK on neurotransmission in this preparation were not fully characterized. Sympathetic neurotransmission in the mouse vas deferens is accomplished via the release of the co-transmitters ATP, noradrenaline and, possibly at higher discharge frequencies, neuropeptide Y (Stjärne *et al.*, 1986; von Kügelgen *et al.*, 1989; Drake & Petersen, 1992). Neurogenic twitch contractions of this preparation can be modulated by a wide variety of mediators, being depressed by α_2-adrenoceptor agonists (Illes & Starke, 1983), purinoceptor agonists (Kurz *et al.*, 1993), opioids (Lord *et al.*, 1977), neuropeptide Y (Stjärne *et al.*, 1986) or calcitonin-gene-related peptide (Manzini & Parlani, 1992), while being increased by endothelins (Rae & Calixto, 1990) or tachykinins (Manzini & Parlani, 1992).

The aim of this study was to characterize, by the use of selective B_1 and B_2 receptor agonists and antagonists, the receptors mediating the effect of BK and related kinins on responses of the mouse vas deferens to electrical field stimulation. We have found that, unlike the vasa deferentia from other species, the mouse preparation displays both B_1 and B_2 receptors which modulate sympathetic neurotransmission in distinct ways.

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Methods

Tissue preparations

Experiments were conducted on male Swiss albino mice (25–35 g), raised in a temperature-controlled (22 ± 2°C) environment with 12 h light/dark cycle, and allowed free access to water and Purina lab chow. Animals were lightly anaesthetized with ether and killed by a sharp blow to the head and cervical dislocation. Both vasa deferentia were carefully excised, placed in a Petri dish containing warm physiological salt solution (see composition below) and freed of adhering connective and adipose tissues. Each vas deferens was set up in 5 ml organ baths containing Krebs solution at 37°C continuously gassed with 95% of O_2 and 5% of CO_2, connected to a strain gauge transducer coupled to a pen recorder (Narco Biosystems, U.S.A.) and submitted to an initial resting tension of 0.5 g. The Krebs solution had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 0.9 and glucose 11 (pH 7.2–7.4). Unless otherwise stated, the Krebs solution also contained captopril (3 μM) to prevent the action of kininase II. A stabilization period of at least 60 min was allowed before drug additions, during which the bath solution was renewed every 15 min. Electrical field stimulation was induced with trains of 4 rectangular 0.5 ms pulses of supramaximal strength (65–70 V) delivered, every 20 s, via a pair of platinum electrodes consisting of a hook below and a ring above each preparation.

Concentration-response curves to bradykinin and related peptides on twitch tension

Following stabilization of the twitch contractions evoked by electrical field stimulation, complete non-cumulative concentration-response curves were obtained to the twitch-potentiating effects of BK, Lys-BK, Met,Lys-BK, [Tyr(Me)^8]-BK or [Hyp^3]-BK, and to the twitch-depressor effect of [des-Arg^9]-BK (0.1 to 300 nM). Each agonist concentration was added to the medium for 2 min followed by 5 renewals of the bathing medium. Electrical field stimulation was interrupted just prior to washout of each agonist concentration and re-initiated 5 min before addition of the next agonist concentration.

Effects of B_1 and B_2 selective receptor antagonists on responses to bradykinin and des-Arg^9-bradykinin

Complete concentration-response curves were obtained for the twitch-potentiating effect of BK (0.1 to 300 nM) in the absence or in the presence of different concentrations of the selective B_2 receptor antagonists, Hoe 140 (1 to 10 nM) or NPC 17731 (3 to 30 nM), incubated with the tissues 5 min before each addition of BK. Only one complete concentration-response curve was carried out for BK in each preparation. Control experiments were carried out in the presence of the vehicle used to dilute the antagonists (phosphate buffer solution, PBS). The apparent pA_2 values (i.e. the co-logarithm of an antagonist's equilibrium dissociation constant or of its K_B) for Hoe 140 and NPC 17731 against BK-induced potentiation of neurogenic contractions were estimated by the rightward shifts they caused, at different concentrations, in the concentration-response curve to BK. The apparent pA_2 value for each antagonist was assessed from a Schild plot of log EC_{50} concentration-ratios minus 1 (CR − 1) versus log concentration of the antagonist, calculated by least square regression analysis (Arunlakshana & Schild, 1959). Antagonism was considered to be competitive in nature if the slope of the Schild regression line did not significantly differ from unity (Kenakin, 1993). Other experiments were performed to evaluate the influence of the selective B_1 receptor antagonist, [Leu^8,des-Arg^9]-BK on responsiveness to BK, in which 1 μM of the antagonist was added to the bathing medium 5 min before each addition of BK.

The effects of [Leu^8,des-Arg^9]-BK (1 μM), as well as those of Hoe 140 (100 nM) or NPC 17731 (100 nM) against the twitch-depressor response to [des-Arg^9]-BK (300 nM) were also assessed. Following a first control challenge with [des-Arg^9]-BK, each antagonist was added to the bathing medium for 5 min and a second challenge with the agonist conducted in its presence. The specificity of the actions of Hoe 140 and NPC 17731 against BK-induced responses was confirmed by testing their influence on twitch potentiations induced by equieffective concentrations of BK (300 nM), Lys-BK (300 nM), substance P (3 nM) or endothelin-1 (1 nM). The specificity of the action of [Leu^8,des-Arg^9]-BK was analysed by testing its influence against twitch inhibition by clonidine (1 nM) or morphine (300 nM). The preparations were usually challenged twice with a given agonist, first in the absence (control) and then in the presence of the antagonist, 30 min later. However, due to the very slow reversibility of endothelin-1-induced twitch potentiation in the mouse vas deferens (Rae & Calixto, 1990), both vasa deferentia from a given animal were each challenged only once with the agonist, one of them in the presence of the B_2 receptor antagonist.

Effects of bradykinin and [des-Arg^9]-bradykinin on contractions induced by noradrenaline and ATP

In other experiments, preparations not subjected to electrical field stimulation were challenged repeatedly, for 1 min at 30 min intervals, with ATP (100 μM) or noradrenaline (10 μM), at concentrations causing contractions similar in size to those evoked by electrical field stimulation. Once the responses stabilized (usually after 2–3 challenges), BK (100 nM) was added to the medium for 30–45 s and a new response to the agonist was obtained in its presence. A final challenge with ATP was also conducted in the presence of BK plus [Leu^8,des-Arg^9]-BK (1 μM), Hoe 140 or NPC 17731 (each at 100 nM), each added 5 min prior to BK. The effects of [des-Arg^9]-BK (300 nM) on responses to both ATP and noradrenaline were also tested. Only one contractile agonist, one kinin and one kinin receptor antagonist were tested in each preparation. All tissues were blotted and weighed after each experiment.

Statistical analysis

Agonist-induced changes in magnitude of twitch responses to electrical field stimulation are expressed as mean ± s.e.mean increases or decreases of twitch tension in mg per 10 mg of wet tissue, relative to the last basal twitch response prior to drug addition. E_{max} and I_{max} indicate maximal increase and maximal decrease of twitch tension induced by a given agonist, respectively. EC_{50} and IC_{50} values in individual experiments (i.e. the concentrations of agonist needed to cause half maximal potentiation or inhibition of twitch responses) were obtained by graphical interpolation and are presented as geometric means accompanied by their 95% confidence limits (Fleming *et al.*, 1972). The apparent pA_2 values are shown as mean ± s.e.mean and the slopes of the Schild plot regression lines as means accompanied by their 95% confidence limits (Kenakin, 1993). Statistical comparisons were performed by analysis of variance followed by Student's t test for paired or unpaired samples, where appropriate, and $P < 0.05$ was considered significant.

Drugs

Drugs used were: BK, Lys-BK, Met,Lys-BK, [Tyr(Me)^8]-BK, [Hyp^3]-BK, [Leu^8,des-Arg^9]-BK, substance P, captopril, clonidine hydrochloride, tetrodotoxin, guanethidine sulphate, ATP disodium, α,β-methylene ATP lithium, noradrenaline

bitartrate and prazosin hydrochloride (all from Sigma Chemical Company, St. Louis, U.S.A.), [des-Arg^9]-BK (Peninsula, Belmont, U.S.A.), endothelin-1 (Peptide Institute, Osaka, Japan), morphine hydrochloride (Merck A.G., Darmstadt, Germany). Hoe 140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8-BK) and NPC 17731 {D-Arg-[Hyp^3,D-Hyp^E(transpropyl)^7,Oic^8]-BK} were kindly given by Hoechst A.G. (Frankfurt, Germany) and Scios/Nova (Baltimore, U.S.A.), respectively. Most stock solutions were made up in PBS, except those of prazosin and noradrenaline, which were made up in 100% ethanol and 0.1 N HCl, respectively. Stock solutions (10 μM to 100 mM) were stored at −18°C and diluted to the desired concentrations with PBS just prior to use. Ethanol did not modify twitch tension or noradrenaline-induced contractions at a concentration of 0.01% (final concentration in the medium when applying prazosin).

Results

Characterization of twitch contractions induced by electrical field stimulation

Contractions of the mouse vas deferens induced by electrical field stimulation, which averaged 539 ± 11 mg of tension per 10 mg of wet tissue ($n = 60$), were of neurogenic and sympathetic origin, as they were abolished by tetrodotoxin (100 nM) or guanethidine (5 μM, $n = 6$ for each; results not shown). Twitch contractions were also fully suppressed following desensitization of P_{2x}-purinoceptors with α,β-methylene ATP (10 μM), but were only partially inhibited (ca. 25%) by the α_1-adrenoceptor blocker, prazosin (100 nM, $n = 6$; results not shown).

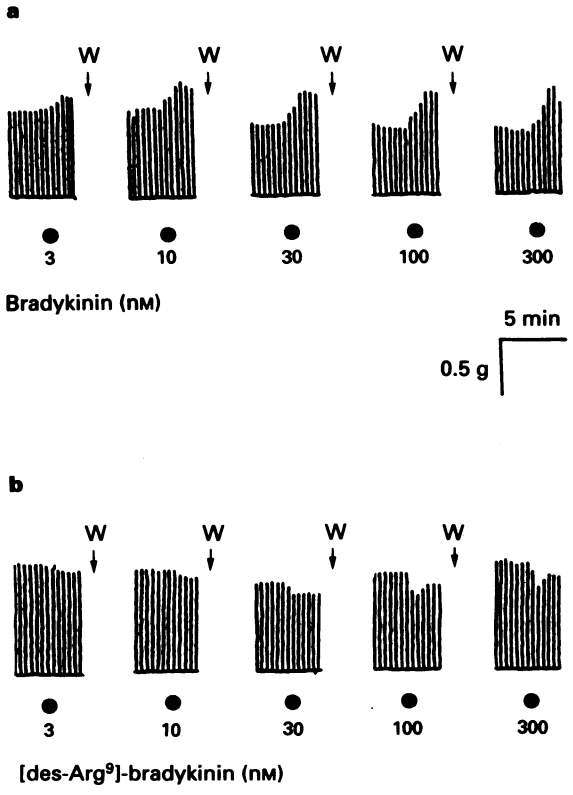

Figure 1 Typical isometric recordings showing the effect of bradykinin (a) and [des-Arg^9]-bradykinin (b) on twitch contractions induced by electrical field stimulation of the mouse isolated vas deferens. Agonists were added to the bathing medium (●) at the concentrations indicated, for 2 min at 30 min intervals. W indicates washout of the agonist. Similar recordings were obtained in another 3 to 5 experiments.

Effects of bradykinin and related peptides on neurogenic contractions

Non-cumulative addition of BK (0.1 nM to 3 μM) caused concentration-dependent potentiations of twitch contractions of the vas deferens (Figures 1 and 2). In the presence of captopril (3 μM), the EC_{50} for this effect was 4.5 nM (1.7–11.6) and the E_{max} 315 ± 19 mg per 10 mg of wet tissue (Table 1). Moreover, repeated additions of BK (30 nM), at 1 h intervals, induced reproducible increases of twitch contractions for up to 8 h after setup ($n = 3$; results not shown). In experiments carried out in the absence of captopril, the potency of BK was reduced 9 fold (EC_{50} 42.9 nM, 29.2–62.8; E_{max} 307 ± 32; $n = 6$). Potentiations of neurogenic twitch contractions were also observed in response to Lys-BK, [Hyp^3]-BK, Met,Lys-BK and the selective B_2 receptor agonist, [Tyr(Me)^8]-BK (0.1 nM to 1 μM), all of which exhibited EC_{50}s and E_{max}s similar to those of BK (Table 1).

In sharp contrast, the selective B_1 receptor agonist, [des-Arg^9]-BK (0.3 to 100 nM) caused concentration-dependent inhibitions of twitches (Figures 1 and 2). The IC_{50} for this effect of [des-Arg^9]-BK was 13.7 nM (10.4–16.1) and the I_{max} 171 ± 11 (Table 1). In other experiments, successive challenges with [des-Arg^9]-BK (300 nM) at 1 h intervals, starting 1 h after setup, induced reproducible depressions of responses to electrical field-stimulation for up to 8 h ($n = 4$; results not shown).

Effects of selective B_1 and B_2 receptor antagonists on responses to bradykinin and [des-Arg^9]-bradykinin

Prior incubation with the selective B_2 receptor antagonists, Hoe 140 (1 to 10 nM) or NPC 17731 (1 to 30 nM), caused parallel rightward displacements of the curve to BK-induced twitch potentiation, yielding apparent pA_2 values (mean ± s.e.mean) of 9.65 ± 0.09 and 9.08 ± 0.13, respectively (Figure 3). The slope values (mean and 95% confidence limits) of the Schild plot regression lines were not statistically different from unity [Hoe 140 1.1 (0.9–1.4); NPC 17731 0.9 (0.8–1.1)]. Neither antagonist displayed partial agonist activity up to 100 nM ($n = 5$; results not shown). Moreover, Hoe 140 and NPC 17731 (100 nM) did not affect twitch potentiations induced by substance P (3 nM) or endothelin-1

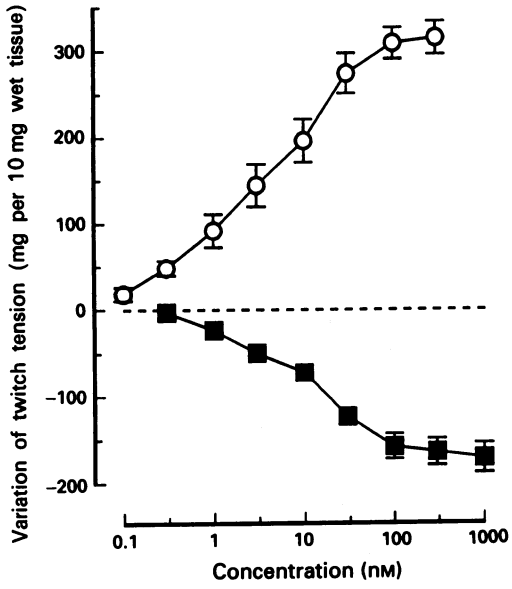

Figure 2 Mean concentration-response curves for the potentiating effect of bradykinin (○) or the inhibitory effect of [des-Arg^9]-bradykinin (■) on twitch contractions evoked by electrical field stimulation of the mouse isolated vas deferens. Values are mean ± s.e.mean of 4 to 6 experiments.

Table 1 Effects of kinins on contractions of the mouse vas deferens induced by electrical field-stimulation

Agonist	*Condition*	n	EC_{50} (nM)[a]	E_{max}[b]
BK	Alone	6	42.9 (29.2–62.8)	+307 ± 32
	+ Captopril 3 μM	6	4.5 (1.7–11.6)	+315 ± 19
Lys-BK	+ Captopril 3 μM	6	5.0 (3.3–7.4)	+332 ± 36
[Tyr(Me)^8]-BK	+ Captopril 3 μM	6	9.5 (5.7–15.7)	+367 ± 50
Met,Lys-BK	+ Captopril 3 μM	6	2.1 (1.0–3.1)	+292 ± 34
[Hyp^3]-BK	+ Captopril 3 μM	6	2.1 (1.1–3.7)	+268 ± 29
[des-Arg^9]-BK	+ Captopril 3 μM	4	13.7 (10.4–16.1)[§]	−171 ± 11[§§]

[a]Geometric means accompanied by 95% confidence limits. [b]Changes in twitch contraction amplitude, expressed in mg of tension per 10 mg wet tissue. [§] IC_{50} for twitch depression. [§§] I_{max} for twitch depression.

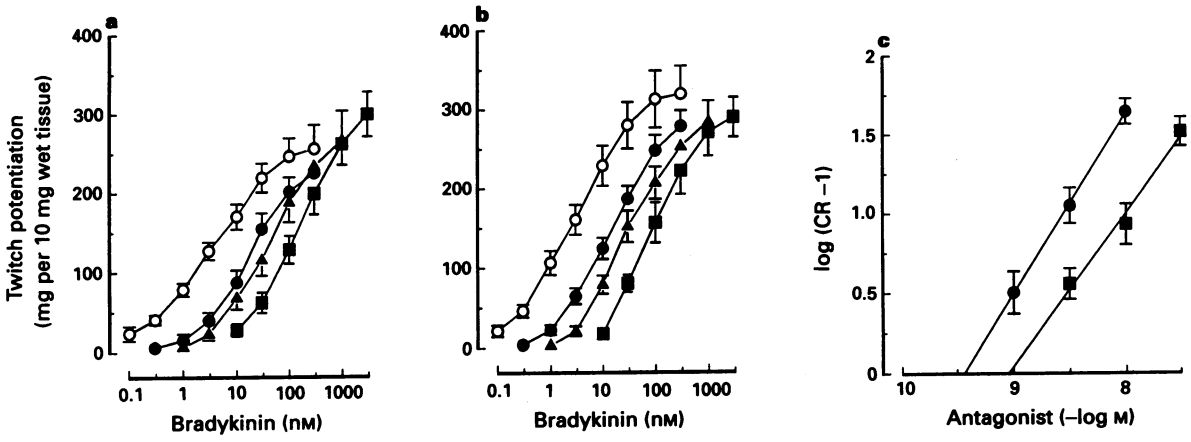

Figure 3 Influence of the selective B_2 receptor antagonists, Hoe 140 and NPC 17731, on bradykinin-induced potentiation of twitch contractions evoked by electrical field stimulation in the mouse isolated vas deferens. Mean concentration-response curves for bradykinin in the absence (○) or in the presence of (a) Hoe 140: 1 (●), 3 (▲) or 10 nM (■), or of (b) NPC 17731: 3 (●), 10 (▲) or 30 nM (■). Panel (c) shows the Schild plots for the blockade of bradykinin-induced twitch potentiation by Hoe 140 (●) or NPC 17731 (■). CR indicates the ratio of EC_{50}s obtained in the presence and absence of the antagonist. Values are mean ± s.e.mean of 6 experiments.

endothelin-1 (1 nM), but virtually abolished similar responses induced by BK (300 nM) or Lys-BK (300 nM) (Figure 4). It is important to point out, however, that neither of these B_2 receptor antagonists, at 100 nM (Figure 4) or at 1 μM ($n = 3$; results not shown), actually reversed the potentiating effect of BK into a depressor effect on twitch contractions, i.e. they did not unmask an inhibitory effect of the agonist.

As shown in Figure 5, the selective B_1 receptor antagonist [Leu^8,des-Arg^9]-BK (1 μM) significantly potentiated the effects of higher concentrations of BK (30 to 300 nM), i.e. induced further augmentation of neurogenic contractions. Furthermore, [Leu^8,des-Arg^9]-BK (1 μM) abolished the inhibition of twitch contractions of the mouse vas deferens caused by [des-Arg^9]-BK (300 nM) (Figure 6), without modifying similar twitch depressions induced by clonidine (1 nM; control 429 ± 38, plus antagonist 394 ± 48; $n = 4$) or morphine (300 nM; control 246 ± 37, plus antagonist 251 ± 26; $n = 3$). On the other hand, twitch depression induced by des-Arg^9-BK (300 nM) was not modified by Hoe 140 (30 nM) or NPC 17731 (100 nM) (Figure 6).

Effects of bradykinin and [des-Arg^9]-bradykinin on contractions induced by ATP and noradrenaline

In non-stimulated preparations, BK (100 nM) increased contractions induced by ATP (100 μM), but did not alter responses to noradrenaline (10 μM; Figure 7). The potentiation of ATP-induced contractions by BK was abolished by preincubation with the selective B_2 receptor antagonists, Hoe 140 (10 nM) or NPC 17731 (30 nM), whereas it was not affected by the selective B_1 receptor antagonist, [Leu^8,des-Arg^9]-BK (1 μM; $n = 3$ in each case; Figure 7a). In contrast to BK, [des-Arg^9]-BK did not modify contractions induced by ATP or noradrenaline (Figure 7b).

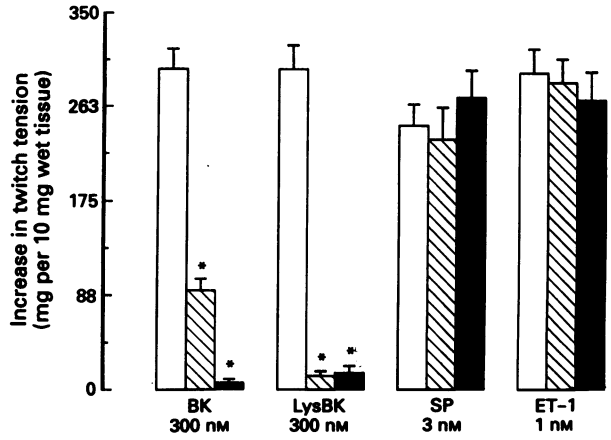

Figure 4 Influences of the selective B_2 antagonists Hoe 140 (100 nM) and NPC 17731 (100 nM) on the potentiation of contractions evoked by electrical field stimulation of the mouse vas deferens induced by bradykinin (BK, 300 nM), Lys-bradykinin (300 nM), substance P (SP, 3 nM) or endothelin-1 (ET-1, 1 nM). Values shown represent responses in the absence (open columns) or presence of Hoe 140 (hatched columns) or NPC 17731 (solid columns) and are the mean ± s.e.mean of 6 experiments. Significance of difference from control value: * $P < 0.05$ (ANOVA followed by Student's paired t test).

Discussion

The present results demonstrate that BK and related peptides modulate sympathetic neurotransmission in the mouse vas deferens in distinct ways, depending on the selectivity of the

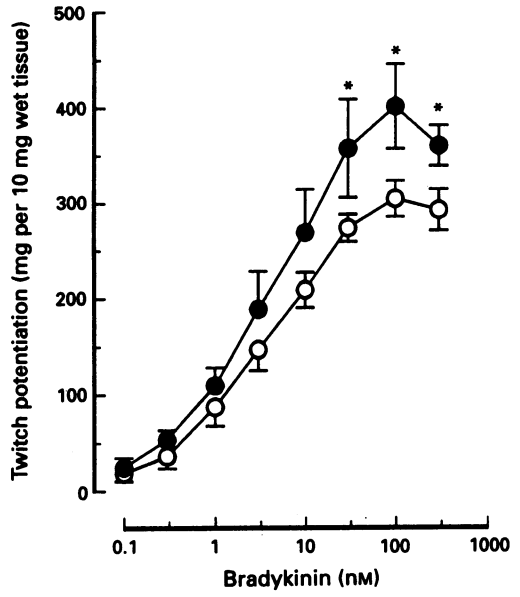

Figure 5 Influence of the selective B_1 receptor antagonist, [Leu^8,des-Arg^9]-BK, on the potentiation of contractions evoked by electrical field stimulation of the mouse vas deferens induced by bradykinin. Mean concentration-response curves for bradykinin in the absence (○) or presence (●) of [Leu^8,des-Arg^9]-BK (1 μM, added 5 min prior to each agonist addition). Values are mean ± s.e.mean of 5 experiments. Significance of difference from control value: * $P<0.05$ (ANOVA followed by Student's paired t test).

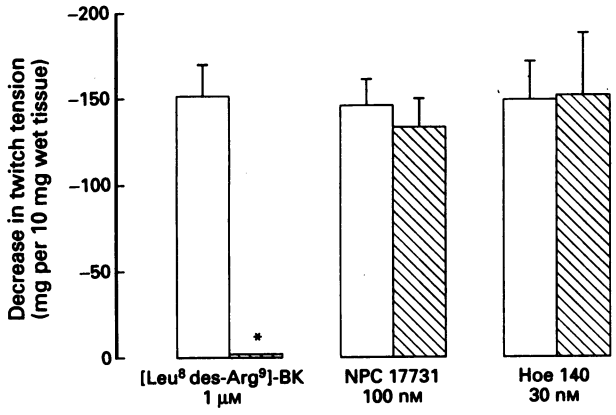

Figure 6 Influence of the selective B_1 receptor antagonist, [Leu^8,des-Arg^9]-BK and of the selective B_2 receptor antagonists, Hoe 140 and NPC 17731, on the inhibitory effect of [des-Arg^9]-bradykinin (300 nM) on twitch contractions induced by field stimulation of the mouse vas deferens. Responses to [des-Arg^9]-bradykinin were obtained in the absence (open columns) or presence (hatched columns) of an antagonist, added 5 min before at the concentration indicated. Values are mean ± s.e.mean of 5 experiments. Significance of difference from control value. * $P<0.001$ (ANOVA followed by Student's paired t test).

agonist towards B_1 or B_2 receptors. Thus, BK, Lys-BK, [Hyp^3]-BK, Met,Lys-BK and the selective B_2 receptor agonist [Tyr(Me)^8]-BK all caused pronounced potentiation of twitch responses, with potencies comparable to those found in other tissues known to express B_2 receptors (Regoli & Barabé, 1980; Bhoola *et al.*, 1992; Hall, 1992). On the other hand, the selective B_1 receptor agonist, [des-Arg^9]-BK, which is inactive in the rat vas deferens (Llona *et al.*, 1987; Asghar *et al.*, 1993), caused a graded inhibition of neurogenic responses of the mouse vas deferens. Another difference between the two species concerns the musculotropic action of BK, which is virtually non-existent in the mouse vas deferens, but robust in the rat vas deferens, particularly in the epididymal portion

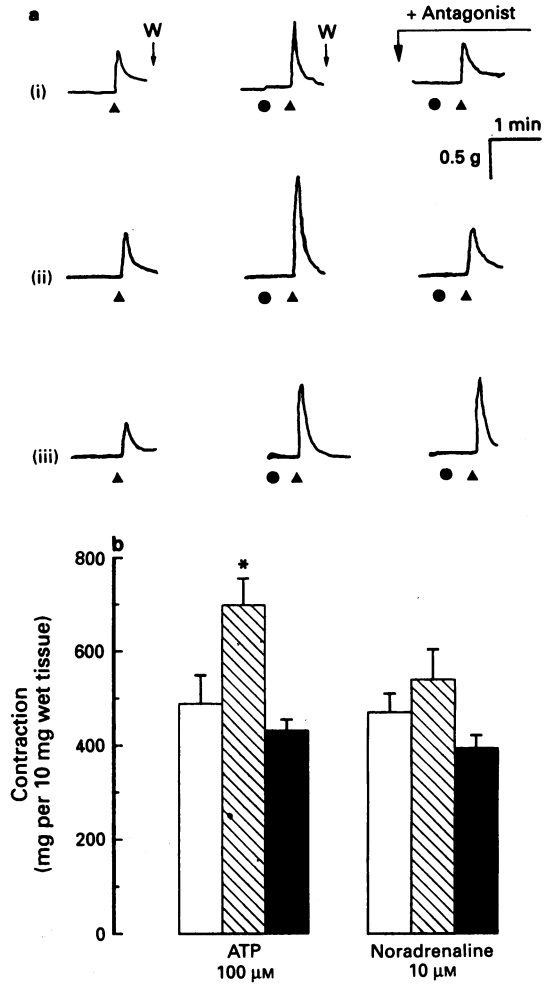

Figure 7 Effects of bradykinin and [des-Arg^9]-bradykinin on contractions induced by ATP and noradrenaline in non-stimulated mouse vas deferens. Panel (a) shows typical isometric recordings of the contractions induced by ATP (100 μM; ▲) alone, in the presence of bradykinin (100 nM added 30–45 s beforehand; ●), and in the presence of bradykinin plus Hoe 140 (10 nM; *i*), NPC 17731 (30 nM; *ii*) or [Leu^8,des-Arg^9]-bradykinin (1 μM; *iii*). Antagonists were added to the medium 5 min before bradykinin. Challenges with ATP were conducted at 30 min intervals and W indicates washout. Similar results were obtained in at least 2 additional experiments of each kind. Panel (b) shows contractions induced by ATP (100 μM) and noradrenaline (10 μM) in the absence (open columns) or in the presence of bradykinin (100 nM; hatched columns) or [des-Arg^9]-bradykinin (300 nM; solid columns). Values are mean ± s.e.mean of 4 to 7 experiments. Significance of difference from control value: * $P<0.05$ (ANOVA followed by Student's paired t test).

(Huidobro-Toro *et al.*, 1986; Rifo *et al.*, 1987; Llona *et al.*, 1987; Donoso & Huidobro-Toro, 1989; Asghar *et al.*, 1993).

The view that BK potentiates neurogenic twitches via B_2 receptors is further substantiated by results showing that Hoe 140 and NPC 17731, two highly potent and selective B_2 receptor antagonists (Hock *et al.*, 1991; Lembeck *et al.*, 1991; Kyle & Burch, 1992; 1993; Burch *et al.*, 1993; Corrêa & Calixto, 1993), induced graded rightward displacements of the twitch potentiation curve to BK without modifying the agonist's E_{max}. Furthermore, Schild regression analysis yielded straight lines with slopes not statistically different from unity, suggesting that BK and both antagonists were interacting with a homogeneous population of receptors, and that Hoe 140 and NPC 17731 act in purely competitive fashion (Kenakin, 1993). Blockade of B_2 receptors with Hoe 140 and NPC 17731 in some tissues is characterized by rightward displacement of the curve to BK allied to a

marked depression of the maximum response (Rhaleb *et al.*, 1992; Griesbacher & Lembeck, 1992; Field *et al.*, 1992; Trifilieff *et al.*, 1993). This discrepancy may be explained by the fact that neither Hoe 140 nor NPC 17731 display any partial agonist activity in the mouse vas deferens. Alternatively, it may result from the different antagonist incubation periods employed. The actions of Hoe 140 and NPC 17731 against responses to BK (and Lys-BK) were clearly specific, as, at concentrations 100 to 300 fold higher than their respective apparent K_B values for B_2 receptors, they did not affect similar twitch potentiations induced by endothelin-1 or substance P.

To our knowledge, the present study is the first to characterize successfully functional B_2 receptors in a murine tissue by use of highly selective antagonists. The apparent pA_2 value for Hoe (9.65) against BK-induced twitch potentiation in the mouse vas deferens is 10 fold larger than that found in an analogous study in the rat vas deferens (Asghar *et al.*, 1993), but is in the same general range found in other tissues expressing B_2 receptors, including guinea-pig and human ileum, rabbit jugular vein, human and hamster urinary bladder and guinea-pig taenia caeci (Hock *et al.*, 1991; Perkins *et al.*, 1991; Field *et al.*, 1992; Hall *et al.*, 1992; Rhaleb *et al.*, 1992; Griesbacher & Lembeck, 1992; Medeiros & Calixto, 1993). The same applies to the apparent pA_2 value we obtained for NPC 17731 (9.08), which is similar to that found in guinea-pig ileum (Kyle & Burch, 1992). Very recently, Hess *et al.* (1994) reported that cloned murine and human B_2 receptors, expressed in Chinese hamster ovary cells, share the same receptor-coupling mechanisms and exhibit similar affinities for B_2 receptor agonists, yet differ markedly in their affinities for B_2 receptor antagonists. The authors concluded that both species express a single B_2 receptor, despite the differences in antagonist binding which are possibly due to species differences in the gene encoding the receptor, as has been shown for other receptor types (Hall *et al.*, 1993).

The kinin receptor mediating the twitch-depressor effect of [des-Arg^9]-BK in the mouse vas deferens is most probably of the B_1 type, as it was resistant to blockade by Hoe 140 or NPC 17731, but was inhibited by the selective B_1 receptor antagonist, [Leu^8,des-Arg^9]-BK, which did not influence twitch depressions induced by clonidine or morphine. Unfortunately, it was not possible to determine the apparent pA_2 value for this effect of [Leu^8,des-Arg^9]-BK, because the relatively small maximal inhibitory response to [des-Arg^9]-BK led to considerable variability in the extent of blockade afforded by low concentrations of the antagonist. The finding that [Leu^8,des-Arg^9]-BK enhanced slightly, but significantly, the potentiation of twitch contractions induced only by high concentrations of BK suggests that the inhibitory B_1 receptors present in the mouse vas deferens display low affinity for BK. Furthermore, the contribution of such B_1 receptors to the overall effect of BK appears to be modest, as BK-induced twitch depressions could not be observed even in presence of a large concentration of the B_2 receptor antagonists, Hoe 140 or NPC 17731 (up to 1 μM).

At least part of the B_2 receptors mediating twitch potentiation appear to be located postjunctionally, as BK also potentiated the responses to an equieffective concentration of exogenous ATP, but not noradrenaline. Furthermore, this effect was also blocked by NPC 11731 and Hoe 140, but not by [Leu^8,des-Arg^9]-BK. Nevertheless, B_2 receptors may also be present on sympathetic nerve terminals of the mouse vas deferens, since BK enhances the electrically-evoked release of [^{3}H]-noradrenaline in this preparation (Llona *et al.*, 1991). Conversely, the location of the B_1 receptors mediating twitch inhibition seems to be mainly prejunctional as [des-Arg^9]-BK failed to affect contractions induced by either ATP or noradrenaline.

Although B_1 receptors in most tissues appear to be inducible (for review see Marceau & Regoli, 1991; Hall, 1992), a variable but significant proportion of constitutive B_1 receptors has been proposed to exist in the rat duodenum (Boschcov *et al.*, 1984), stomach fundus (Calixto & Medeiros, 1992) and portal vein (Campos & Calixto, 1994). It is tempting to propose that both B_1 and B_2 receptors in the mouse vas deferens are constitutive, as responses mediated by these receptor types were obtained immediately after the end of the equilibration period and remained reproducible for up to 8 h after setup. However, the present evidence is still insufficient to make such a proposal.

In conclusion, we have shown that, in contrast to what has been reported in rat vas deferens, kinins exert both facilitatory and inhibitory influences on sympathetic neurotransmission in the mouse vas deferens. While the facilitatory influence seems to involve both pre- and postjunctional B_2 receptors, twitch inhibition is mediated by stimulation of B_1 receptors, which are presumably prejunctional. Further studies are in progress to clarify the cellular mechanisms triggered by activation of both types of kinin receptors and their possible relevance for transmission at this autonomic neuromuscular junction.

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Different effects of salmeterol, formoterol and salbutamol on cholinergic responses in the ferret trachea

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1 In the present study, the inhibitory effects of the selective β_2-adrenoceptor agonists, salmeterol, formoterol and salbutamol, have been investigated on contractions of ferret trachea induced both by endogenous and exogenous acetylcholine. The aim of the study was to evaluate quantitative and/or qualitative differences in response which may indicate both pre-and post-junctional sites of action. The non-selective β-antagonist, sotalol, was used to estimate β-adrenoceptor involvement.

2 Isometric tension was measured in ferret isolated tracheal strips. The inhibitory effects of the drugs were studied on tonic contractions induced by pre-junctional activation with electrical field stimulation (EFS) (2 Hz, 700 mA) or post-junctional activation with exogenous acetylcholine (ACh) (0.5 μM, about EC_{80}), giving a similar degree of smooth muscle response.

3 Concentration-response experiments were performed with formoterol (0.3 nM–0.3 μM) and salmeterol and salbutamol (10 nM–10 μM). The experiments ended with the addition of sotalol (10 μM).

4 All three β-agonists inhibited the contractions in a concentration-dependent manner. Salbutamol, formoterol and salmeterol inhibited the EFS-induced contractions by 66(8)%, 105(5)% and 103(8)% (mean(s.e.mean)) respectively. ACh-induced contractions were inhibited by 37(6)%, 72(11)% and 33(8)%. Theophylline (10 nM–3 mM) inhibited the contractions to the same degree.

5 β-Adrenoceptor blockade by sotalol significantly antagonized the inhibitory effects of salbutamol and formoterol on both EFS- and ACh-induced contractions. The effect of salmeterol on ACh-induced contraction was also significantly antagonized, whereas the inhibition of EFS-induced contraction was virtually unaffected.

6 In conclusion, salbutamol, salmeterol and formoterol produced greater inhibitory effects in preparations contracted by EFS than in preparations contracted by exogenously-added ACh. In the case of formoterol and salbutamol, the effects on both levels are most probably due to β-adrenoceptor stimulation, whereas for salmeterol the dominant pre-junctional effect is probably not mediated via β-adrenoceptors. This non-β-mediated effect could represent an additional relaxant mechanism for salmeterol.

Keywords: β-Agonists; cholinergic responses; pre-junctional β-adrenoceptors; salmeterol; formoterol; salbutamol

Introduction

Two long-acting β-adrenoceptor agonists, salmeterol and formoterol, have recently been developed. The duration of the bronchodilator effect in human subjects is about 12 h compared with 4–6 h for earlier available β-agonists. The mechanism for the long duration is not fully understood and could be explained by a combination of properties. Lipophilicity is probably one important factor (Anderson *et al.*, 1994), but other properties, such as binding to an exosite close to the receptor have also been suggested (Ball *et al.*, 1991).

Lipophilicity is probably also important for the unspecific, non-β-mediated effects which has been shown for β-adrenoceptor ligands (Ijzerman *et al.*, 1987). In the case of salmeterol, several observations have been published showing non-β-mediated effects in both the airway smooth muscle and in other cells (Baker & Fuller, 1990; Jeppsson *et al.*, 1992; Lindén *et al.*, 1993; Coleman *et al.*, 1994; Nials *et al.*, 1994). For formoterol and salbutamol, there are no data concerning non-β-mediated effects.

The bronchodilator effect for β-agonists has been attributed to the stimulation of β-adrenoceptors on the smooth muscle. However, in several functional *in vitro* studies it has been suggested that β-agonists also exert a pre-junctional effect on cholinergic nerves, leading to a reduced output of acetylcholine (Vermeire & Vanhoutte 1979; Baker & Don 1987; Danser *et al.*, 1987; Ito, 1988; Rhoden *et al.*, 1988; Janssen & Daniel, 1990).

In the present study, we evaluated the relaxant effects of salmeterol and formoterol in the ferret isolated trachea. Possible non-β-mediated effects were estimated after treatment with the non-selective β-antagonist, sotalol. Contractions were induced either by exogenously added acetylcholine (ACh) or by electric field stimulation (EFS). ACh-induced contractions can only be inhibited by post-junctional mechanisms, whereas EFS-induced contractions can be inhibited by both post-junctional and pre-junctional mechanisms. To facilitate studies of the pre-junctional effects, we chose the ferret trachea, a preparation containing relatively few β_2-adrenoceptors mediating the post-junctional β-mediated relaxation (Skoogh *et al.*, 1989).

Thus, the aim of this study was to investigate the inhibitory effects of salmeterol, formoterol and salbutamol on cholinergic nerve-induced contractions (EFS) and exogenously-added ACh contractions in order to evaluate differences concerning the magnitude of the effects as well as to what extent these effects are β-adrenoceptor mediated.

Methods

The study was approved by the Animal Ethics Committee at the Medical Faculty at Göteburg University (DNr:260/88).

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Preparation

Eighteen male ferrets were killed instantly by electrocution and then exsanguinated. The trachea was rapidly removed and immediately immersed in oxygenated Krebs-Ringer (KR) solution with the following composition (in mM): NaCl 118, KCl 5.9, $CaCl_2$ 2.5, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25.5 and glucose 5.6. Ascorbic acid (0.3 mM) was added to the KR as an anti-oxidant. The trachea was dissected free and cut into 8 rings, each 7 mm wide. The rings were opened by cutting the cartilage opposite the smooth muscle and then connected to steel hooks. The epithelium covering the muscle was removed by gentle dissection in order to avoid the influences of epithelium-derived relaxant substances (Ullman *et al.*, 1988; 1990a,b).

The strips were mounted in 7 ml organ baths with KR maintained at 37°C and aerated with 94% O_2-6% CO_2 which produces a pH of 7.4. The baths were constantly flushed with KR, 1.5 ml min^{-1}, during the experiment. The isometric muscle tension was continuously recorded via Grass force transducers (FT03) connected to an NB-MIO-16 analogue/digital converting board and a MacIntosh II computer using the Labview signal-processing software (National Instruments, Austin, Texas, USA) (Ullman *et al.*, 1990a). This system permits the real-time monitoring of the experiment and the simultaneous and continuous saving of data for subsequent evaluation.

Experimental design

The tension was initially set at 8 g. After 60 min the tension was readjusted to 6 g (Skoogh *et al.*, 1982). A tonic contraction was induced either by exogenous acetylcholine (ACh) causing a post-junctional activation or by electric field stimulation (EFS) causing a pre-junctional activation of the cholinergic nerves. ACh was added in an EC_{80} concentration (0.5 μM) according to preliminary experiments and the parameters for EFS were chosen to match this degree of contraction. All the strips were treated with guanethidine (3 μM) (Löfdahl *et al.*, 1986; Brock & Cunnane 1988) to deplete the sympathetic nerves of neurotransmitter, and abolish sympathetic responses to EFS.

EFS was delivered by constant current generators, via rectangular platinum electrodes (5 × 30 mm) mounted parallel to the airway preparations (10 mm apart). Biphasic square waves with a pulse duration of 0.5 ms were applied at a frequency of 2 Hz. The current used was 700 mA. The cholinergic nature of the contractile response was demonstrated in separate experiments by addition of 1 μM atropine which abolished the response.

Thirty minutes after the administration of ACh and the start of EFS, the concentration-response experiments were performed. One animal (8 strips) was used in every experiment, and two treatments of the following four were used. Salmeterol, formoterol, salbutamol or vehicle were chosen randomly in each experiment. Salmeterol has a slow onset of action (Ball *et al.*, 1991; Jeppsson *et al.*, 1992; Ullman *et al.*, 1992) and, to guarantee maximum effect, a one-hour interval between successive administrations of agonist was chosen. This made it impossible to produce the entire cumulative concentration-response curve in the same experiment, and the experiment was therefore divided into two parts each using a separate animal. In the first group, we used 10 nM and 1 μM of salmeterol and salbutamol and 0.3 nM and 30 nM of formoterol. In the second group we used 0.1 μM and 10 μM salmeterol and salbutamol and 3 nM and 0.3 μM formoterol. All experiments ended with the addition of the non-selective β-adrenoceptor antagonist, sotalol (10 μM), to estimate β-adrenoceptor involvement (Figure 1).

In order to ascertain that the contractions induced by EFS and ACh were comparable, and thus equally inhibited by a drug with no pre-junctional effects, we performed concentration-response experiments with theophylline. The EFS and ACh contractions were induced in the manner described above and increasing concentrations of theophylline (10 nM–3 mM) were added cumulatively every 10 min or when the maximum effect was obtained. Isoprenaline (0.1 mM) was finally added to obtain maximum relaxation.

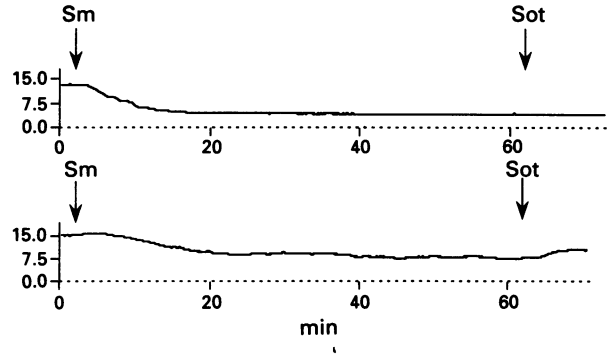

Figure 1 Original recording of the tension (in g) in the ferret tracheal strips. The upper panel shows the electrical-field-stimulation-induced contraction, and the lower panel shows the acetylcholine-induced contraction. Abbreviations: Sm; addition of 10 μM of salmeterol and Sot; addition of 10 μM of sotalol.

Drugs

Acetylcholine chloride (Sigma), atropine sulphate (Sigma), guanethidine sulphate (gift from Ciba-Geigy), (±)-sotalol hydrochloride (gift from Astra Hässle), (±)-isoprenaline hemisulphate (Sigma) and theophylline (Theofyllin inj. 20 mg ml^{-1}; gift from Astra Draco) were dissolved in distilled water. Salmeterol base (racemate) and salbutamol sulphate (gifts from Glaxo) and formoterol fumarate (racemate, pure R- and S-enantiomers; gift from Ciba-Geigy) were initially dissolved in 20 μl of glacial acetic acid and diluted in distilled water and frozen as stock solution.

Data analysis

The drug effects are presented as percentage inhibition of the initial contraction induced by either exogenous ACh or EFS. The concentration-response curves are constructed from the maximum effect seen during the incubation period. All the results are expressed as the mean (s.e.mean). Student's *t* distribution (two-tailed) for differences between data (paired or unpaired) was determined at 95% confidence intervals. The confidence intervals are presented in Table 1 and are referred to by capital letters in the text. *n* equals the number of airway preparations.

Results

There was no statistically-significant difference in the magnitude of the initial contraction in preparations contracted with exogenous ACh (15.9(0.8) g) compared with preparations contracted with EFS (15.1(0.8) g) (Table 1, **A**).

The inhibitory effects of theophylline were similar on contractions induced by EFS and exogenously-added ACh, as shown in the concentration-response curves in Figure 2.

Control strips

The EFS-contracted-preparations showed a slightly (17.9%) lower level of contraction at the end of the experiment than ACh-treated preparations (Table 2), and this small difference was significant (Table 1, **B**). When sotalol was added, there was a small increase in the contractile level, which was significant in EFS-contracted preparations (Table 1, **C**) but not in ACh-contracted preparations (Table 1, **D**). This small

Table 1 Statistical analysis of the results obtained in the study calculated as the difference in the level of inhibition after various treatments

	Comparison	*Mean and (95% confidence intervals) for differences between inhibitions (% inhibition of initial contraction)*	n
A	Degree of initial contraction ACh v. EFS	0.7 (−1.5 to 3.0) NS	61 and 60
B	Control strips ACh v. EFS (60 min after dose 2)	17.9 (11.0 to 24.9)	16
C	Control strips EFS: before v. after addition of sotalol, 10 μM	−8.6 (−13.5 to −3.6)	16
D	Control strips ACh: before v. after addition of sotalol, 10 μM	−3.0 (−6,3 to 0,3) NS	16
E	Salbutamol 10 μM ACh v. EFS	29.3 (7.4 to 51.2)	8
F	Salbutamol 10 μM ACh: before v. after addition of sotalol 10 μM	−35.3 (−21.7 to −49.0)	8
G	Salbutamol 10 μM EFS: before v. after addition of sotalol 10 μM	−43.8 (−34.2 to −53.4)	8
H	Formoterol 0.3 μM ACh v. EFS	33.1 (6.5 to 59.7)	7
I	Formoterol 0.3 μM ACh before v. after addition of sotalol 10 μM	−42.8 (−26.7 to −58.9)	7
J	Formoterol 0.3 μM EFS: before v. after addition of sotalol 10 μM	−58.5 (−44.8 to −72.3)	7
K	Salmeterol 10 μM ACh v. EFS	70.1 (44.8 to 95.4)	7 and 8
L	Salmeterol 10 μM ACh: before v. after addition of sotalol 10 μM	−19.9 (−29.7 to −10.1)	8
M	Salmeterol 10 μM EFS: before v. after addition of sotalol 10 μM	−11.7 (−23.6 to 0.2) NS	7

ACh; acetylcholine-induced contraction, EFS; electric-field-stimulation-induced contraction; NS; non-significant.

effect might be explained by a small amount of residual sympathetic nerve activation in spite of pre-treatment with guanethidine.

Salbutamol

Salbutamol (1−10 μM) caused a concentration-dependent inhibition of the contractions induced both by EFS and by ACh (Figure 3). The inhibition by 10 μM was significantly more pronounced in preparations contracted by EFS (66(8)% inhibition) than in preparations contracted by exogenous ACh (37(6)% inhibition) (Table 1, **E**).

Sotalol (10 μM) at the end of the experiment significantly reduced the salbutamol effect to 22(8)% inhibition in EFS-contracted preparations and totally blocked the effect in exogenous ACh-contracted preparations (1(1)% inhibition) (Figure 3) (Table 1, **F** and **G**).

Formoterol

Formoterol (3−300 nM) also caused a concentration-dependent inhibition of the contractions (Figure 3). At 300 nM, the inhibition in EFS-contracted preparations was total (105(5)%) and in ACh-contracted preparations the inhibition was 72(11)%, which was significantly less than in EFS-contracted preparations (Table 1, **H**).

Sotalol (10 μM) at the end of the experiment, significantly reduced the effect of formoterol to 47(6)% inhibition in EFS-induced contractions and to 29(8)% inhibition in ACh-induced contractions (Figure 3)(Table 1, **I** and **J**).

Salmeterol

Salmeterol (1−10 μM) caused a concentration-dependent inhibition of the EFS-induced contractions, whereas only the highest concentration (10 μM) inhibited ACh-induced contractions (Figure 3). At 10 μM, the inhibition was total (103(8)%) in EFS-contracted preparations, whereas it reached 33(8)% inhibition in ACh-treated preparations. This difference was significant (Table 1, **K**).

Sotalol (10 μM) at the end of the experiment had no significant effect on the salmeterol effect in preparations contracted by EFS (to 91(13)% inhibition), thereby indicating that this effect was not mediated by β-adrenoceptors (Table 1, **M**). In preparations contracted by ACh, however, the salmeterol effect (10 μM) was significantly reduced to 13(4)% inhibition (Table 1, **L**).

Discussion

In the present study we found that the inhibitory effects of salbutamol, formoterol and salmeterol on EFS-induced contractions were greater than those on ACh-induced contractions. Sotalol significantly counteracted the inhibition of all three drugs in ACh-contracted strips. However, in EFS-contracted strips, sotalol only antagonized the inhibition induced by salbutamol and formoterol, in contrast to salmeterol where no significant antagonism was found.

The discrepancy regarding the antagonist effect of sotalol in EFS-contracted preparations is probably an indication of

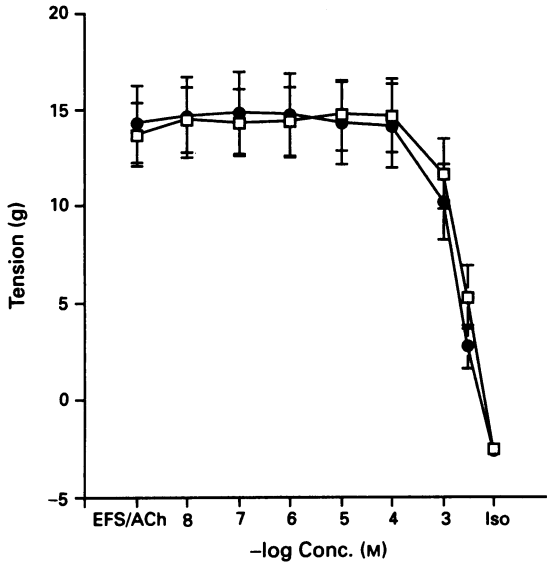

Figure 2 Inhibition of contraction induced by electric field stimulation, EFS (2 Hz, 700 mA) (●), or exogenously-added acetylcholine, ACh (0.5 μM) (□) by cumulatively added concentrations of theophylline. Abbreviations: Iso; 0.1 μM isoprenaline to obtain maximum relaxation.

Table 2 Degree of contraction in control strips without β-agonist treatment, 60 min after the time of β-agonist addition and 5 min after sotalol (10 μM) treatment

	Acetylcholine (% of initial contraction)	*Electric field stimulation* (% of initial contraction)
Dose 1	105.7 (2.1) $n = 16$	98.7 (2.0) $n = 14$
Dose 2	102.1 (1.8) $n = 16$	86.2 (2.9) $n = 16$
Sotalol (10 μM)	107.1 (1.8) $n = 16$	94.8 (2.6) $n = 16$

Values are means with s.e.mean in parentheses.

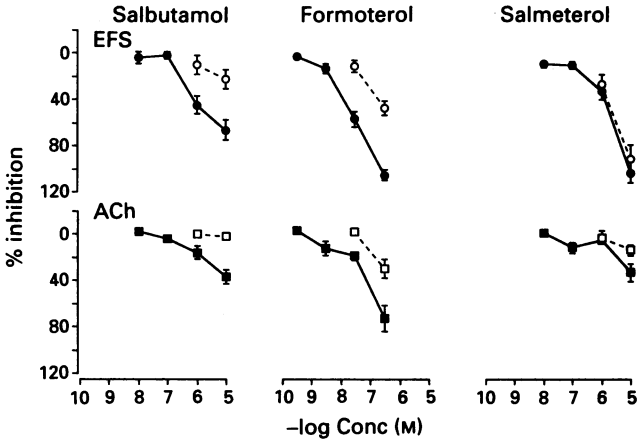

Figure 3 Inhibition of contraction induced by electrical field stimulation (2 Hz, 700 mA) or acetylcholine (0.5 μM), by increasing concentrations of salbutamol, formoterol and salmeterol (filled symbols). Values after sotalol (10 μM) with open symbols.

a non-β-mediated effect of salmeterol. Another possible explanation is that the sotalol concentration was too low to overcome the salmeterol effect. However, we think this is unlikely since theoretical calculations suggest a one log step shift of the salmeterol curve in the presence of 10 μM of sotalol (McKay, 1978). The shift in salmeterol-treated preparations is therefore expected to be similar to the shift seen in formoterol-treated preparations (Figure 3). As this is not the case, the dominant pre-junctional inhibitory effect of salmeterol is probably not mediated by β-adrenoceptors.

A non-β-mediated effect of salmeterol has been described before. In guinea-pig trachea, a sotalol-resistant relaxant effect of salmeterol was noted in both carbachol-induced contraction (Lindén *et al.*, 1993) and nerve-stimulated contraction (Jeppsson *et al.*, 1992; Coleman *et al.*, 1994). Furthermore, Baker & Fuller (1990) found an anti-inflammatory effect on human alveolar macrophages which was not reduced by propranolol.

The origin of the non-β-mediated effect is not known, but it is tentatively suggested that it could be a local anaesthetic effect on the cell membrane. A positive correlation between lipophilicity and local anaesthetic effects for β-adrenoceptor ligands has been demonstrated (Ijzerman *et al.* 1987), and salmeterol is highly lipophilic, as demonstrated in a study in which the octanol/water lipophilic distribution coefficient was measured (Jeppsson *et al.*, 1989a).

The non-β-mediated effect of salmeterol has not been studied in human subjects, normals or asthmatics. Although the concentrations of salmeterol used in these experiments are quite high, the result might still be relevant for man since the local concentration in the lungs after inhalation is also high, although the exact concentration is difficult to determine. Theoretical calculations have suggested concentrations of 0.1–1 mM in the airway lumen (Kerrebijn, 1990). Furthermore, one *in vitro* study involving a tracheal tube preparation showed that the concentration-difference between lumen and tracheal tissue is about tenfold after the intraluminal administration of a lipophilic substance. The concentration in the surrounding bath was close to nil for the lipophilic substance whereas 20% of a hydrophilic substance was found there (Jeppsson *et al.*, 1989b). Thus, *in vitro* studies at least suggest high concentrations in the tissue and an ability of lipophilic substance to remain in the tissue. In our study, inhibitory non-β-mediated effects on the cholinergic nerves were seen with 1 μM of salmeterol. We therefore suggest that this possible mechanism of action for salmeterol should be evaluated in human studies.

In the present study we found that the EFS-induced contractions were more effectively inhibited than the ACh-induced contractions by all the agonists. There was no significant difference in the magnitude of the initial contraction and theophylline inhibited the contractions in a similar way. We have found no reports in the literature about pre-junctional effects of theophylline in addition to its post-junctional effects and our own results do not indicate pre-junctional effects. This argues that the difference between the effects of the β-agonists on responses to EFS and ACh really reflect an additional β-effect on the pre-junctional level.

In our study, we used guanethidine to prevent sympathetic nerve effects. In spite of this, addition of sotalol resulted in a small increase in the response to EFS, indicating that a small remaining sympathetic nerve activation was present. This effect is, however, too small to explain the findings with the β-agonists showing a more pronounced inhibition of the EFS-contraction compared to the ACh-contraction. Besides, if the sympathetic effect were responsible for the difference, the same difference should have been found in the theophylline experiments which was not the case.

The more pronounced inhibition in EFS-contracted preparations indicates a pre-junctional mechanism in addition to the post-junctional β_2-adrenoceptor effect on the smooth muscle. In formoterol- and salbutamol-treated preparations this appears to be a β-mediated mechanism, as it was antagonized by sotalol. In a functional study, it is however, impossible to determine the exact mechanism. It could be directly mediated by β-adrenoceptors on the cholinergic nerves leading to a reduced output of ACh, or alternatively indirectly via release of other endogenous

mediators. The present study indicates, however that β_2-adrenoceptors are involved, since specific β_2-agonists were used, although this does not exclude the existence of a β_1-mediated mechanism as well.

The evidence in favour of a β-mediated inhibitory effect on cholinergic nerves is mostly indirect. Several authors have shown in functional studies in canine isolated airways and human isolated airways that β-agonists inhibit cholinergic nerve-induced contractions in concentrations that have little effect on comparable contractions induced by exogenous acetylcholine (Vermeire & Vanhoutte 1979; Danser *et al.*, 1987; Rhoden *et al.*, 1988; Ito, 1988; Janssen & Daniel, 1990). In addition to functional studies, excitatory junctional potentials have been measured, and these results also indicate pre-junctional receptors (Ito, 1988; Janssen & Daniel, 1990). The most direct method would be to evaluate the ACh release from cholinergic nerves. Martin & Collier (1986) found in muscle preparations from guinea-pig ileum that there was a significant inhibition of the ACh output after administration of noradrenaline. In canine isolated airways, however, they failed to find a significant inhibition of the release of ACh, although there was a tendency towards a decline in the trachea. The discrepancy could be due to a lack of sensitivity in the method, since the amount of ACh released was 20–40 times lower in the airways than in the ileum (Martin & Collier, 1986).

In conclusion, we have found a more pronounced inhibitory effect by salbutamol, salmeterol and formoterol in preparations contracted by EFS than in preparations contracted by exogenously added ACh. For all three β-agonists, the post-junctional effect is due to β-adrenoceptor stimulation. The pre-junctional effect of salbutamol and formoterol is also at least largely due to β-adrenoceptor stimulation, whereas the dominant pre-junctional effect of salmeterol is probably not mediated via β-adrenoceptors. The non-β-mediated effect could represent an additional relaxant mechanism for salmeterol.

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Delayed-type hypersensitivity-induced increase in vascular permeability in the mouse small intestine: inhibition by depletion of sensory neuropeptides and NK_1 receptor blockade

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1 This study investigates the effects of capsaicin-induced depletion of sensory neuropeptides and of neurokinin$_1$ (NK_1) receptor blockade on delayed-type hypersensitivity (DTH)-induced changes of vascular permeability in the small intestine of the mouse.

2 The DTH reaction in the small intestine was elicited by dinitrofluorobenzene (DNFB)-contact sensitization followed by oral dinitrobenzene sulphonic acid (DNBS) challenge. To assess vascular leakage the accumulation of the plasma marker, Evans blue (EB), was measured 2, 24 and 48 h after the challenge.

3 The small intestinal DTH reaction was characterized by a significant increase in vascular permeability 24 h after the challenge of previously sensitized mice when compared to vehicle-sensitized mice ($P<0.05$, ANOVA). Capsaicin-induced depletion of sensory neuropeptides, two weeks before the sensitization, completely inhibited the DTH-induced increase in small intestinal vascular permeability at 24 h ($P<0.05$, ANOVA). Vehicle/control: 108.2 ± 8.6 ng EB mg^{-1} dry weight; vehicle/DTH 207.8 ± 25.1 ng EB mg^{-1} dry weight; capsaicin/control: 65.8 ± 11.9 ng EB mg^{-1} dry weight; capsaicin/DTH: 84.3 ± 7.6 ng EB mg^{-1} dry weight.

4 The tachykinins, substance P and neurokinin A (1.5 to 50×10^{-11} mol per mouse, i.v.), induced an increase in vascular leakage in the small intestine of naive mice. The specific NK_1 receptor antagonist, RP67580 (10^{-9} mol per mouse, i.v.) was the most effective in reducing the substance P-induced plasma extravasation when compared with other NK receptor antagonists, FK224 and FK888.

5 Treatment of DNFB-sensitized mice with RP67580 (10^{-9} mol per mouse, i.v.) immediately before and 1 h after the DNBS challenge resulted in a significant reduction of the DTH-induced increase in vascular permeability at 24 h (vehicle/control: 107.5 ± 8.8 ng EB mg^{-1} dry weight; RP67580/control: 95.4 ± 5.4 ng EB mg^{-1} dry weight; vehicle/DTH: 206.6 ± 22.6 ng EB mg^{-1} dry weight; RP67580/DTH: 132.6 ± 13.6 ng EB mg^{-1} dry weight, $P<0.05$, ANOVA).

6 These results suggest that sensory nerves are involved in the development of small intestinal DTH reactions in the mouse. NK_1 receptors could play an important role in the initiation of the DTH-induced changes in vascular leakage.

Keywords: Small intestinal vascular permeability; delayed-type hypersensitivity; mouse small intestine; sensory neuropeptides; capsaicin; tachykinins

Introduction

It has been proposed that delayed-type hypersensitivity (DTH) reactions in the gastrointestinal tract resemble some of the pathological features of inflammatory bowel diseases (IBD) in man (Kagnoff, 1987). These pathological conditions of inflammation are characterized by an increased T cell activation and abnormalities in intestinal T cell function (Fais *et al.*, 1987; MacDonald *et al.*, 1990; Schreiber *et al.*, 1991). It has been suggested that DTH reactions require lymphocyte (T cell) and mast cell activation (Askenase & van Loveren, 1983). Both cell types are sensitive to sensory neuropeptides. In the periphery, sensory efferent neurones innervate the gastrointestinal tract and are found in close association with lymphocytes and mucosal mast cells (Polak, 1978; Skofitsch *et al.*, 1985; Stead *et al.*, 1987a,b; 1989; Arizono *et al.*, 1990; Dvorak *et al.*, 1992). In addition, neuroendocrine modulation of the intestinal immune system during inflammatory reactions has been demonstrated (Bienenstock *et al.*, 1988; Shanahan & Anton, 1988; Koch *et al.*, 1991). The described effects of neuropeptides on particular immunological functions are variable and frequently opposing: e.g. substance P enhances mitogen-stimulated lymphocyte proliferation (Stanisz *et al.*, 1986; Scicchitano *et al.*, 1988), whereas vasoactive intestinal peptide, somatostatin and calcitonin gene-related peptide inhibit mitogen-stimulated lymphocyte proliferation (Stanisz *et al.*, 1986; Umeda *et al.*, 1988). Substance P has the ability to activate mucosal mast cells and substance P-induced inflammatory responses have been shown to be mast cell-dependent (Joos *et al.*, 1993; Shanahan *et al.*, 1985; Matsuda *et al.*, 1989; Yano *et al.*, 1989; Joos & Pauwels, 1993). The role of sensory neuropeptides in cell-mediated DTH reactions has been studied in the skin using capsaicin to deplete sensory nerves (Girolomoni & Tigelaar, 1990). Capsaicin is a neurotoxin and induces an initial excitation with a massive release of mediators of unmyelinated sensory C fibres rapidly followed by a desensitization phase with depletion of neuropeptides (Jancso *et al.*, 1967; Buck & Burks, 1986).

In the present study, a DTH reaction was elicited in the small intestine of mice with dinitrofluorobenzene (DNFB) as

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the contact sensitizing hapten followed by an oral challenge with dinitrobenzene sulphonic acid (DNBS). The protocol used to induce small intestinal inflammation was similar to that previously described in the rat (Kraneveld *et al.*, 1993). To investigate the role of sensory nerves, the influence of capsaicin-induced depletion of sensory neuropeptides on the DTH-induced changes of small intestinal vascular permeability was examined. Furthermore, the importance of tachykinins in the development of the effector phase of the small intestinal DTH reaction was studied by use of selective tachykinin receptor antagonists.

Methods

Male Balb/c mice (6–8 week, Central Animal Laboratory (GDL) Utrecht University, Utrecht, The Netherlands) were used in all experiments. The experiments were approved by the Animal Care Committee, Utrecht University.

Induction of the DTH response in the small intestine

Dinitrofluorobenzene (DNFB, 5 mg ml^{-1}, 100 µl) or vehicle (acetone:olive oil = 4:1) was applied on the surface of the skin of the shaven thorax and feet of anaesthetized mice (sodium pentobarbitone, 30 mg kg^{-1} i.p., n = 5/6) on 2 consecutive days. On day 5 the animals were challenged intranasally with dinitrobenzene sulphonic acid (DNBS, 6 mg ml^{-1}, 50 µl). In preliminary investigations, it was observed that Evans blue dye administered intranasally was found in substantial amounts in the gastrointestinal tract of mice, demonstrating that drugs administered intranasally gain access to the intestine.

Small intestinal vascular permeability changes induced by DTH reaction

To assess vascular leakage, Evans blue dye (EB, 12.5 mg ml^{-1} in sterile saline, 50 µl) was administered intravenously to anaesthetized mice (i.p. sodium pentobarbitone 30 mg kg^{-1}) 2 h before the animals were killed. Evans blue dye binds to plasma proteins and can be used as a marker of plasma protein extravasation (Udaka *et al.*, 1970). Shortly before the mice were killed heparin was administered i.v. (10 u ml^{-1} blood) and a blood sample was taken. After the animal had been killed the intestines were vascularly perfused with warm saline (5 ml) to eliminate the excess of vascular Evans blue. The Evans blue dye was extracted from tissue-segments of the small intestine overnight in formamide at 40°C and the amount of dye in extracts and plasma samples was determined with a spectrophotometer at 620 nm (Rogers *et al.*, 1989). The amount of Evans blue dye was expressed as ng per mg small intestinal dry weight and corrected for the plasma EB concentration. Intestinal vascular leakage was examined 0–2, 22–24 and 46–48 h after the DNBS challenge. The following groups were studied: DNFB/DNBS: DNFB sensitization and DNBS challenge and control DNBS: sham sensitization and DNBS challenge. In addition, mice which were DNFB-sensitized and sham-challenged (DNFB control) and mice which were sham-sensitized and sham-challenged (naive) were also studied.

Effect of depletion of capsaicin-sensitive sensory nerves on DTH-induced changes in vascular permeability

Capsaicin was used to deplete neuropeptides from unmyelinated sensory C fibres. The mice were anaesthetized with sodium pentobarbitone, 30 mg kg^{-1}, and received s.c. injections of capsaicin (25 mg kg^{-1}) on 2 consecutive days. Controls were treated with vehicle (alcohol:Tween 80:saline = 2:1:7) alone. The mice were used 2 weeks after pretreatment with capsaicin. Table 1 shows the groups which were examined to investigate the role of capsaicin-sensitive sensory nerves in DTH-induced changes in small intestinal vascular permeability 2, 24 and 48 h after the challenge.

Table 1 Capsaicin pretreatment of mice

Pretreatment	n	*Sensitization*	*Challenge*
Vehicle	5	DNFB	DNBS
Vehicle	6	Vehicle	DNBS
Capsaicin	5	DNFB	DNBS
Capsaicin	5	Vehicle	DNBS

Pretreatment with capsaicin: 25 mg kg^{-1} s.c. on 2 consecutive days 2 weeks before the sensitization. DNFB: dinitrofluorobenzene; DNBS: dinitrobenzene sulphonic acid; vehicle:alcohol:Tween 80:saline = 2:1:7.

To assess the degree of neuropeptide depletion, we studied a neurogenic inflammatory cutaneous reaction in separate groups of capsaicin- or vehicle-pretreated mice. Neurogenic inflammation was induced by topical administration of 40 µl capsaicin solution (1 mg ml^{-1}) onto one ear. The inflammatory oedema formation in the skin was assessed by punching out ear discs (diameter: 8 mm) and differences between the weight of the capsaicin-challenged ear and the non-challenged ear were expressed in mg.

Direct effect of tachykinins, substance P and neurokinin A on small intestinal vascular permeability in naive mice

The tachykinins, substance P and neurokinin A, were given intravenously to non-sensitized and non-challenged 'naive' animals (1.5×10^{-11}–5×10^{-10} mol per mouse in sterile saline, n = 5/6 mice). In addition the effects of three different neurokinin receptor antagonists were studied on the basal small intestinal vascular leakage and on the substance P-induced change in small intestinal permeability. The following neurokinin receptor antagonists were tested: the selective NK_1 receptor antagonist FK888 (10^{-9} mol per mouse); the selective NK_1 receptor antagonist RP67580 or its inactive enantiomer RP68651 (10^{-9} mol per mouse); the nonselective NK_1/NK_2 receptor antagonist FK224 (5×10^{-9} mol per mouse). Each neurokinin receptor antagonist was injected i.v. simultaneously with substance P (5×10^{-11} mol per mouse). In addition, RP67580 was also tested on the NKA (5×10^{-11} mol per mouse, i.v.) and PAF (2×10^{-11} mol per mouse, i.v.)-induced increase in vascular leakage. After 1 h the small intestinal vascular leakage was assessed with Evans blue dye as described above.

Effect of NK_1 receptor antagonist, RP67580 treatment on the DTH-induced changes in small intestinal vascular permeability

In further experiments, the effect of the NK_1 receptor antagonist, RP67580, on the DTH-induced changes of vascular permeability in the small intestine was studied 24 h after the DNBS challenge in DNFB-sensitized or control animals. RP67580 (10^{-9} mol per mouse per injection, n = 5/6 mice per treatment) was administered intravenously using three treatment regimes: (i) immediately before and 1 h after the challenge; (ii) 22 and 23 h after the challenge and (iii) immediately before and 1 h, 22 and 23 h after the challenge. We also studied the effect of the inactive enantiomer RP68651 (10^{-9} mol per mouse; treatment regime (iii)) on the DTH-induced increase in vascular leakage 24 h after the challenge.

Materials

DNFB, substance P and neurokinin A were purchased from Sigma (St. Louis, MO, U.S.A.). DNBS was obtained from Eastman Kodak Co. (Rochester New York, U.S.A.). Evans blue dye, capsaicin and Tween 80 were obtained from Fluka

Chemika (Buchs, Switzerland). Sodium pentobarbitone (Nembutal) was from Sanofi (France). The nonpeptide NK_1 receptor antagonist, RP67580, (3aR, 7aR)-7,7-diphenyl-2-(1-imino-2-(2-methoxyphenyl)ethyl)perhydroisoindol-4-one, and its inactive enantiomer RP68651, (3aS, 7aS)-7,7-diphenyl-2-(1-imino-2-(2-methoxyphenyl)ethyl)perhydroisoindol-4-one, were gifts from Rhône-Poulenc (Rorer, France). The dipeptide NK_1 receptor antagonist, FK888, N^2[(4R)-4-hydroxy-1-(1-methyl-1H-indol-3-yl)carbonyl-L-propyl]-*N*-methyl-*N*-phenyl methyl-3-(2-naphthyl)L-alaninamide, and the peptide NK_1 and NK_2 receptor antagonist, FK224, N-[N^2-[N-[N-[N-[2,3-didehydro-*N*-methyl-*N*-[N-[3-(2-penthylphenyl)-propionyl]-L-threonyl]tyrosyl]-L-leucynyl]-D-phenylalanyl]-L-*allo*-threonyl]-L-asparaginyl]-L-serine-*v*-lactone, were gifts from Fujisawa Pharmaceuticals Co (Osaka, Japan). RP67580, RP68651, FK224 and FK888 were dissolved in ethanol and stored in stock solutions of 10^{-3} M. Further dilutions were made in sterile saline.

Data analysis

The results are presented as mean ± s.e.mean. The DTH-induced changes in small intestinal permeability were analysed by analysis of variance (ANOVA), and the statistical significance of differences between means was determined with the Newman-Keuls test. The assessment of neuropeptide depletion in the skin, the effects of substance P, neurokinin A and the effects of the neurokinin receptor antagonists on small intestinal vascular leakage were evaluated statistically with Student's *t* test (unpaired). All *P* values <0.05 were considered to reflect a statistically significant difference.

Results

DTH-induced changes in small intestinal vascular permeability in the mouse

Basal small intestinal vasopermeability in naive mice was 46.4 ± 3.6 ng EB mg^{-1} dry weight. Figure 1a shows that 0–2 h after the DNBS challenge there were no significant differences in small intestinal vascular leakage values between DNFB/DNBS mice and the control group (control/DNBS). The DNBS challenge alone induced a significant increase in vascular leakage 24 and 48 h after the challenge when compared to basal level (Figure 1a). However, a more profound significant increase in vascular permeability was found 22–24 h after DNBS challenge in DNFB-sensitized mice (DNFB/DNBS: 207.8 ± 25.1 ng EB mg^{-1} dry weight compared to the control/DNBS: 108.2 ± 8.6 ng EB mg^{-1} dry weight, $P<0.01$). It was noted that the DTH-induced vascular leakage response had returned to control levels 46–48 h after the challenge. In addition, the vascular permeability values of mice, which were DNFB-sensitized and sham-challenged, did not differ significantly from the values of non-sensitized and non-challenged mice (data not shown).

Effect of depletion of capsaicin-sensitive sensory nerves on DTH-induced changes in vascular permeability

As demonstrated in Table 2, topical administration of capsaicin resulted in a significant increase in ear weight indicating neurogenic inflammation in the skin. Pretreatment with capsaicin 2 weeks before the topical capsaicin challenge prevented this neurogenic inflammation. Therefore, it was concluded that the pretreatment with capsaicin on 2 consecutive days resulted in a depletion of neuropeptides in the mouse after 2 weeks.

This protocol was used to investigate further the role of capsaicin-sensitive nerves in the DTH-induced small intestinal inflammation. Pretreatment with capsaicin completely inhibited the profound increase in small intestinal vascular permeability observed 22–24 h after the DNBS challenge in DNFB-sensitized mice (Figure 1b). In addition, depletion of capsaicin-sensitive sensory nerves also abolished the increased vascular permeability caused by DNBS challenge in non-sensitized mice 24 and 48 h after the challenge (Figure 1b).

Direct effect of tachykinins substance P and neurokinin A on small intestinal vascular permeability in naive mice

The direct effects of intravenously injected substance P (SP) and neurokinin A (NKA) were investigated on small intestinal vascular leakage in naive mice. Both tachykinins induced an increase in vascular permeability as reflected by an increase in Evans blue tissue accumulation (Table 3). This substance P response was dose-dependent (vehicle: 64.6 ± 4.0; 1.5×10^{-11} mol per mouse: 92.7 ± 8.1; 5×10^{-11} mol per

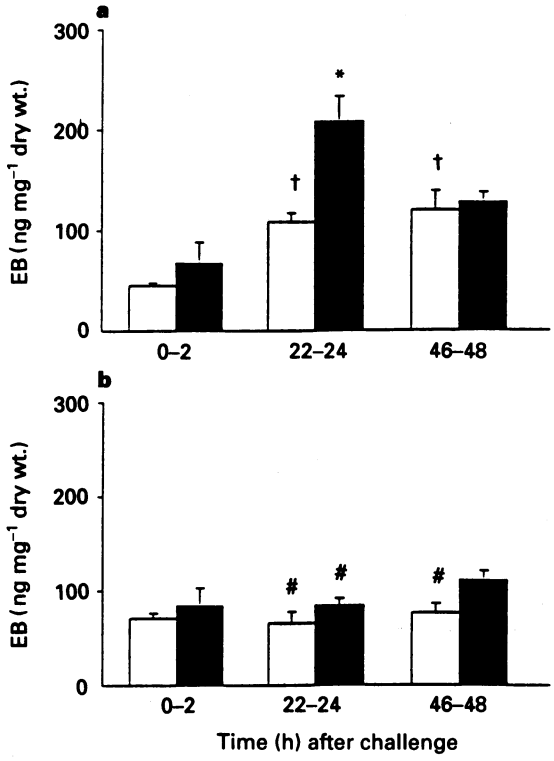

Figure 1 DTH reaction in the small intestine of vehicle-(a) and capsaicin-(b) pretreated mice 2, 24 and 48 h after challenge. Pretreatment with capsaicin, 25 mg kg^{-1}, s.c. or saline on 2 consecutive days was performed 2 weeks before the sensitization. Changes in small intestinal vascular leakage after intranasal administration of DNBS in DNFB-sensitized mice (DNFB/DNBS: solid column) and control mice (control/DNBS: open column) are expressed as Evans blue tissue accumulation (ng mg^{-1} dry weight) for 5 or 6 mice per group and shown as mean ± s.e.mean. *$P<0.05$ compared to control groups; †$P<0.05$ compared to basal vascular leakage (46.4 ± 3.6 ng EB mg^{-1} dry weight) and #$P<0.05$ compared to vehicle pretreatment, ANOVA, Newman-Keuls test.

Table 2 Effect of capsaicin-induced neuropeptide depletion on neurogenic inflammatory responses to topical capsaicin (1 mg ml^{-1}, 40 µl) applied to the mouse ear

Pretreatment	n	*Ear-disk weight difference* (mg)
Vehicle	6	7.28 ± 0.87
Capsaicin	9	0.43 ± 0.28*

Mice were pretreated with capsaicin (25 mg kg^{-1}, s.c.) on 2 consecutive days 2 weeks prior to these tests. The results are expressed as mean ± s.e.mean for *n* mice. *$P<0.05$ compared with vehicle pretreatment, Student's *t* test.

Table 3 Effects of the NK_1/NK_2 receptor antagonist, FK224, the NK_1 receptor antagonist, FK888, and the NK_1 receptor antagonist, RP67580, and its inactive enantiomer, RP68651, on basal small intestinal vascular leakage and on substance P-induced increase of small intestinal vascular leakage (RP67580 was also tested on NKA- and PAF-induced increase in vascular leakage)

	Evans blue tissue accumulation (ng mg^{-1} dry weight)			
Antagonist	*Saline*	(n)	*Substance P*	(n)
Vehicle	65.1 ± 2.4	(11)	122.9 ± 10.8*	(11)
FK224	100.1 ± 5.2#	(6)	100.9 ± 6.0#	(6)
FK888	71.1 ± 4.7	(6)	94.9 ± 6.3*,#	(6)
RP67580	73.3 ± 6.3	(10)	77.0 ± 5.7#	(10)
RP68651	69.5 ± 8.0	(5)	134.7 ± 16.7*	(5)
	Saline	(n)	*NKA*	(n)
Vehicle	73.8 ± 7.3	(4)	118.8 ± 7.3*	(4)
RP67580	66.8 ± 5.8	(4)	70.4 ± 3.5#	(4)
	Saline	(n)	*PAF*	(n)
Vehicle	61.9 ± 2.4	(5)	115.6 ± 8.2*	(5)
RP67580	62.0 ± 2.9	(5)	116.8 ± 5.7*	(5)

The mice were injected intravenously. Doses: substance P and NKA: 5×10^{-11} mol per mouse; PAF: 2×10^{-11} mol per mouse; FK224: 5×10^{-9} mol per mouse, FK888, RP67580 and RP68651: 10^{-9} mol per mouse. The results are expressed as mean ± s.e.mean Evans blue tissue accumulation (ng mg^{-1} dry weight) for n mice per group. $*P<0.05$ compared to saline-treated mice and $\#P<0.05$ compared to vehicle pretreatment, Student's t test.

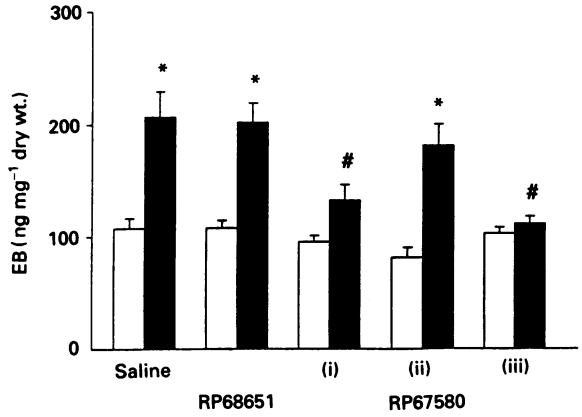

Figure 2 The effects of treatment with the NK_1 receptor antagonist, RP67580 (10^{-9} mol per mouse per i.v. injection) and with the inactive enantiomer, RP68651 (10^{-9} mol per mouse per i.v. injection) on the DNFB/DNBS-induced DTH reaction in the small intestine 22–24 h after DNBS challenge in DNFB- (solid columns) or vehicle- (open columns) sensitized mice. Three regimens of treatments with RP67580 were applied: (i) immediately before and 1 h after the challenge; (ii) 22 and 23 h after the challenge and (iii) immediately before and 1 h, 22 and 23 h after the challenge. RP68651 was administered according to treatment regimen (iii). The vascular leakage responses are expressed as Evans blue tissue accumulation (ng mg^{-1} dry weight) for 5 or 6 mice per group. Means ± s.e.mean are given. $*P<0.05$ compared to control group and $\#P<0.05$ compared to saline/DTH group, ANOVA, Newman-Keuls test.

mouse: 122.9 ± 10.8; 50×10^{-11} mol per mouse: 109.9 × 8.2 ng EB mg^{-1} dry weight, $P<0.05$ compared to vehicle-treatment for all substance P doses, Student's t test) and maximal at a dose of 5×10^{-11} mol per mouse. The increase in small intestinal vascular leakage induced by the tachykinins in naive mice, provided us with a system to test several NK_1 and NK_1/NK_2 receptor antagonists. Table 3 shows that the specific NK_1 receptor antagonist, RP67580 (10^{-9} mol per mouse), reduced the substance P- (5×10^{-11} mol per mouse) induced vascular leakage most profoundly by 94%, whereas its inactive enantiomer RP68651 (10^{-9} mol per mouse) did not influence the substance P response. RP67580 also inhibited the NKA vascular response by 91%. Moreover, RP67580 did not have an effect on the PAF-induced increase in vascular leakage. FK888 (10^{-9} mol per mouse), another specific NK_1 receptor antagonist, reduced the substance P-induced vascular leakage in the small intestine by 57%. The two NK_1 receptor antagonists, FK888 and RP67580, did not affect basal vascular leakage in the small intestine. The nonspecific NK_1/NK_2 receptor antagonist FK224 (5×10^{-9} mol per mouse) induced a small but nonsignificant inhibition of the substance P-induced vascular permeability change. However, intravenous injection of FK224 alone resulted in a significant increase in vascular leakage compared to control. In further experiments RP67580 was used to investigate the role for tachykinins in the DTH response in the mouse small intestine.

Effect of NK_1 receptor antagonist RP67580 treatment on the DTH-induced changes in small intestinal vascular permeability

Figure 2 shows that intravenous administration of RP67580 (10^{-9} mol per mouse per injection) significantly reduced the DTH-induced vascular response by 63% when given immediately before and 1 h after the DNBS challenge (treatment regime (i)). Administration of RP67580 at 22 and 23 h after the challenge (treatment regime (ii)) did not have a significant effect on the DTH response. Treatment with RP67580 immediately before and 1, 22 and 23 h after the challenge (treatment regime (iii)) reduced the DTH-induced increase in vascular permeability by 91%. In contrast, treatment with the inactive enantiomer RP68651 (treatment regime (iii)) did not affect the DTH response. The compounds, RP67580 and RP68651, had no influence on small intestinal vascular leakage in control/DNBS mice.

Discussion

The major finding in the present study is that depletion of sensory neuropeptides and blockade of the NK_1 receptor inhibit DTH-induced changes of small intestinal vasopermeability in the mouse. In addition, we have shown that the tachykinins, substance P and neurokinin A, are able to induce an increase in small intestinal vascular leakage and that this leakage could be blocked by NK_1 receptor antagonists. These results demonstrate that the sensory neuropeptides, especially tachykinins, are involved in the development of DTH-induced increase in plasma extravasation in the small intestine of the mouse.

The DTH response in the small intestine of the mouse was characterized by an increase in vascular permeability 24 h after challenge. The time course of the DTH reaction was different in rats, which showed a significant DNFB/DNBS-induced increase of vascular leakage 48 h after the challenge (Kraneveld *et al.*, 1993). This could be the result of species differences, or difference in dose of the DNBS challenge or in its pharmacokinetics.

In this study it was demonstrated that depletion of sensory neuropeptides by pretreatment with capsaicin abolished the DTH-induced increase in vascular permeability in the small intestine of the mouse. Interestingly, capsaicin pretreatment also inhibited the non-specific DNBS-induced increase in vascular permeability in non-sensitized animals. This non-immunological increase in small intestinal vascular leakage could be the result of an irritant effect of the hapten in which sensory neuropeptides play a role. It has been reported that

single intrarectal installation of the hapten, trinitrobenzene sulphonic acid (TNBS), produces a damaged colon in unsensitized rats; however, in these studies considerably higher doses of the hapten were used (Rachmilewitz *et al.*, 1989; Yamaki *et al.*, 1992).

Several studies have shown that the *in vivo* manipulation of the nervous system resulted in altered immune responses, including those involved in DTH reactions (Macris *et al.*, 1970; Jankovic & Isakovic, 1973; Maric & Jankovic, 1987; Josefsson *et al.*, 1991). More recently, in an immediate-type hypersensitivity reaction in the small intestine, it was shown that tetrodotoxin-induced neuronal blockade inhibited the antigen-induced increase in intestinal permeability in sensitized rats (Crowe *et al.*, 1993). Few reports have described the contribution of peptidergic neurones in the expression of DTH reactions. In the skin, Giolomi & Tigelaar (1990) have shown that early ear swelling induced by the hapten DNFB in mice, 2 h after the challenge, was markedly reduced by capsaicin pretreatment. In the intestinal tract, Maggi *et al.* (1987) showed that depletion of sensory neuropeptides from capsaicin-sensitive nerves significantly increased the degree and incidence of ulceration of the rat duodenum. However, they also reported that in the same intestinal segment, capsaicin-induced increase in vascular permeability was absent in depleted rats.

To characterize further the involvement of sensory neuropeptides in DTH-induced intestinal inflammation, the role of tachykinins in the effector phase of the DTH reaction was investigated. Firstly, substance P and neurokinin A were shown to induce an increase in small intestinal vascular leakage in naive mice. However, the DTH-induced increase in vasopermeability was more profound than the tachykinin-induced increase in vascular leakage. The NK_1 receptor antagonist, RP67580, was most effective in reducing the substance P-induced increase in small intestinal vascular leakage when compared with FK224 and FK888. Since the inactive enantiomer RP68651 did not influence the substance P response and RP67580 did not affect PAF-induced increase in small intestinal vascular leakage, it can be concluded that substance P acts selectively via the NK_1 receptor. In addition, RP67580 significantly inhibited the NKA vascular response in the mouse small intestine. The effects of tachykinins on vascular permeability are to a large extent mediated by NK_1 receptors on endothelial cells (Ohkubo & Nakanishi, 1989). Autoradiographically, it was demonstrated that binding sites for tachykinins exist on the vasculature of the digestive tract. Submucosal arterioles and venules in the intestine were shown to express only NK_1 receptors, whilst NK_2 binding sites were totally absent (Gates *et al.*, 1988; Holzer, 1992). Recently, Beaujouan *et al.* (1993) have reported a higher potency of RP67580 in the mouse and the rat compared with other NK_1 receptor antagonists. Our results suggest that NK_1 receptors are involved in the substance P- and NKA-induced changes in vascular leakage in the mouse small intestine.

RP67580 was used to investigate the role of NK_1 receptors in the DNFB-induced small intestinal DTH response in the mouse. Treatment with RP67580 immediately before and 1 h after the DNBS challenge in DNFB-sensitized mice resulted in a significant reduction of small intestinal vascular leakage at 24 h. In contrast, administration of RP67580 at 22 and 23 h after the challenge did not have a significant effect. Since the inactive enantiomer RP68651 did not affect the DTH response, it can be concluded that the NK_1 receptor plays a role in the initiation of the DTH-induced vascular responses.

It is reasonable to speculate that neuropeptides have an important role in the initiation, maintenance and symptoms associated with inflammatory responses. The gastrointestinal tract is densely innervated by sensory nerves. Immunohistological studies have demonstrated substance P immunoreactivity in nerves throughout the wall of the gastrointestinal tract (Furness & Costa, 1987; Costa *et al.*, 1987; 1991). In addition, it was demonstrated that receptors for substance P were upregulated on blood vessels and lymphoid follicles in Crohn's disease and ulcerative colitis (Mantyh *et al.*, 1988). In animal models of gastrointestinal inflammation, substance P levels were raised during infection (Swain *et al.*, 1992). Karmeli *et al.* (1991) showed that ethanol-induced gastric mucosal damage is mediated by substance P.

Several possibilities can explain the effects of the NK_1 receptor blockade on the DTH reaction. Firstly, many reports have described the close association between mast cells and sensory nerves within the gastrointestinal tract (Skofitsch *et al.*, 1985; Stead *et al.*, 1987b; Crivellato *et al.*, 1991). The mast cell in the gut wall has been reported to be an important immune effector cell, especially in the initiation of DTH responses. It has been hypothesized that stimulation of mast cells leads to the initiation of a cascade of cellular infiltration to develop a DTH response (Askenase & van Loveren, 1983). In addition, substance P induces mediator release from different mast cell types *in vitro* (Shanahan *et al.*, 1985; Assem *et al.*, 1989). The observation of the release of airway mast cell mediators *in vivo* by substance P and neurokinin A, supports the growing belief that mast cells are under sensory nervous control (Joos & Pauwels, 1993). Furthermore, the existence of NK_1 receptors on mast cells has been reported (Joos *et al.*, 1993). Very recently, it has been demonstrated that intestinal mucosal mast cells are activated 30 min after DNBS challenge of DNFB-sensitized rats (Kraneveld *et al.*, 1994). Depletion of sensory neuropeptides or treatment with a NK_1 receptor antagonist immediately before and 1 h after the challenge could, therefore, inhibit the initiating role of the mast cell in our model of gastrointestinal inflammation. Secondly, neuropeptides have a range of effects on the proliferation, migration and activation of lymphocytes. In general, it seems that the tachykinins, substance P and neurokinin A have predominantly stimulatory activity, whereas calcitonin gene-related peptide, vasoactive intestinal peptide and somatostatin have predominantly inhibitory activities (Stanisz *et al.*, 1986). The T lymphocyte is one of the key inflammatory cells in DTH responses. In addition, receptors for substance P have been found on T lymphocytes, although they were not characterized into NK_1, NK_2 or NK_3 receptors (Croitoru *et al.*, 1990). Thus, inhibition of the stimulatory action of substance P on lymphocytes by NK_1 receptor blockade or neuropeptide depletion, could underly the marked reduction of inflammatory response observed in DTH-induced small intestinal inflammation. Finally, a role of neuropeptides in the increase in vascular leakage in DNFB-sensitized and DNBS-challenged mice can be explained by the direct inflammatory actions of neuropeptides: vasodilatation and plasma extravasation, mediated via NK_1 receptors on endothelial cells. One would expect that NK_1 receptor blockade at the time of the DTH response would have an effect. This phenomenon was not observed.

The relevant biological actions together with the close association between sensory nerves, mast cells and immune cells in the gastrointestinal tract, suggests a possible function for neuropeptides in inflammatory responses. In this study, an important role for sensory nerves and for the NK_1 receptor in the DNFB/DNBS-induced DTH reaction in the small intestine of the mouse is demonstrated. Tachykinins could play an important role in the initiation of DTH-induced vascular leakage either indirectly via mast cells or lymphocytes or directly on endothelial cells.

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Effect of inhibition of nitric oxide synthesis on the uptake of LDL and fibrinogen by arterial walls and other organs of the rat

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1 The effects of administering 3 mg ml^{-1} N^{G}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO), on the uptake of low density lipoprotein (LDL), fibrinogen and blood pressure were determined in conscious, unrestrained, cannulated normotensive and spontaneously hypertensive (SHR) Wistar rats.

2 The uptake of LDL and fibrinogen, labelled respectively with ^{125}I or ^{131}I via the adduct tyramine cellobiose ([^{125}I]TC-LDL and [^{131}I]TC-fibrinogen), were compared in aortic walls, heart, skeletal muscle, lung, liver, kidney and adrenal during the final 24 h of 6 days' administration of L-NAME in the drinking water.

3 In control normotensive rats, the systolic blood pressure did not change significantly over 6 days, while administration of L-NAME in normotensive rats increased the blood pressure progressively and significantly to about 170 mmHg over the same period.

4 In normotensive rats L-NAME increased significantly the uptake of both LDL and fibrinogen by aortic walls and heart, but not by muscle, lung, liver, kidney and adrenal.

5 The blood pressure in SHR was about 170 mmHg before administration of L-NAME and did not increase significantly after 6 days of treatment. In these rats the uptake of LDL or of fibrinogen was increased only in the heart but not in aortic walls nor in any of the other organs.

6 In normotensive rats the increase in blood pressure caused by inhibition of NO generation was associated with increases in the uptake of the atherogenic plasma proteins LDL and fibrinogen by the wall of the aorta and by the heart but not by the skeletal muscle, lung, liver, kidney and adrenal. The slightly higher blood pressure of SHR treated with L-NAME was not associated with increased uptake of LDL or fibrinogen by aorta nor by any organ except heart. Thus, while in normotensive rats the increase in blood pressure caused by inhibition of NO generation was associated with increase in the uptake of atherogenic plasma proteins LDL and fibrinogen by the wall of the aorta and by the heart, in SHR which showed uptakes similar to the normotensive animals, the non-significant increase in blood pressure induced by inhibition of NO generation was not associated with increased uptake of LDL or fibrinogen in any organ except the heart.

7 These results are consistent with effects, demonstrated earlier, of infusing the pressor agents noradrenaline, adrenaline and angiotensin II, which produce increases in uptake of LDL and fibrinogen by aortic wall, whereas this is not so in spontaneously hypertensive rats.

Keywords: N^{G}-nitro-L-arginine methyl ester (L-NAME); blood pressure; low density lipoprotein (LDL); fibrinogen; uptake; rat, normotensive and hypertensive

Introduction

Previous experiments have shown that infusions of noradrenaline or adrenaline into carotid arteries of anaesthetized rabbits to produce moderate increases in their local blood concentrations increase the uptake of atherogenic plasma proteins, low density lipoproteins (LDL) and fibrinogen by the artery walls (Shafi *et al.*, 1989; Cardona-Sanclemente & Born, 1992a). Furthermore, systemic infusions of adrenaline or angiotensin II into conscious unrestrained rats, producing slight to moderate rises in blood pressure, increase the uptake of LDL and fibrinogen by aortic walls (Cardona-Sanclemente & Born, 1992b; Cardona-Sanclemente *et al.*, 1994a). However, in spontaneously hypertensive rats of the same age in which the blood pressure was higher than in those infused with the pressor agents, the uptake of LDL and fibrinogen was the same as in the controls, and was also not increased by infusions of angiotensin II (Cardona-Sanclemente *et al.*, 1994b).

Thus the mechanism(s) responsible for the increased uptake of the atherogenic plasma proteins and, specifically, the involvement of blood pressure is not clear. We now show that yet another means of increasing blood pressure, namely infusion of an inhibitor of endogenous nitric oxide (NO) formation, is also associated with increases in LDL and fibrinogen uptake by aortic walls in conscious, unrestrained normotensive but not in hypertensive (SHR) Wistar rats.

The formation of nitric oxide by the inducible synthase has been demonstrated in several tissues and cells (Moncada, 1992). We, therefore, took the opportunity to determine the effect of inhibition of NO formation on the uptake of LDL and fibrinogen by other organs.

Methods

Animals

Fourteen adult male Wistar rats (300 ± 14 g) and seven SHR (293 ± 5 g) were fed *ad libitum* on standard diet. The rats were kept in separate wire-bottomed cages in an air-conditioned animal house with a light period lasting from 08 h 00 min to 20 h 00 min.

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L-NAME administration

A group of 7 Wistar and the 7 SHR rats received N^G-nitro-L-arginine methyl ester (L-NAME; Sigma Chemical Company, Dorset) in the drinking water for six days. The controls were 7 weight-matched Wistar rats drinking tap water only. L-NAME was dissolved in the drinking water at a concentration of 3.0 mg ml^{-1}. In preliminary experiments, this dose elicited the most reproducible increases in blood pressure.

Fibrinogen

Lyophilized human fibrinogen (KabiVitrum, Sweden) was dissolved in physiological saline to give a concentration of 100 mg ml^{-1}.

Low density lipoprotein preparation

Human blood was obtained by venepuncture from healthy students or laboratory personnel with lipid levels in the normal range. After mixing with 0.15 M EDTA, the blood was centrifuged at 8700 *g* for 30 min at 4°C. LDL (density 1.019–1.063 g ml^{-1}) was separated by floatation after sequential centrifugation at 105000 *g* for 24 h at 4°C on KBr solutions adjusted to a final density of 1.019 and 1.063 (Havel *et al.*, 1955; Koga *et al.*, 1969; Fidge & Poulis, 1978; Nagelkerke *et al.*, 1984; Cardona-Sanclemente *et al.*, 1988). The final LDL fraction was dialysed at 4°C against phosphate buffered saline containing 0.3 nM EDTA at pH 7.5 to remove excess salts. Protein content was measured by the BCA assay reagent (Pierce Chemical Co., U.S.A.).

Labelling and iodination of LDL and fibrinogen

An adduct of cellobiose and tyramine (TC) was radioiodinated with ^{125}I or ^{131}I and attached covalently to LDL or fibrinogen respectively. The labelled TC remains *in situ* when the lipoprotein or fibrinogen undergo degradation, so that the radioactivity is a measure of the proteins which were present in the tissues (Pittman *et al.*, 1983). The labelled proteins were dialysed in cold phosphate-buffered saline pH 7.5 containing EDTA. The tyramine cellobiose-labelled LDL ([^{125}I]-TC-LDL) and fibrinogen ([^{131}I]-TC-fibrinogen) had specific activities of 100–400 c.p.m. ng^{-1} protein and free ^{125}I content of 1–4% for the LDL fraction, and of 50–150 c.p.m. ng^{-1} protein and free ^{131}I content of 1–3.5% for the fibrinogen preparation.

Surgical procedures

Rats were anaesthetized with intramuscular Hypnorm (Janssen) 0.3 ml kg^{-1} and intraperitoneal methyl diazepin-one (Diazepam, Phoenix Pharm.) 2.5 mg kg^{-1}. An incision was made in the neck and the right common carotid exposed. A fine polythene cannula was tied into the carotid artery. The cannula, previously closed with a pin, was passed through a subcutaneous tunnel in the skin to exit dorsally at the back of the neck, and the ventral incision was closed by suture. After surgery each rat was housed individually and allowed to recovery overnight before beginning the experiment proper. The patency of the cannula was maintained by a daily injection of 0.2 ml hirudin (10 μg ml^{-1}). Five days after surgery the rats were anaesthetized with Hypnorm alone and the labelled proteins injected through the femoral vein. Twenty-four hours later the rats were anaesthetized and killed by exsanguination via the abdominal aorta. After puncturing the abdominal aorta for collecting blood and for washing out the cardiovascular system with saline, the whole aorta was exposed from the heart to the bifurcation and excised intact. Surrounding fat and loose connective tissue were removed under a stereomicroscope. The vessel was washed 5 times in ice-cold buffer and placed in preweighed tubes. Samples of other organs and tissues were excised and their radioactivities the samples were dried at 60°C to constant weight. Results are expressed as ng apo B protein per mg dry weight. All procedures were approved by the Animal Care and Safety Committee and conformed to guidelines established by the Home Office.

Blood pressure measurements

The rats' blood pressure and heart rate were determined daily in the conscious, unrestrained animals by connecting the indwelling catheter in the carotid artery to a transducer coupled to a Multitrace 2 Recorder 5022 (Lectromed, U.K.).

Statistical analysis

The significance of differences among groups of animals was assessed by Student's two-tailed *t* test for unpaired samples. A *P* value of less than 0.05 was taken as significant. Results are shown as mean ± s.e.mean.

Results

When normotensive rats were given L-NAME in the drinking water at doubling concentrations from 0.75 to 3.0 mg ml^{-1}, the aortic uptake of LDL was significantly increased only by the highest concentration (Figure 1). Normotensive and hypertensive rats treated with L-NAME at this concentration appeared to remain as healthy as the control rats of the same age, although the body weights of the hypertensive rats decreased very slightly and not significantly compared to the normotensive rats, the body weights of which remained constant (Figure 2). The body weight of normotensive rats receiving L-NAME decreased by about 17% compared to control rats during the experiment.

Administration of L-NAME *ad libitum* in the drinking water at a concentration of 3.0 mg ml^{-1} decreased the oral water intake by 27% (11.3 ml day^{-1}) in normotensive rats and by 16% (13.0 ml day^{-1}) in SHR compared with the control group (15.5 ml day^{-1}). Because of these decreases in water consumption, the daily doses of L-NAME were

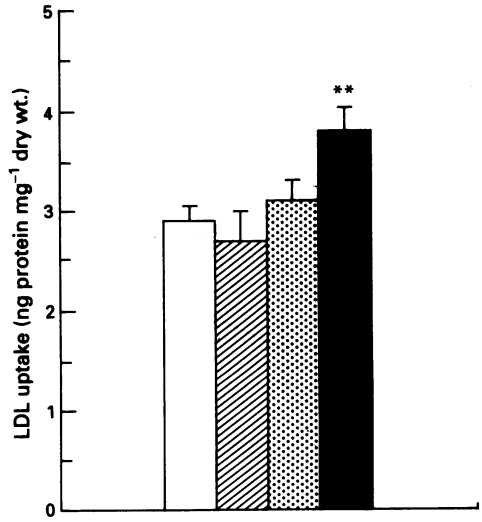

Figure 1 Dose-response effect of N^G-nitro-L-arginine methyl ester (L-NAME) on aortic uptake of human low density lipoprotein (LDL) by normotensive rats. L-NAME was administered for 6 days in the drinking water at concentrations of 0.75 ($n = 5$), 1.5 ($n = 6$) and 3.0 ($n = 6$) mg ml^{-1}, shown respectively by the increasingly shaded columns from left to right. The open column represents the controls, i.e. no L-NAME in the drinking water. The values represent means ± s.e.mean: $*P<0.05$; $**P<0.01$.

33.9 mg day^{-1} in the normotensive and 39.0 mg day^{-1} in the spontaneously hypertensive rats.

During 6 days administration of L-NAME to normotensive rats the systolic blood pressure increased progressively from 131 ± 8 to 173 ± 9 mmHg (mean ± s.e.mean), i.e. an increase of 32% compared to controls (Figure 3). In SHR treated with L-NAME the systolic blood pressure began higher than in the normotensive rats receiving L-NAME and rose during the first three days from 170 ± 3 to 198 ± 8 mmHg followed, by a decrease almost to the initial value at 185 ± 10 mmHg.

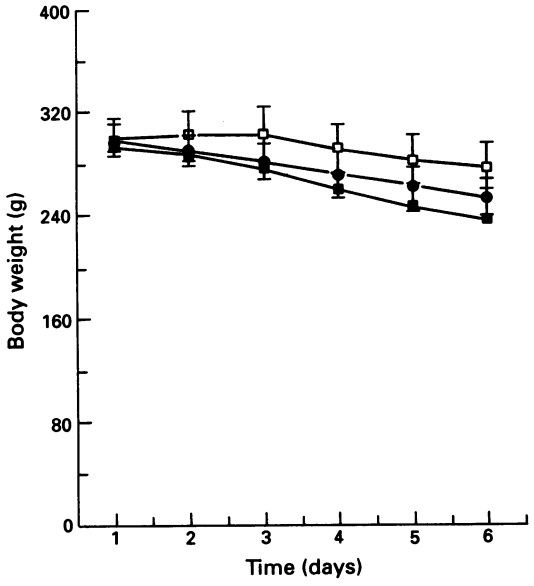

Figure 2 Changes in body weight of conscious, unrestrained normotensive and hypertensive Wistar rats during administration of N^G-nitro-L-arginine methyl ester (L-NAME) in the drinking water for 6 days. Control, (□), $n = 6$; normotensive rats + L-NAME, (●), $n = 7$; and SHR + L-NAME, (■), $n = 7$. L-NAME was administered in the water at a concentration of 3 mg ml^{-1}. The values represent means ± s.e.mean.

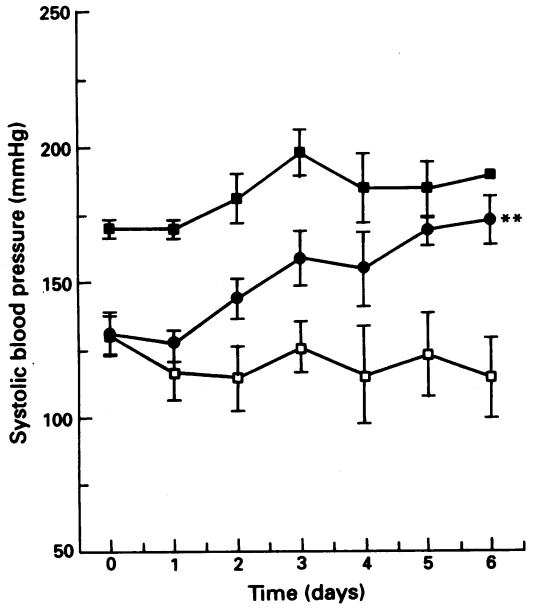

Figure 3 Effect of administration of N^G-nitro-L-arginine methyl ester (L-NAME) in the drinking water for 6 days on diastolic blood pressure of conscious, unrestrained normotensive and hypertensive Wistar rats. Control, (□), $n = 6$; normotensive rats + L-NAME, (●), $n = 7$; and SHR + L-NAME, (■), $n = 7$. The values represent means ± s.e.mean: $^*P<0.05$; $^{**}P<0.01$.

In the normotensive rats administration of L-NAME for 6 days produced significant increases in the uptake by the aorta of both LDL and fibrinogen over the final 24 h period (Figures 4 and 5). By contrast, similar administration of L-NAME to SHR rats did not increase the uptake by aorta of either LDL or fibrinogen over that in the control rats (Figures 4 and 5).

In control rats the amounts of LDL taken up per mg dry weight increased in the order aorta < heart < muscle < lung < kidney < adrenal < liver, the hepatic uptake being about 15 times greater than the aortic. For fibrinogen the uptake was aorta ≤ heart < adrenal ≤ lung ≤ muscle << liver ≤ kidney, the hepatic and renal uptakes being about 18 times greater than the aortic.

Administration of L-NAME brought about highly significant increases in uptake of both LDL and fibrinogen in the hearts of normotensive rats as well as significant increases in the hearts of SHR compared to normotensive controls.

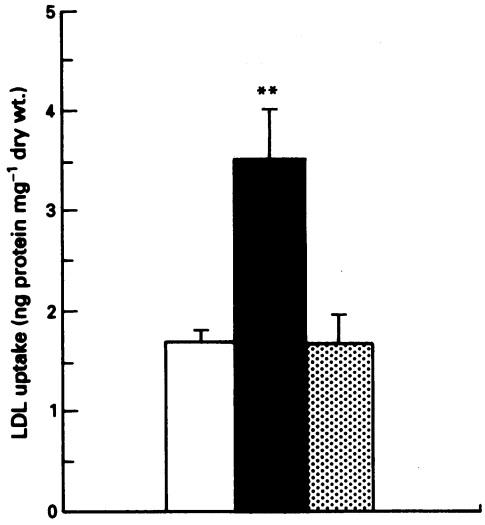

Figure 4 Aortic uptake of human low density lipoprotein (LDL) in the final 24 h of six days administration of N^G-nitro-L-arginine methyl ester (L-NAME) in the drinking water. Control, open column, $n = 6$; control rats + L-NAME, solid column, $n = 7$; and SHR + L-NAME, stippled column, $n = 7$. The values represent means ± s.e.mean: $^*P<0.05$; $^{**}P<0.01$.

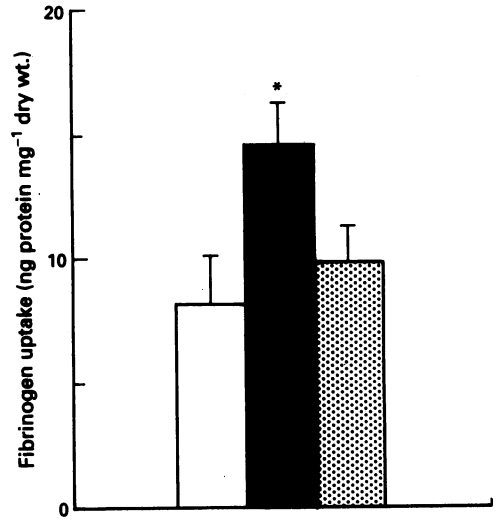

Figure 5 Aortic uptake of human fibrinogen in the final 24 h of six days administration of drinking water (control, open column, $n = 6$); normotensive rats (control + L-NAME, solid column, $n = 7$); and SHR (SHR + L-NAME, stippled column, $n = 7$). The values represent means ± s.e.mean: $^*P<0.05$; $^{**}P<0.01$.

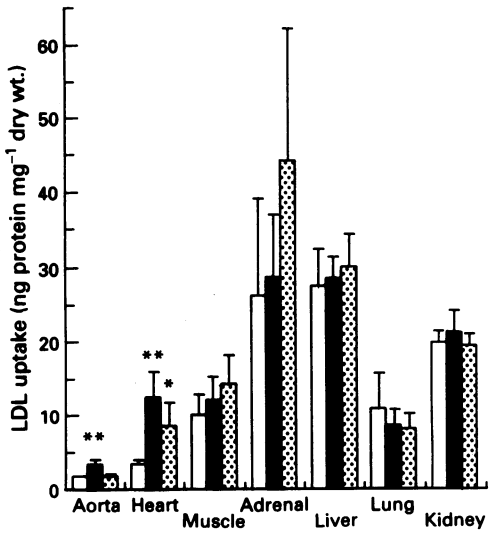

Figure 6 Uptake of human low density lipoprotein (LDL) by different organs in the final 24 h of six days administration of N^G-nitro-L-arginine methyl ester (L-NAME) in the drinking water. Control rats, open column, $n = 6$; control rats receiving L-NAME solid column, $n = 7$; and SHR receiving L-NAME, stippled column, $n = 7$. The values represent means ± s.e.mean: $*P<0.05$; $**P<0.01$.

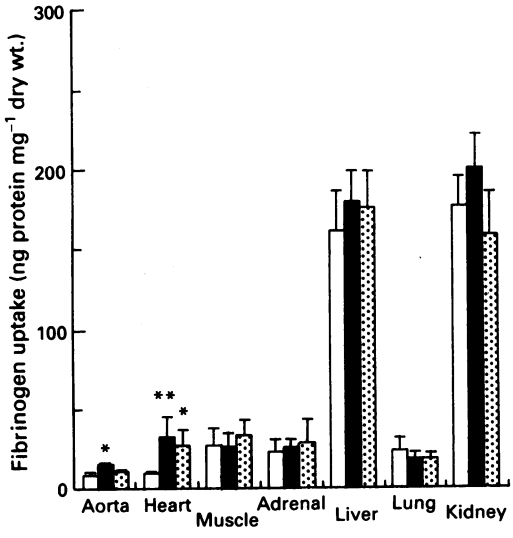

Figure 7 Uptake of human fibrinogen into different organs in the final 24 h of six days administration of N^G-nitro-L-arginine methyl ester (L-NAME) in the drinking water. Control rats, open column, $n = 6$; control rats receiving L-NAME, solid column, $n = 7$; and SHR receiving L-NAME, stippled column, $n = 7$. The values represent means ± s.e.mean: $*P<0.05$; $**P<0.01$.

However, L-NAME did not increase the uptake of either protein in any of the other organs, viz. skeletal muscle, lung, kidney, liver and adrenal (Figures 6 and 7).

Discussion

The technique of labelling LDL and fibrinogen with radioiodine via tyramine cellobiose ([^{125}I]-TC-LDL and [^{131}I]-TC-fibrinogen respectively) means that the radioactivities measured in aortic walls and other organs do not distinguish between labelled breakdown products accumulating over the 24 h periods during which the labelled LDL and fibrinogen were circulating, and whatever amounts of intact labelled LDL and fibrinogen were present at the time of measurement. We are aware that the parameter we term uptake is predominantly a measure of such accumulation. Experiments now underway will also quantify the amounts of labelled unmetabolized LDL in the wall.

The present results show that in conscious, unrestrained, normotensive rats the uptake of LDL and fibrinogen by the aortic walls is increased by administration of L-NAME. L-NAME inhibits the formation of the potent vasodilator substance nitric oxide (NO) from L-arginine by endothelial cells (Moncada *et al.*, 1988). Our rats showed the expected progressive rise in blood pressure (Rees *et al.*, 1990), so that moderate hypertension accompanied the increased uptake of the atherogenic plasma proteins by aortic walls. In this respect, therefore, our findings with L-NAME resemble those previously obtained with other pressor agents viz. noradrenaline, adrenaline and angiotensin II. Indeed, this previously observed association of uptake and blood pressure increases was a major reason for determining the effect of L-NAME as yet another means of raising blood pressure.

At first sight, therefore, the latest results would support some direct connection between the two effects. Such a connection would also plausibly emerge from observations showing that the labelling of vascular endothelial cell vesicles with ferritin is increased with increasing intralumenal pressure (Moffitt *et al.*, 1992). However, some of our own findings are inconsistent with such a conclusion. Thus, angiotensin II infused at a concentration sufficiently low as to cause only a very small and temporary rise in blood pressure still produced highly significant increases in LDL and fibrinogen uptake (Cardona-Sanclemente *et al.*, 1994b). Even more striking was the absence of any increase in uptake in age-matched spontaneously hypertensive rats in which the blood pressure was higher than that produced by any of the administered pressor agents, although still well within the range occurring in clinical hypertension. For this there are, of course, several possible explanations. SHR begin to be hypertensive soon after birth, so that three-months' old animals may have developed some kind of adaptation to the mechanism(s) responsible for the uptake increases produced by administered pressor agents after five or six days. The striking lack of the uptake-increasing effect of L-NAME, as of angiotensin II, in the SHR also suggests the development of an adaptive process in these rats. Alternatively, although both control and spontaneously hypertensive rats were Wistars, there may have been genetic factors to account for the difference.

The determination of uptake by internal organs revealed interesting differences. Thus, the uptake of LDL was greatest in the liver, as expected from the normal metabolic routing of most circulating LDL via the hepatic high affinity receptors. The similarly large uptake by the adrenals was less expected because, in the rat, most of the cholesterol for steroid production comes from high density lipoprotein (HDL) (Gwynne & Strauss, 1982). LDL uptake was much less in the heart and even less in the aorta, presumably reflecting their comparative deficiency in high affinity receptors (Vasile *et al.*, 1983). If, therefore, LDL uptake by aorta and, perhaps, by heart was mostly by the non-receptor-mediated pathway underlying atherogenesis (Vasile *et al.*, 1983; Born, 1991), this would appear to be the mechanism potentiated by L-NAME and other pressor agents. This conclusion is supported by the fact that the uptake of fibrinogen, which is not known to involve any receptor mechanism, was increased by L-NAME only in the aorta and heart.

The large uptake of fibrinogen by liver and kidney was unexpected and appears to be a new observation (Hantgan *et al.*, 1994). The concentration of fibrinogen in the plasma is a major risk factor for coronary heart disease and stroke (Heinrich *et al.*, 1994; Wilhelmsen *et al.*, 1984). In relation to this, great importance attaches to factors which control this concentration, particularly the rates of biosynthesis of fibrinogen and of its removal from the circulation, and the uptake by liver and kidney presumably represents a major mechanism of fibrinogen removal.

The greatly increased uptake of LDL and fibrinogen brought about by L-NAME in the heart but in none of the other organs may be of great significance. This effect was so large that it was present also, although reduced, in the hearts of the SHR. What aorta and other arteries have in common with the heart and what differentiates them from other organs are the large, constantly repeated motions due to the heart beat. The results suggest, therefore, that reducing nitric oxide increases the effect of pulsatility on macromolecular flux into heart and arteries. In the heart, moreover, the increased uptake was so great as to make it unlikely to have occurred only in the coronary arteries but possibly in the myocardium as well.

Considering our experimental findings in relation to human atherosclerotic disease, it is nevertheless interesting that the risk of both coronary heart disease and stroke is increased more than additively when both LDL or fibrinogen and systolic or diastolic blood pressures are high (Wilhelmsen *et al.*, 1984; Heinrich *et al.*, 1994). If our work should after all point to some mechanistic connection between blood pressure and the accumulation of atherogenic plasma proteins in arterial walls they would provide, at least in principle, an explanation for these epidemiological facts.

Whether or not this is so, our findings indicate that nitric oxide limits the flux of the atherogenic plasma proteins into artery walls. In this, the effect of nitric oxide is similar to that of the net negative charge on arterial endothelium (Görög & Born, 1982; Ludlam *et al.*, 1991) although of course, through entirely different mechanisms. Our results indicate a novel anti-atherosclerotic effect of nitric oxide and so another factor in atherogenesis.

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Comparison of the transplacental transfer of enalapril, captopril and losartan in sheep

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1 The transplacental transfers of three drugs (enalapril, captopril and losartan) which block the renin angiotensin system and have different lipophilicities were studied in chronically catheterized foetal sheep (125–139 days gestation).

2 The ability of the foeto-placental unit to convert enalapril to enalaprilat was studied in two chronically catheterized foetuses. Enalapril (3 mg kg^{-1}, 7.9 μmol kg^{-1}) given i.v. to the foetuses abolished the foetal pressor response to 5 μg angiotensin I (AI) in one foetus and attenuated the pressor response in the other.

3 Enalapril (100 mg, 5.7 μmol kg^{-1}) given i.v. to the ewe ($n = 5$) abolished the maternal pressor response to 2.5 μg AI ($n = 1$) and attenuated the maternal pressor response to 5 μg AI ($n = 5$, $P < 0.001$). The foetal pressor response to 5 μg AI ($n = 2$) and 10 μg AI ($n = 3$) did not change. The maternal and foetal pressor responses to angiotensin II (AII; $n = 5$) did not change.

4 Foetal pressor responses to 5 μg AI ($n = 1$) and 10 μg AI ($n = 2$) were attenuated within 11 min of their mothers ($n = 3$) being given i.v. captopril (15 mg, 1.5 μmol kg^{-1}). Foetal pressor responses to 5 μg AII ($n = 1$) and to 10 μg AII ($n = 2$) did not change.

5 Losartan (10 mg kg^{-1}, 21.7 μmol kg^{-1}) given i.v. to the foetus ($n = 9$) attenuated the foetal pressor response to 5 μg AII ($P < 0.001$) but the maternal pressor response to 5 μg AII did not change.

6 Losartan (100 mg, 21.7 μmol kg^{-1}) given i.v. to the ewe ($n = 5$) attenuated the maternal pressor response to 5 μg AII ($P < 0.002$) but the foetal pressor response to 5 μg AII did not change.

7 It is concluded that the foeto-placental unit of the sheep can metabolize enalapril to enalaprilat. Captopril readily crosses the sheep placenta but enalapril and losartan do not. Thus, the transplacental transfer of these drugs does not parallel their lipid solubilities. Furthermore the results show that AT_1 receptors are important in mediating the vasoconstrictor effects of AII in the foetus.

Keywords: Enalapril; captopril; losartan; transplacental transfer; foetus; foetal sheep

Introduction

The transport of substances across the placenta may occur by the following mechanisms: ultrafiltration, simple and facilitated diffusion, active transport, pinocytosis and breaks in placental villi (Parke, 1984). For transport of drugs, simple diffusion is usually the most important mechanism (Goldstein *et al.*, 1974; Parke, 1984; Benet & Sheiner, 1985) and the lipid solubility of a drug is considered a good index of its ability to cross the placenta, i.e. the more lipid soluble the drug the more readily it should cross the placenta (Mirkin, 1973; Parke, 1984; Benet & Sheiner, 1985; Wang *et al.*, 1985). The lipid solubility of a drug is indicated by its octanol/water partition coefficient (Kp), where:

$$Kp = \frac{\text{drug concentration in organic phase}}{\text{drug concentration in aqueous phase}}$$

Kp varies with the degree to which the drug is ionized and is therefore dependent on the pH and the p*K*a of the ionizable groups present within the molecule. Oh & Mirkin (1971) showed that sodium salicylate, which is completely ionized at physiological pH, crossed placental membranes more readily than it penetrated the central nervous system. Hence, although lipoprotein in nature, the placental barrier cannot be assumed to have the same properties as other biological membranes.

Drugs that block the renin angiotensin system (RAS) are amongst the most effective antihypertensive agents available. Enalapril (its active metabolite, enalaprilat) and captopril are nonpeptidic angiotensin converting enzyme (ACE) inhibitors. They have very low Kp values and low protein binding (Table 1). Enalapril is more potent and longer acting than captopril (Biollaz *et al.*, 1981; Gavras *et al.*, 1981; Sweet *et al.*, 1981). Cushman *et al.* (1989) compared the relative potencies of enalapril maleate and captopril in spontaneously hypertensive rats and found that in *ex vivo* studies, an oral dose of 20 mg kg^{-1} (41 μmol kg^{-1}) enalapril maleate produced the same ACE-inhibitory effect as 30 mg kg^{-1} (138 μmol kg^{-1}) captopril and in *in vitro* studies enalapril had an I_{50} of 2.8 nmol l^{-1} while captopril had an I_{50} of 9.7 nmol l^{-1}.

However, captopril given i.v. to the pregnant ewe has been shown to rapidly cross the placenta and block the foetal pressor response to angiotensin I (AI) (Kingsford &

Table 1 Comparison of molecular weights (mol. wt.), octanol-water partition coefficients (Kp) at pH 7 and serum protein binding of enalapril, enalaprilat, captopril and losartan

Compound	*Mol. wt.*	*Kp* (pH 7)	*Serum protein binding* (%)
Enalapril	376.46	0.071 (Randive *et al.*, 1992)	NA
Enalaprilat	348.4	<0.001 (Randive *et al.*, 1992)	40% (Lin *et al.*, 1988)
Captopril	217.9	0.004 (Randive *et al.*, 1992)	25–30% (Kubo & Cody, 1985)
Losartan	461.01	15 ± 1 (MSD)	97–99% (MSD)

NA = not available; MSD = data supplied by Merck Sharp & Dohme.

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Lumbers, 1989; Lumbers *et al.*, 1992). Broughton Pipkin & Wallace (1986) reported that enalapril given i.v. to the pregnant ewe did not appear to cross the sheep placenta. They tested maternal ACE activity by giving bolus injections of 5 or 10 μg AI and by measuring ACE levels. However, although foetal ACE levels were measured, foetal pressor responses to AI were not tested. Enalapril is a prodrug with little or no direct biological activity (Gross *et al.*, 1981). In the adult, it is desterified in the liver to the very potent and long-lasting ACE inhibitor, enalaprilat (Ulm, 1983). Thus it is important to establish whether or not the foeto-placental unit is capable of converting enalapril to enalaprilat.

ACE inhibitors not only block the production of angiotensin II (AII) but also increase endogenous bradykinin levels (Yang *et al.*, 1970; 1971; Das & Soffer, 1975; Ondetti *et al.*, 1977; Swartz *et al.*, 1979) and prostaglandin release (McGiff *et al.*, 1972; Murthy *et al.*, 1978; Swartz *et al.*, 1980; Moore *et al.*, 1981). Therefore if studies are designed to investigate the role of the RAS, a more specific method would be to inhibit the actions of AII by receptor blockade. Losartan is a nonpeptide, AT_1 receptor antagonist with a high Kp (Table 1) that lacks agonist, kinin and prostaglandin inducing properties (Wong *et al.*, 1990a,b). Losartan is metabolized from a competitive AT_1 receptor antagonist to a primary metabolite, EXP3174, which is a non-competitive receptor antagonist (Smith *et al.*, 1992). EXP3174 has been shown to be partly responsible for the hypotensive effect of losartan in rats (Wong *et al.*, 1990c) and man (Ohtowa *et al.*, 1993) but not dogs (Wong *et al.*, 1991). There is no information on the metabolism of losartan to EXP3174 in sheep. There are no reports in the current literature on the transplacental transfer of losartan.

Although a mixture of both AT_1 and AT_2 receptor populations have been reported in the foetus, the majority of receptors appear to be AT_2 (Tsutsumi *et al.*, 1991a,b; Grady *et al.*, 1991; Viswanathan *et al.*, 1991; Grone *et al.*, 1992). All known biological actions of AII appear to be mediated via AT_1 receptors and currently no physiological functions have been definitely associated with the AT_2 receptor. Therefore, before assessing the transplacental transfer of losartan we established that i.v. administration of losartan to the foetus attenuated the foetal pressor response to AII, i.e. the ovine foetus had functional AT_1 receptors.

Because drugs which block the RAS are potentially very effective antihypertensive agents and because the lipid solubility of a drug is generally accepted as a good index of its ability to cross the placenta, we assessed the transplacental transfer of three drugs which inhibit the RAS and which have different Kps. These drugs were the ACE inhibitors, captopril and enalapril and the AT_1 receptor antagonist, losartan. As the foetal RAS is important in the maintenance of normal foetal renal function (Lumbers & Stevens, 1987; Lumbers *et al.*, 1993) it can be inferred that adverse foetal renal effects will be applicable to all inhibitors of the RAS that cross the placenta.

In these experiments the ability of the ovine foetus to convert enalapril to enalaprilat was assessed by testing foetal pressor responses to AI before and after i.v. administration of enalapril to the foetus. The effects of enalapril and captopril on the transplacental blockade of ACE activity were assessed by testing maternal and foetal pressor responses to AI before and after i.v. administration of these ACE inhibitors to the ewe. The transplacental transfer of losartan was assessed by examining maternal and foetal pressor responses to AII before and after i.v. administration of losartan to the ewe.

Methods

Surgery

Pregnant ewes were anaesthetized with 1.5 g sodium thiopentone, i.v. Anaesthesia was maintained with 1–3% halothane in oxygen; the foetus was also anaesthetized by these agents. Under aseptic conditions, polyvinyl catheters (1.5 mm O.D., 1.0 mm I.D.) were inserted into a foetal femoral artery and both recurrent tarsal veins. Polyvinyl catheters (2.7 mm O.D., 1.5 mm I.D.) were inserted into a maternal femoral vein and femoral artery and into the amniotic cavity. At the end of surgery and for the next 2 days, 3 ml of a mixture of procaine penicillin (600 mg) and dihydrostreptomycin sulphate (750 mg) were given i.m. to the ewe and intra-amniotically to the foetus. The ewes were housed in metabolic cages in a laboratory maintained at 18–20°C. They had free access to water and were given a diet of lucerne chaff (up to 2 kg day^{-1}), oats (140 g day^{-1}) and 6 g NaCl. These experiments were approved by the Animal Care and Ethics committee of the University of New South Wales.

Experimental protocol

Experiments were carried out in 12 chronically catheterized pregnant ewes and their foetuses (gestation age 125–139 days). Intra-amniotic and maternal and foetal arterial pressures were monitored continuously with Bell and Howell pressure transducers connected to a Grass Polygraph recorder. Pressor responses to i.v. bolus doses of AI and AII in ewes and their foetuses were staggered in time and were tested at least in duplicate. When AI and AII were given to the ewe, foetal arterial pressure was unaffected; likewise when AI and AII were given to the foetus, maternal arterial pressure did not change.

Enalapril

The ability of the foeto-placental unit of the sheep to convert enalapril to enalaprilat was tested in two chronically catheterized foetal sheep (135 days gestation). Foetal pressor responses to 5 μg AI and 5 μg AII were measured before and for up to 30 min after the i.v. administration of enalapril to the foetus (3 mg kg^{-1}, 7.9 μmol kg^{-1}).

Placental transfer of enalapril was tested in 5 sheep aged 126–139 days gestation (mean ± s.e.mean: 133 ± 2 days). Maternal and foetal pressor responses to AI and AII were measured before and after the ewes were given i.v. enalapril (100 mg, 5.7 μmol kg^{-1}). Maternal ACE activity was assessed by measuring the maternal pressor response to 2.5 μg AI ($n = 1$) and 5 μg AI ($n = 5$). Foetal ACE activity was assessed by measuring the foetal pressor responses to 5 μg AI ($n = 2$) and 10 μg AI ($n = 3$). Maternal pressor responses to 5 μg AII ($n = 5$) and foetal pressor responses to 5 μg AII ($n = 2$) or 10 μg AII ($n = 3$) were also measured. Pressor responses were tested for up to 121 min (mean ± s.e.mean: 49 ± 18 min) following the administration of enalapril to the ewe.

Captopril

Three of the ewes (133, 134 and 139 days gestation) were then given an i.v. bolus of captopril (15 mg, 1.46 μmol kg^{-1}), 36 ± 3 min after enalapril, and maternal pressor responses to 5 μg AI ($n = 3$) and 5 μg AII ($n = 3$) were measured. In two of these sheep, maternal pressor responses to 5 μg AI and 5 μg AII and foetal pressor responses to 10 μg AI and 10 μg AII were measured for up to 38 min. In the other sheep, maternal and foetal pressor responses to 5 μg AI and 5 μg AII were measured for 33 min.

Losartan

In nine sheep aged 125–132 days gestation (130 ± 1 days) maternal and foetal pressor responses to 5 μg AII were measured before and 1 h after foetuses were given an i.v. bolus of losartan (10 mg kg^{-1}, 21.7 μmol kg^{-1}). These foetuses were given a second i.v. bolus of losartan

(10 mg kg^{-1}, 21.7 μmol kg^{-1}) and again, after 1 h, maternal and foetal pressor responses to 5 μg AII were measured.

In five sheep aged 126–135 days gestation (131 ± 2 days) maternal and foetal pressor responses to 5 μg AII were measured before and after ewes were given an i.v. bolus of losartan (10 mg kg^{-1}, 21.7 μmol kg^{-1}). Pressor responses were measured for up to 97 min (60 ± 14 min) after administration of losartan to the ewe.

In another sheep, maternal and foetal pressor responses to 2.5 μg AII and 5 μg AII were measured before and for 1 h after the ewe was given an i.v. bolus of losartan (200 mg, 9.2 μmol kg^{-1}). The foetus was given an i.v. bolus of losartan (200 mg, 144.6 μmol kg^{-1}), 103 min after losartan had been given to the ewe, and the maternal pressor responses to 2.5 μg AII and 5 μg AII and the foetal pressor responses to 2.5 μg AII, 5 μg AII and 100 μg AII were tested. In a second sheep maternal and foetal pressor responses to 2.5 μg AII, 5 μg AII and 10 μg AII were measured before the ewe was given an i.v. bolus of losartan (300 mg, 13.8 μmol kg^{-1}). Then the maternal pressor responses to 10 μg AII and the foetal pressor responses to 2.5 μg AII, 5 μg AII and 10 μg AII were tested. The foetus was given an i.v. bolus of losartan (18.9 mg, 13.7 μmol kg^{-1}), 40 min after losartan was given to the ewe, and the maternal pressor responses to 10 μg AII and the foetal pressor responses to 2.5 μg AII, 5 μg AII and 10 μg AII were tested.

Foetal body weight (FBW) at the time of the experiment was calculated from the formula:

$$FBW = e^{\ln(\text{gestational age, days}) \times 2.94182 - 6.27298}$$

(Gibson & Lumbers, 1993)

Analysis of data

Values are for systolic and diastolic pressures (unless otherwise stated) and are expressed as mean ± standard error of the mean (s.e.mean). Foetal systolic and diastolic pressures were corrected for IAP prior to determining the pressor responses. Data were analysed with an IBM compatible PC and the software package SPSS/PC (Nie *et al.*, 1982). Student's paired *t* test was used to test for statistical significance before and after treatment. As only two foetuses were given enalapril, individual results for these experiments are given. In the study in which foetuses were given two doses of losartan, two-way analysis of variance was used to analyse data and Newman-Keuls test was used to find the means which differed (Zar, 1984).

Drugs

Sodium thiopentone (Pentothal, Abbott Australasia Pty. Ltd.; Sydney, N.S.W., Australia) and halothane (Fluothane, ICI; Macclesfield, Cheshire, England) were used as anaesthetic agents. Procaine penicillin and dihydrostreptomycin mixture (Hydropen, Bomac Laboratories; Asquith, N.S.W., Australia) was administered post-operatively. Enalapril (pure substance; Merck Sharp & Dohme; South Granville, N.S.W., Australia), captopril (Squibb; Noble Park, Victoria, Australia) and losartan (potassium salt; The Du Pont Merck Pharmaceutical Company; Wilmington, DE, U.S.A.), angiotensin I (Boehringer Mannheim Australia Pty. Ltd.; Sydney, N.S.W., Australia) and angiotensin II (Hypertensin, Ciba Geigy; Basle, Switzerland) were dissolved in 0.15 M NaCl on the day of use. Sodium pentobarbitone i.v. (Lethobarb, Arnolds of Reading; Victoria, Australia) was used to kill the ewe and foetus.

Results

Enalapril

The ability of the foeto-placental unit to convert enalapril to enalaprilat was tested in two foetuses. In one foetus the pressor response to 5 μg AI decreased from 11.9/7.0 mmHg during control to 4.2/6.3 mmHg 6 min after enalapril and was abolished 8 min after enalapril was given to the foetus. The pressor response to 5 μg AII did not change from control values of 18.3/13.3 mmHg. In the other foetus, the pressor response to 5 μg AI fell from 18.2/15.2 to 8.2/5.9 after 10 min and was 5.4/4.1 mmHg after 30 min. The foetal pressor response to 5 μg AII did not change from control values of 19.1/14.7 mmHg.

The i.v. administration of enalapril to pregnant ewes completely blocked the maternal pressor response to 2.5 μg AI ($n = 1$) and attenuated the maternal pressor responses to 5 μg AI ($n = 5$, $P < 0.001$; Figure 1a). The foetal pressor response to 5 μg AI ($n = 2$) and 10 μg AI ($n = 3$) did not change (Figure 1b). In one of these sheep the maternal pressor response to 2.5 μg AI (27.5/30.0 mmHg) was abolished within 6 min of administration of enalapril (Figure 1a) and the maternal pressor response to 5 μg AI was markedly attenuated by 12 min (from 41.9/41.3 to 10.0/6.7 mmHg) and by 78 min it was 8.5/6.0 mmHg. The foetal pressor response to 10 μg AI was unchanged throughout this period; before enalapril was given to the ewe it was 14.6/9.6 and after 68 min it was 13.25/8.25 mmHg. The maternal pressor response to 5 μg AII ($n = 5$) and the foetal pressor responses to 5 μg AII ($n = 2$) and 10 μg AII ($n = 3$) did not change (Figure 2).

Captopril

Pressor responses to AI were attenuated in each of the foetuses whose mothers were given captopril. In one foetus, the pressor responses to 5 μg AI had fallen from 14.4/9.4 to 4.6/3.8 mmHg, 11 min after captopril was given i.v. to the ewe but the foetal pressor response to 5 μg AII was

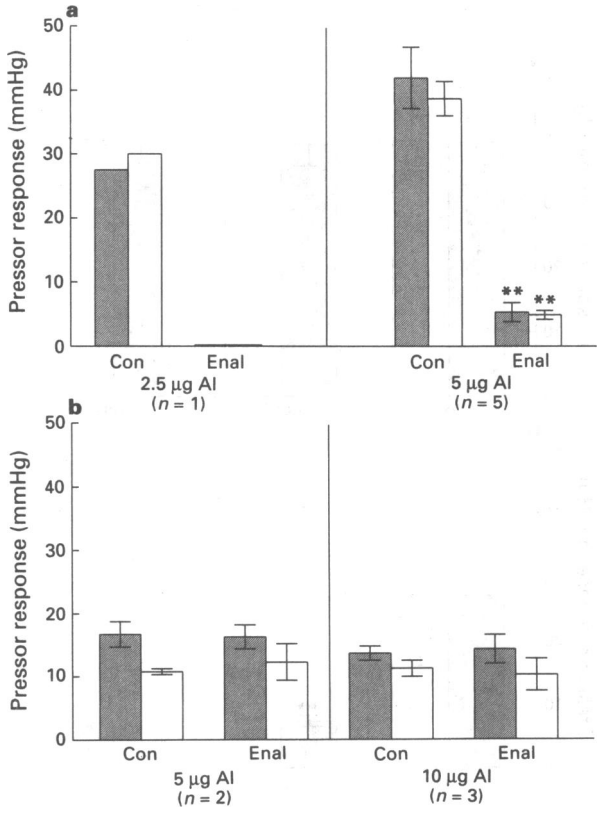

Figure 1 Mean ± s.e.mean for systolic (hatched columns) and diastolic (open columns) pressor responses of (a) the ewe to 2.5 μg angiotensin I (AI) and 5 μg AI and (b) the foetus to 5 μg AI and 10 μg AI before (Con) and after (Enal) the ewe was given i.v. enalapril (100 mg, 5.7 μmol kg^{-1}). **$P < 0.001$ when compared with control.

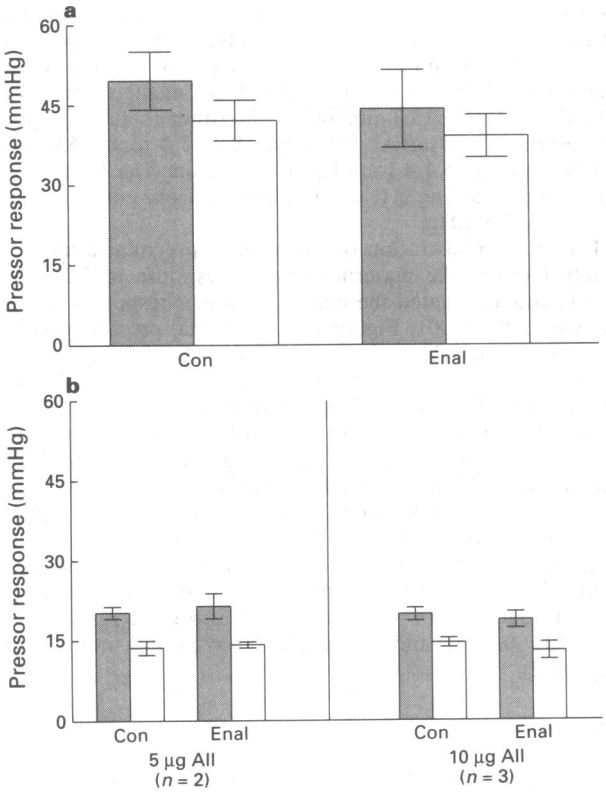

Figure 2 Mean ± s.e.mean for systolic (hatched columns) and diastolic (open columns) pressor responses of (a) the ewe to 5 μg angiotensin II (AII) ($n = 5$) and (b) the foetus to 5 and 10 μg AII before (Con) and after (Enal) the ewe was given i.v. enalapril (100 mg, 5.7 μmol kg^{-1}).

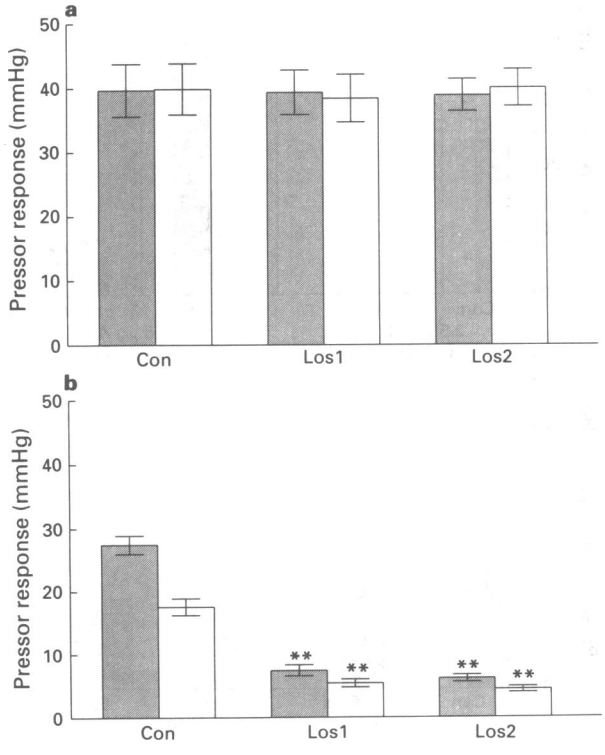

Figure 3 Mean ± s.e.mean for systolic (hatched columns) and diastolic (open columns) pressor responses of (a) the ewe and (b) the foetus to 5 μg angiotensin II (AII) before (Con), 1 h (Los1) and 2 h (Los2) after the i.v. administration of losartan (10 mg kg^{-1}, 21.7 μmol kg^{-1}) to the foetus. $^{**}P < 0.001$ when compared with control ($n = 9$).

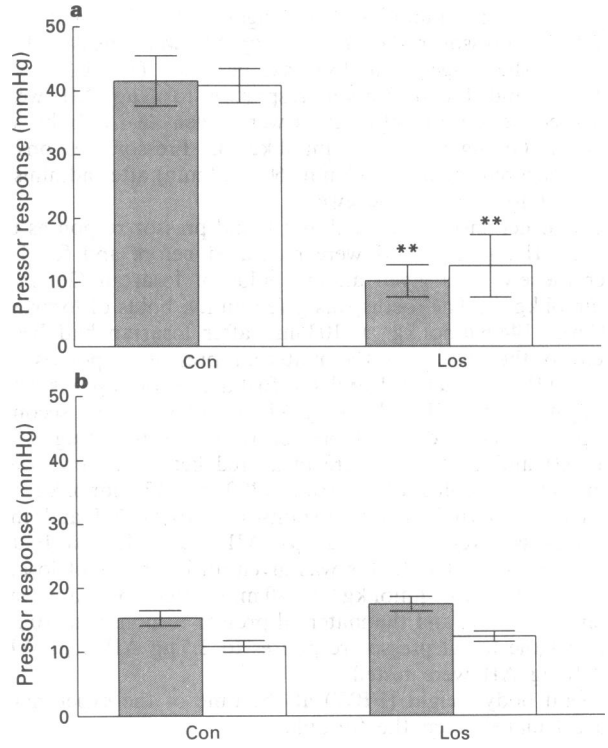

Figure 4 Mean ± s.e.mean for systolic (hatched columns) and diastolic (open columns) pressor responses of (a) the ewe and (b) the foetus to 5 μg angiotensin II (AII) before (Con) after the i.v. administration of losartan (10 mg kg^{-1}, 21.7 μmol kg^{-1}) to the ewe. $^{**}P < 0.002$ when compared with control ($n = 5$).

unchanged from control values of 23.8/13.6 mmHg. In another foetus, the pressor response to 10 μg AI fell from 17.0/14.5 to 8.8/10.0 mmHg, 7 min after captopril was given i.v. to the ewe and the pressor response to 10 μg AII was unchanged from control values of 19.9/15.6 mmHg. In the third foetus, the pressor response to 10 μg AI fell from 11.7/11.1 to 6.9/5.6 mmHg, 8 min after captopril was i.v. given to the ewe and the foetal pressor response to 10 μg AII was unchanged from control values of 15.7/10.1 mmHg.

Losartan

The i.v. administration of losartan (10 mg kg^{-1}, 21.7 μmol kg^{-1}) to foetuses attenuated the foetal pressor response to 5 μg AII. The foetal pressor response fell from 27.4 ± 1.5/17.5 ± 1.3 to 7.4 ± 0.9/5.4 ± 0.6 mmHg ($P < 0.001$) 1 h after the first dose of losartan and was 6.1 ± 0.6/4.4 ± 0.5 mmHg ($P < 0.001$, compared with control) 1 h after the second dose of losartan (Figure 3b). There was no difference between the foetal pressor responses at 1 h and 2 h. Maternal pressor responses to 5 μg AII were not changed (Figure 3a).

The i.v. administration of losartan (10 mg kg^{-1}, 21.7 μmol kg^{-1}) to pregnant ewes attenuated the maternal pressor responses to 5 μg AII ($P < 0.002$; Figure 4a); the foetal pressor responses to 5 μg AII did not change (Figure 4b).

In two other sheep, pressor responses to a number of doses of AII were established. In one sheep an i.v. bolus of losartan (200 mg, 9.2 μmol kg^{-1}) to the ewe attenuated the maternal pressor response to 2.5 μg AII (from 50.0/30.0 to 10.0/15.0 mmHg) and to 5 μg AII (from 55.0/33.0 to 35.0/25.0 mmHg); but did not change the foetal pressor responses to 2.5 μg AII (from 22.5/12.5 to 25.0/12.5 mmHg) and to 5 μg AII (from 35.0/20.0 to 35.0/22.0 mmHg). In the same animal, an i.v. bolus of losartan (200 mg, 144.6 μmol kg^{-1}) to the foetus abolished the foetal pressor response to 2.5 μg

AII and to 5 μg AII. The foetal pressor response to 100 μg AII was only 10.0/10.0 mmHg, 17 min after administration of losartan to the foetus. The maternal pressor responses to AII were not attenuated by losartan administration to the foetus (2.5 μg AII: from 25.0/20.0 to 30.0/30.0 mmHg; 5 μg AII: from 35.0/25.0 to 45.0/35.0 mmHg).

In the second sheep administration of an i.v. bolus of losartan (300 mg, 13.8 μmol kg^{-1}) to the ewe abolished the maternal pressor response to 10 μg AII. The foetal pressor responses to 2.5 μg, 5 μg and 10 μg AII were not changed from control values of 23.0/14.0, 25.0/16.3 and 46.5/29.4 mmHg, respectively (after losartan they were 22.5/15.0, 25.0/17.5 and 50.0/26.0 mmHg, respectively). The maternal pressor response to 10 μg AII was still completely blocked 48 min after losartan had been administered to the ewe. The i.v. administration of losartan (18.9 mg, 13.7 μmol kg^{-1}) to the foetus completely blocked the foetal pressor response to 2.5 μg AII and 5 μg AII and markedly attenuated the foetal pressor response to 10 μg AII from 46.5/29.4 to 13.5/8.5 mmHg.

Discussion

In these experiments the transplacental transfers of enalapril, captopril and losartan were determined by direct bioassay. Direct bioassay not only provides an assessment of the transplacental transfer of a drug but also indicates the biological impact of a particular dose of a drug given to the mother on both mother and foetus.

Because enalapril is a prodrug with little or no direct biological activity (Gross *et al.*, 1981) it was necessary to establish whether or not the foeto-placental unit of the sheep was capable of converting enalapril to enalaprilat. The results show that the foeto-placental unit of the sheep is capable of this conversion.

Administration of 100 mg (5.7 μmol kg^{-1}) enalapril i.v. to the pregnant ewe inhibited the maternal but not the foetal pressor response to AI (Figure 1). These results are in agreement with the findings of Broughton Pipkin & Wallace (1986) and indicate that enalapril does not cross the sheep placenta.

Although enalapril given i.v. to the ewe did not inhibit the foetal pressor response to AI, captopril given i.v. to the ewe attenuated, within 11 min, the foetal pressor response to AI in all three foetuses in which it was studied. Captopril was given to the ewes 36 ± 3 min following enalapril and pressor responses tested for up to 38 min. Enalapril was shown to have no effect on foetal pressor responses to AI within this time period. Therefore the inhibition of the foetal pressor response to AI following administration of captopril to the ewe are due to the effects of captopril alone.

The rapid inhibition of the foetal pressor response to AI following administration of captopril to the ewe is similar to results previously reported (Kingsford & Lumbers, 1989; Lumbers *et al.*, 1992). It has also been shown that captopril given i.v. to the chronically catheterized pregnant ewe (15 mg i.v. bolus followed by a continuous infusion at 6 mg h^{-1}) not only crosses the placenta and inhibits the foetal pressor response to 5 μg AI but also causes a fall in foetal mean arterial pressure (Lumbers *et al.*, 1992; 1993). Broughton Pipkin *et al.* (1982) also showed that captopril (2.8–3.5 mg kg^{-1}) given as an i.v. bolus to the pregnant ewe caused a fall in foetal arterial pressure within 10 min of administration.

Since losartan given i.v. to the foetus inhibited the foetal pressor response to AII, AT_1 receptors must be important in mediating the pressor effect of AII in the foetus. Thus it is valid to test for the transplacental transfer of losartan by measuring pressor responses to AII. There was no evidence that losartan crossed the sheep placenta.

Enalapril, enalaprilat and captopril have very low lipid solubilities whereas losartan is very lipid soluble (Table 1). Both enalaprilat and captopril exhibit similarily low levels of protein binding (Table 1). Enalapril, enalaprilat, captopril and losartan all have molecular weights <600 (Table 1) and such molecules should cross the placenta more readily if sufficiently lipid soluble (Parke, 1984). Since the lipophilicities and protein bindings of enalaprilat and captopril are similar, the more rapid rate of transfer of captopril (in sufficient amounts to be biologically effective) compared with enalapril cannot be explained on the basis of different lipophilicity or protein binding. The molecular weight of captopril is about half that of losartan but captopril is also several orders of magnitude less lipid soluble than losartan (Table 1). It is unlikely that difference in molecular weight alone could explain the rapid transplacental transfer of captopril. It is possible that the rapid inhibition of the foetal RAS following captopril administration to the ewe is due to active transport of captopril across the placenta via an amino acid carrier-mediated system. There is some evidence that the rapid rate of absorption of captopril from the gastrointestinal tract (despite its low lipid solubility) is due to an amino acid carrier-mediated system in rats (Hu & Amidon, 1988). A variety of transport systems for individual amino acids exist in the placenta (Verhaeghe & Assche, 1992). Finally, metabolism of enalapril, enalaprilat and losartan by the placenta into inactive metabolites cannot be excluded.

The sheep placenta is an epitheliochorial placenta whereas the placenta of man is haemomonochorial (Faber & Thornburg, 1983). Thus it should not be assumed that these results obtained in the sheep would be the same in man. However, the pregnant ewe and her foetus is one of the most popular models in developmental biology and pharmacology. In 1982 Broughton Pipkin *et al.* reported that captopril given to pregnant ewes and rabbits increased the rate of still-births in both species. The clinical use of captopril to treat pregnancy associated hypertension has been associated with foetal (Knott *et al.*, 1989) and neonatal anuria (Guignard *et al.*, 1981; Rothberg & Lorenz, 1984). Studies by Lumbers *et al.* (1993) showed that captopril given to the pregnant ewe rapidly crossed the placenta and disrupted foetal renal function and explained why the use of captopril in human pregnancy is associated with anuria. Schubiger *et al.* (1988) reported acute renal failure in a neonate whose mother was treated with enalapril for pregnancy-associated hypertension suggesting that enalapril, like captopril, crosses the human placenta. However, Broughton Pipkin & Wallace (1986) found that enalapril given i.v. to the ewe (1 mg kg^{-1}) did not cross the sheep placenta. Our results also show that captopril, but not enalapril or losartan, rapidly crosses the sheep placenta. Because the lipid solubility of a drug is so widely accepted as a good clinical index of its ability to cross the placenta (Mirkin, 1973; Goldstein *et al.*, 1974; Parke, 1984; Wang *et al.*, 1985; Benet & Sheiner, 1985) the results described above are of great interest because captopril, enalapril and losartan did not cross the sheep placenta as would be predicted from their lipid solubilities.

Thus, it is concluded that the foetal sheep can metabolize enalapril to enalaprilat and that AT_1 receptors are important in mediating the vasoconstrictor effects of AII in the foetus. Captopril with a very low Kp crosses the epitheliochorial placenta of the sheep whereas enalapril, which also has a low Kp, and losartan which has a high Kp do not cross. Thus Kp cannot be used as a measure of the ease with which these drugs cross the sheep placenta.

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Modulation of acetylcholine release at mouse neuromuscular junctions by interaction of three homologous scorpion toxins with K^+ channels

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1 The effects of three scorpion toxins, charybdotoxin (CTX), iberiotoxin (IbTX), and noxiustoxin (NTX) have been studied on acetylcholine release and on K^+ channels by means of twitch tension and electrophysiological recording techniques using isolated skeletal muscle preparations and by a radioligand binding assay using ^{125}I-labelled dendrotoxin I (DpI) and rat brain synaptosomal membranes.

2 On chick biventer cervicis preparations, CTX and IbTX (125 nM) augmented the twitch responses to indirect muscle stimulation. Further, the increase (about 70–80% of control twitch height) was fast in onset, reaching a maximum within 25–30 min. NTX at 125 nM produced a slower augmentation of the twitch responses to indirect muscle stimulation, with the maximum response being seen after 40–50 min.

3 On mouse triangularis sterni preparations, CTX (300 nM after 35–40 min) and IbTX (100 nM after 15 min) increased quantal content of the evoked endplate potentials (e.p.p.) by about two fold. However, NTX (300 nM) caused only a small increase in e.p.p. amplitude, which was followed by repetitive e.p.ps in response to single shock nerve stimulation after 40–50 min.

4 Extracellular recording of nerve terminal current waveforms in triangularis sterni preparations revealed that CTX and IbTX (3–100 nM), but not NTX (100 nM), blocked the Ca^{2+}-activated K^+ current, $I_{K\text{-}Ca}$. However, there was no major change in the portion of the nerve terminal waveform associated with voltage-dependent K^+ currents, I_{Kv}.

5 In the radioligand binding assay, NTX potently displaced labelled [^{125}I]-DpI, whereas CTX produced only partial displacement. However, IbTX did not displace [^{125}I]-DpI from its binding sites on rat brain synaptosomal membranes.

6 We conclude that these three structurally homologous scorpion toxins act on different K^+ channels and that this leads to different patterns of facilitation of acetylcholine release. IbTX acts selectively on high conductance Ca^{2+}-activated K^+ channels, leading to an increase in the amplitude of e.p.ps without any other changes. NTX acts on voltage-dependent K^+ channels that are sensitive to dendrotoxin and causes repetitive e.p.ps. CTX shares amino acid residues that exist in the structures of IbTX and NTX; CTX acts on both Ca^{2+}- and voltage-dependent K^+ channels.

Keywords: Neurotoxins; potassium channels; neuromuscular junction; acetylcholine release; dendrotoxin; charybdotoxin; iberiotoxin; noxiustoxin

Introduction

Potassium channels with distinctive properties and pharmacological sensitivites play critical physiological roles in many types of cells. Although several different genes encoding K^+ channel proteins have been cloned and expressed in oocytes, it is still unclear which gene products are important in regulation of such processes as neurotransmitter release. One approach to the study of channel function *in situ* is to examine the effects of highly selective channel blockers. Polypeptide toxins from several Old World scorpions block voltage-gated inactivating K^+ channels (for review, see Harvey *et al.*, 1993). Moreover, some toxins from *Leiurus quinquestriatus hebraeus* (e.g. charybdotoxin, CTX) block high-conductance Ca^{2+}-activated K^+ channels (Miller *et al.*, 1985) as well as voltage-gated inactivating K^+ channels (Schneider *et al.*, 1989; Schweitz *et al.*, 1989; Blaustein *et al.*, 1991). In contrast, venoms from several New World scorpions contain polypeptides that selectively block only voltage-gated, non-inactivating K^+ channels (Blaustein *et al.*, 1991). The present paper describes the interactions of three homologous scorpion toxins with K^+ channels that lead to increase in transmitter release from cholinergic nerve terminals.

Noxiustoxin (NTX), a 39-residue-chain peptide toxin from the scorpion *Centruroides noxius*, is known to block neuronal K^+ channels (Carbone *et al.*, 1987). It facilitated acetylcholine release in chick biventer cervicis preparations and displayed binding of a radiolabelled dendrotoxin from synaptosomal membranes from rat brain (Harvey *et al.*, 1992). NTX probably blocks dendrotoxin-sensitive voltage-dependent K^+ channels in rat brain synaptosomal membranes (Harvey *et al.*, 1992) and at higher concentrations it also blocks Ca^{2+}-activated K^+ channels from T-tubules of skeletal muscle (NTX's $K_d = 450$ nM) (Valdivia *et al.*, 1988).

Iberiotoxin (IbTX) from *Buthus tamulus* venom consists of a single polypeptide chain (37 residues) that can block $I_{K\text{-}Ca}$ selectively; it does not block other types of K^+ channels (Galvez *et al.*, 1990). IbTX displays 68% sequence identity with charybdotoxin (CTX). However, IbTX possesses four more acidic and one less basic amino acid residue than does CTX, making this toxin much less positively charged than the other peptide (Galvez *et al.*, 1990). IbTX interacts with CTX at a distinct site on the channel and modulates CTX binding by an allosteric mechanism (Galvez *et al.*, 1990; Candia *et al.*, 1992). IbTx has recently been reported to

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increase acetylcholine release at frog neuromuscular junctions (Robitaille *et al.*, 1993).

CTX is a 37-amino acid peptide from the venom of the scorpion *Leiurus quinquestriatus hebraeus*, which was originally described as a potent blocker of high-conductance Ca^{2+}-activated K^+ channels (Miller *et al.*, 1985). Subsequently, it was shown that CTX can also block the inactivating voltage-dependent K^+ channel that is present in rat brain and human T lymphocytes, but not other types of K^+ channels (Gimenez-Gallego *et al.*, 1988; Stuhmer *et al.*, 1989; Price *et al.*, 1989; Oliva *et al.*, 1991). CTx also binds to dendrotoxin-sensitive proteins from brain membranes, implying an action on voltage-dependent K^+ channels (Schweitz *et al.*, 1989; Harvey *et al.*, 1989). CTx has been found to increase acetylcholine release at frog neuromuscular junctions (Robitaille & Charlton, 1992), although an earlier study on a mouse preparation reported no effect under normal recording conditions (Anderson *et al.*, 1988).

Neuronal membranes contain several distinct K^+ conductances that can be distinguished by their voltage-dependency and interactions with blocking agents (Rogawski, 1985). Potassium channels contribute to the repolarization of the membrane after an action potential and to the regulation of the excitability of neurones, and they help to regulate the release of transmitter from nerve terminals. Their blocking by a K^+ channel blocker (e.g., 3,4-diaminopyridine) can cause prolongation of the time course of depolarization and increase transmitter release. The importance of particular subtypes of K^+ channel to nerve terminal function cannot be examined by use of agents such as 3,4-diaminopyridine or tetraethylammonium because of their low selectivity. It was hoped that a comparison of effects of the more selective blocking agents IbTx, CTX and NTX would reveal the physiological contributions of voltage- and Ca^{2+}-dependent K^+ channels at motor nerve terminals in mouse skeletal muscle.

Methods

Twitch tension recording

Biventer cervicis nerve-muscle preparations (Ginsborg & Warriner, 1960) were isolated from 3–14 day old chicks and mounted with a resting tension of 0.5–1 g in 2 or 10 ml tissue baths containing physiological salt solution of the following composition (mM): NaCl 118.5, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_2 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 11.1. The solution was maintained at 33°C and was bubbled with 95% O_2 plus 5% CO_2 to buffer at pH 7.3. Muscle was indirectly stimulated through ring electrodes every 10 s with pulses of 0.2 ms duration and a voltage greater than that which produced a maximal twitch.

Electrophysiology

Experiments were performed at room temperature (20–25°C) on the left triangularis sterni nerve-muscle preparation (McArdle *et al.*, 1981) isolated from male mice (Balb C, 20–25 g). The complete dissection of the muscle, was performed under continuous perfusion at a rate of 12–20 ml min^{-1} with physiological solution of the above composition.

To study evoked transmitter release, the muscle was prevented from twitching by using low Ca^{2+} (0.5 mM) and high Mg^{2+} (1.5–5.0 mM) physiological solution. The recording electrode (3 M KCl, 8–20 Megohms) was placed in a muscle cell at an endplate region, as judged by the appearance of miniature endplate potentials (m.e.p.ps) with a rise time of 1 ms or less. In most experiments, 150–250 m.e.p.ps and 80–150 e.p.ps evoked by stimulation of 0.5 Hz were recorded from each endplate, at each time period. The same recording site was used throughout each experiment.

For extracellular recording, presynaptic waveforms were recorded with a glass microelectrode (2 M NaCl, 16–30 Megohms) placed inside the perineural sheath (near endplate areas) of one of the branches of an intercostal nerve (Mallart, 1985; Penner & Dreyer, 1986; Anderson *et al.*, 1988). Usually 50–70 waveforms were recorded at each time period. Preparations were stimulated at 0.5 Hz. Recording sites were rejected if signal amplitude varied by more than 10% during the first 15 min prior to addition of the test substance. Physiological salt solution (10–20 ml) containing toxins was aerated and recirculated through the tissue bath during electrophysiological recording.

Radioligand binding assay

Synaptosomal membranes were prepared from the brains of 200–250 g male Sprague-Dawley rats (Harvey *et al.*, 1989). Each brain was homogenized in 10 ml homogenization buffer (32 mM sucrose, 2 mM Tris.HCl; pH 7.4) at 4°C. The suspension was centrifuged at 29000 r.p.m. for 10 min. The resulting pellet was lysed by addition of 10 ml lysis buffer (5 mM Tris.HCl; pH 8.1). The suspension was centrifuged at 20000 r.p.m. for a further 20 min, and the pellet was finally resuspended in 25 ml synaptosomal buffer of the following composition (mM): NaCl 130, KCl 3, $CaCl_2$ 2, $MgCl_2$ 2, and Tris.HCl 20, pH 7.4 Aliquots (5 × 5 ml) were stored frozen until needed. For competition binding experiments, 200 µl of membrane suspension was incubated at room temperature (19–21°C) with a standard amount (1–3 nM) of ^{125}I-toxin I and various amounts of competing toxin. The final volume was 0.5 ml. The membranes were collected by centrifugation on a microfuge and radioactivity determined.

Source of toxins

NTX was isolated as described previously (Possani *et al.*, 1982). CTX and IbTX were synthesized by Dr C. Vita (DIEP, CEA Saclay, Gif sur Yvette, France). [^{125}I]-DpI was prepared by F.A. De-Allie and Dr P.N. Strong (Neuromuscular Research Unit, Hammersmith Hospital, London).

Results

Twitch tension experiments

CTX, IbTX and NTX were tested on indirectly stimulated chick biventer cervicis preparations. CTX (125 nM) induced a large increase in the responses of indirectly stimulated nerve-muscle preparations. Maximum twitch augmentation after 25 min was 85 ± 2% of control twitch height (Figure 1). IbTX (125 nM) augmented indirectly stimulated muscle twitches by 75 ± 7% of control twitch height. The maximum responses were observed after 30 min (Figure 1). NTX (125 nM) augmented indirectly stimulated muscle twitches by 70 ± 5% of control twitch height. The maximum responses were observed after 40–50 min (Figure 1).

Electrophysiology

Extracellular recording experiments Under normal conditions in the absence of 3,4-diaminopyridine (3,4-DAP), CTX (100–300 nM) did not change the first or second negative deflection of the perineural waveform, which are associated with Na^+ currents and voltage-dependent K^+ currents, respectively (see Mallart, 1985). Adding 3,4-DAP (400 µM) revealed a positive component followed by a delayed negative component, which can be seen to correspond to $I_{K\text{-}Ca}$ because of its sensitivity to tetraethylammonium (TEA) (Mallart, 1985; Anderson *et al.*, 1988) (Figure 2a). CTX, at 3–100 nM, induced a concentration-dependent blockade of $I_{K\text{-}Ca}$ within

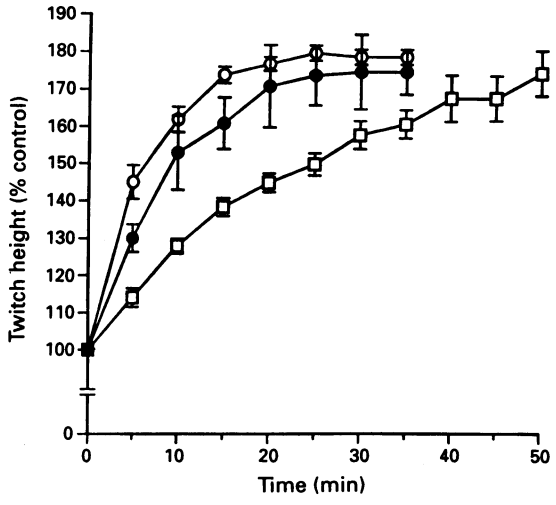

Figure 1 Effects of three scorpion toxins on chick biventer cervicis nerve muscle preparations. Charybdotoxin (○), iberiotoxin (●), and noxiustoxin (□) at 125 nM increased responses to indirect stimulation. Points represent the means of 4–6 experiments with s.e.mean. At the time of maximum twitch augmentation, responses to acetylcholine, carbachol and KCl were unchanged (data not shown).

4–5 min exposure (Figure 2b). IbTX (100 nM) did not display any effects on the first and second negative deflection of the perineural waveform recorded in the absence of 3,4-DAP. In the presence of a maximally effective concentration of 3,4-DAP (400 μM), IbTX (3–100 nM) reduced the amplitude of the Ca^{2+}-dependent K^+ component of the perineural waveform in a concentration-dependent manner (Figure 2c). IbTX appeared to be slightly more active than CTX at blocking $I_{K\text{-}Ca}$, and IbTX was faster in onset than CTX. NTX (up to 300 nM) blocked neither first and second negative deflections (recorded in the absence of 3,4-DAP), nor the $I_{K\text{-}Ca}$ (recorded in the presence of 3,4-DAP). Adding TEA (3 mM) or CTX (100 nM) to the recycling solution after 50 min exposure to NTX revealed a long positive deflection (Figure 2d). In the absence of 3,4-DAP, repetitive firings were detected in the presence of NTX.

Intracellular recording In Mg^{2+}-paralysed preparations, CTX (100–300 nM) and IbTX (100 nM) increased the amplitude of evoked e.p.ps with no significant change in e.p.p. time course (Figure 3a and b). As CTX and IbTX had no effect on the mean amplitude or time course of m.e.p.ps, CTX and IbTX increased quantal content of e.p.ps. The increase in quantal content was observed after 15 min and reached a plateau, which was about two fold of control, after

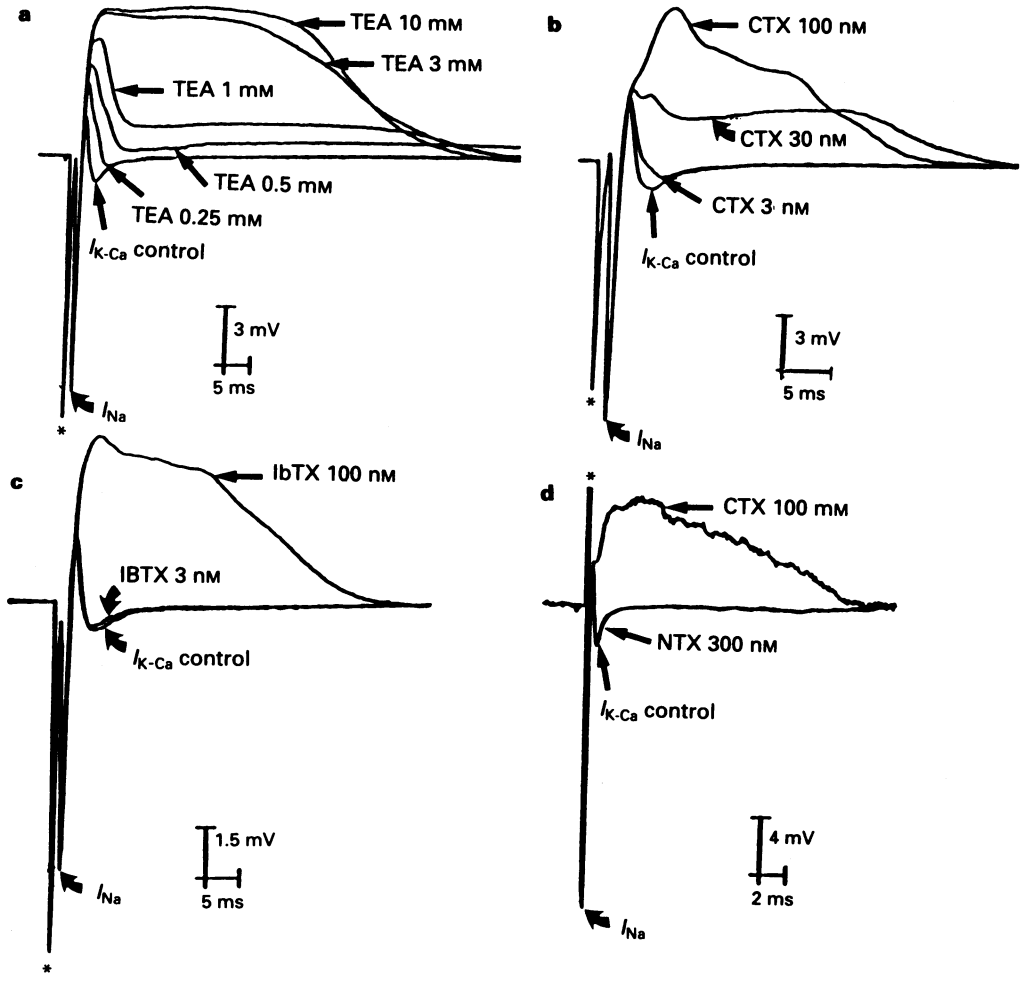

Figure 2 Effects of the three toxins on extracellulary recorded nerve terminal action potentials. Voltage-dependent K^+-channels were blocked by 400 μM 3,4-diaminopyridine to reveal deflection corresponding to a Ca^{2+}-activated K^+ current ($I_{K\text{-}Ca}$). (a) Tetraethylammonium (TEA) progressively blocks the $I_{K\text{-}Ca}$ to reveal a Ca^{2+}-dependent plateau current. (b) Charybdotoxin (CTX) also blocks $I_{K\text{-}Ca}$ and induces a plateau current when added cumulatively from 3 to 100 nM. (c) Iberiotoxin (IbTX) blocks $I_{K\text{-}Ca}$ and induces a plateau current. (d) Noxiustoxin (NTX) up to 300 nM did not affect the $I_{K\text{-}Ca}$. In the presence of NTX, CTx (100 nM) could still induce a plateau response. The peak of the waveform corresponding to the Na^+ current is indicated by the arrows marked I_{Na}, * indicates the stimulus artefacts.

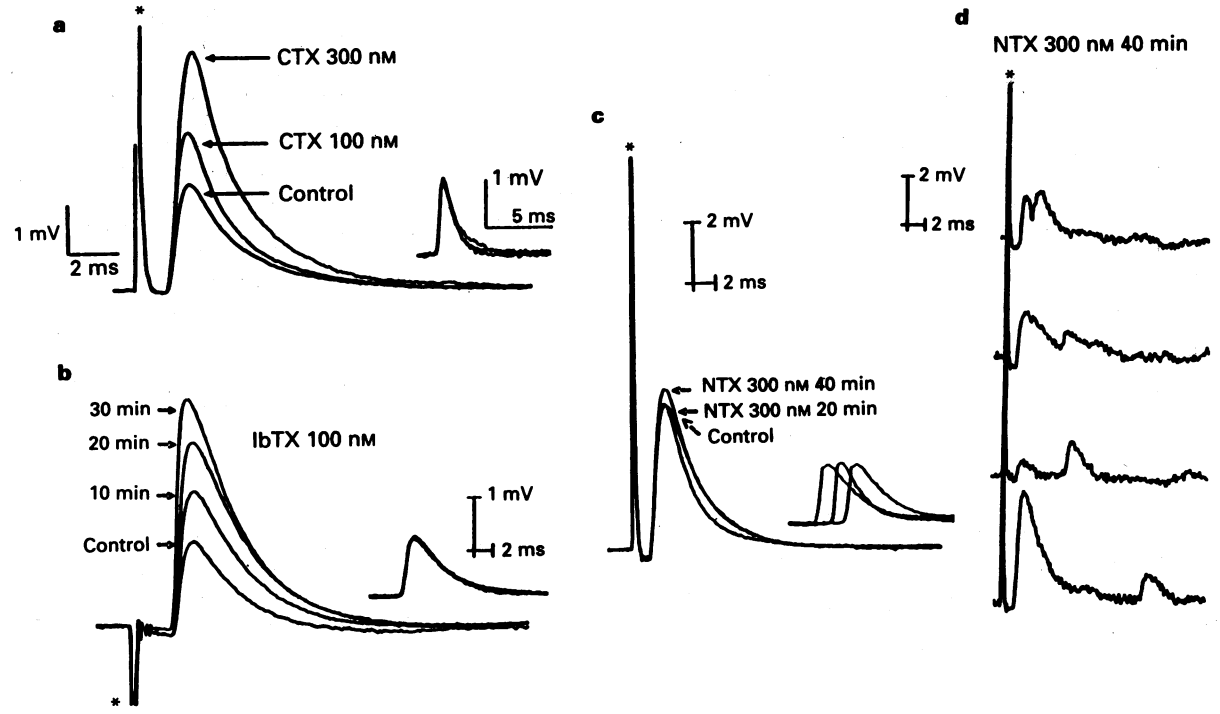

Figure 3 Effects of the three toxins on intracellularly recorded endplate potentials and miniature endplate potentials. (a) Superimposed averaged e.p.ps in the absence (control) and presence of charybdotoxin (CTX) at 100 and 300 nM. Inset: superimposed averaged m.e.p.ps in absence and presence of CTX (100 and 300 nM). (b) Superimposed e.p.ps before and at different times after the addition of 100 nM iberiotoxin (IbTX). Inset: superimposed averaged m.e.p.ps before and in the presence of IbTX. (c) Superimposed e.p.ps before and in the presence of 300 nM noxiustoxin (NTX). Inset: aligned averaged m.e.p.ps before and after 20 and 40 min exposure to NTX (from left to right). (d) Four successive nerve stimulations after 40 min exposure to 300 nM NTX to show the repetitive activity after the evoked e.p.p. * Stimulus artefacts.

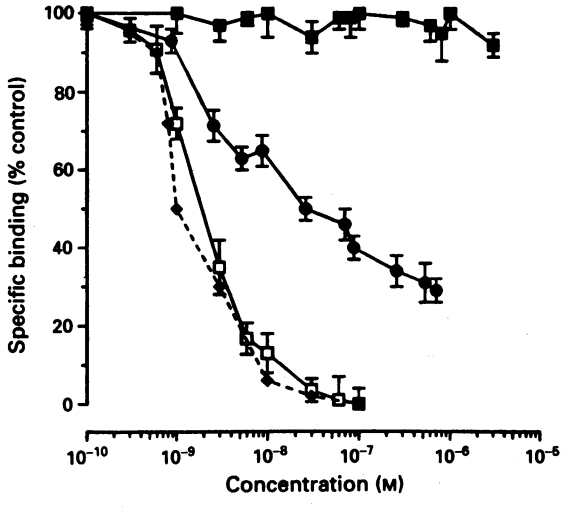

Figure 4 Displacement of specific binding of [^{125}I]-dendrotoxin I from rat brain synaptosomal membranes: (□) native dendrotoxin I; (●) charybdotoxin; (◆) noxiustoxin; (■) iberiotoxin.

Charybdotoxin	ZFTNVSCTTSKECWSVCQRL
Iberiotoxin	ZFTDVDCSVSKECWSVCKDL
Noxiustoxin	TIINVKCTSPKQCSKPCKEL
	-HNTSRGKCMNKKCRCYS
	-FGVDRGKCMGKKCRCYQ
	YGSSAGAKCMNGKCKCYNN

Figure 5 Amino acid sequences of charybdotoxin, iberiotoxin and noxiustoxin. Standard single letter amino acid abbreviations used: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine, K, lysine, L, leucine, M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine; Z, pyroglutamic acid.

30–45 min. IbTX was faster in onset and more potent than CTX.

NTX (300 nM) produced a small increase in the amplitude of evoked e.p.ps with no significant change in the other characteristics of e.p.ps or m.e.p.ps after 40 min (Figure 3c). However, after 40–50 min exposure to NTX, single shock stimulation gave rise to repetitive firing of e.p.ps (Figure 3d).

Competition binding assay studies

[^{125}I]-DpI exhibits saturable binding to the dendrotoxin receptor protein in rat brain synaptosomal membrane preparations. CTX was able to compete with [^{125}I]-toxin I for its binding sites on rat brain synaptosomal membranes (Figure 4). The IC_{50} value for CTX was about 50 nM, and the apparent K_i was 3.8 ± 0.43 nM ($n = 6$). However, CTx displaced only about 70% of the specific binding of [^{125}I]-DpI. IbTX, up to 3 μM, was not able to displace labelled dendrotoxin from its binding sites (Figure 4). As previously shown, NTX at 100 nM completely displaces the labelled dendrotoxin from its binding sites (Harvey *et al.*, 1992). The K_i for NTX was about 0.5×10^{-11} M.

Discussion

The effects of three homologous scorpion toxins CTX, IbTX, and NTX (Figure 5) were studied on both Ca^{2+}-activated K^+ current and dendrotoxin-sensitive voltage-dependent K^+ currents at mouse motor nerve terminals. These toxins contain 37–39 amino acid residues, with over 44% identity in sequence, and they have similar 3-dimensional conforma-

tions. Despite their structural homology, the three toxins had different effects on neuromuscular transmission. IbTx caused an immediate increase in quantal content, while CTX produced a similar increase with a slower onset. Neither IbTx nor CTX caused repetitive firing. In contrast, NTX augmented quantal content to a lesser effect than did IbTx and CTX, but induced repetitive e.p.ps in response to single shock stimulation. An earlier study with NTX (Harvey *et al.*, 1992) had failed to find effects on acetylcholine release in mouse diaphragm preparations; however, higher concentrations were effective in the present study.

The effects on acetylcholine release appear to correlate with the effects of the toxins on different types of K^+ channels in neurones. NTX binds with high affinity to dendrotoxin-sensitive sites on synaptosomal membranes, and dendrotoxins are known to induce repetitive e.p.ps at neuromuscular junctions (Anderson & Harvey, 1988). NTX had no effect on $I_{K\text{-}Ca}$ recorded at motor nerve terminals, although it can block other Ca^{2+}-activated K^+ currents (Valdivia *et al.*, 1988). IbTx had no effect on dendrotoxin binding or on voltage-dependent K^+ currents at the mouse motor nerve terminal. However, IbTx was a potent blocker of $I_{K\text{-}Ca}$ at the motor nerve terminal. CTX was less specific in its effects: it has an allosteric interaction at dendrotoxin binding sites (Harvey *et al.*, 1989), and it blocks $I_{K\text{-}Ca}$ at motor nerve terminals (Anderson *et al.*, 1988). Although a previous study failed to find effects of CTX on acetylcholine release at neuromuscular junctions (Anderson *et al.*, 1988), the present results demonstrate that long exposures to a high concentration of CTX increase quantal content at a mouse neuromuscular junction. Previously, CTX had been shown to facilitate acetylcholine release at frog neuromuscular junctions (Robitaille & Charlton, 1992).

From the effects of the three scorpion toxins, it appears that both Ca^{2+}-activated and voltage-gated K^+ channels are involved in regulating acetylcholine release at the mouse motor nerve terminal. Although blockade of each channel type causes an increase in transmitter release, blockade of $I_{K\text{-}Ca}$ is faster (as has been seen with IbTX) and increases the amplitude of e.p.ps evoked by single nerve stimulation. Blockade of voltage-gated K^+ channel in contrast is slower to affect transmitter release, and leads to repetitive firing (as seen with NTX).

We thank Drs F.A. De-Allie and P.N. Strong (Royal Postgraduate Medical School, London) for the gift of [^{125}I]-DpI, and Dr C. Vita (DIEP, CEN Saclay, France) for the gift of iberiotoxin and charybdotoxin, and Dr L.D. Possani (UNAM, Cuernavaca, Mexico) for the gift of noxiustoxin.

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In vitro characterization of tripitramine, a polymethylene tetraamine displaying high selectivity and affinity for muscarinic M_2 receptors

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1 The antimuscarinic effects of tripitramine were investigated *in vitro* in isolated driven left (force) and spontaneously beating right (force and rate) atria as well as in the ileum of guinea-pig and rat and in the trachea and lung strip of guinea-pig and compared with the effects of methoctramine.

2 Tripitramine was a potent competitive antagonist of muscarinic M_2 receptors in right and left atria. The pA_2 values ranged from 9.14 to 9.85. However, in the guinea-pig and rat left atria but not in guinea-pig right atria, tripitramine at lower concentrations (3–10 nM) produced a less than proportional displacement to the right of agonist-induced responses owing to the presence of a possible saturable removal process.

3 Tripitramine was about three orders of magnitude less potent in ileal and tracheal than in atrial preparations (pA_2 values ranging from 6.34 to 6.81) which makes it more potent and more selective than methoctramine.

4 Another intriguing finding was the observation that the pA_2 value of 7.91 observed for tripitramine in guinea-pig lung does not correlate with that found at both muscarinic M_2 and M_3 receptor subtypes, which clearly indicates that the contraction of guinea-pig lung strip is not mediated by these muscarinic receptor subtypes.

5 A combination of tripitramine with atropine resulted in addition of the dose-ratios for left atria as required for two antagonists interacting competitively with the same receptor site, whereas the same combination gave a supra-additive antagonism on guinea-pig ileum which suggests that tripitramine interacts with a second interdependent site.

6 Tripitramine was more specific than methoctramine since, in addition to muscarinic receptors, it inhibited only frog rectus abdominis muscular (pIC_{50} value of 6.14) and rat duodenum neuronal (pIC_{50} value of 4.87) nicotinic receptors among receptor systems investigated, namely α_1-, α_2-, and β_1-adrenoceptors, H_1- and H_2-histamine receptors, and muscular and neuronal nicotinic receptors.

Keywords: Tripitramine; methoctramine; polymethylene tetraamines; muscarinic M_2 receptors; muscarinic receptor subtypes; saturable removal process

Introduction

Molecular biology studies have clearly established that muscarinic receptors are heterogeneous and are comprised of at least five subtypes (m_1 to m_5) (Bonner, 1989; Hulme *et al.*, 1990; Caulfield, 1993). Four of them have been pharmacologically characterized and are designated as M_1, M_2, M_3 and M_4 according to their affinity for selective antagonists (Hulme *et al.*, 1990; Caulfield, 1993; Friebe *et al.*, 1993; Wess, 1993). For example, muscarinic M_1 receptors are characterized by high affinity for pirenzepine and are found in high density in neuronal tissues and autonomic ganglia (Hammer *et al.*, 1980); muscarinic M_2 receptors have low affinity for pirenzepine and high affinity for methoctramine (Melchiorre *et al.*, 1987b), AF-DX 116 (11[[2-[(diethylamino)-methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b]-[1,4]benzodiazepin-6-one) (Hammer *et al.*, 1986) and himbacine (Anwar-ul *et al.*, 1986), and are located mainly in cardiac tissue, whereas muscarinic M_3 receptors display low affinity for pirenzepine and high affinity for 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine methiodide) (Barlow *et al.*, 1976) and *p*-fluoro-hexahydrosiladiphenidol (Lambrecht *et al.*, 1988), and are found in smooth muscles and glands. At present, no selective antagonist is available for the muscarinic M_4 receptor subtype which has been characterized, however, in rat striatum (Waelbroeck *et al.*, 1991). Furthermore, this receptor subtype has been found in NG 108-15 cells (Michel *et al.*, 1989; Lazareno *et al.*, 1990; Caulfield & Brown, 1991), chicken heart, rabbit lung (Lazareno *et al.*, 1990) and guinea-pig uterus (Dörje *et al.*, 1990). However, Eglen *et al.* (1989) and Bognar *et al.* (1992) have argued that guinea-pig uterus contains muscarinic M_2 rather than M_4 receptors. Recently, Bognar *et al.* (1992) have suggested the existence in rabbit iris sphincter of a muscarinic receptor subtype differing from muscarinic M_1–M_4 receptors.

Among available muscarinic receptor antagonists, methoctramine, although its discovery dates from only 1987, has contributed significantly to muscarinic receptor subtype characterization and classification owing to its high affinity for muscarinic M_2 receptors, low affinity for muscarinic M_3 receptors and intermediate affinity for muscarinic M_1 receptors (Melchiorre *et al.*, 1987b; 1989; Melchiorre, 1988; 1990). In spite of these properties, methoctramine lacks receptor specificity since it inhibits, albeit with modest potency, other receptor systems, such as α_1- (Melchiorre *et al.*, 1987b) and β_1-adrenoceptors (Eglen *et al.*, 1988) and muscular (Melchiorre *et al.*, 1987b) and neuronal nicotinic receptors (Watson *et al.*, 1992). In an effort to improve affinity and selectivity of polymethylene tetraamines we started a study by performing structural modifications on methoctramine such as to incorporate in its tetraamine backbone a 11-acetyl-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one moiety of AQ-RA 741 (11-[[4-[4-(diethylamino)-butyl]-1-piperidinyl]acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one) (Eberlein *et al.*, 1989; 1992), an analogue of pirenzepine

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which is claimed to display high selectivity for muscarinic M_2 receptors. This investigation has led, very recently, to the discovery of tripitramine (Figure 1) that, to the best of our knowledge, represents the most potent and the most selective muscarinic receptor antagonist known until now in both functional (M_2 and M_3) and binding (M_1 to M_4 and m_1 to m_5) assays (Melchiorre *et al.*, 1993; Maggio *et al.*, 1994).

We now describe further *in vitro* experiments with tripitramine in comparison to methoctramine in atrial, tracheal and ileal preparations derived from guinea-pig and rat and with different agonists. The guinea-pig lung strip preparation was also included in this study to verify whether the contraction of this tissue is mediated by muscarinic M_2-like receptors (Roffel *et al.*, 1993). Furthermore, antagonist combination experiments with atropine (Clark & Mitchelson, 1976; Stockton *et al.*, 1983) were performed to verify the mechanism of action of tripitramine. In addition, receptor specificity was assessed by investigating tripitramine in comparison to methoctramine on other receptor systems, namely rat vas deferens α_1- and α_2-adrenoceptors, guinea-pig atrial β_1-adrenoceptors, guinea-pig right atrial H_2-histamine receptors, guinea-pig ileal H_1-histamine receptors, rat duodenum neuronal nicotinic receptors and frog rectus abdominis muscular nicotinic receptors. The data were analysed quantitatively according to well established pharmacological methods, namely Schild analysis (Arunlakshana & Schild, 1959; Tallarida *et al.*, 1979; Tallarida & Murray, 1991) and assessment of interaction between antagonists according to the dose-ratio method (Paton & Rang, 1965).

Methods

Guinea-pigs (200–400 g), rats (150–200 g) or frogs (10–20 g) were killed by cervical dislocation under ketamine anaesthesia and the organs required were set up rapidly under a suitable resting tension in 15 ml organ baths containing physiological salt solution kept at appropriate temperature (see below) and aerated with 5% CO_2:95% O_2 at pH 7.4. Dose-response curves were constructed by cumulative addition of the agonist (Van Rossum, 1963). The concentration of agonist in the organ bath was increased approximately five fold at each step, with the exception of guinea-pig lung strip and frog rectus abdominis preparations, with each addition being made only after the response to the previous addition had attained a maximal level and remained steady. Contractions were recorded by means of a force displacement transducer (FT.03 Grass and 7003 Basile) connected to a four-channel pen recorder (Battaglia-Rangoni KV 380). In all cases, parallel experiments in which tissues did not receive any antagonist were run in order to check any variation in sensitivity. It was always verified that the EC_{50} values for the agonist in tissues receiving only the solvent were not significantly different ($P > 0.05$) from control values. In all other cases experiments were discarded.

Guinea-pig left atria

The hearts of guinea-pigs of either sex were rapidly removed, washed by perfusion through the aorta with oxygenated physiological salt solution and right and left atria were separated out. The left atria were mounted under 0.2–0.3 g tension at 35°C in Tyrode solution of the following composition (mM): NaCl 136.9, KCl 5.4, $MgSO_4 \cdot 7H_2O$ 1.0, $CaCl_2$ 2.52, NaH_2PO_4 0.4, $NaHCO_3$ 11.9 and glucose 5.5. Tissues were stimulated through platinum electrodes by square-wave pulses (0.6–0.8 ms, 1 Hz, 1–5 V). Inotropic activity was recorded isometrically. Tissues were equilibrated for 1h, and cumulative dose-response curves to carbachol (0.01–1 μM), oxotremorine (1–100 nM), methoctramine (10–500 μM) or tripitramine (10–500 μM) were constructed. Following incubation with the antagonist for 30–180 min, a new dose-response curve to carbachol or oxotremorine was obtained. In combination experiments (simultaneous administration) of tripitramine (0.1–0.5 μM) with atropine (0.5–1 μM), the contact time for antagonists was always 60 min. The end of the tripitramine effect was determined by the following procedure. A control dose-response curve to carbachol was obtained followed by a curve in the presence of tripitramine (50 nM). The antagonist was then removed from the bath and a dose-response curve to carbachol was constructed after a washing period of 60 min. The start of tripitramine effect was investigated by exposing the atria to tripitramine (10, 50 and 100 nM) for 30, 60, 120 and 180 min, respectively, before a second dose-response curve to carbachol was obtained. To verify whether a polyamine oxidase is involved in tripitramine degradation, a few experiments were also performed in the presence of N,N′-*bis*(2,3-butadienyl)-1,4-butanediamine dihydrochloride (10 μM). When determining methoctramine- and tripitramine-induced positive inotropic responses in atrial tissue isolated from guinea-pigs pretreated with reserpine, reserpine was injected i.p., at single doses of 2 mg kg^{-1} 48 and 24 h before the animals were killed in order to eliminate the influence of noradrenaline, which might be released from sympathetic nerve terminals (Temma *et al.*, 1977).

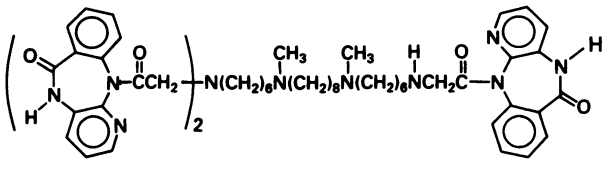

Figure 1 Structural formula of tripitramine.

Rat left atria

The heart of male rats was rapidly removed and right and left atria were separated out. The left atria were mounted under 0.1–0.2 g tension and stimulated as described for guinea-pigs. Inotropic activity was recorded isometrically. Tissues were equilibrated for 1 h, and cumulative dose-response curves to carbachol (0.01–1 μM) were constructed. Following incubation with the antagonist for 60 (> 10 nM) or 120 min (≤ 10 nM), a new dose-response curve to carbachol was obtained.

Spontaneously beating guinea-pig right atria

Spontaneously beating right atria were suspended under 0.15–0.20 g tension at 35°C in Tyrode solution as for left atria. Heart rate and size of contractions were recorded isometrically with carbachol (0.01–1 μM), isoprenaline (1–100 μM) or histamine (0.1–50 μM) as agonists. The contact time for antagonists was 60 min except when determining antimuscarinic activity of tripitramine. In this case, the contact time was 120 min for concentrations ≤ 10 nM.

Guinea-pig ileum

The terminal portion of the ileum was excised after discarding the 4–6 cm nearest to the ileo-caecal junction. The tissue was cleaned and segments of approximately 2 cm were set up under 1 g tension at 37°C in organ baths containing Tyrode solution of the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, $MgSO_4$ 1.2, KH_2PO_4 1.19, $NaHCO_3$ 25 and glucose 11. Tension changes were recorded isotonically. Tissues were allowed to equilibrate for at least 30 min during which time the bathing solution was changed every 10 min. Dose-response curves to carbachol (0.01–0.5 μM), oxotremorine (0.01–1 μM) or histamine (0.01–5 μM) were obtained at 30 min intervals, the first one being discarded and the second one taken as control. Following incubation with the antagonist for 60 min, a new dose-response curve to the agonist was obtained.

Rat ileum

The procedure described for guinea-pig ileum was followed with carbachol (0.1–5 μM) used as the agonist. Following incubation with the antagonist for 60 min, a new dose-response curve to the agonist was obtained.

Frog rectus abdominis

The rectus abdominis muscle was set up at 25°C in an organ bath containing Clark frog Ringer solution of the following composition (mM): NaCl 111, KCl 1.88, $CaCl_2$ 1.08, NaH_2PO_4 0.08, $NaHCO_3$ 2.38 and glucose 11.1. Dose-response curves to carbachol (1–100 μM) were obtained at 30 min intervals, the first one being discarded and the second one taken as control. Following incubation with the antagonist for 60 min, a new dose-response curve to the agonist was obtained. The end of the tripitramine effect was determined by the following procedure. A control dose-response curve to carbachol was obtained followed by a curve in the presence of tripitramine (3 μM). The antagonist was then removed from the bath and a dose-response curve to carbachol was constructed after a washing period of 60 min.

Rat duodenum

The procedure described by Irie *et al.* (1994) was followed. Briefly, duodenum of adult rats, 9–11 weeks old, was dissected out and segments of approximately 2.2 cm of the proximal portion but not the bulb were set up under 1 g tension at 35°C in organ baths containing modified Locke solution of the following composition (mM): NaCl 154, KCl 4.02, $CaCl_2$ 1.36, $MgCl_2$ 0.9, $NaHCO_3$ 2.97 and glucose 5.56. Tension changes were recorded isotonically. Tissues were equilibrated for 1 h, and the effect of a single dose of 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP, 100 μM), taken as control, was recorded. Following 30-min recovery and incubation with the antagonist for 60 min, a new response to a single dose of DMPP (100 μM) was obtained.

Rat vas deferens

Both vasa deferentia from male albino rats were isolated, freed from adhering connective tissue and transversely bisected. Prostatic, 12 mm in length, and epididymal portions, 14 mm in length, were prepared and mounted individually at 37°C in organ baths containing Krebs solution of the following composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.52, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 11.1. $MgSO_4$ concentration was reduced to 0.6 mM when the twitch response to field stimulation was studied. The loading tension used to assess α_1- or α_2-blocking activities was 0.4 g or 0.5–0.8 g, respectively. The tissues were allowed to equilibrate for at least 1 h before addition of any drug. Field stimulation of the tissue was carried out by means of two platinum electrodes, placed near the top and bottom of the vas deferens, at 0.1 Hz with square pulses of 3 ms duration at voltage of 10–35 V. The stimulation voltage was fixed throughout the experiments. Propranolol hydrochloride (1 μM) and desipramine hydrochloride (10 nM) were present in the Krebs solution throughout the experiments outlined below to block β-adrenoceptors and neuronal uptake mechanisms, respectively. The α_1-adrenoceptor blocking activity was determined on the epididymal portion of the vas deferens. Noradrenaline (0.01–5 μM) dose-response curves were obtained cumulatively, the first one being discarded and the second one taken as control. After incubation with the antagonist for 60 min, a third dose-response curve was obtained. The α_2-adrenoceptor blocking activity was assessed on the prostatic portion of the vas deferens by antagonism to clonidine. A first clonidine (1–100 nM) dose-response curve, taken as control, was obtained cumulatively avoiding the inhibition of more than 90% of twitch responses, while the concentration of clonidine causing 100% inhibition was deduced from the second dose-response curve obtained from parallel experiments. Under these conditions it was possible to obtain a second dose-response curve which was not significantly different from the first one. Thus, after incubation with antagonist for 60 min a dose-response curve was obtained.

Guinea-pig trachea

The trachea was cut transversally between the segment of cartilage and four groups of tracheal segments, each one made up of three rings, were tied together and mounted under a tension of 1 g at 37°C in organ baths containing Krebs-Ringer solution of the following composition (mM): NaCl 95, KCl 4.7, $CaCl_2$ 2.50, $MgSO_4$ 1.0, KH_2PO_4 1.17, $NaHCO_3$ 25 and glucose 10.6. The tissues were allowed to stabilize for 90 min. The tension was recorded isometrically. Cumulative dose-response curves to carbachol (0.01–1 μM) were constructed. Following incubation with the antagonist for 60 min, a new dose-response curve to the agonist was obtained.

Guinea-pig lung

The procedure described by Roffel *et al.* (1993) was followed. Briefly, strips of peripheral lung tissue, approximately 15 × 2 × 2 mm, were cut either from the body of a lower lobe with the longitudinal axis of the strip parallel to the bronchus or from the peripheral margin of the lobe and set up under 0.3 g tension at 37°C in organ baths containing Krebs-Henseleit buffer solution of the following composition (mM): NaCl 118.78, KCl 4.32, $CaCl_2$ 2.52, $MgSO_4$ 1.18, KH_2PO_4 1.28, $NaHCO_3$ 25 and glucose 5.5. Tension changes were recorded isotonically. After a 60 min equilibration period the strips were precontracted twice by cumulative administration of carbachol (0.1, 1, 10 and 0.1, 1, 10, 100 μM with washing periods of 45 min) before determination of the control dose-response curve. After a 45-min washing period, a consecutive dose-response curve was made in the presence of antagonist. The contact time for antagonists was 60 min.

Statistical and data analysis

Dose ratios (DR) at the EC_{50} values of the agonists were calculated at one to six antagonist concentrations and each concentration was tested from four to eight times. Dissociation constants (pA_2 values) were estimated by Schild plots (Arunlakshana & Schild, 1959) constrained to slope −1.0, as required by the theory (Tallarida *et al.*, 1979). When applying this method, it was always verified that the experimental data generated a line whose derived slope was not significantly different from unity ($P > 0.05$). An apparent dissociation constant (K_B) for the antagonists at a single concentration was derived from the equation K_B = [antagonist]/(DR − 1) (Furchgott, 1972). pIC_{50} values represent the negative logarithm of that dose of antagonist which inhibits 50% of the maximum response to an agonist. Data are presented as means ± s.e. of n experiments. Differences between mean values were tested for significance by Student's t test.

Drugs

All drugs used, carbachol (carbamoylcholine chloride, EGA-Chemie), atropine sulphate (Merck), (±)-ketamine hydrochloride, (±)-isoprenaline hydrochloride, clonidine hydrochloride and desipramine hydrochloride (Sigma), histamine dihydrochloride (C. Erba), R-(−)-noradrenaline hydrochloride, (±)-propranolol hydrochloride, oxotremorine (1-[4-[1-pyrrolidinyl]-2-butynyl]-2-pyrrolidinone sesquifumarate), DMPP (1,1-dimethyl-4-phenylpiperazinium iodide) and tyramine hydrochloride (Aldrich-Chemie), N,N′-*bis*(2,3-butadi-

enyl)-1,4-butanediamine dihydrochloride, tripitramine (1,1,24-tris[[5,11-dihydro-6-oxo-6*H*-pyrido[2,3-*b*][1,4]-benzodiazepin-11-yl)carbonyl]methyl]-8,17-dimethyl-1,8,17,24-tetraazatetracosane tetraoxalate) and methoctramine tetrahydrochloride (synthesized in our laboratory), were dissolved in twice distilled water with the exception of reserpine (Fluka) which was dissolved in 50% ethanol containing 0.8 mg of ascorbic acid for 2 mg of reserpine.

Results

Affinity of tripitramine at muscarinic receptor subtypes

Tripitramine (0.015–3 μM) antagonized potently the negative inotropic effects induced by carbachol or oxotremorine at muscarinic M_2 receptors in electrically stimulated left atria from rat and guinea-pig. There was a dose-dependent parallel shift to the right of the agonist dose-response curves without either basal tension or maximum response being affected (shown for carbachol in guinea-pig left atria in Figure 2). However, tripitramine at concentrations lower than 15 nM in the range 3–10 nM gave a parallel shift to the right of the agonist dose-response curves but the shift was not proportional to the concentration being markedly lower than the expected value (Figure 2). Schild analysis of the results gave linear plots in the range 0.015–3 μM with slopes which were not significantly different from unity (Table 1). However, in guinea-pig left atrial preparations and in the concentration-range 3–10 nM, nonlinear Schild plots were obtained and the slopes were much higher than unity (3.68 ± 0.03 and 3.53 ± 0.05 for carbachol and oxotremorine as agonists, respectively). Similar results were obtained in rat left atria (Figure 3).

In guinea-pig left atria, the maximum effect of tripitramine was reached within 60 min for concentrations > 10 nM and within 120 min for concentrations ≤ 10 nM (Figure 4). Experiments performed in the presence of N,N'-*bis*(2,3-butadienyl)-1,4-butanediamine (10 μM), which is known to block polyamine oxidase (Bey *et al.*, 1985), did not modify the maximum effect of tripitramine (10 nM) (results not shown).

As for methoctramine (Melchiorre *et al.*, 1987a), tripitramine antagonism was easily reversible in guinea-pig left atria, since 60 min washing of the tissues after incubation for 60 min with tripitramine (50 nM) brought the EC_{50} value (± 95% C.F.) for carbachol [81 (75–89) nM ($n = 5$)] to the control value [73 (65–82) nM ($n = 5$)]. The carbachol- or oxotremorine-induced responses in left atria were antagonized by tripitramine in the range 0.015–3 μM with pA_2 values (Table 1) that closely resembled the pK_i value

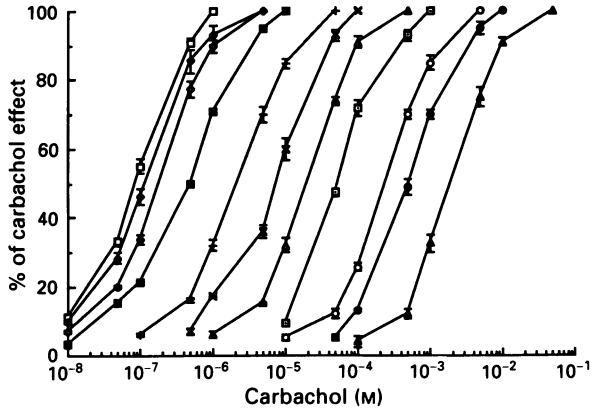

Figure 2 Antagonism of carbachol-induced negative inotropic effect at muscarinic M_2 receptors in guinea-pig left atria. Dose-response curves for carbachol were obtained before (□) and after exposure to 3 (◆), 5 (◇), 7 (■), 10 (+), 15 (×), 50 (△), 100 (⊡), 500 (○), 1000 (●) and 3000 nM (▲) tripitramine for 120 (3–10 nM) or 60 min (15–3000 nM). Each point is the mean (± s.e.mean) of five to eight observations.

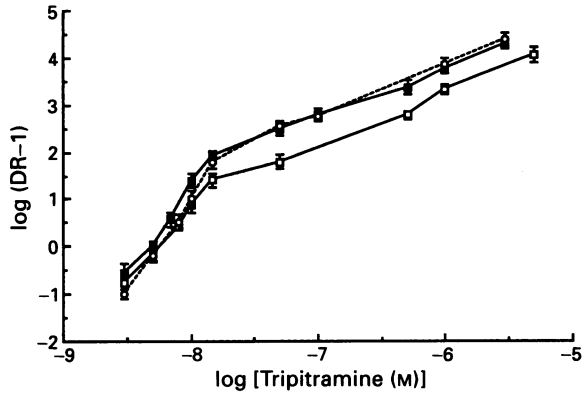

Figure 3 Schild plot for the antagonism between carbachol or oxotremorine and tripitramine at cardiac muscarinic M_2 receptors in guinea-pig (carbachol, ■; oxotremorine, ○) and rat (carbachol, □) left atrium. Each point is the mean (± s.e.mean) of five to eight observations.

Table 1 Antagonist affinities at guinea-pig atria, ileum, trachea and lung, and rat ileum and left atrium muscarinic receptors

Preparation[a]	Concentration (μM)	*Tripitramine* pA_2[b]	Slope[c]	*Methoctramine* pA_2[b]	Slope[c]
Guinea-pig					
Left atrium	0.015, 0.05, 0.1, 0.5, 1, 3	9.69 ± 0.02	1.01 ± 0.04	7.78 ± 0.01	0.99 ± 0.02
Left atrium[d]	0.015, 0.05, 0.1, 1, 3	9.85 ± 0.04	1.07 ± 0.04	7.89 ± 0.04	1.01 ± 0.03
Right atrium					
(force)	0.003, 0.005, 0.01, 0.1, 1	9.20 ± 0.12	1.02 ± 0.05	7.56 ± 0.08	1.06 ± 0.03
(rate)	0.003, 0.005, 0.01, 0.1	9.34 ± 0.02	0.99 ± 0.01	7.47 ± 0.10	1.09 ± 0.07
Ileum	5, 10, 50, 100	6.50 ± 0.05	1.00 ± 0.02	6.28 ± 0.06	1.04 ± 0.02
Ileum[d]	10, 50, 100	6.81 ± 0.02	1.03 ± 0.03	6.35 ± 0.07	1.02 ± 0.04
Trachea	10, 50, 100	6.34 ± 0.04	1.03 ± 0.06	6.30 ± 0.02	1.06 ± 0.07
Lung	0.05, 0.1, 1, 10	7.91 ± 0.03	1.02 ± 0.02	6.97 ± 0.07	1.05 ± 0.04
Rat					
Left atrium	0.015, 0.05, 0.5, 1, 5	9.14 ± 0.09	1.08 ± 0.04	7.80 ± 0.08	1.03 ± 0.04
Ileum	3, 5, 50	6.45 ± 0.06	1.05 ± 0.02	5.99 ± 0.03	1.02 ± 0.02

[a]The agonist was carbachol unless otherwise indicated. [b]Each pA_2 value was obtained from 12 to 36 dose-ratios at the indicated concentrations for tripitramine and at 0.05, 0.5 and 5 μM (atria) or 5, 10 and 50 μM (ileum) concentrations for methoctramine and determined by the method of constrained plot (Tallarida *et al.*, 1979; Tallarida & Murray, 1991). Results are presented as the mean ± s.e. [c]Slopes were estimated by Schild plots (Arunlakshana & Schild, 1959) and were not significantly different ($P > 0.05$) from unity. [d]The agonist was oxotremorine.

(9.54 ± 0.08) obtained in heart membranes (Melchiorre *et al.*, 1993). Under the same experimental conditions methoctramine was about two orders of magnitude less potent than tripitramine (Table 1).

In spontaneously beating right atria, tripitramine (0.003–1 μM, force; 0.003–0.1 μM, rate) caused a dose-dependent inhibition of the negative chronotropic and inotropic effects induced by carbachol without modifying basal rate or tension. The Schild plots, unlike the left atria, were linear over all the range of concentrations investigated and the slopes of the regression lines were not significantly different from unity (Figure 5, Table 1). pA_2 values of 9.20 ± 0.12 and 9.34 ± 0.02 were found at muscarinic M_2 receptors in atrial tissue and sinus atrial pacemaker cells, respectively, which were not significantly different and slightly lower than the pA_2 value obtained in guinea-pig left atria (Table 1).

Tripitramine (5–100 μM) caused a dose-dependent parallel shift to the right of carbachol dose-response curves of guinea-pig and rat ileum with no changes in the maximum response (shown for guinea-pig ileum in Figure 6). Similarly, tripitramine (10–100 μM) antagonized in a dose-dependent fashion oxotremorine-induced contractions in guinea-pig ileum without affecting the maximum response (not shown). The Schild plots were linear and the slopes of the regression lines were not significantly different from unity pointing to competitive antagonism (Figure 5, Table 1). Irrespective of agonist and species used, the pA_2 values were similar, ranging from 6.45 ± 0.06 to 6.81 ± 0.02. Methoctramine was slightly less potent than tripitramine, the pA_2 values ranging from 5.99 ± 0.03 to 6.35 ± 0.07.

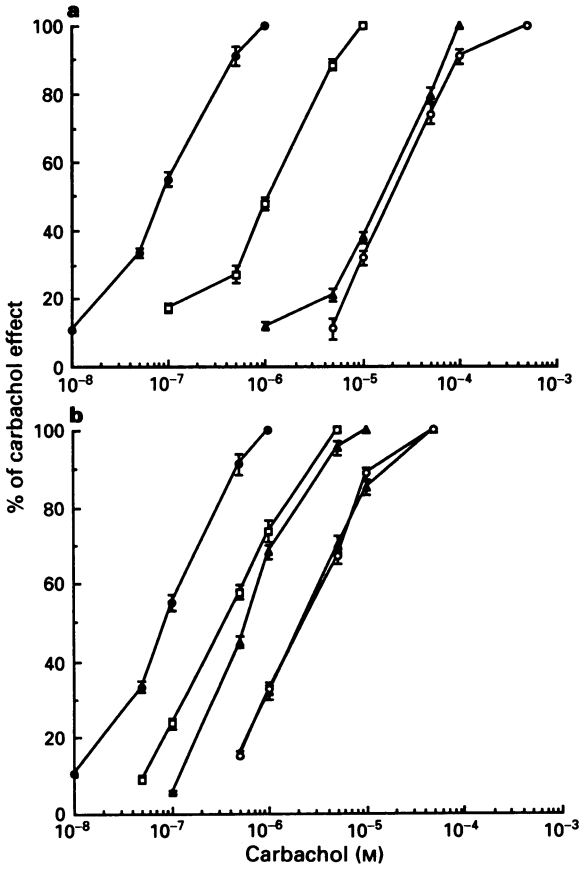

Figure 4 Time course of tripitramine effect on carbachol-induced negative inotropic effect at muscarinic M_2 receptors in guinea-pig left atria. Dose-response curves for carbachol were obtained before (●) and after exposure (a) to 50 nM tripitramine for 30 (□), 60 (▲) and 120 min (○), and (b) to 10 nM tripitramine for 30 (□), 60 (△), 120 (○) and 180 min (▲). Each point is the mean (± s.e.mean) of four to eight observations.

Tripitramine (0.05–10 μM) caused a dose-dependent inhibition of carbachol-induced contractions of guinea-pig lung strip. The agonist dose-response curves were displaced in a parallel fashion with no changes in the maximum response (Figure 7). The data derived from Schild analysis point to competitive antagonism (Figure 5, Table 1). A pA_2 value of 7.91 ± 0.01 was found at muscarinic receptors in guinea-pig lung strip, which was markedly lower than the value obtained at muscarinic M_2 receptors in atria while being significantly higher than that at muscarinic M_2 receptors in smooth muscle (ileum and trachea). This finding clearly suggests that muscarinic receptors mediating contraction in guinea-pig lung strip can be classified neither as M_2 nor as M_3 subtypes. Under the same experimental conditions methoctramine gave a pA_2 value of 6.97 ± 0.07 which is similar to the value (7.30 ± 0.06) obtained by Roffel *et al.* (1993).

In guinea-pig tracheal rings, tripitramine (10–100 μM) caused a dose-dependent inhibition of carbachol-induced contraction with no changes in the maximum response (not shown). The Schild plot was linear and the slope of the regression line was not significantly different from unity pointing to an apparent competitive antagonism (Table 1; Figure 5). The pA_2 value (6.43 ± 0.04) was similar to that found in the ileum. Methoctramine was slightly less potent than tripitramine, the pA_2 value amounting to 6.30 ± 0.02.

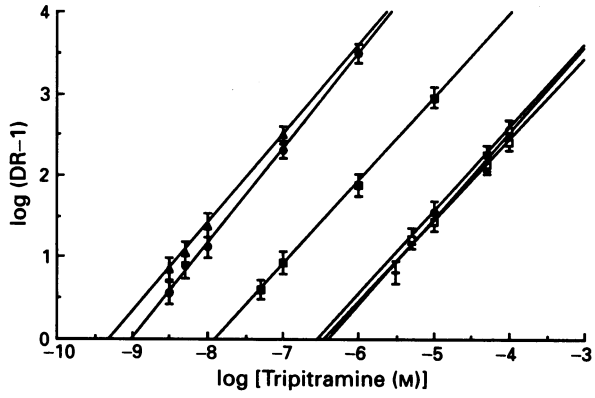

Figure 5 Schild plot for the antagonism between carbachol and tripitramine at cardiac (M_2) and smooth muscle (M_3) muscarinic receptors in guinea-pig right atrium (rate, ▲; force, ●), lung strip (■), trachea (□) and ileum (○), and rat ileum (+). Each point is the mean (± s.e.mean) of four to seven observations.

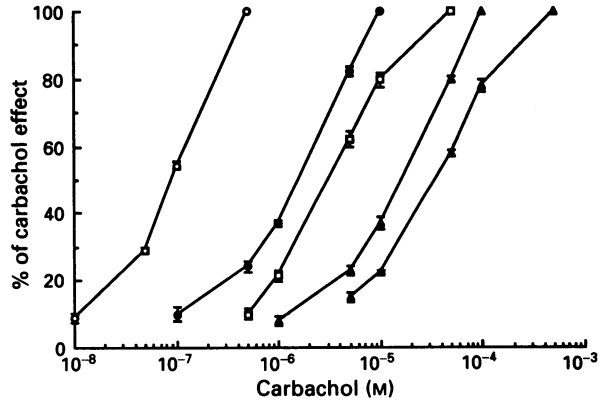

Figure 6 Antagonism of carbachol-induced contractions at muscarinic M_3 receptors in guinea-pig ileum. Dose-response curves for carbachol were obtained before (○) and after exposure to 5 (●), 10 (□), 50 (▲) and 100 μM (△) tripitramine for 60 min. Each point is the mean (± s.e.mean) of four to seven observations.

Antagonism at muscarinic M_2 and M_3 receptors by tripitramine and atropine in combination

The mutual competition between tripitramine and atropine was investigated on the basis of dose-ratio analysis (Paton & Rang, 1965).

In the guinea-pig left atria, the experimental dose-ratios of the combination of tripitramine (0.5 and 0.1 μM) with atropine (1 and 0.5 μM) amounted to 6329 ± 433 and 2073 ± 189, respectively, and were not significantly different from those expected for a combination of two competitive antagonists (6184 and 1897) (Table 2).

Guinea-pig ileal preparations were exposed simultaneously to tripitramine (5 and 100 μM) and atropine (0.005 and 0.1 μM). The experimental dose-ratios of the combination resulted 132 ± 7.5 and 5926 ± 160, respectively, and were significantly higher than those expected for a combination of two competitive antagonists (36 and 635) (Table 3).

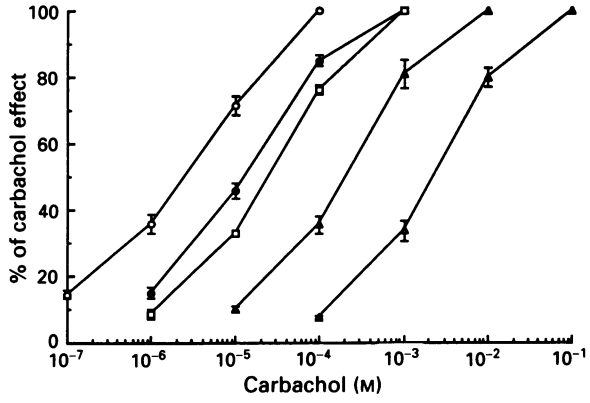

Figure 7 Antagonism of carbachol-induced contractions at muscarinic receptors in guinea-pig lung strip. Dose-response curves for carbachol were obtained before (○) and after exposure to 0.05 (●), 0.1 (□), 1 (▲) and 10 μM (△) tripitramine for 60 min. Each point is the mean (± s.e.mean) of four to six observations.

Specificity and selectivity of tripitramine vs methoctramine

Table 4 shows the selectivity ratios for tripitramine and methoctramine. It is evident that tripitramine is more than one order of magnitude more selective than methoctramine for muscarinic M_2 receptors. Furthermore, tripitramine is two orders of magnitude more potent than methoctramine. It should be noted, however, that the selectivity ratios (atria vs. ileum and trachea) may be inaccurate owing to the finding that tripitramine did not behave as a competitive antagonist in the ileum in combination experiments with atropine. For this reason, the M_2/M_3 selectivity ratios reported in Table 4 are only apparent values.

Table 5 shows the receptor specificity of tripitramine in comparison to methoctramine. Methoctramine (10–500 μM) induced a concentration-dependent positive inotropic response at β_1-adrenoceptors in guinea-pig left atrial preparations. At the maximum concentration employed (500 μM), a percentage of 209 ± 3.1 ($n = 5$) in developed tension was observed. The EC_{50} value was not calculated since no maximum response was attained (Figure 8). Under the same conditions, tripitramine (10–500 μM) did not give a significant increase in developed tension. In tissues isolated from animals pretreated with reserpine, noradrenaline depletion was determined by exposing isolated atria to a single dose of tyramine (10 μg ml^{-1}) before starting experiments (Muskus, 1962). Responses to tyramine were abolished. The EC_{50} value (± 95% C.F.) for tyramine control was 11.5 (10.8–12.4) μM ($n = 4$). In pretreated tissues, the response to methoctramine was lower than that observed in tissues from untreated animals, whereas tripitramine was completely devoid of activity (Figure 8).

Tripitramine and methoctramine (10 μM) did not antagonize isoprenaline- or histamine positive chronotropic responses at β_1-adrenoceptors or H_2-histamine receptors in sinus atrial pacemaker cells from guinea-pig right atria, respectively. The EC_{50} values (± 95% C.F.) for isoprenaline in the presence of tripitramine [2.11 (1.50–2.90) nM] ($n = 4$) or methoctramine [1.92 (1.60–2.40) nM] ($n = 4$) were not significantly different from that of control [3.25 (2.50–3.90) nM] ($n = 8$). Similarly, the EC_{50} values for histamine in the

Table 2 Dose-ratios obtained with tripitramine and atropine alone and in combination on muscarinic receptors of guinea-pig left atrium with carbachol as agonist

Tripitramine concentration (μM)	*Tripitramine dose-ratio* (DR_1)	*Atropine concentration* (μM)	*Atropine dose-ratio* (DR_2)	*Experimental combination dose-ratio*	*Expected combination dose-ratio* ($DR_1 + DR_2 - 1$)
0.5	3344 ± 315 ($n = 5$)	1	2841 ± 263 ($n = 5$)	6329 ± 433[a] ($n = 7$)	6184
0.1	890.4 ± 102 ($n = 5$)	0.5	1008 ± 35 ($n = 5$)	2073 ± 189[a] ($n = 6$)	1897

Each dose-ratio (DR) value is the mean ± s.e. and the number of observations is given in parentheses.
[a]Not significantly different ($P > 0.05$) from expected combination dose-ratio for two competitive antagonists.

Table 3 Dose-ratios obtained with tripitramine and atropine alone and in combination on muscarinic receptors of guinea-pig ileum with carbachol as agonist

Tripitramine concentration (μM)	*Tripitramine dose-ratio* (DR_1)	*Atropine concentration* (μM)	*Atropine dose-ratio* (DR_2)	*Experimental combination dose-ratio*	*Expected combination dose-ratio* ($DR_1 + DR_2 - 1$)
100	356 ± 42 ($n = 4$)	0.1	280 ± 37 ($n = 5$)	5926 ± 160[a] ($n = 7$)	635
5	30 ± 2.5 ($n = 4$)	0.005	6.3 ± 2.7 ($n = 5$)	132 ± 7.5[a] ($n = 6$)	36

[a]Significantly different ($P < 0.05$) from expected combination dose-ratio for two competitive antagonists.

absence [0.66 (0.40–0.90) μM] ($n = 8$) and in the presence of 10 μM methoctramine [0.74 (0.60–1.0) μM] ($n = 4$) or tripitramine [0.66 (0.50–0.90) μM] ($n = 4$) were not significantly different.

Tripitramine and methoctramine (10 μM) did not antagonize histamine-induced responses at H_1-histamine receptors of guinea-pig ileum. The EC_{50} values (± 95% C.F.) for histamine in the absence [0.21 (0.17–0.27) μM] ($n = 10$) and in the presence of tripitramine [0.15 (0.12–0.19) μM] ($n = 5$) or methoctramine [0.18 (0.14–0.23) μM] ($n = 5$) were not significantly different.

Tripitramine (0.3–3 μM) caused a dose-dependent inhibition of the maximum contraction induced by carbachol at muscular nicotinic receptors in frog rectus abdominis with a pIC_{50} value of 6.14 ± 0.07 (Figure 9). This antagonism was reversible, since 60 min washing of the tissues after incubation with tripitramine (1 μM) brought the EC_{50} value (± 95% C.F.) for carbachol [7.03 (6.49–7.90) μM ($n = 5$)] back to the control value [6.85 (6.19–7.60) μM ($n = 5$)]. Under the same experimental conditions methoctramine behaved similarly, the pIC_{50} value was 6.52 ± 0.05 in agreement with results previously reported (Melchiorre *et al.*, 1987b).

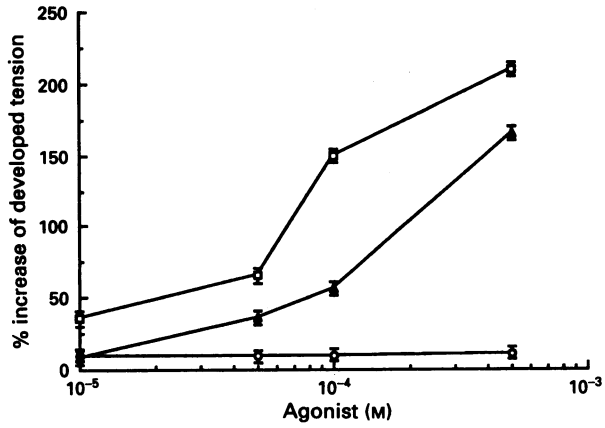

Figure 8 Increase in isometric tension developed by methoctramine (□, △) and tripitramine (○) at β_1-adrenoceptors in electrically driven guinea-pig left atrium. Data from reserpine-pretreated animals are shown only for methoctramine (△) because tripitramine was devoid of activity as in unpretreated animals. Each point is the mean (± s.e.mean) of five observations.

Table 4 Selectivity ratios of tripitramine and methoctramine expressed as the antilog of the difference between the pA_2 values for atrial, tracheal and ileal muscarinic receptors with carbachol used as agonist unless otherwise indicated

Preparation[a]	*Selectivity ratio* *Tripitramine*	*Methoctramine*
Left atrium/ileum	1549	32
Left atrium/ileum[b]	1096	35
Left atrium/trachea	2239	30
Right atrium (force)/ileum	501	19
Right atrium (force)/trachea	724	18
Right atrium (rate)/ileum	692	15
Right atrium (rate)/trachea	1000	15
Right atrium (rate)/right atrium (force)	1	1
Left atrium/lung	60	6
Right atrium (force)/lung	19	4
Right atrium (rate)/lung	27	3
Lung/ileum	26	5
Lung/trachea	37	5
Ileum/trachea	1	1
Rat left atrium/rat ileum	490	65

[a]Tissues were from guinea-pigs unless otherwise indicated. [b]The agonist was oxotremorine in both preparations.

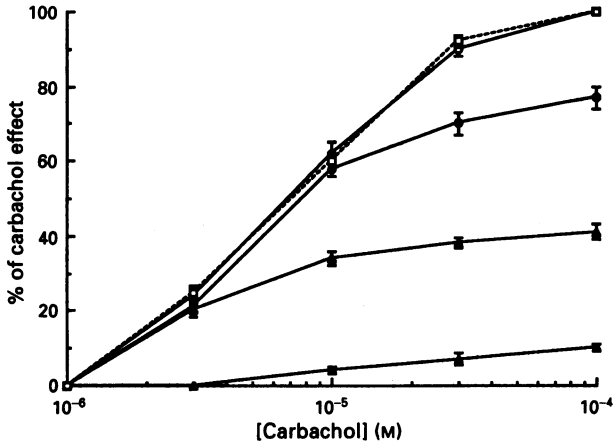

Figure 9 Noncompetitive antagonism of carbachol-induced contractions at muscular nicotinic receptors in frog rectus abdominis. Dose-response curves for carbachol were obtained before (○) and after exposure to 0.3 (●), 1 (▲) and 3 μM (△) tripitramine for 60 min, and after exposure to 1 μM (□) tripitramine for 60 min followed by 60 min washing. Each point is the mean (± s.e.mean) of four to six observations.

Table 5 Receptor systems affected by tripitramine and methoctramine

Receptor gene or subtype	*Tripitramine*	*Methoctramine*
m_1-muscarinic	yes (pK_i, 8.80[a])	yes (pK_i, 7.30[a])
M_1-muscarinic	yes (pK_i, 7.63[b])	yes (pK_i, 7.43[b])
m_2-muscarinic	yes (pK_i, 9.57[a])	yes (pK_i, 7.84[a])
M_2-muscarinic	yes (pA_2, 9.14–9.85; pK_i, 9.54[b])	yes (pA_2, 7.47–7.89; pK_i, 7.84[b])
m_3-muscarinic	yes (pK_i, 7.42[a])	yes (pK_i, 6.56[a])
M_3-muscarinic	yes (pA_2, 6.45–6.81; pK_i, 6.19[b])	yes (pA_2, 5.99–6.35; pK_i, 5.96[b])
m_4-muscarinic	yes (pK_i, 8.19[a])	yes (pK_i, 7.42[a])
M_4-muscarinic	yes (pK_i, 7.93[b])	yes (pK_i, 7.58[b])
m_5-muscarinic	yes (pK_i, 7.47[a])	yes (pK_i, 6.50[a])
α_1-adrenoceptor	no	yes (pK_B, 5.66)
α_2-adrenoceptor	no	no
β_1-adrenoceptor	no	yes[c]
H_1-histamine	no	no
H_2-histamine	no	no
muscular nicotinic	yes (pIC_{50}, 6.14)	yes (pIC_{50}, 6.52)
neuronal nicotinic	yes (pIC_{50}, 4.87)	yes (pIC_{50}, 4.96)

Data from this study and [a]Maggio *et al.* (1994), [b]Melchiorre *et al.* (1993). [c]Methoctramine behaved as a weak agonist. The EC_{50} value was not calculated since no maximum response was attained. At the maximum concentration used (500 μM), an increase in developed tension of 209% was obtained.

Tripitramine (5, 10 and 30 μM) inhibited the maximum relaxation induced at neuronal nicotinic receptors in rat duodenum by 100 μM DMPP with a pIC_{50} value of 4.87 ± 0.08. Under the same experimental conditions methoctramine behaved similarly, the pIC_{50} value amounting to 4.96 ± 0.05.

Tripitramine (10 μM) did not antagonize noradrenaline-induced contraction or clonidine-induced inhibition of twitch responses at α_1- and α_2-adrenoceptors in epididymal and prostatic portions of rat vas deferens, respectively. The EC_{50} values (± 95% C.F.) for noradrenaline or clonidine in the absence [0.18 (0.14–0.23) μM ($n = 5$) and 4.12 (3.50–4.72) nM ($n = 4$), respectively] and in the presence of tripitramine [0.19 (0.12–0.28) nM ($n = 5$) and 2.83 (2.30–3.63) nM ($n = 4$), respectively] were not significantly different. Under the same experimental conditions methoctramine (10 μM) did not affect clonidine-induced responses whereas it caused a parallel shift to the right of noradrenaline dose-response curves without affecting the maximum response. The EC_{50} values (± 95% C.F.) for noradrenaline in the absence and presence of methoctramine were [0.18 (0.14–0.23) μM ($n = 5$)] and [1.01 (0.94–1.10) μM ($n = 4$)] which allowed calculations of a pK_B value of 5.66 ± 0.03.

Discussion

The heterogeneity of muscarinic receptors is well documented and multiple receptor subtypes have been characterized (Caulfield, 1993). For this reason in the last decade there has been a resurgence of interest in muscarinic receptors owing to the observation that multiple subtypes may exist both in the central nervous system and periphery, which may represent potential targets for therapeutically useful drugs (Hulme *et al.*, 1990; McKinney & Coyle, 1991). Our effort to determine the structural elements of polymethylene tetraamines which confer selectivity toward a muscarinic receptor subtype, rather than to another (Melchiorre *et al.*, 1987b; 1989; Melchiorre, 1990) led to the design of tripitramine (Melchiorre *et al.*, 1993). This new tetraamine incorporates (Figure 1) the structural features of not only methoctramine but also pirenzepine, a selective muscarinic M_1 receptor antagonist (Hammer *et al.*, 1980), and of its analogues AF-DX 116 and AQ-RA 741, selective muscarinic M_2 receptor antagonists (Eberlein *et al.*, 1992; Hammer *et al.*, 1986). Preliminary results showed that tripitramine is not only more potent but also significantly more selective than the two prototypes methoctramine and AQ-RA 741 for muscarinic M_2 receptors (Melchiorre *et al.*, 1993). Furthermore, tripitramine displayed a unique binding profile for muscarinic receptor subtypes: $M_2 > M_4 \geq M_1 > M_3$. Clearly, it was able to distinguish among M_2 and all other muscarinic receptor subtypes investigated, M_2 to M_4, as well as between M_1 and M_3, and M_4 and M_3 subtypes (Melchiorre *et al.*, 1993). Furthermore, tripitramine displayed a similar binding profile ($m_2 > m_1 > m_4 > m_3 = m_5$) at human cloned muscarinic receptors (Table 5) (Maggio *et al.*, 1994). Thus, it was demonstrated that tripitramine discriminates markedly between both native and cloned muscarinic M_2 and M_4 receptor subtypes with a selectivity ratio value ranging from 24 to 41 (Melchiorre *et al.*, 1993; Maggio *et al.*, 1994). This finding may have relevance since all selective muscarinic M_2 receptor antagonists available to date, bind to muscarinic M_2 and M_4 receptors with similar affinities. Methoctramine displayed only 2 fold higher affinity for muscarinic m_2 compared to m_4 receptors. Similarly, Dörje *et al.* (1991) found that methoctramine, himbacine, AF-DX 250, AF-DX 384 and AQ-RA 741, all classified as selective muscarinic M_2 receptor antagonists, possess similar affinities at both muscarinic m_2 and m_4 receptor subtypes. We have now investigated further the antimuscarinic effects of tripitramine in comparison to methoctramine in atrial, tracheal, lung strip and ileal preparations derived from different species and with different agonists. Furthermore, antagonist combination experiments were carried out with atropine in order to verify the mechanism of action of tripitramine. In addition, tripitramine effects were determined also on other receptors, namely α_1-, α_2-, and β_1-adrenoceptors, H_1- and H_2-histamine receptors, and muscular and neuronal nicotinic receptors to assess its receptor specificity.

Tripitramine was an apparently simple competitive muscarinic receptor antagonist in all preparations investigated, namely right and left atria, ileum, trachea and lung strip, since it produced antagonism that was reversible and surmountable over a wide range of concentrations (Figures 2, 6 and 7). Schild analysis of the data yielded slopes that were not significantly different from unity (Table 1). It is known that the slope of a Schild regression is of paramount importance to assess the type of antagonism to an agonist response in a tissue. Competitive antagonism requires linear Schild regressions with a slope equal to unity. It is not unusual, however, that Schild regressions for competitive antagonists are not linear or have slopes significantly different from unity and several factors may be accounted for. Irrespective of agonist and species used, tripitramine failed to produce a linear Schild regression over the concentration-range investigated in left atria (Figure 3). At higher concentrations (0.015–3 μM) tripitramine gave linear plots with slopes not significantly different from unity as required for a competitive antagonist (Table 1). However, at lower concentrations (< 0.015 μM) a deviation from linearity was observed and in the 3–10 nM range the Schild plots had a slope of the regression line significantly higher than unity. Slopes greater than unity may be obtained when (a) there are nonequilibrium steady states or (b) a saturable removal mechanism for the antagonist is present in the tissue (Kenakin, 1993). In the first hypothesis, low K_B estimates were obtained and this phenomenon is particularly true for low concentrations which may require longer time of onset of equilibrium than high concentrations. It was observed indeed that the maximum effect of tripitramine was reached within 60 min for concentrations > 10 nM and 120 min for concentrations ≤ 10 nM (Figure 4). Although the tissues were incubated for the time necessary to reach the maximum effect of tripitramine, a nonlinear Schild plot (Figure 3) was obtained for lower concentrations. It appears that the low K_B estimates for tripitramine (≤ 10 nM) can hardly be ascribed to nonequilibrium steady states. As a consequence, it may well be that guinea-pig and rat left atria have a saturable removal mechanism for tripitramine. In this case the concentration of tripitramine in the receptor compartment will be considerably lower than that in the organ bath because of the presence of a removal site for the antagonist thus accounting for the low K_B estimates until the concentration of tripitramine reaches a value (> 10 nM) sufficient to saturate the removal process. If confirmed, this phenomenon would not be surprising since it has already been observed for atropine in rabbit ileum (Kenakin, 1993; Kenakin & Beek, 1987) and mouse urinary bladder as well as for N-methylatropine in mouse urinary bladder (Durant *et al.*, 1991). These tissues possess an esterase enzyme that degrades atropine leading to nonlinear Schild regression with a slope greater than unity and an anomalous pA_2 value. The regression of the line was reduced to unity by blocking the enzyme with an excess of 4-methylbutyrate which acted as an alternative substrate preventing atropine degradation. Interestingly, atropinesterase was not found in guinea-pig ileum (Kenakin, 1993; Kenakin & Beek, 1987). Similarly, the presence of a possible saturable removal mechanism for tripitramine in guinea-pig left atria as well as in rat left atria was shown but not in guinea-pig right atria. However, atropinesterase cannot be the enzyme responsible for tripitramine degradation since tripitramine lacks an ester function. Considering the structure of tripitramine, two functionalities might be the target of enzymes, that is four amine functions and six amide groups. Since polyamines such as spermidine and spermine are

biodegraded by the enzyme polyamine oxidase, we investigated whether this enzyme might also be responsible for tripitramine degradation. However, experiments performed in the presence of N,N'-*bis*(2,3-butanedienyl)-1,4-butanediamine, an irreversible inhibitor of polyamine oxidase (Bey *et al.*, 1985), ruled out this possibility since its presence in the bath did not modify the effect of tripitramine at low concentrations. Further experiments are needed to clarify the mechanism underlying tripitramine removal from receptor compartment. However, we can safely exclude the possibility that tripitramine removal is due to an artefact, such as binding to glassware used in the experiments at low concentrations of tripitramine, because the phenomenon was not observed in guinea-pig right atria with the same glassware and, what is more important, the same low concentrations (up to 3 nM). Whatever the reason is, either a nonequilibrium steady state or a saturable removal process, it can be assumed that the pA_2 values, ranging from 9.14 ± 0.09 to 9.85 ± 0.04, calculated at high concentrations (0.015–3 μM) may represent the true dissociation constant of tripitramine in rat and guinea-pig left atria.

Clearly, the affinity of tripitramine at muscarinic M_2 receptors is about three orders of magnitude greater than that at muscarinic M_3 receptors in ileal and tracheal preparations (Table 1). Tripitramine displayed a selectivity ratio ranging from 490 to 1820 (Table 4), which is in agreement with results previously reported (Melchiorre *et al.*, 1993). Thus, the M_2/M_3 selectivity profile of tripitramine is qualitatively analogous to that of methoctramine, but it is evident that the cardioselectivity of tripitramine is much greater (Table 4). Furthermore, a comparison between tripitramine and AQ-RA 741, another muscarinic receptor antagonist classified as M_2-selective, reveals that tripitramine has a better affinity profile since AQ-RA 741 displayed a M_2/M_3 selectivity ratio value of about 80 and a pA_2 value around 8 for cardiac muscarinic M_2 receptors (Eberlein *et al.*, 1989) which are markedly lower than those of tripitramine.

One most intriguing finding of the present investigation was the result obtained in guinea-pig lung strip preparation. Using a number of subtype selective muscarinic receptor antagonists, Roffel *et al.* (1993) suggested that cholinergic contraction of the guinea-pig lung strip is mediated by muscarinic M_2-like receptors. The affinity of tripitramine for muscarinic receptor subtypes in guinea-pig lung does not appear, however, in agreement with the conclusion reached by Roffel *et al.* (1993). The pA_2 values of 7.91 ± 0.03 for tripitramine in guinea-pig lung does not correlate with the affinity found for both muscarinic M_2 and M_3 receptor subtypes in atria and smooth muscles (ileum and trachea) where pA_2 values of 9.14–9.85 and 6.45–6.81 were obtained, respectively (Table 1). In parallel experiments in guinea-pig lung strips, methoctramine displayed an affinity value of 6.97 ± 0.07 which is similar to that (pA_2, 7.30 ± 0.06) reported by Roffel *et al.* (1993). An analysis of the results shown in Table 5 reveals that the functional affinity of tripitramine in guinea-pig lung correlates well with the affinity at either native or cloned muscarinic receptors only for the M_4 subtype. However, the affinity of tripitramine for cloned muscarinic m_3 and m_5 receptors is only 3 fold lower than that in guinea-pig lung strip. Nevertheless, on the basis of tripitramine affinity profile, it can be suggested that the contraction of guinea-pig lung strip is not mediated by muscarinic M_2 receptors. It remains to be established, however, whether the M_4 subtype or, as suggested by Roffel *et al.* (1993), a novel subtype or a mixture of muscarinic receptor subtypes is involved in the contractile process of guinea-pig lung strip.

The mutual competition between tripitramine and atropine was investigated in electrically stimulated left atria and in ileum according to the dose-ratio method (Paton & Rang, 1965). As shown in Table 2, in the atria a combination of tripitramine and atropine resulted in dose-ratios equal to the sum of the dose-ratios produced by the two antagonists when tested alone. This suggests that tripitramine and atropine compete for the same receptor site. These results are very similar to those reported for a combination of methoctramine and atropine (Melchiorre *et al.*, 1987a).

Experiments performed in guinea-pig ileum with a combination of tripitramine and atropine gave results which were not consistent with a competitive mode of action of tripitramine (Table 3). In fact, the combination dose-ratios were somewhat greater than expected, but these results are not consistent with the multiplication of dose-ratios which would be expected if two antagonists interacted exclusively at independent sites. It follows that tripitramine and atropine compete for two distinct but interdependent sites. Thus tripitramine may interact with muscarinic M_3 receptors in an allosteric fashion and, consequently, its dissociation constants derived in guinea-pig and rat ileum and guinea-pig trachea may be incorrect and should be considered as apparent pA_2 values. Again these results are very similar to those reported for a combination of methoctramine and atropine (Melchiorre *et al.*, 1987a).

Since methoctramine was shown to antagonize rat vas deferens α_1-adrenoceptors (Melchiorre *et al.*, 1987b), muscular (Melchiorre *et al.*, 1987b) and neuronal nicotinic receptors (Watson *et al.*, 1992), and to display positive inotropic activity at β_1-adrenoceptors in guinea-pig left atria (Eglen *et al.*, 1988), tripitramine was investigated also in other receptor preparations to assess its receptor specificity in comparison to methoctramine. Experiments performed in electrically stimulated guinea-pig left atria revealed that tripitramine, unlike methoctramine, did not induce positive inotropic responses (Figure 8). Methoctramine elicited a modest positive inotropic activity which was more pronounced in tissues from unpretreated than from reserpine-pretreated animals in contrast with results previously reported (Eglen *et al.*, 1988). However, it should be noted that, though the chronic pretreatment with reserpine increases sensitivity to β-adrenoceptor agonists, reserpine induced changes may be species-dependent since guinea-pigs failed to reveal a significant receptor increase, whereas rats did (Chess-Williams *et al.*, 1986). In addition, the time course and dosage of reserpine may be of relevance, since an increase in receptor number in rat cardiac tissue was found after seven days but not after one or three days of treatment (Chess-Williams *et al.*, 1986).

Experiments performed on frog rectus abdominis showed that tripitramine, like methoctramine (present results and Melchiorre *et al.*, 1987b), inhibited muscular nicotinic receptors with a noncompetitive mechanism because the maximum response to carbachol was depressed (Figure 9). Furthermore, this blockade was reversed after tissue washing. Tripitramine displayed a pIC_{50} value of 6.14 ± 0.07 that was slightly lower than that obtained for methoctramine (6.52 ± 0.05). Watson *et al.* (1992) reported that methoctramine has antagonistic activity in the micromolar range at ganglionic nicotinic receptors. Very recently, Irie *et al.* (1994) showed that the action at neuronal nicotinic receptors can be easily investigated in rat isolated duodenum. It turned out indeed that both tripitramine and methoctramine inhibited with modest potency neuronal nicotinic receptors with pIC_{50} values of 4.87 ± 0.08 and 4.96 ± 0.05, respectively. In all other experiments performed tripitramine did not antagonize α_1-, α_2-, and β_1-adrenoceptors, and H_1- and H_2-histamine receptors (Table 5). Methoctramine gave similar results but it antagonized noradrenaline-induced responses in rat vas deferens α_1-adrenoceptors with a pK_B value of 5.66 ± 0.03, which is in agreement with results previously reported (Melchiorre *et al.*, 1987b).

As shown in Table 5, it is evident that tripitramine has not only an outstanding affinity and selectivity for M_2 muscarinic receptors but also a superior receptor specificity compared with methoctramine. Furthermore, in guinea-pig lung strip, tripitramine displayed a marked lower potency than that observed at cardiac muscarinic M_2 receptors allowing the

conclusion that the contraction in the guinea-pig lung strip is not mediated by muscarinic M_2-like receptors. Beside muscarinic receptors, tripitramine antagonized with a modest potency, among receptor systems investigated so far, only muscular and neuronal nicotinic receptors whereas methoctramine affected, in addition, α_1- and β_1-adrenoceptors.

In conclusion, tripitramine represents the most potent and the most selective muscarinic M_2 receptor antagonist at present available. It may serve as a powerful tool for the characterization of muscarinic receptor subtypes.

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Characterization of histamine H_3 receptors regulating acetylcholine release in rat entorhinal cortex

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1 The pharmacological properties and location of H_3 receptors modulating acetylcholine release have been investigated in non-superfused slices and synaptosomes of rat entorhinal cortex preloaded with [^{3}H]-choline.

2 (R)α-methylhistamine, an H_3-receptor agonist, potently inhibited the K^+-evoked tritium release from slices, an effect antagonized by thioperamide, an H_3-receptor antagonist, with nanomolar potency.

3 The K^+-evoked tritium release from synaptosomes remained unaltered in the presence of the potent and selective H_3-receptor agonists, imetit and (R)α-methylhistamine, suggesting that H_3 receptors modulating acetylcholine release are not presynaptically located on cholinergic nerve terminals.

4 Phenylbutanoylhistamine and phenylpropylhistamine, two H_3-receptor antagonists of moderate potency, failed to antagonize the inhibitory effects of (R)α-methylhistamine observed in slices. Unexpectedly, both compounds when used alone, inhibited tritium release from slices and synaptosomes with micromolar potency and to the same extent (by approximately 50% when added at a final concentration of 200 μM). This inhibitory effect did not involve H_1, H_2 or H_3 receptors and was not mediated by an unknown histamine receptor site, since histamine used at a high concentration neither reproduced nor antagonized the effect of phenylbutanoylhistamine. It remained unaltered in the presence of scopolamine and was neither mimicked nor antagonized by vasoactive intestinal peptide, previously shown to be colocalized with acetylcholine in some neurones.

5 It is concluded that acetylcholine release in rat entorhinal cortex is modulated by H_3 receptors presumably not located on cholinergic axon terminals. It remains to be established whether these H_3 receptors belong to a receptor subtype different from those previously described since the potency of phenylbutanoylhistamine and phenylpropylhistamine as H_3-receptor antagonists was probably greatly underestimated by additional properties of both drugs.

Keywords: Acetylcholine release; entorhinal cortex; slices; synaptosomes; histamine H_3 receptors

Introduction

The histamine H_3 receptor was initially detected as an autoreceptor modulating histamine synthesis and release in the brain (Arrang *et al.*, 1983; 1985; 1987a,b). However, autoradiography of H_3 receptors in rat and monkey brain together with lesion experiments (Martinez Mir *et al.*, 1990; Cumming *et al.*, 1991; Pollard *et al.*, 1993) indicate that the majority of central H_3 receptors are not autoreceptors. In agreement with this view, functional studies performed in brain have indicated the occurrence of presynaptic inhibitory H_3 receptors on axon terminals not only of histaminergic but also noradrenergic (Schlicker *et al.*, 1989), 5-hydroxytryptaminergic (Fink *et al.*, 1990), dopaminergic (Schlicker *et al.*, 1993) and peptidergic neurones (Matsubara *et al.*, 1992). Also, it has been recently demonstrated that activation of H_3 receptors inhibits the release of tritium, presumably [^{3}H]-acetylcholine, from slices of rat entorhinal cortex preloaded with [^{3}H]-choline (Clapham & Kilpatrick, 1992) and the pharmacological profile of this response led the authors to postulate the existence of H_3-receptor subtypes. The existence of multiple H_3-receptor subtypes was also suggested based upon the comparison of functional and radioligand binding studies (Arrang *et al.*, 1990; West *et al.*, 1990a,b; Hey *et al.*, 1992; Schlicker *et al.*, 1992).

We have used non-superfused slices and synaptosomes of entorhinal cortex to reinvestigate this possibility as well as to assess the presynaptic localization of H_3 receptors on acetylcholine-releasing terminals themselves.

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Methods

Release from slices

Slices (0.3 mm thick) from the entorhinal cortex of male Wistar rats (180-200 g, Iffa Credo, France) were prepared with a McIlwain tissue chopper and suspended in modified Krebs-Ringer bicarbonate medium (mM): NaCl 120, KCl 0.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 27.5 and glucose 10, pH 7.4 gassed wtih O_2/CO_2 (95:5). The slices were preincubated for 45 min at 37°C in the presence of 0.1 μM [^{3}H]-choline for [^{3}H]-acetylcholine synthesis. The slices were then washed with fresh Krebs solution (4 × 20 ml) in order to remove excess radioactivity, and transferred to a small basket (Arrang *et al.*, 1983). The basket was subsequently immersed six times (for 10 min periods each) in fresh Krebs solution. After the last washing period, 250 μl aliquots of the slice suspension (1-3 mg of protein) were distributed into tubes, preincubated in each experiment for 5 min in the presence of various drugs tested as histamine agonists or antagonists and subsequently incubated for 2 min with 2, 20 or 30 mM K^+ (final concentration). Incubations were stopped by short centrifugation. The pellets were homogenized in 500 μl 0.01 M HCl and radioactivity present in tissue and medium was measured by liquid scintillation spectrometry.

Release from synaptosomes

A crude synaptosomal fraction was prepared after homogenization of the entorhinal cortex in 0.32 M sucrose at 4°C according to the method of Whittaker (1966). Synaptosomes were suspended in Krebs solution and incubated for 45 min with [^{3}H]-choline as described above for slices. After six

extensive washings by successive centrifugations and suspensions in fresh medium (2 mM K^+), synaptosomes (0.3–1 mg of protein) were distributed into tubes, incubated in each experiment for 5 min in the presence, when required, of the various drugs tested as agonists or antagonists and subsequently incubated for 2 min with 2, 10 or 20 mM K^+ (final concentration). Incubations were ended and radioactivity determined as for slice experiments. Protein contents were determined according to the method of Lowry *et al.* (1951) with bovine serum albumin used as the standard.

Analysis of data

Radioactivity (in d.p.m.) was estimated by liquid scintillation spectrometry at 45% counting efficiency.

Values for tritium release elicited by tissue (slices or synaptosomes) depolarization with high K^+ solutions were calculated in each experiment as the difference between mean tritium levels in high K^+ and 2 mM K^+ media of the same experiment, respectively, and are given as percentages of the total tritium present in tissue plus medium.

For determination of the means and standard errors of EC_{50} and IC_{50} values, the total curves were analysed with an iterative computer least-squares method derived from Parker & Waud (1971) (except Figure 3). The apparent dissociation constant (K_i value) of thioperamide was calculated from its IC_{50} value, assuming a competitive antagonism and neglecting the possible effect of endogenous histamine, according to the equation of Cheng & Prusoff (1973): $K_i = IC_{50}/(1 + S/EC_{50})$, where S represents the concentration of (R)α-methylhistamine (1 μM) and EC_{50} the concentration of (R)α-methylhistamine eliciting a half-maximal inhibitory effect on K^+-evoked release of tritium.

Statistical evaluation of the results was by Student's *t* test. Any significant difference obtained for tritium release was determined from pairs of values (representing the mean ± s.e.mean) obtained from experiments carried out in parallel and calculated as percentages before or after subtraction of the mean basal efflux and was checked by statistical analysis (*t* test) of the same parameters expressed in d.p.m. mg^{-1} protein of tritium measured in the media.

Chemicals and drugs

[^{3}H]-choline chloride (75 Ci $mmol^{-1}$) was purchased from the Radiochemical Centre (Amersham, UK). Drugs and their sources were as follows: mepyramine hydrochloride (Specia, Paris, France), ranitidine hydrochloride (Glaxo, Greenford, UK), burimamide and impromidine trihydrochloride (Smith Kline Beecham, London, UK), (R)α-methylhistamine dihydrogenmaleate and thioperamide (Bioprojet, Paris, France), histamine dihydrochloride, oxotremorine sesquifumarate and scopolamine dihydrochloride (Sigma, St Louis, MO, U.S.A.), vasoactive intestinal polypeptide (VIP) (porcine) and thyrotropin releasing hormone (TRH) (Bachem, Bubendorf, Switzerland). Imetit dihydrobromide was synthesized by Prof. C.R. Ganellin (University College London, England) and phenylbutanoylhistamine and phenylpropylhistamine were synthesized by Prof. W. Schunack (Free University, Berlin, Germany).

Results

Release from slices

The spontaneous efflux of tritium from slices of rat entorhinal cortex into the medium in the presence of 2 mM K^+, 1 μM mepyramine and 10 μM ranitidine represented 2031 ± 338 d.p.m. mg^{-1} protein, i.e. 8.8 ± 0.5% of the total (tissue plus medium) tritium, per 2 min. In the presence of 20 mM K^+, 1 μM mepyramine and 10 μM ranitidine, the release of tritium in the medium represented 14.0 ± 0.7% of the total per 2 min, giving an evoked release of 5.2 ± 0.4% ($n = 16$ separate experiments) over spontaneous efflux. The K^+-induced release was inhibited by (R)α-methylhistamine in a concentration-dependent and saturable manner (Figure 1). The maximal inhibition of release was 39 ± 4% and the EC_{50} was 25 ± 10 nM. As previously reported (Clapham & Kilpatrick, 1992), in the absence of mepyramine and ranitidine, the EC_{50} was increased by about one order of magnitude with a similar maximal inhibition (not shown). Therefore, all the experiments with slices were carried out in the presence of 1 μM mepyramine and 10 μM ranitidine. In the presence of 30 mM K^+, 1 μM mepyramine and 10 μM ranitidine, the release of tritium over spontaneous efflux represented 7.6 ± 0.4% of the total ($n = 3$ experiments) and was not significantly inhibited (by 12 ± 2%) in the presence of 2 μM (R)α-methylhistamine.

Thioperamide at increasing concentrations progressively antagonized the inhibitory effect of (R)α-methylhistamine with an IC_{50} value of 0.9 ± 0.6 μM (Figure 2), leading to a mean apparent K_i value of 22 ± 15 nM. Thioperamide, in high concentrations (10–100 μM), tended to enhance by about 20% the K^+-evoked release of tritium over the non-inhibited level (100%, Figure 2). Phenylbutanoylhistamine (200 μM) failed to antagonize the inhibitory effect of (R)α-methylhistamine but, when added alone in the presence of 20 mM K^+, 1 μM mepyramine and 10 μM ranitidine, significantly inhibited the K^+-evoked release of tritium by 44% (Table 1).

Release from synaptosomes

The basal tritium efflux from synaptosomes into the medium, evaluated in the presence of 2 mM K^+ represented 6290 ±

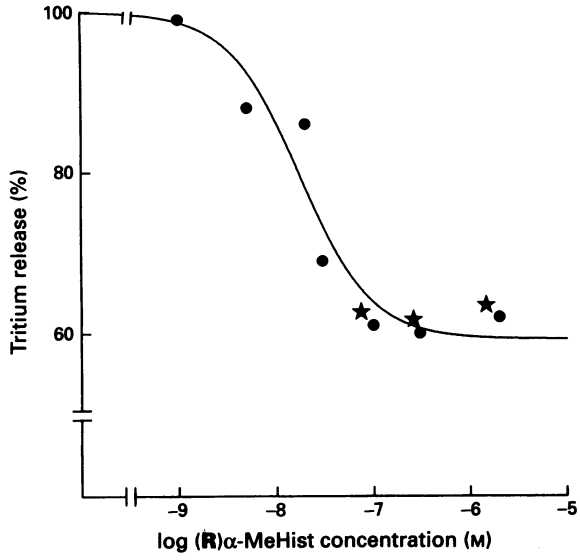

Figure 1 Effect of (R)α-methylhistamine ((R)α-MeHist) on K^+-evoked tritium release from slices of rat entorhinal cortex. Slices were allowed to synthesize [^{3}H]-acetylcholine during a 45 min preincubation in the presence of [^{3}H]-choline. After several washing steps, slices were distributed into tubes, incubated for 5 min in the presence of mepyramine (1 μM) and ranitidine (10 μM) with or without (R)α-MeHist, and subsequently incubated for 2 min in the presence of 2 or 20 mM K^+ (final concentration). The basal tritium efflux (2 mM K^+) represented 9.0 ± 0.5% of total tritium per 2 min and was not significantly modified in the presence of (R)α-MeHist. The release of tritium induced by 20 mM K^+ was calculated in each experiment as the difference between mean tritium levels in 20 and 2 mM K^+ media, respectively, and represented, in the absence of (R)α-MeHist, 3.5 ± 0.4% of the total (tissue plus medium) tritium. Results are expressed as percentages of this value. Each point represents the results from three separate experiments with 3–4 determinations. *$P < 0.05$ as compared with 20 mM K^+ in the absence of (R)α-MeHist.

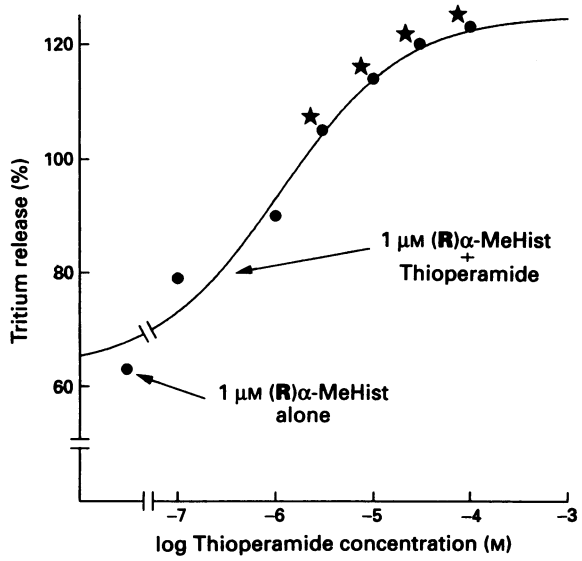

Figure 2 Effect of thioperamide on the inhibition by (R)α-methylhistamine ((R)α-MeHist) of the K^+-evoked tritium release from slices of rat entorhinal cortex. The spontaneous efflux of tritium (2 mM K^+) represented 9.5 ± 0.5% of total per 2 min, and tritium release induced by 20 mM K^+ over spontaneous efflux (expressed as a percentage of total (tissue plus medium) tritium) represented 5.8 ± 0.6%. Results are expressed as a percentage of this value. Thioperamide in increasing concentrations was added, in the presence of 1 μM mepyramine and 10 μM ranitidine, to (R)α-MeHist in a fixed concentration (1 μM) which, alone, inhibited the K^+-evoked tritium release from slices by 37%. Each point represents the results from three separate experiments with 4 determinations. *$P<0.05$ as compared with 1 μM (R)α-MeHist alone.

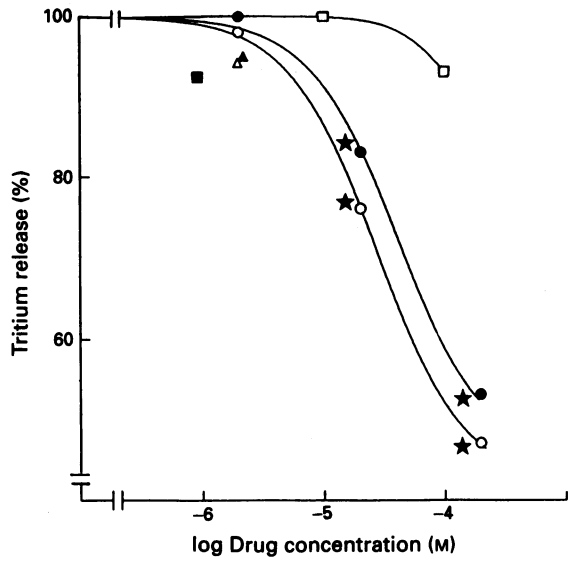

Figure 3 Modulation by various imidazole derivatives of tritium release from synaptosomes of rat entorhinal cortex depolarized with 10 mM K^+: (□) histamine; (●) phenylbutanoylhistamine; (○) phenylpropylhistamine; (■) imetit; (△) (R)αmethylhistamine; (▲) thioperamide. The spontaneous efflux of tritium (2 mM K^+) represented 10.0 ± 0.8% of total tritium per 2 min. In the absence of added agents, the release of tritium induced over basal efflux by incubation for 2 min with 10 mM K^+ represented 4.4 ± 0.6% of the total. Results are expressed as percentages of this value. Each point represents mean results from two separate experiments with quadruplicate determinations. Similar results were obtained in the presence of 1 μM mepyramine and 10 μM ranitidine. *$P<0.05$ as compared with 10 mM K^+ alone.

Table 1 Influence of (R)α-methylhistamine and phenylbutanoylhistamine on K^+-induced tritium release from slices and synaptosomes of rat entorhinal cortex

	% tritium release	
	Slices	*Synaptosomes*
Control	5.8 ± 0.6	5.4 ± 0.3
(R)α-methylhistamine (2 μM)	3.7 ± 0.6* (−36%)	5.0 ± 0.4 (−7%)
Phenylbutanoylhistamine (200 μM)	3.2 ± 0.5* (−44%)	3.1 ± 0.3** (−43%)

Slices and synaptosomes were depolarized for 2 min in the presence of 1 μM mepyramine, 10 μM ranitidine and either 20 mM K^+ (slices) or 10 mM K^+ (synaptosomes) (final concentration), respectively. Values represent the K^+-induced tritium release over spontaneous efflux, i.e. the difference between mean tritium levels in 20 mM K^+ (slices) or 10 mM K^+ (synaptosomes) and 2 mM K^+ media, respectively, expressed as a percentage of total (tissue plus medium) tritium. The spontaneous efflux (2 mM K^+) represented 9.1 ± 0.3% (slices) and 13.8 ± 0.2% (synaptosomes) of total tritium, per 2 min. Values in parentheses indicate percentage changes as compared with 10 mM K^+ or 20 mM K^+ alone (controls). Values are the mean ± s.e.mean of 3–9 determinations in two separate experiments. *$P<0.05$; **$P<0.01$ as compared with respective controls.

448 d.p.m. mg^{-1} protein, i.e. 13.0 ± 0.8% of the total tritium (present in tissue plus medium), per 2 min. The release elicited by 10 mM K^+ over basal efflux corresponded to 5.0 ± 0.3% of the total ($n = 11$ experiments) (Table 1 and Figure 3) and was not significantly modified in the presence of 1 μM imetit, 2 μM (R)α-methylhistamine, 100 μM histamine or 2 μM thioperamide tested in the absence (Figure 3) or presence (Table 1 and data not shown) of 1 μM mepyramine and 10 μM ranitidine. It was significantly ($P<0.05$) reduced by 32 ± 6% ($n = 3$ experiments) in the presence of 200 μM oxotremorine and was inhibited in a concentration-dependent manner by phenylbutanoylhistamine and phenylpropylhistamine (Figure 3). The inhibition by 46 ± 4% ($n = 7$ experiments) observed in the presence of 200 μM phenylbutanoylhistamine was not significantly diminished in the presence of 1 mM histamine, 1 μM mepyramine, 10 μM ranitidine, 200 μM burimamide, 60 μM impromidine, 2 μM thioperamide or 1 μM scopolamine (not shown). It was neither mimicked nor antagonized in the presence of 0.1 μM VIP or TRH (not shown). The inhibitory effect of 200 μM phenylbutanoylhistamine was less marked (−19 ± 1%, $n = 3$ experiments), although still significant ($P<0.05$), in the presence of 20 mM K^+. Although of limited magnitude, a reproducible and significant ($P<0.01$) decrease (by 11 ± 1%) of the basal tritium efflux was observed after a 2 min incubation of synaptosomes with 200 μM phenylbutanoylhistamine or phenylpropylhistamine in 2 mM K^+ ($n = 5$ experiments).

Discussion

It was recently reported that histamine H_3 receptors modulate the release of [^{3}H]-acetylcholine from superfused slices of entorhinal cortex (Clapham & Kilpatrick, 1992). The present data, obtained in a non-superfused slice system (Arrang *et al.*, 1983), confirm this observation. The tritium release from depolarized slices, previously shown to consist mainly of [^{3}H]-acetylcholine (Barnes *et al.*, 1989), was potently inhibited by (R)α-methylhistamine, a selective H_3-receptor agonist (Figure 1). The response to (R)α-methylhistamine was inhibited by the selective H_3-receptor antagonist thioperamide (Figure 2), with a mean apparent K_i value (22 ± 15 nM) not significantly different from its K_i values at H_3 receptors modulating [^{3}H]-histamine release from brain slices ($K_i = 5.6 \pm 1.0$ nM, Arrang *et al.*, 1987a) or synaptosomes ($K_i = 5.6 \pm 1.4$ nM, Garbarg *et al.*, 1992). In

contrast, its potency was increased approximately 10 fold in the presence of the H_1- and H_2-receptor antagonists, mepyramine (1 μM) and ranitidine (10 μM). Although both drugs did not significantly affect the K^+-evoked release, Clapham & Kilpatrick (1992) postulated from this result that H_1 and H_2 receptor stimulation might enhance acetylcholine release. Such a stimulation might also occur *in vivo* since it was recently shown by microdialysis that endogenous histamine stimulates endogenous acetylcholine release in rat hippocampus, mainly via interaction with H_2 receptors (Mochizuki *et al.*, 1994). It has also been suggested that stimulation of H_2 receptors releases endogenous noradrenaline in rat hypothalamus (Blandina *et al.*, 1989) and endogenous enkephalin in mouse striatum (Garbarg *et al.*, 1991).

In synaptosomes of entorhinal cortex, however, tritium release remained unaltered in the presence of two H_3-receptor agonists (Table 1 and Figure 3). Previous studies performed on the regulation of histamine release by presynaptic H_3-autoreceptors (Arrang *et al.*, 1985) or galanin receptors (Arrang *et al.*, 1991) and using very similar experimental conditions have shown that the maximal inhibitory effect observed in synaptosomes was smaller than in slices. However, although it cannot be entirely ruled out, the existence of a small modulation in synaptosomes seems to be unlikely since it was not observed in conditions (10 mM K^+) previously shown to increase markedly the maximal inhibitory effect of galanin on histamine release in rat hypothalamus and hippocampus (Arrang *et al.*, 1991). Moreover, the expected modulation by oxotremorine of tritium release from synaptosomes preloaded with [^{3}H]-choline (Raiteri *et al.*, 1984; Marchi & Raiteri, 1985) was found in the same experiments (not shown). Therefore, these data with synaptosomes strongly suggest that H_3 receptors modulating acetylcholine release in slices are not, for the most part, presynaptically located on cholinergic nerve terminals, but are found either on non-cholinergic nerve endings impinging on the former, or postsynaptically on intrinsic perikarya. The postsynaptic location of these H_3 receptors may account, at least partially, for the decrease in the number of H_3 receptors observed in cerebral cortex and striatum after local infusion of neurotoxins (Cumming *et al.*, 1991; Pollard *et al.*, 1993). Moreover, a physiological function of these receptors in the control of acetylcholine release is suggested by the participation of H_3 receptors in the endogenous modulation of acetylcholine release as shown by the facilitatory effect of thioperamide observed both *in vitro* (Figure 2, Clapham & Kilpatrick, 1992) and *in vivo* (Mochizuki *et al.*, 1994). However, the weak and relatively short-lasting effects of H_3 ligands observed *in vivo* on acetylcholine release as measured by microdialysis (Mochizuki *et al.*, 1994) suggest that this tonic modulation of acetylcholine release is probably minor, when compared to the modulation of histamine release (Arrang *et al.*, 1987a).

In contrast to thioperamide, phenylbutanoylhistamine, an H_3-receptor antagonist with moderate potency (Lipp *et al.*, 1988; 1992; Stark *et al.*, 1990; 1994) failed to antagonize the inhibitory effect of (R)α-methylhistamine on acetylcholine release from slices of rat entorhinal cortex (Clapham & Kilpatrick, 1992). In addition phenylbutanoylhistamine failed to antagonize the (R)α-methylhistamine-induced inhibition of non-adrenergic non-cholinergic contractions of the guinea-pig ileum (Taylor & Kilpatrick, 1992) and it was suggested from these data that this compound might discriminate several H_3-receptor subtypes. In order to reinvestigate this hypothesis, we further analysed the effects of phenylbutanoylhistamine as well as its amine derivative, phenylpropylhistamine, a compound with a ten times lower affinity at H_3 receptors (Lipp *et al.*, 1988; 1992; Stark *et al.*, 1990; 1994). Surprisingly, phenylbutanoylhistamine, when added alone, inhibited acetylcholine release from slices (Table 1) and both compounds inhibited, with a similar potency and to the same extent (by about 50% when added at a final concentration of 200 μM), acetylcholine release from synaptosomes (Figure 3). Although the inhibitory effect of both drugs was concentration-dependent, no plateau was reached and the EC_{50} values and maximal effect (which was probably higher than that observed in the presence of 200 μM of both drugs) could not be determined (Figure 3). However, their potency could be roughly evaluated to be in the micromolar range. This effect was not mediated by H_3 receptors, being neither mimicked by (R)α-methylhistamine or imetit in synaptosomes, nor blocked by three H_3-receptor antagonists added at a high concentration. It did not involve H_1 or H_2 receptors since it was not reversed by mepyramine or ranitidine. The involvement of a histamine receptor site, different from the known H_1, H_2 or H_3 receptors, was also ruled out since no inhibition was observed in synaptosomes in the presence of 100 μM histamine and no antagonism of the effect of phenylbutanoylhistamine was found in the presence of 1 mM histamine. The inhibitory effect was not mediated by muscarinic autoreceptors, being unchanged in the presence of a high concentration of scopolamine and receptors for VIP, a peptide colocalized with acetylcholine in one type of cholinergic innervation in the cortex (Eckenstein & Baughman, 1984) are probably not involved. Even whether both drugs act via interaction with receptors or via another mechanism is unclear. The small modulation of the basal tritium efflux from synaptosomes observed in their presence might suggest an unspecific mechanism. However, the partially depolarized state of synaptosomes incubated in the presence of 2 mM K^+ has been previously suggested to account for a receptor-mediated modulation observed under basal conditions (Arrang *et al.*, 1987b). Hence the mechanisms responsible for the inhibition by phenylbutanoylhistamine and phenylpropylhistamine remain to be identified. Whatever the mechanism, these properties of both drugs probably lead to a large underestimation of their moderate H_3-antagonist potency and do not allow any conclusion to be drawn as to the possible existence of H_3-receptor subtypes.

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VOLUME 114 (7) APRIL 1995

SPECIAL REPORT

P. Klemm, T.D. Warner, D. Willis, A.R. Moore & J.R. Vane. Coronary vasoconstriction *in vitro* in the hearts of polyarthritic rats: effectiveness of *in vivo* treatment with the endothelin receptor antagonist SB 209670 **1327**

PAPERS

A.J. Lawrence, E. Krstew & B. Jarrott. Functional dopamine D_2 receptors on rat vagal afferent neurones **1329**

T.A. Swierkosz, J.A. Mitchell, T.D. Warner, R.M. Botting & J.R. Vane. Co-induction of nitric oxide synthase and cyclo-oxygenase: interactions between nitric oxide and prostanoids **1335**

D.T. Walsh, V.B. Weg, T.J. Williams & S. Nourshargh. Substance P-induced inflammatory responses in guinea-pig skin: the effect of specific NK_1 receptor antagonists and the role of endogenous mediators **1343**

M. Alegret, E. Cerqueda, R. Ferrando, M. Vázquez, R.M. Sánchez, T. Adzet, M. Merlos & J.C. Laguna. Selective modification of rat hepatic microsomal fatty acid chain elongation and desaturation by fibrates: relationship with peroxisome proliferation **1351**

P.M. Beart, A. Schousboe & A. Frandsen. Blockade by polyamine NMDA antagonists related to ifenprodil of NMDA-induced synthesis of cyclic GMP, increases in calcium and cytotoxicity in cultured neurones **1359**

G.R. May, W.S. Rowand, J.G. McCormack & R.D. Sheridan. Neuroprotective profile of lifarizine (RS-87476) in rat cerebrocortical neurones in culture **1365**

H. Bult, G.R.Y. de Meyer & A.G. Herman. Influence of chronic treatment with a nitric oxide donor on fatty streak development and reactivity of the rabbit aorta **1371**

B. Battistini, M. Woods, L.J.D. O'Donnell, T.D. Warner, R. Corder, A. Fournier, M.J.G. Farthing & J.R. Vane. Contractile activity of endothelin precursors in the isolated gallbladder of the guinea-pig: presence of an endothelin-converting enzyme **1383**

J.E. Nally, C.C. Docherty, R.A. Clayton & N.C. Thomson. Bronchodilator and pre-protective effects of urodilatin in bovine bronchi *in vitro*: comparison with atrial natriuretic peptide **1391**

M. Kurosawa, K. Messlinger, M. Pawlak & R.F. Schmidt. Increase of meningeal blood flow after electrical stimulation of rat dura mater encephali: mediation by calcitonin gene-related peptide **1397**

P. Soares-da-Silva, M. Pestana, M.A. Vieira-Coelho, M.H. Fernandes & A. Albino-Teixeira. Assessment of renal dopaminergic system activity in the nitric oxide-deprived hypertensive rat model **1403**

J.U. Chowdhury, Y.-W. Pang, S.-M. Huang, M. Tsugeno & T. Tomita. Sustained contraction produced by caffeine after ryanodine treatment in the circular muscle of the guinea-pig gastric antrum and rabbit portal vein **1414**

F. Orallo, A.F. Alzueta, M. Campos-Toimil & J.M. Calleji. Study of the *in vivo* and *in vitro* cardiovascular effects of (+)-glaucine and N-carbethoxysecoglaucine in rats **1419**

R. Tavakoli & N. Frossard. 5-HT_2-mediated bronchial responses after syngeneic lung transplantation in the rat **1428**

E. Kelly, P.K. Harrison & R.J. Williams. Effects of acute and chronic ethanol on cyclic AMP accumulation in NG108-15 cells: differential dependence of changes on extracellular adenosine **1433**

S.A. Kilfeather, S. Tagoe, A.C. Perez, K. Okona-Mensa, R. Matin & C.P. Page. Inhibition of serum-induced proliferation of bovine tracheal smooth muscle cells in culture by heparin and related glycosaminoglycans **1442**

F. de Ponti, M. Cosentino, A. Costa, M. Girani, G. Gibelli, L. D'Angelo, G. Frigo & A. Crema. Inhibitory effects of SR 58611A on canine colonic motility: evidence for a role of β_3-adenoceptors **1447**

Y.-X. Wang, S.L. Lim & C.C.Y. Pang. Increase by N^G-nitro-L-arginine methyl ester (L-NAME) of resistance to venous return in rats **1454**

E. Talpain, R.A. Armstrong, R.A. Coleman & C.J. Vardey. Characterization of the PGE receptor subtype mediating inhibition of superoxide production in human neutrophils **1459**

L.A. Miller, D.E. Cochrane, R.E. Carraway & R.S. Feldberg. Blockade of mast cell histamine secretion in response to neurotensin by SR 48692, a nonpeptide antagonist of the neurotensin brain receptor **1466**

J. Maas, G.A. Rae, J.P. Huidobro-Toro & J.B. Calixto. Characterization of kinin receptors modulating neurogenic contractions of the mouse isolated vas deferens **1471**

A. Bergendal, A. Lindén, J. Lötvall, B.-E. Skoogh & C.-G. Löfdahl. Different effects of salmeterol, formoterol and salbutamol on cholinergic responses in the ferret trachea **1478**

A.D. Kraneveld, T.L. Buckley, D. van Heuven-Nolsen, Y. van Schaik, A.Sj. Koster & F.P. Nijkamp. Delayed-type hypersensitivity-induced increase in vascular permeability in the mouse small intestine: inhibition by depletion of sensory neuropeptides and NK_1 receptor blockade **1483**

L.E. Cardona-Sanclemente & G.V.R. Born. Effect of inhibition of nitric oxide synthesis on the uptake of LDL and fibrinogen by arterial walls and other organs of the rat **1490**

K.M. Stevenson, K.J. Gibson & E.R. Lumbers. Comparison of the transplacental transfer of enalapril, captopril and losartan in sheep **1495**

H. Vatanpour & A.L. Harvey. Modulation of acetylcholine release at mouse neuromuscular junctions by interaction of three homologous scorpion toxins with K^+ channels **1502**

A. Chiarini, R. Budriesi, M.L. Bolognesi, A. Minarini & C. Melchiorre. *In vitro* characterization of tripitramine, a polymethylene tetraamine displaying high selectivity and affinity for muscarinic M_2 receptors **1507**

J.M. Arrang, G. Drutel & J.-C. Schwartz. Characterization of histamine H_3 receptors regulating acetylcholine release in rat entorhinal cortex **1518**

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BRITISH JOURNAL OF PHARMACOLOGY VOLUME 114 (7) 1995

SPECIAL REPORT

P. Klemm, T.D. Warner, D. Willis, A.R. Moore & J.R. Vane. Coronary vasoconstriction *in vitro* in the hearts of polyarthritic rats: effectiveness of *in vivo* treatment with the endothelin receptor antagonist SB 209670 **1327**

PAPERS

A.J. Lawrence, E. Krstew & B. Jarrott. Functional dopamine D_2 receptors on rat vagal afferent neurones **1329**

T.A. Swierkosz, J.A. Mitchell, T.D. Warner, R.M. Botting & J.R. Vane. Co-induction of nitric oxide synthase and cyclo-oxygenase: interactions between nitric oxide and prostanoids **1335**

D.T. Walsh, V.B. Weg, T.J. Williams & S. Nourshargh. Substance P-induced inflammatory responses in guinea-pig skin: the effect of specific NK_1 receptor antagonists and the role of endogenous mediators **1343**

M. Alegret, E. Cerqueda, R. Ferrando, M. Vázquez, R.M. Sánchez, T. Adzet, M. Merlos & J.C. Laguna. Selective modification of rat hepatic microsomal fatty acid chain elongation and desaturation by fibrates: relationship with peroxisome proliferation **1351**

P.M. Beart, A. Schousboe & A. Frandsen. Blockade by polyamine NMDA antagonists related to ifenprodil of NMDA-induced synthesis of cyclic GMP, increases in calcium and cytotoxicity in cultured neurones **1359**

G.R. May, W.S. Rowand, J.G. McCormack & R.D. Sheridan. Neuroprotective profile of lifarizine (RS-87476) in rat cerebrocortical neurones in culture **1365**

H. Bult, G.R.Y. de Meyer & A.G. Herman. Influence of chronic treatment with a nitric oxide donor on fatty streak development and reactivity of the rabbit aorta **1371**

B. Battistini, M. Woods, L.J.D. O'Donnell, T.D. Warner, R. Corder, A. Fournier, M.J.G. Farthing & J.R. Vane. Contractile activity of endothelin precursors in the isolated gallbladder of the guinea-pig: presence of an endothelin-converting enzyme **1383**

J.E. Nally, C.C. Docherty, R.A. Clayton & N.C. Thomson. Bronchodilator and pre-protective effects of urodilatin in bovine bronchi *in vitro*: comparison with atrial natriuretic peptide **1391**

M. Kurosawa, K. Messlinger, M. Pawlak & R.F. Schmidt. Increase of meningeal blood flow after electrical stimulation of rat dura mater encephali: mediation by calcitonin gene-related peptide **1397**

P. Soares-da-Silva, M. Pestana, M.A. Vieira-Coelho, M.H. Fernandes & A. Albino-Teixeira. Assessment of renal dopaminergic system activity in the nitric oxide-deprived hypertensive rat model **1403**

J.U. Chowdhury, Y.-W. Pang, S.-M. Huang, M. Tsugeno & T. Tomita. Sustained contraction produced by caffeine after ryanodine treatment in the circular muscle of the guinea-pig gastric antrum and rabbit portal vein **1414**

F. Orallo, A.F. Alzueta, M. Campos-Toimil & J.M. Calleji. Study of the *in vivo* and *in vitro* cardiovascular effects of (+)-glaucine and N-carbethoxysecoglaucine in rats **1419**

R. Tavakoli & N. Frossard. 5-HT_2-mediated bronchial responses after syngeneic lung transplantation in the rat **1428**

E. Kelly, P.K. Harrison & R.J. Williams. Effects of acute and chronic ethanol on cyclic AMP accumulation in NG108-15 cells: differential dependence of changes on extracellular adenosine **1433**

S.A. Kilfeather, S. Tagoe, A.C. Perez, K. Okona-Mensa, R. Matin & C.P. Page. Inhibition of serum-induced proliferation of bovine tracheal smooth muscle cells in culture by heparin and related glycosaminoglycans **1442**

F. de Ponti, M. Cosentino, A. Costa, M. Girani, G. Gibelli, L. D'Angelo, G. Frigo & A. Crema. Inhibitory effects of SR 58611A on canine colonic motility: evidence for a role of β_3-adenoceptors **1447**

Y.-X. Wang, S.L. Lim & C.C.Y. Pang. Increase by N^G-nitro-L-arginine methyl ester (L-NAME) of resistance to venous return in rats **1454**

E. Talpain, R.A. Armstrong, R.A. Coleman & C.J. Vardey. Characterization of the PGE receptor subtype mediating inhibition of superoxide production in human neutrophils **1459**

L.A. Miller, D.E. Cochrane, R.E. Carraway & R.S. Feldberg. Blockade of mast cell histamine secretion in response to neurotensin by SR 48692, a nonpeptide antagonist of the neurotensin brain receptor **1466**

J. Maas, G.A. Rae, J.P. Huidobro-Toro & J.B. Calixto. Characterization of kinin receptors modulating neurogenic contractions of the mouse isolated vas deferens **1471**

A. Bergendal, A. Lindén, J. Lötvall, B.-E. Skoogh & C.-G. Löfdahl. Different effects of salmeterol, formoterol and salbutamol on cholinergic responses in the ferret trachea **1478**

A.D. Kraneveld, T.L. Buckley, D. van Heuven-Nolsen, Y. van Schaik, A.Sj. Koster & F.P. Nijkamp. Delayed-type hypersensitivity-induced increase in vascular permeability in the mouse small intestine: inhibition by depletion of sensory neuropeptides and NK_1 receptor blockade **1483**

L.E. Cardona-Sanclemente & G.V.R. Born. Effect of inhibition of nitric oxide synthesis on the uptake of LDL and fibrinogen by arterial walls and other organs of the rat **1490**

K.M. Stevenson, K.J. Gibson & E.R. Lumbers. Comparison of the transplacental transfer of enalapril, captopril and losartan in sheep **1495**

H. Vatanpour & A.L. Harvey. Modulation of acetylcholine release at mouse neuromuscular junctions by interaction of three homologous scorpion toxins with K^+ channels **1502**

A. Chiarini, R. Budriesi, M.L. Bolognesi, A. Minarini & C. Melchiorre. *In vitro* characterization of tripitramine, a polymethylene tetraamine displaying high selectivity and affinity for muscarinic M_2 receptors **1507**

J.M. Arrang, G. Drutel & J.-C. Schwartz. Characterization of histamine H_3 receptors regulating acetylcholine release in rat entorhinal cortex **1518**

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