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SPECIAL REPORT

Characterization of two new ET_B selective radioligands, [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 in human heart

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Two new endothelin receptor radioligands, [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1, were characterized in tissue sections of human right atrium and left ventricle. Both radioligands had high affinity ([^{125}I]-BQ3020 right atrium: $K_D = 0.145 \pm 0.037$ nM, left ventricle: $K_D = 0.107 \pm 0.004$ nM; [^{125}I]-[Ala^{1,3,11,15}]ET-1 right atrium: $K_D = 0.239 \pm 0.036$ nM, left ventricle: $K_D = 0.199 \pm 0.027$ nM). Competition binding experiments were performed in the left ventricle. The selective ET_A receptor compound BQ123 competed with low affinity against [^{125}I]-BQ3020 ($K_D = 28.7 \pm 2.7$ μM) and [^{125}I]-[Ala^{1,3,11,15}]ET-1 ($K_D = 28.5 \pm 4.2$ μM). The selective ET_B receptor compound BQ3020 competed with high affinity against [^{125}I]-BQ3020 ($K_D = 40.8 \pm 6.6$ pM) and [^{125}I]-[Ala^{1,3,11,15}]ET-1 ($K_D = 0.276 \pm 0.099$ nM). Another selective ET_B receptor compound, [Ala^{1,3,11,15}]ET-1 also competed with high affinity against [^{125}I]-BQ3020 ($K_D = 0.663 \pm 0.120$ nM) and [^{125}I]-[Ala^{1,3,11,15}]ET-1 ($K_D = 0.643 \pm 0.124$ nM). These results indicate that [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 are selective ET_B receptor radioligands. [Ala^{1,3,11,15}]ET-1 competed with the non-selective radioligand [^{125}I]-ET-1 in left ventricle and revealed the presence of ET_A and ET_B receptors in the proportions of 76:24% respectively in the human left ventricle.

Keywords: Endothelin; BQ3020, [Ala^{1,3,11,15}]ET-1; BQ123; [^{125}I]-BQ3020; [^{125}I]-[Ala^{1,3,11,15}]ET-1; [^{125}I]-ET-1; ET_A receptor; ET_B receptor; human heart

Introduction Endothelin-1 produces a number of important effects in the heart including positive inotropic and chronotropic responses (Davenport *et al.*, 1989; Reid *et al.*, 1989), stimulation of myocyte hypertrophy and renin release (Shubeita *et al.*, 1990). These effects may be produced by one or more endothelin receptors; two subtypes, ET_A and ET_B have been cloned from cDNA libraries (Sakurai *et al.*, 1992). Recently three highly selective ET_A and ET_B receptor compounds have become available, BQ123 cyclo[D-Asp-L-Pro-D-Val-L-Leu-D-Trp-] (ET_A, Ihara *et al.*, 1992; Nakamichi *et al.*, 1992), [Ala^{1,3,11,15}]ET-1 (ET_B, Saeki *et al.*, 1991) and BQ3020 [Ala^{11,15}]Ac-ET-1(6-21) (ET_B). These compounds were used to characterize two new radioligands, [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 in human heart.

Methods Human right atrium and left ventricular free wall were obtained from recipient patients undergoing cardiac transplantation at the Papworth Everard Hospital. Four hearts in total were used, three with ischaemic heart disease and one with the Eisenmengers syndrome. Cardiac tissue was snap frozen in liquid nitrogen and stored at − 70°C until use. Tissues were mounted in O.C.T. compound and sections cut (10 μm) and mounted onto gelatin/chromic potassium sulphate coated microscope slides. Slide-mounted tissue sections (10 μm) were incubated with (3-[^{125}I]iodotyrosyl^{13})-endothelin (ET)-1, (3-[^{125}I]iodotyrosyl^{13})-[Ala^{11,15}]Ac-ET-1(6-21) or (3-[^{125}I]iodotyrosyl^{13})-[Ala^{1,3,11,15}]ET-1 (Amersham International plc, U.K.) in the absence or presence of competing agents for 120 min, except for association experiments where increasing time periods (0–240 min) were used at 22°C as previously described for [^{125}I]-ET-1 (Davenport *et al.*, 1989). Non-specific binding was determined with the corresponding unlabelled ligand (1 μM). Sections were rinsed in Tris-HCl (0.05 M), pH 7.4, 4°C (3 × 5 min) and counted in a gamma counter. Association binding data were analysed with REAP (Gamma Research Systems, Knoxfield, Australia), saturation and competition binding data were analysed with EBDA (McPherson, 1983) and LIGAND (Munson & Rodbard, 1980). Data files were run simultaneously with LIGAND to obtain final parameter estimates. The presence of 1 or 2 sites was tested using the *F*-ratio test in LIGAND. The model adopted was that which provided the significantly best fit ($P < 0.05$).

Drugs BQ3020 and [Ala^{1,3,11,15}]ET-1 were synthesized by solid phase *t*-Boc chemistry, purified by gel filtration and the sequences confirmed by amino acid analysis. Peptide concentration was determined by u.v. spectrophotometry. BQ123 was a gift from Parke-Davis Pharmaceutical Division, Ann Arbor, Michigan, U.S.A.

Results [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 binding to sections of human left ventricle was time-dependent and reached equilibrium after 120 min at 18°C with observed association rate constants of 0.0245 ± 0.0003 min^{-1} and 0.0205 ± 0.0002 min^{-1} respectively.

[^{125}I]-BQ3020 (2 pM–8 nM) and [^{125}I]-[Ala^{1,3,11,15}]ET-1 (6 pM–7 nM) binding to sections of human right atrium and left ventricle was concentration-dependent. Non-specific binding for [^{125}I]-BQ3020 ranged from 24–52% at 2 pM, 19–30% at 0.15 nM and 53–93% at 8 nM and for [^{125}I]-[Ala^{1,3,11,15}]ET-1, 21–62% at 6 pM, 13–22% at 0.3 nM and 70–74% at 7 nM. The Hill coefficients for both radioligands were less than unity (Table 1) suggesting the presence of multiple binding sites. Analysis of binding over the lower concentrations ([^{125}I]-BQ3020 2 pM–0.6 nM; [^{125}I]-[Ala^{1,3,11,15}]-ET-1 6 pM–1.5 nM) resulted in Hill coefficients close to unity. Analysis with LIGAND revealed one binding site for both radioligands (Table 1). A two-binding site model was tested for each radioligand but was not preferred to a one-site model ($P > 0.05$).

[^{125}I]-BQ3020 (0.1–0.2 nM) and [^{125}I]-[Ala^{1,3,11,15}]ET-1 (0.1–0.7 nM) were used in competition binding experiments

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Table 1 Saturation binding analysis for endothelin receptor radioligands in human right atrium and left ventricle

	n	*nH*	K_D	B_{max}
[^{125}I]-BQ3020	RA 3	0.46 ± 0.14	0.145 ± 0.037	25.7 ± 6.8
	LV 3	0.43 ± 0.06	0.107 ± 0.004	18.9 ± 4.8
[^{125}I]-[Ala^{1,3,11,15}]ET-1	RA 3	0.77 ± 0.01	0.239 ± 0.036	22.2 ± 3.4
	LV 3	0.72 ± 0.03	0.199 ± 0.027	12.4 ± 2.2

Hill coefficients (nH), dissociation constants (K_D, nM) and maximal density of receptors (B_{max}, fmol mg^{-1} protein) for [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 in human right atrial (RA) and left ventricular (LV) sections. Values are mean ± s.e.mean from *n* experiments.

against ET_A and ET_B selective compounds in human left ventricle. These concentrations were selected as they provide a clear signal with a high proportion of specific binding to total binding. These concentrations also lie within the range which provided Hill coefficients close to unity in saturation binding experiments. The ET_B-selective compound, BQ3020, competed with [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 with high affinity. Another ET_B-selective compound, [Ala^{1,3,11,15}]ET-1, also competed with [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 with high affinity (Table 2). The ET_A-selective compound, BQ123, competed with low affinity for both radioligands in human left ventricle (Table 2). BQ123 (100 μM) produced 55 ± 2% and 78 ± 2% inhibition of [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 binding.

[Ala^{1,3,11,15}]ET-1 was investigated further in human left ventricle. It competed with the non-selective radioligand, [^{125}I]-ET-1, and revealed the presence of a high affinity binding site corresponding to the ET_B receptor and a lower affinity binding site corresponding to the ET_A receptor in the proportions of 24% (ET_B) and 76% (ET_A, Table 2).

Discussion The new endothelin receptor radioligands, [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1, selectively label ET_B receptors with high affinity. The results of this study suggest that [Ala^{1,3,11,15}]ET-1 is 2500 fold selective for the ET_B receptor. Similar studies (Molenaar *et al.*, unpublished observations) indicate that BQ3020 is approximately 1500 fold selective for the ET_B receptor and BQ123 is 33000 fold selective for the ET_A receptor.

Hill coefficients for [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 in saturation binding experiments were less than unity suggesting the presence of another binding site at higher concentrations. This site is unlikely to be an ET_A receptor in view of the affinities of [Ala^{1,3,11,15}]ET-1 (4.52 μM) and BQ3020 (2.04 μM, Molenaar *et al.*, unpublished observations) for the ET_A receptor. Higher concentrations of [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 than those used in the present study will be needed to characterize fully the low affinity site. The concentrations of [^{125}I]-BQ3020 (0.1–0.2 nM) and [^{125}I]-[Ala^{1,3,11,15}]ET-1 (0.1–0.7 nM) used in competition binding experiments were considered optimal for labelling the ET_B receptor in human cardiac sections. Lower concentrations result in higher levels of non-specific binding and a low 'signal' while higher concentrations also result in an increase in non-specific binding and multiple receptor binding. In other studies using rat cerebellum which has a higher proportion of ET_B receptors (> 85%, Davenport *et al.*, unpublished observations) Hill coefficients for both radioligands are close to unity. The shallow competition binding curves produced by the ET_A-selective antagonist ligand, BQ123, against [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 remain to be explained but may indicate heterogeneity of binding sites.

In the human left ventricular tissue studied both ET_A and ET_B receptors were detected in the proportions of 76%:24% respectively. Although hearts of two pathological conditions were used in the present study no differences were detected in the radioligand binding assays.

Human heart comprises myocytes, specialized pacemaker and conducting regions, blood vessels, neuronal, connective and adipose tissue; however, the precise anatomical localization of endothelin receptor subtypes is unclear at the present time. The availability of highly selective radioligands and compounds will assist in the determination of the function, cellular localization and quantitation of these receptor subtypes.

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Table 2 Competition binding data analysis between endothelin receptor radioligands and selective competing agents

	n	*nH*	K_D			
[^{125}I]-BQ3020						
BQ3020	3	0.97 ± 0.18	40.8 ± 6.6 pM			
[Ala^{1,3,11,15}]ET-1	3	0.91 ± 0.03	0.66 ± 0.120 nM			
BQ123	3	0.53 ± 0.08	28.7 ± 2.7 μM			
[^{125}I][Ala^{1,3,11,15}]ET-1						
BQ3020	3	0.92 ± 0.15	0.276 ± 0.099 nM			
[Ala^{1,3,11,15}] ET-1	3	0.92 ± 0.06	0.643 ± 0.124 nM			
BQ123	3	0.62 ± 0.01	28.5 ± 4.2 μM			
[^{125}I]-ET-1						
	n	*nH*	$K_D ET_B$	$K_D ET_A$	$\%ET_B$	$\%ET_A$
[Ala^{1,3,11,15}]ET-1	4	0.45 ± 0.04	1.82 ± 1.35 nM	4.52 ± 0.49 μM	24 ± 3	76 ± 3

Pseudo Hill coefficient values (nH) and dissociation constant values (K_D) for BQ3020 and BQ123 and [Ala^{1,3,11,15}]ET-1 at endothelin-1 receptors in human left ventricle. Shown also is the percentage of ET_A and ET_B receptors determined with [Ala^{1,3,11,15}]ET-1. Values are mean ± s.e.mean from *n* experiments.

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SPECIAL REPORT

Modulation of the induction of nitric oxide synthase by eicosanoids in the murine macrophage cell line J774

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The effect of eicosanoids on the induction of nitric oxide synthase in the murine macrophage cell line J774 has been studied. We found that prostaglandin E_2 (PGE_2) and iloprost (a stable analogue of prostacyclin) both at nanomolar concentrations inhibited the lipopolysaccharide stimulated induction of NO synthase. In contrast $PGF_{2\alpha}$, U46619, a stable analogue of thromboxane A_2, leukotrienes B_4 and C_4 had no effect. These data demonstrate that the L-arginine: NO pathway in macrophages may be modulated by prostanoids.

Keywords: J774 macrophages; eicosanoids; nitric oxide synthase; lipopolysaccharide

Introduction Macrophages activated with bacterial lipopolysaccharide (LPS) express inducible nitric oxide (NO) synthase and produce large amounts of NO which has been reported to be an effector molecule of the cytostatic/cytotoxic properties of these cells (Hibbs *et al.*, 1988).

Macrophages are also an important source of eicosanoids which may modulate their activation state. Thus the cytostatic/cytotoxic effect of activated macrophages appears to be inhibited by some cyclo-oxygenase products like prostaglandin E_2 (PGE_2) and PGI_2 and increased by lipoxygenase metabolites like leukotriene B_4 (LTB_4) and LTC_4 (Bonta & Ben-Efraim, 1987).

In the light of these findings we decided to investigate the effect of eicosanoids on the induction of NO synthase in J774 macrophages.

Methods The murine monocyte/macrophage cell line J774 (American Tissue Culture catalogue T1B 67 page 231) was grown in Dulbecco's modified Eagle's medium (Gibco) at 37°C as previously described (Di Rosa *et al.*, 1990). These cells were plated in 24 well culture plates (Falcon) at a concentration of 2.5×10^5 cells ml^{-1} and allowed to adhere at 37°C in 5% CO_2/95% air for 2 h. Thereafter the medium was replaced with fresh medium and cells were activated by LPS (0.1 $\mu g\,ml^{-1}$) from *Salmonella thyphosa* (Difco). Then cells were incubated in the presence or absence of various concentrations (see Results) of test compounds: PGE_2, $PGF_{2\alpha}$, LTB_4, LTC_4 (Sigma), U46619 (Cayman Chemicals), or iloprost (Schering). After 24 h nitrite (NO_2^-) and eicosanoid levels were assessed in the culture media.

Nitrites were measured with the Griess reagent as previously described (Di Rosa *et al.*, 1990) and PGE_2, 6-keto-$PGF_{1\alpha}$, LTB_4 and LTC_4 by enzyme-immunoassay (Cayman Chemicals). Results are expressed as nmol (NO_2^-) and pmol (eicosanoids) released by 10^6 cells in 24 h.

Data are expressed as mean ± s.e.mean. Comparisons were made by the unpaired two-tailed Student's *t* test. The level of statistically significant difference was defined as $P<0.01$.

Results The production of NO_2^- by unstimulated J774 macrophages was undetectable (<1 nmol per 10^6 cells in 24 h). Incubation of the cells with LPS (0.1 $\mu g\,ml^{-1}$) caused a substantial release of NO_2^- (60.5 ± 1.9 nmol). The basal levels of PGE_2 and 6-keto-$PGF_{1\alpha}$ were 0.44 ± 0.03 pmol and 0.6 ± 0.04 pmol respectively. When the cells were incubated with LPS the levels rose to 8 ± 0.4 pmol for PGE_2 and 11.6 ± 0.5 pmol for 6-keto-$PGF_{1\alpha}$. The level of LTB_4 and LTC_4 in the culture media of unstimulated cells was 0.28 ± 0.03 pmol for LTB_4 and 0.16 ± 0.05 pmol for LTC_4. Both these levels remained unchanged following stimulation of the cells with LPS.

When J774 macrophages were stimulated with the same amount of LPS in the presence of 0.0001–1 μM PGE_2 or of iloprost, a stable PGI_2 analogue, a concentration-dependent inhibition of NO_2^- generation was observed (Figure 1). Iloprost was approximately 10 times more potent that PGE_2. Neither compound at any of the concentrations tested affected NO_2^- generation when added to the cells 6 h after LPS challenge (Figure 1). In contrast, $PGF_{2\alpha}$, U46619 (a stable analogue of thromboxane A_2, TXA_2), LTB_4 and LTC_4, all tested at concentrations ranging from 0.01 to 1 μM, did not significantly affect NO_2^- release when added concomitantly with LPS or 6 h later (data not shown).

Discussion Our results show that J774 macrophages exposed to LPS produce large amounts of both NO_2^- and arachidonic acid metabolites generated by the cyclo-oxygenase pathway, whereas the levels of lipoxygenase pathway metabolites are not affected. These results are in agreement with previously reported data (Humes *et al.*, 1982; Di Rosa *et al.*, 1990).

Here we present evidence that PGE_2 and PGI_2 (iloprost), both at nanomolar concentrations, inhibit LPS-induced

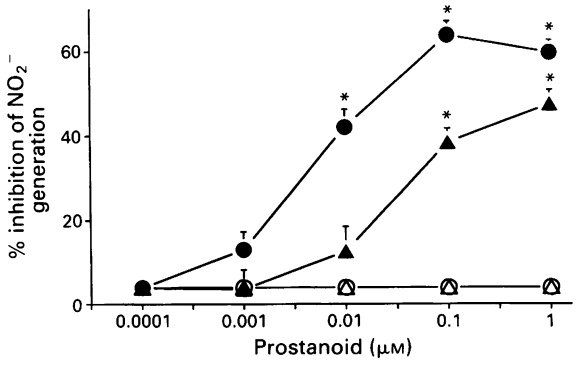

Figure 1 Effect of prostaglandin E_2 (PGE_2) and iloprost on NO_2^- production by lipopolysaccharide (LPS)-activated J774 cells. Solid symbols refer to experiments in which PGE_2 (▲) or iloprost (●) were added to the culture medium concomitantly with LPS. Open symbols refer to experiments in which PGE_2 (△) or iloprost (○) were added to the culture medium 6 h after LPS. The vertical bars show standard errors ($n = 6$).
*$P<0.01$.

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NO_2^- production by J774 cells. Interestingly the concentrations of these eicosanoids which inhibit NO_2^- production are in the same range of those we found in the culture medium of LPS-stimulated J774 cells. Both PGE_2 and iloprost act in a concentration-dependent fashion as inhibitors of the NO synthase induction process since they do not directly inhibit the activity of the enzyme once it has been expressed. Other prostanoids like $PGF_{2\alpha}$ and U46619 did not modify either the induction of NO synthase or the activity of the enzyme.

Nitric oxide has been identified as an effector molecule of the cytotoxic properties of activated macrophages (Hibbs *et al.*, 1988). Our finding of the inhibitory action of PGE_2 and PGI_2 on the induction of NO synthase by LPS is consistent with previously reported observations showing that nanomolar concentrations of PGE_2 inhibited the LPS-induced tumouricidal activity of mouse resident peritoneal macrophages (Taffet *et al.*, 1981).

We could not detect any significant action of exogenously added LTB_4 and LTC_4 on NO_2^- generation by LPS-stimulated J774 macrophages. Our results are in agreement with other studies suggesting that products of the lipoxygenase pathway do not modulate macrophage tumouricidal activity (Schultz *et al.*, 1985). However since other studies have shown that LTB_4 and LTC_4 both increase macrophage tumouricidal activity (Bonta & Ben-Efraim, 1987) our results may suggest that these eicosanoids may not modulate the cytotoxic effect of activated J774 cells.

Our experiments do not explain the mechanism by which PGE_2 and PGI_2 inhibit the induction of NO synthase in J774 cells. Since it is well known that both PGE_2 and PGI_2 stimulate adenylate cyclase in activated macrophages, resulting in an increase in adenosine 3';5'-cyclic monophosphate (cyclic AMP) levels, the mechanism of action of these prostanoids in the regulation of NO synthase induction could be related to this cyclic nucleotide. This hypothesis is supported by the fact that both $PGF_{2\alpha}$ and U46619, which do not enhance cyclic AMP levels, have no action on NO generation. Furthermore, it is interesting to note that nanomolar concentrations of PGE_2, which we have shown to inhibit LPS-induced NO_2^- generation, also inhibit tumour necrosis factor α (TNFα) release from LPS-activated murine macrophages (Renz *et al.*, 1988) and rat Kupffer cells (Peters *et al.*, 1990). Since it has been shown that TNFα induces NO synthase in murine macrophages (Drapier *et al.*, 1988) the inhibition by prostaglandins of the release of TNFα and probably other cytokines could also be considered as a possible explanation of our findings. In this respect the relationship between eicosanoids and the L-arginine: NO pathway, as well as the relevance of this interaction in the host defence mechanism, deserves further investigation.

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Central neuropeptide Y and the sigma ligand, JO 1784, reverse corticotropin-releasing factor-induced inhibition of gastric acid secretion in rats

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1 The central interactions between the sigma ligand, JO 1784, [(+)-N-cylcopropylmethyl-N-methyl-1,4-diphenyl-1-ethylbut-3-en-1-ylamine hydrochloride], or neuropeptide Y (NPY) and corticotropin-releasing factor (CRF)-induced inhibition of gastric acid secretion were investigated in rats anaesthetized with urethane. Drugs were injected intracisternally (i.c.) or into specific hypothalamic nuclei. Gastric acid secretion was measured by the flushed technique under basal and pentagastrin (10 $\mu g\,kg^{-1}\,h^{-1}$, i.v.) stimulated conditions.

2 Intracisternal injection of CRF (10 μg), bombesin (0.1 μg) and human recombinant interleukin-1β (hIL-1β, 0.1 μg) inhibited gastric acid response to pentagastrin by 72%, 56% and 62%, respectively. NPY (0.5 μg) or JO 1784 (0.5 μg) injected i.c. did not alter acid secretion but completely prevented the inhibitory effect of CRF. The antagonistic effect of NPY and JO 1784 against CRF was dose-related (0.01–0.5 μg) and peptide-specific since NPY and JO 1784 did not alter the antisecretory action of bombesin or hIL-1β.

3 The putative sigma receptor antagonist, BMY 14802, (1 $mg\,kg^{-1}$, s.c.) did not influence pentagastrin-stimulated acid secretion nor CRF-induced inhibition of gastric acid secretion; however, BMY 14802 administered s.c. 20 min before JO 1784 or NPY, abolished the antagonistic effect of both JO 1784 and NPY.

4 CRF (3 μg) microinjected into the hypothalamic paraventricular nucleus (PVN) and the lateral hypothalamus (LH) inhibited pentagastrin-stimulated gastric acid secretion by 61% and 51%; NPY (0.03 μg) or JO 1784 (0.03 μg) microinjected into the PVN had no effect by themselves but blocked CRF antisecretory action. Microinjection into the LH had no effect.

5 In conclusion, NPY and JO 1784 (a sigma ligand) interacts with CRF in the PVN to block CRF-induced inhibition of pentagastrin-stimulated gastric acid secretion. The central action of JO 1784 and NPY is specific to CRF and may involve sigma binding sites.

Keywords: Neuropeptide Y; sigma ligand; bombesin; interleukin-1β; corticotropin-releasing factor; paraventricular nucleus; lateral hypothalamus; gastric acid secretion in rats; central nervous system

Introduction

Corticotropin-releasing factor (CRF) injected into the cerebrospinal fluid or selective brain nuclei inhibits gastric acid secretion and motor function (Taché *et al.*, 1983; 1987; Lenz *et al.*, 1988a,b; Stephens *et al.*, 1988) and increases gastric and duodenal bicarbonate secretion (Lenz, 1989; Gunion *et al.*, 1990), and colonic motor function in rats (Williams *et al.*, 1987; Jiménez & Bueno, 1990; Gué *et al.*, 1991; Mönnikes *et al.*, 1992). The alterations of gastrointestinal function induced by centrally administered CRF have physiological relevance since central injection of the CRF antagonist, α-helical CRF9-41, prevents similar changes in gastrointestinal secretory and motor function induced by exposure to various stressors (Williams *et al.*, 1987; Lenz *et al.*, 1988b; Stephens *et al.*, 1988; Taché *et al.*, 1991; Gué *et al.*, 1991; Mönnikes *et al.*, 1992).

Recently, it has been reported that both neuropeptide Y (NPY) and the sigma ligand, JO 1784, [(+)-N-cyclopropyl-methyl-N-methyl-1,4 diphenyl-1-ethyl-but-3-en-1-ylamine, hydrochloride] administered into the lateral ventricle block CRF- and stress-induced increases in colonic motility (Jiménez & Bueno, 1990; Junien *et al.*, 1991). Furthermore, the antagonistic action of NPY and JO 1784 on stress- and CRF-related colonic motor disturbances involved a G_i protein (Junien *et al.*, 1991) and was reversed by the putative sigma receptor antagonist, BMY 14802 (Gué *et al.*, 1992). JO 1784 is a potent and specific ligand for sigma sites labelled with (+)-N-allylnormetazocine (NANM) with no affinity for phencyclidine (PCP) sites in the rat brain (Roman *et al.*, 1989; 1990). BMY 14802 has been described as a potential antipsychotic agent with selective and stereospecific inhibition of binding at sigma sites and with no affinity for PCP or D_2 or D_1 binding sites (Walker *et al.*, 1990; Taylor *et al.*, 1991).

The present study was undertaken to investigate the interaction between NPY, the sigma ligand, JO 1784, and the central action of CRF in inhibiting acid secretion. In particular, we have demonstrated that NPY and JO 1784 injected intracisternally block intracisternal CRF-induced inhibition of gastric acid secretion in rats. In addition, the specificity of NPY and JO 1784 action and the influence of BMY 14802 were examined. Lastly, hypothalamic nuclei previously reported to be sites of action for CRF-induced inhibition of gastric acid secretion (Taché *et al.*, 1983; Gunion & Taché, 1987a; Gunion *et al.*, 1990) were explored for possible sites of NPY, JO 1784 and CRF interactions.

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Methods

Animals

Male Sprague-Dawley albino rats (Simonsen Laboratories, Gilroy, CA, U.S.A.) weighing 250–350 g were housed in group cages under conditions of controlled temperature (22 ± 1°C) and illumination (06 h 00 min to 18 h 00 min). Animals were maintained on Purina Laboratory Chow *ad libitum* (diet No. 5001; Ralston-Purina, St Louis, MO, U.S.A.) and tap water. Animals were deprived of food for 18 h but given free access to water before the start of the study. All experiments were performed in rats under urethane anaesthesia (1.25 g kg^{-1}, i.p.).

Measurement of acid secretion

For measurement of gastric acid secretion a cannula was inserted into the trachea of urethane-anaesthetized rats and a laparotomy was performed. The pylorus was ligated and a double-lumen cannula was acutely inserted through a small incision into the forestomach. Care was taken not to occlude or damage major vessels. The abdominal musculature and the skin were closed with silk sutures. Thirty minutes following surgery, gastric acid output was monitored for a 40 min basal period before central injection of drugs, then throughout the 140 min experimental period post-treatment. Acid output was determined by flushing the gastric lumen every 10 min with two 5 ml boluses of saline and one bolus of air at the end of each 10 min period. The flushed perfusate was titrated with 0.1 N NaOH to pH 7.0 on an automatic titrator (Radiometer, Copenhagen).

Experimental protocol

After basal measurement of gastric acid secretion in rats under urethane anaesthesia, two intracisternal injections were given at 10 min intervals of either saline and saline; saline and CRF (10 μg); NPY (0.01, 0.1 or 0.5 μg) and saline; or NPY (0.01, 0.1 or 0.5 μg) and CRF (10 μg); JO 1784 (0.01, 0.1 or 0.5 μg) and saline; JO 1784 (0.01, 0.1 or 0.5 μg) and CRF (10 μg). The doses of NPY and JO 1784 were selected according to previous reports showing an inhibitory effect on CRF-induced increase in colonic motility (Jiménez & Buéno, 1990; Junien *et al.*, 1991). To determine whether NPY and JO 1784 action is CRF-specific, intracisternal injection of saline, NPY (0.5 μg) or JO 1784 (0.5 μg) were followed 10 min later by the intracisternal injection of bombesin (0.1 μg) or interleukin-1β (0.1 μg). Doses for intracisternal injection of CRF, bombesin and interleukin-1β were selected to induce 50–70% inhibition of acid secretion as shown in previous dose-response studies (Taché *et al.*, 1983; Gunion & Taché, 1987b; Saperas *et al.*, 1990).

In the second experiment, the putative sigma receptor antagonist, BMY 14802 (1 mg kg^{-1}) was given subcutaneously 20 min before the first intracisternal injection of either saline, JO 1784 (0.5 μg) or NPY (0.5 μg). Ten minutes later CRF (10 μg) was injected intracisternally. A similar regimen of BMY 14802 administration was shown to exert central actions (Taylor *et al.*, 1991; Gué *et al.*, 1992).

In all these studies, 10 min after the second intracisternal injection, pentagastrin (10 μg kg^{-1} h^{-1}) was infused through the femoral vein for 2 h.

In the last study, 7 days before the experiment, rats were anaesthetized using a mixture of ketamine (75 mg kg^{-1}, i.p.; Fort Dodge, IA, U.S.A.) and xylazine (5 mg kg^{-1}, i.p.; Mobay, Shawnee, KS, U.S.A.) and a 26 G stainless steel guide cannula (Plastic Products, Roanoke, VA, U.S.A.), was positioned unilaterally (left side) 3 mm above either the paraventricular nucleus of the hypothalamus (PVN) or the lateral hypothalamus (LH) according to the coordinates of Paxinos & Watson (1986). On the day of the study, rats under urethane-anaesthesia were microinjected into the PVN or LH with saline, NPY (0.03 μg) or JO 1784 (0.03 μg), 10 min before microinjection of CRF (3 μg) into the same respective nucleus. Microinjection of saline, CRF or JO 1784 into hypothalamic brain sites was performed by use of a 1 μl Hamilton microsyringe connected by polyethylene tubing (PE-20) to a 33 G cannula (Plastic Products, Roanoke, VA, U.S.A.) which was lowered 3 mm below the end of the guide cannula. Substances were delivered in 50 nl volume over 1 min and the injection cannula was left in place for another 2 min.

Ten minutes after the last microinjection, pentagastrin was infused. At the end of the experiment, the hypothalamic sites were marked with 50 nl of 0.05% Alcian blue 8GX microinjected under the same conditions. Each rat was then perfused intracardially with 200 ml of saline followed by 200 ml of a 9% formalin solution. Brains were removed, stored overnight in 20% sucrose solution and sectioned serially at a thickness of 32 μm with a freezing microtome. The sections were stained with toluidine blue and examined microscopically. The injection sites were identified and mapped on drawings taken from Paxinos and Watson's stereotaxic atlas (Paxinos & Watson, 1986).

Drugs and treatments

The following drugs were used: human/rat NPY (Penninsula Laboratories, Inc. Belmont, CA, U.S.A.), JO 1784 [(+)-N-cyclopropylmethyl-N-methyl-1, 4-diphenyl-1-ethyl-but-3-en-1-ylamine, hydrochloride, (Department of Therapeutic Chemistry, Jouveinal Research Institute, Fresnes, France)], bombesin and rat CRF (Salk Institute, La Jolla, CA, U.S.A.; courtesy of Dr Jean Rivier). Before the experiment, drugs in powder form were freshly dissolved in 0.9% saline. Recombinant human interleukin-1β (hIL-1β; Glaxo Institute for Molecular Biology, Geneva, Switzerland) was diluted in 100 mM Tris-HCl, 2 mM sodium azide pH 7.8 at an initial concentration of 1 μg 10 μl^{-1} and stored at −70°C; the stock solution was diluted in saline before use. Intracisternal injections of drugs or saline were given in 5 μl by direct puncture of the occipital membrane with a 50 μl Hamilton syringe in rats maintained in the stereotaxic apparatus. BMY-14802, [(α-(4-fluorophenyl)-4-(5-fluoro-2-pyrimidinyl)-1-piperazine-butanol hydrochloride; Bristol-Meyers Squibb Co., Wallingford, CT, U.S.A.] was dissolved in 0.9% saline and injected subcutaneously in 0.2 ml. Pentagastrin (Peptavlon, Ayerst Lab. New York, NY, U.S.A.) was diluted in saline and infused through the femoral vein at a rate of 1.1 ml 2 h^{-1}.

Statistics

Gastric acid output expressed in μmol 90 min^{-1} represents the integrated acid response during the plateau from 30 to 120 min following pentagastrin infusion. Data were analyzed by Dunnett's procedure for multiple comparisons after a two-way or a multiple-way ANOVA. $P<0.05$ was considered statistically significant.

Results

Effect of intracisternal injection of JO 1784 and NPY on CRF, bombesin and hIL-1β-induced inhibition of pentagastrin-stimulated acid secretion

In urethane-anaesthetized rats, basal gastric acid secretion was low (4.2 ± 0.3 μmol 10 min^{-1}); intravenous infusion of pentagastrin (10 μg kg^{-1} h^{-1}) in saline-injected rats stimulated gastric acid secretion. A plateau was reached 30 min after the beginning of the infusion and was maintained at 20 ± 2 μmol 10 min^{-1} for the 90 min experimental period (Figure 1). Intracisternal injection of CRF (10 μg) significantly reduced by 72%, pentagastrin-stimulated gastric

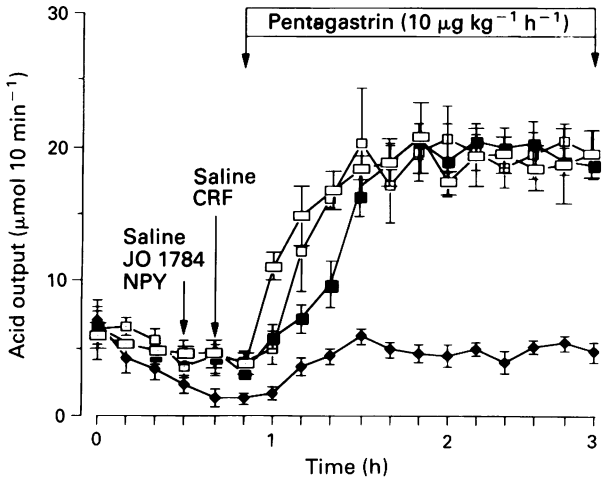

Figure 1 Time course of the action of neuropeptide Y (NPY) and JO 1784 on intracisternal corticotropin releasing factor (CRF)-induced inhibition of pentagastrin-stimulated acid secretion in urethane-anaesthetized rats. Rats were given two intracisternal injections at 10 min apart of saline + saline (□, $n = 8$), saline + CRF, 10 μg, (◆, $n = 6$), JO 1784, 0.5 μg + CRF, 10 μg, (■, $n = 6$) and NPY, 0.5 μg, + CRF, 10 μg, (▭, $n = 6$). Ten minutes later, pentagastrin was infused throughout the experiment. Each point shows mean with vertical bars indicating the s.e.mean.

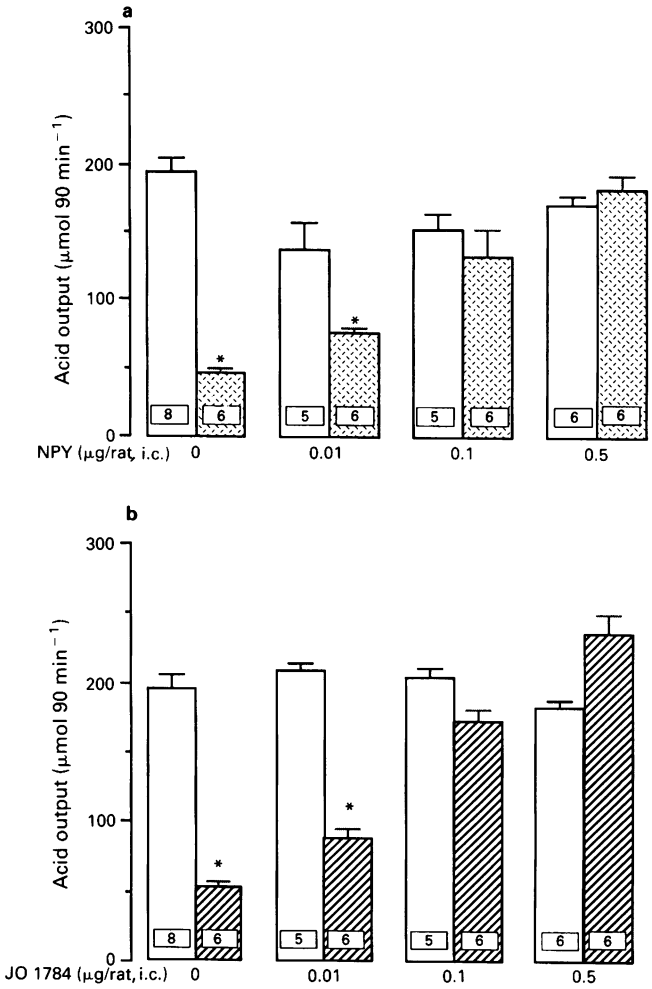

Figure 2 Dose-related antagonism of intracisternal corticotropin releasing factor (CRF)-induced inhibition of gastric acid secretion by neuropeptide Y (NPY) and JO 1784 in urethane-anaesthetized rats. Intracisternal injections of saline, or various doses of NPY (a) or JO 1784 (b) were followed 10 min later by intracisternal injection of saline (open columns) or CRF, 10 μg, (hatched and stippled columns). All animals were infused for 2 h with pentagastrin (10 μg kg⁻¹ h⁻¹) 10 min after the second intracisternal injection. Each column represents the mean with vertical bars indicating the s.e.mean of gastric acid output during the 30 to 120 min collection period after the beginning of pentagastrin perfusion. Total outputs were analyzed by a two-way analysis of variance followed by Dunnett's test.
$P < 0.05$ compared to respective saline-treated groups.

acid output in rats (Figure 1). In CRF-treated rats, the mean gastric acid output (μmol 90 min⁻¹, calculated 30 min after beginning of pentagastrin infusion) was 51 ± 4 vs 180 ± 16 ($P < 0.01$) in saline-treated group (Figure 2).

Intracisternal injection of NPY (0.01, 0.1 and 0.5 μg) and JO 1784 (0.01, 0.1 and 0.5 μg) did not modify pentagastrin-stimulated gastric acid secretion (Figure 2). NPY (0.01, 0.1 and 0.5 μg) and JO 1784 (0.01, 0.1 and 0.5 μg) injected intracisternally 10 min before CRF (10 μg), dose-dependently prevented CRF-induced inhibition of gastric acid secretion (Figure 2). Intracisternal injection of bombesin (0.1 μg) or hIL-1β (0.1 μg) reduced by 56% and 62% respectively gastric acid output stimulated by pentagastrin (Table 1). Intracisternal injection of 0.5 μg of NPY or JO 1784 did not influence the antisecretory action of bombesin and IL-1β (Table 1).

BMY 14802 (1 mg kg⁻¹, s.c.) did not influence pentagastrin-stimulated gastric acid secretion, or the antisecretory action of intracisternal CRF (10 μg) (Figure 3). However, in the BMY 14802-treated group, intracisternal JO 1784 and NPY (0.5 μg) no longer abolished CRF-induced inhibition of pentagastrin-stimulated acid secretion (Figure 3). After injection of BMY 14802, gastric acid output (μmol 90 min⁻¹) in JO 1784- and NPY-treated groups were 97 ± 9 and 102 ± 10 respectively; this was not different from those of CRF-treated rats (99 ± 4) (Figure 3).

Effects of hypothalamic microinjection of NPY and JO 1784 on CRF-induced inhibition of gastric acid secretion

When microinfused unilaterally into the PVN or the LH, CRF (3 μg) inhibited by 61% and 51%, respectively, the gastric acid secretion stimulated by pentagastrin (Figure 4). Microinjection of NPY (0.03 μg) or JO 1784 (0.03 μg) into the PVN, 10 min before microinfusion of CRF (3 μg) into the same nucleus, completely ($P < 0.05$) prevented the antisecretory effect of CRF injected into the PVN (Figure 4). The gastric acid output (μmol 90 min⁻¹) was 189 ± 13 and 184 ± 18 respectively vs 76 ± 8 in CRF-treated group. When infused at the same dose (0.03 μg) into the LH, neither NPY nor JO 1784 influenced the antisecretory action of CRF-microinjected into the LH (Figure 4).

CRF microinjected outside of the PVN or lateral hypothalamus, namely the zona incerta ($n = 2$) and tuber cinereum ($n = 3$) did not significantly ($P > 0.05$) modify the gastric acid secretion stimulated by pentagastrin.

Discussion

Intracisternal injection of CRF (10 μg), bombesin (0.1 μg) and hIL-1β (0.1 μg) 10 min before pentagastrin injection inhibited by 72%, 56% and 62%, respectively, the plateau of secretory response to pentagastrin in urethane-anaesthetized rats. These data confirm previous reports showing that CSF injections of CRF, bombesin and hIL-1β inhibit pentagastrin-stimulated gastric acid secretion in conscious or anaesthetized rats (Taché *et al.*, 1983; Gunion & Taché, 1987b; Lenz *et al.*, 1989). The present data show that the inhibitory effect of CRF was completely prevented by NPY injected intracisternally. The antagonistic effect of NPY injected intracisternally was dose-related (0.01–0.5 μg) and occurred at doses that did

Table 1 Effects of intracisternal injection of neuropeptide Y (NPY) and JO 1784 on bombesin- and interleukin-1β-(hIL-1β) induced inhibition of gastric acid secretion in urethane-anaesthetized rats

Treatment	n	*Gastric acid output* (μmol 90 min^{-1})
Saline + saline	10	202 ± 6
Saline + bombesin	10	88 ± 7*
NPY + bombesin	5	104 ± 14*
JO 1784 + bombesin	6	97 ± 10*
Saline + hIL-1β	10	77 ± 3*
NPY + hIL-1β	6	84 ± 13*
JO 1784 + hIL-1β	5	85 ± 5*

Fasted rats under urethane anaesthesia were injected intracisternally with saline, NPY (0.5 μg) or JO 1784 (0.5 μg) and 10 min later with bombesin (0.1 μg) or hIL-1β (0.1 μg). Ten minutes later pentagastrin (10 μg kg^{-1} h^{-1}) was infused. Results are shown as gastric acid output mean ± s.e.mean of 90 min period starting 30 min after pentagastrin infusion.
*$P<0.05$, test compared to 'saline + saline'-treated group.

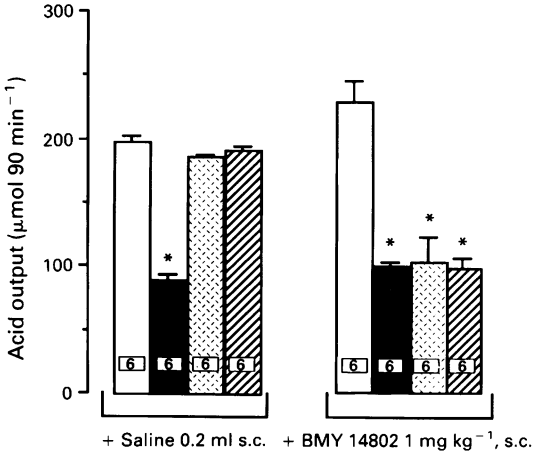

Figure 3 Influence of the sigma antagonist, BMY 14802, on JO 1784 and neuropeptide Y (NPY)-induced blockade of corticotropin releasing factor (CRF) action on gastric acid secretion in urethane-anaesthetized rats. Rats were injected intracisternally twice at an interval of 10 min wtih saline + saline (open columns), saline + CRF, 10 μg, (solid columns), NPY, 0.5 μg + CRF, 10 μg, (stippled columns) or JO 1783, 0.5 μg, + CRF, 10 μg, (hatched columns). BMY 14802 was injected subcutaneously 20 min before intracisternal injection of JO 1784 and NPY. Ten minutes after the second intracisternal injection, animals were infused for 2 h with pentagastrin (10 μg kg^{-1} h^{-1}). Each column represents the mean with vertical bars indicating the s.e.mean of gastric acid output during the 30 to 120 min collection period after the beginning of pentagastrin perfusion.
*$P<0.05$ compared with 'saline + saline'-treated group (ANOVA followed by Dunnett's test).

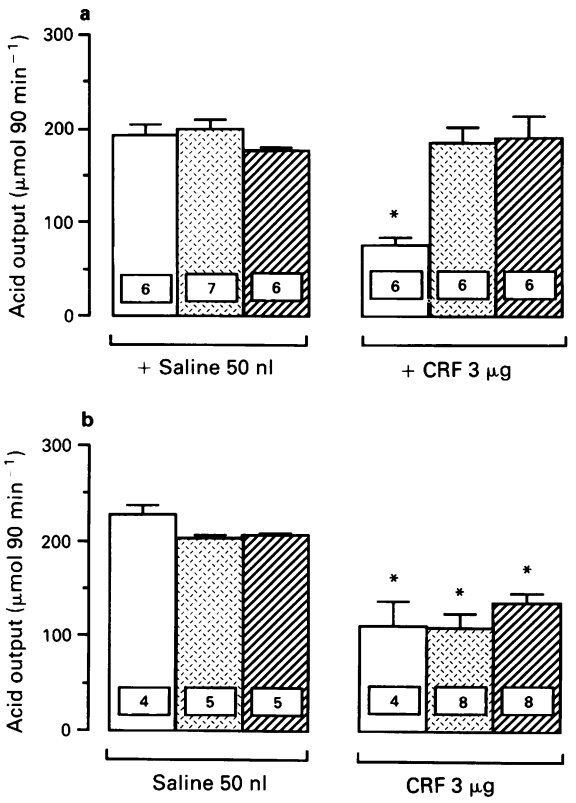

Figure 4 Effect of neuropeptide Y (NPY) and JO 1784 microinfused into the paraventricular nucleus (a) of the hypothalamus and lateral hypothalamus (b) on corticotropin releasing factor (CRF)-induced inhibition of gastric acid secretion in anaesthetized rats. Paraventricular nucleus (PVN) and lateral hypothalamus (LH) microinjections of saline (open columns), NPY, 0.03 μg, (stippled columns) or JO 1784, 0.03 μg, (hatched columns) were followed 10 min later by PVN microinjection of saline or CRF, 3 μg. All animals were infused for 2 h with pentagastrin (10 μg kg^{-1} h^{-1}) 10 min after the intrahypothalamic injections. Each column represents the mean with vertical bars indicating the s.e.mean of gastric acid output during the 30 to 120 min collection period after the beginning of pentagastrin perfusion.
$P<0.05$ compared with respective saline-microinjected group

not influence basal and pentagastrin-stimulated gastric acid secretion. In line with the latter findings, injection of NPY (0.2–0.8 μg) into the 3rd ventricle was previously reported to have no effect on basal gastric acid secretion in rats (Humphreys *et al.*, 1988). At higher doses (2.5 and 35 μg), however, intracisternal or intracerebroventricular injection of NPY increased basal or pentagastrin-stimulated gastric acid secretion in urethane-anaesthetized rats (Matsuda *et al.*, 1991; Geoghegan *et al.*, 1992). The interaction between NPY and CRF is peptide specific since intracisternal injection of NPY at a dose preventing CRF antisecretory action did not alter bombesin- or hIL-1β-induced inhibition of pentagastrin-stimulated acid secretion. The lack of an antagonistic effect of NPY cannot be related to differences in the magnitude of the antisecretory action of the peptides since bombesin and hIL-1β were given intracisternally at doses inducing lower or similar inhibition of pentagastrin-stimulated acid secretion (56% and 62%) as CRF (72%).

NPY injected into the rat lateral brain ventricle at a similar dose-range was previously shown to prevent intracerebroventricular injection of CRF- and psychological stress-induced stimulation of colonic motor activity (Jiménez & Buéno, 1990; Junien *et al.*, 1991). Increased colonic transit in response to stress exposure involves endogenous release of CRF in the brain (Williams *et al.*, 1987; Lenz *et al.*, 1988a; Gué *et al.*, 1991; Mönnikes *et al.*, 1992). Taken together these data indicate that NPY can antagonize the alterations of gastrointestinal secretory and motor function induced by exogenous or endogenous CRF. Another central interaction between NPY and CRF has also been shown in relation to ingestive behaviour (Morley *et al.*, 1987) suggesting a broader modulatory action of NPY on centrally mediated biological activities of CRF.

The central CRF-sensitive neuronal elements involved in the inhibition of gastric acid secretion have been previously localized in the PVN and lateral hypothalamus (Taché *et al.*, 1983; Gunion & Taché, 1987a). These specific hypothalamic areas were chosen to investigate further the anatomical sites

of the functional interaction between NPY and CRF. Unilateral microinjection of CRF (3 μg) into the PVN or lateral hypothalamus inhibited pentagastrin-stimulated acid secretion by 61% and 51% respectively as previously described (Taché et al., 1983; Gunion & Taché, 1987a). NPY (0.03 μg) microinjected into these hypothalamic nuclei under the same conditions did not influence the acid response to pentagastrin. At higher doses (0.2–0.8 μg), NPY inhibits basal gastric acid secretion when microinjected into the PVN but not into the lateral hypothalamus (Humphreys et al., 1988). When microinjected into the PVN at doses that did not influence basal or pentagastrin-stimulated acid secretion, NPY completely prevented the antisecretory action of CRF microinjected into the PVN. The antagonistic action of NPY against CRF appears to be site-specific since when microinjected into the lateral hypothalamus before CRF, NPY did not alter the antisecretory effect of CRF. NPY-induced behavioural, endocrine, cardiovascular and respiratory responses have been assigned to a primary action into the PVN whereas the lateral hypothalamus was less or not responsive (Morley et al., 1987; Harland et al., 1988; Leibowitz, 1991). Taken together, these results indicate that the PVN is an important site for central biological actions of NPY.

Neuroanatomical and electrophysiological findings support a possible physiological relevance of the observed interactions between NPY and CRF in the PVN. Anatomical studies indicate that the PVN, including the parvicellular part which regulates autonomic function (Sawchenko & Swanson, 1982), contains a dense innervation with NPY immunoreactive fibres and terminals (Sawchenko & Pfeiffer, 1988; Lenz, 1989) and high density of receptors (Martel et al., 1986; Dumont et al., 1992) compared with the lateral hypothalamus. Moreover, NPY terminals are morphologically associated with CRF-containing cell bodies in the PVN (Liposits, 1990). Electrophysiological studies indicate that CRF directly excites neurones in the PVN whereas NPY inhibits excitatory synaptic transmission in several neuronal preparations and tissues tested (Colmers et al., 1985; Yamashita et al., 1991). In addition, NPY action is observed at pmol doses which is in the same range as the NPY content in the PVN (Allen et al., 1983), and endogenous CRF in the PVN has recently been reported to be involved in stress-related alterations of gut function (Mönnikes et al., 1992).

The underlying cellular mechanisms through which central NPY exerts its antagonist effect on centrally mediated biological actions of CRF remains to be investigated. Present data obtained with the recently characterized sigma ligand, JO 1784 (Roman et al., 1989; 1990) and the putative sigma antagonist, BMY 14802 (Walker et al., 1990; Taylor et al., 1991) indicate a possible interaction with sigma binding sites in the brain. JO 1784 injected into the cisterna magna or PVN under similar conditions as NPY, mimicked the antagonistic effects of NPY on CRF-induced inhibition of acid response to pentagastrin. Moreover, the antagonistic effects of intracisternal injection of both JO 1784 and NPY on intracisternal CRF-induced antisecretory effect was completely blocked by the administration of BMY 14802. The responsive site of action of the sigma ligand in the PVN is well correlated with the high density of sigma binding sites in this hypothalamic nucleus (Grundlach et al., 1986). In addition, peripheral injection of BMY 14802 has been associated with a marked increased in neuronal activity in the PVN and motor nuclei in the brainstem (Puppa & London, 1989). The nature of the functional interation between NPY and sigma binding sites needs to be clarified further. Previous reports that NPY can act directly at the sigma binding sites (Roman et al., 1989; 1990) have been challenged by other groups who failed to demonstrate that NPY binds to brain sigma receptors (Tam & Mitchell, 1991; Quirion et al., 1991).

In summary, these data indicate that the central action of CRF that inhibits gastric secretory function can be blocked by NPY. The NPY antagonistic action is specific to CRF and is exerted at a specific hypothalamic site, mainly in the PVN. In addition, data using the selective sigma ligand, JO 1784, and the putative sigma antagonist, BMY 14802, suggest a functional interaction between NPY and sigma binding sites that modulates the central action of CRF.

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Preconditioning of the ischaemic myocardium; involvement of the L-arginine nitric oxide pathway

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1 Short periods of coronary artery occlusion protect the heart against the effects of a subsequent prolonged period of ischaemia. This phenomenon is known as preconditioning of the ischaemic myocardium.

2 In mongrel, chloralose-urethane anaesthetized open-chest dogs, within a restricted body weight range, two 5 min periods of occlusion of the anterior descending branch of the left coronary artery markedly reduced the severity of the early ischaemic arrhythmias resulting from a prolonged (25 min) occlusion of the same coronary artery starting 20 min later. Thus, the number of ventricular premature beats (VPBs) was reduced from 528 ± 140 in controls to 78 ± 27 in preconditioned dogs, the incidence of ventricular fibrillation (VF) was reduced from 47% to 0% and the incidence of ventricular tachycardia (VT) from 100% to 20%. ST-segment elevation recorded from electrodes within the ischaemic area, and the degree of inhomogeneity of conduction within the ischaemic area were markedly reduced in these preconditioned dogs.

3 The incidence of VF following reperfusion of the ischaemic myocardium at the end of the 25 min occlusion period was reduced in the preconditioned dogs from 100% to 60%; there was thus a 40% survival from the combined ischaemia-reperfusion insult compared with 0% in the controls.

4 N^G-nitro-L-arginine methyl ester (L-NAME) an inhibitor of the L-arginine nitric oxide pathway, given in a dose of 10 mg kg^{-1} intravenously on two occasions, both before the initial preconditioning occlusion and then again before the prolonged occlusion, partially attenuated the protective effects of preconditioning. There were more VPBs (220 ± 75), a higher incidence of VT (60%) and more episodes of VT (11.5 ± 6.0 compared to 0.7 ± 0.3 episodes in the preconditioned dogs not given L-NAME); none of the animals survived reperfusion (incidence of VF 100%). The improvement in the severity of the degree of inhomogeneity which resulted from preconditioning was abolished by L-NAME administration.

5 L-NAME itself elevated blood pressure (from 96 ± 5 mmHg diastolic to 119 ± 7 mmHg), reduced heart rate (from 155 ± 7 to 144 ± 4 beats min^{-1}) but did not change LVEDP, $LVdP/dt_{max}$, coronary blood flow, ST-segment elevation or the degree of inhomogeneity of conduction. When given 10 min before the prolonged coronary artery occlusion in dogs not subjected to preconditioning, L-NAME had no significant effect on the severity of arrhythmias except for more periods of VT (a mean of 11.7 ± 4.7 episodes per dog).

6 It is concluded from these studies that the generation of nitric oxide contributes to the marked antiarrhythmic effects of preconditioning in the canine myocardium, probably through elevation of cyclic GMP.

Keywords: Nitric oxide; N^G-nitro-L-arginine methyl ester (L-NAME); preconditioning; ventricular arrhythmias; myocardial ischaemia; reperfusion; inhomogeneity of conduction; endogenous myocardial protective substances

Introduction

There has been considerable recent interest in the possibility that the heart is capable of rapidly adapting to brief periods of ischaemic stress, whether induced by transient coronary artery occlusion (Murry *et al.*, 1986; Henrichs *et al.*, 1987; Komori *et al.*, 1990; Li *et al.*, 1990; Vegh *et al.*, 1990; 1992a) or by rapid ventricular pacing (Vegh *et al.*, 1991a) in such a way that the severity of subsequent, more prolonged, periods of ischaemia (and the arrhythmic consequence of reperfusion; Shiki & Hearse, 1987) is much reduced. This phenomenon is known as preconditioning of the ischaemic myocardium. The mechanism(s) of this protection are unknown. Suggestions have included the opening up of coronary collateral vessels by the preconditioning occlusions (such that myocardial blood flow is higher during the subsequent prolonged occlusion), a reduction in the rate of utilization of high-energy phosphates, myocardial 'stunning', enhanced potassium uptake, the opening of ATP-dependent K^+ channels and inhibition of cardiac responsiveness to sympathetic neurotransmitters. The evidence for these, which is not completely convincing, has been recently reviewed (Vegh *et al.*, 1992b). An alternative suggested mechanism is that brief periods of ischaemia stimulate the heart to produce 'endogenous myocardial protective substances' (Parratt, 1987) which, in some way, protect the heart against subsequent, more severe ischaemic episodes. There is recent evidence for a major role of adenosine in the reduction in ultrastructural damage resulting from preconditioning (Van Winkle *et al.*, 1991; Liu *et al.*, 1991) and, since the antiarrhythmic effects of preconditioning are largely lost if the cyclo-oxygenase pathway of arachidonic acid metabolism is inhibited (Vegh *et al.*, 1990), the generation of prostanoids, and perhaps especially prostacyclin, also seems to be involved in this protection.

Because of the close interrelationship between adenosine, prostanoids, bradykinin (which is also cardioprotective; Martorana *et al.*, 1990; Vegh *et al.*, 1991b) and nitric oxide (NO), we have examined, using an inhibitor of the L-arginine nitric

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oxide pathway, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME; Moore *et al.*, 1990) whether this substance might also play a role in ischaemic preconditioning. Preliminary accounts of these results have been given to meetings of the International Society for Heart Research (Vegh *et al.*, 1991c) and the Physiological Society (Vegh *et al.*, 1992c).

Methods

We used mongrel dogs, mainly Hungarian alsations, of either sex (evenly distributed between the groups in a ratio of 2.5 males:1 female) and with a body weight in excess of 17 kg (mean 22.8 ± 1.5 kg). The dogs were anaesthetized with a mixture of chloralose and urethane (60 and 200 mg kg^{-1} respectively given intravenously) and ventilated with room air using a Harvard Respirator at a rate/volume sufficient to maintain arterial blood gases and pH within normal limits (Vegh *et al.*, 1990). The temperature was measured from the oesophagus and maintained, by a heating pad, between 36.8 and 37.5°C.

A thoracotomy was performed at the fifth intercostal space and the anterior descending branch of the left coronary artery (LAD) prepared for occlusion just proximal to the first main diagonal branch. Epicardial ST-segment changes and the degree of inhomogeneity of activation were measured from the left ventricular wall distal to the proposed coronary artery occlusion with unipolar electrodes and a 'composite electrode' previously described (Vegh *et al.*, 1987). This gives a summarised recording of R-waves from 30 epicardial measuring points. In the adequately perfused and oxygenated myocardium all sites are activated simultaneously, resulting in a single large spike. However, following occlusion, widening and fractionation of this summarized R-wave occurs indicating that adjacent fibres are not simultaneously activated because of inhomogeneity of conduction. We expressed inhomogeneity of conduction as the greatest delay in activation (in ms) within the ischaemic area, i.e. between the first and last burst. This reflects in part, local changes in blood flow.

Blood flow in the left circumflex coronary artery was measured in some of the experiments with a 2.0 mm electromagnetic flow probe and a Statham SP2202 flow meter. These parameters, together with a limb lead electrocardiogram, systemic arterial pressure and left ventricular (LV) pressure (Statham P23Dp transducers) and LV$dP/dt$ were recorded on an eight channel Medicor R81 recorder. At the end of the experiment patent blue V dye was infused into the occluded LAD coronary artery to estimate the area at risk. This was expressed as a percentage of the left ventricular free wall.

Ventricular arrhythmias during ischaemia and reperfusion were analysed as outlined by Vegh *et al.* (1992a). No distinction was made between couplets and salvos, which were included as single ventricular ectopic (premature) beats (VPBs), and we defined ventricular tachycardia (VT) as a run of four or more ectopics at a rate faster than the resting sinus rate. We also estimated the number of episodes of ventricular tachycardia during coronary artery occlusion in each animal. To limit the variability in the severity of arrhythmias during coronary artery occlusion, we used dogs with a body weight in excess of 17 kg since smaller dogs have less severe arrhythmias following occlusion. For example, in a separate control group using dogs with a body weight less than 14 kg (mean weight 11.8 ± 0.3 kg) the number of premature beats was 280 ± 152 (compare 497 ± 109 in the present study; $P<0.01$) and the incidence of VF was 20% (compare 47% in the present series; $P<0.05$).

Data are expressed as means (± s.e.mean) and differences between means were compared by Student's t test, corrected for multiple comparisons, or by the Mann-Whitney U test (for arrhythmias). To compare between-group differences in VT and ventricular fibrillation (VF) and in survival from the combined ischaemia-reperfusion episode, the Fisher exact probability test was used. Differences between groups were considered significant at a level of $P<0.05$. Although these experiments were carried out in Szeged the protocol complies with the UK Home Office requirement (Project Licence No. 60/00307).

The protocols were as follows:-

(1) *Group 1 (controls)* These 15 animals served as controls and were allowed to stabilize after surgery for 1 h; the LAD coronary artery was then occluded for 25 min, after which the ischaemic area was reperfused.

(2) *Group 2 (preconditioned)* These 10 animals were preconditioned by two 5 min coronary artery occlusions, with a 20 min reperfusion period between, followed 20 min after the second preconditioning coronary artery occlusion, by a prolonged (25 min) occlusion. The ischaemic area was then reperfused.

(3) *Group 3 (preconditioned + L-NAME)* These 10 animals, were preconditioned in the same manner as the Group 2 dogs but also received L-NAME (10 mg kg^{-1} intravenously) both 10 min before the first 5 min preconditioning occlusion and 10 min before the prolonged (25 min) occlusion. These dogs were also reperfused at the end of the 25 min prolonged occlusion period.

(4) *Group 4 (controls plus L-NAME)* These ten dogs were given L-NAME (10 mg kg^{-1}) 10 min before a prolonged LAD occlusion in order to determine whether inhibition of NO generation itself modified post-occlusion arrhythmias. One of these animals died before occlusion. In this group the occlusion period was maintained for 60 rather than 25 min in order to determine if ventricular ectopic activity was maintained beyond the usual 20–25 i.e. whether inhibition of NO generation 'spread' the early ischaemic arrhythmias over a longer time span (Vegh *et al.*, 1992a).

Results

Haemodynamic changes induced by coronary artery occlusion and by L-NAME

Occlusion of the LAD coronary artery resulted, during the first 5 min, in a small decrease in mean arterial blood pressure (of 5.4 ± 1.8 mmHg; i.e. from 100 ± 5 to 95 ± 4 mmHg; $P<0.01$) and a marked increase in LVEDP (of 8.9 ± 1.3 mmHg i.e. from 5.6 ± 0.7 to 14.5 ± 1.2; $P<0.001$). Heart rate was unchanged by occlusion (132 ± 3 to 131 ± 3 beats min^{-1}). There was also a transient and significant ($P<0.05$) decrease in LV$dP/dt$ (of − 180 ± 71 mmHg s^{-1}). One of the most pronounced and immediate effects of occlusion of the anterior descending coronary artery was a 'compensatory' increase in blood flow in the circumflex coronary artery (of 6.3 ± 1.5 ml min^{-1} from a resting value of 37 ± 3 ml min^{-1}) and a decrease in coronary vascular resistance (diastolic arterial pressure divided by diastolic coronary blood flow) of − 0.7 ± 0.14 units.

The administration of L-NAME resulted in a marked, and long lasting, increase in systemic arterial blood pressure (from 140 ± 8 mmHg systolic and 96 ± 5 mmHg diastolic to 160 ± 8 mmHg and 119 ± 7 mmHg after 5 min; $P<0.05$) and a reduction in heart rate (from 155 ± 7 to 144 ± 4 beats min^{-1}; $P<0.05$). There were no significant changes in LVEDP (8.5 ± 1 mmHg to 10.0 ± 1.1 mmHg) or in LV$dP/dt_{max}$ (2139 ± 115 to 2203 ± 133 mmHg s^{-1}). There was also no significant change in coronary blood flow (35 ± 3 ml min^{-1} to 37 ± 3 ml min^{-1}); calculated vascular resistance was thus increased by L-NAME from 3.3 ± 0.3 to 3.9 ± 0.3 units ($P<0.05$). The administration of L-NAME resulted in no change either in epicardial electrocardiograms or in the degree of inhomogeneity of electrical activation. In those dogs (Group 3) given 2 doses of L-NAME, the haemodynamic effects of the second dose were significantly less than

those of the first (e.g. increase in mean arterial blood pressure 7.0 ± 1.7 mmHg (cf 22 ± 3 mmg Hg; $P<0.01$) and a reduction in heart rate of −2 ± 1 beats min^{-1} (cf 11 ± 3 beats min^{-1}; $P<0.001$). This perhaps implies that NO generation was still markedly inhibited at the commencement of the prolonged occlusion.

The haemodynamic effects of coronary artery occlusion in dogs treated with L-NAME were similar to those in the controls. For example, there was a small decrease in mean arterial blood pressure (of 5.3 ± 2.7 mmHg) and an increase in LVDEP of 4.0 ± 0.8 mmHg. Heart rate was unchanged (137 ± 6 to 144 ± 7 beats min^{-1}).

Ventricular arrhythmias during the preconditioning occlusions and the influence of L-NAME

There were occasional ventricular premature beats (VPBs) during the preconditioning occlusions in the Group 2 dogs. Thus, during the first 5 min preconditioning occlusion there was a mean of 14 ± 13 ventricular premature beats (including 1 episode of VT) and 2 ± 1 VPBs (and 1 period of VT) during reperfusion; during the second occlusion there were 12 ± 7 VPBs (2 episodes of VT) and a mean of 8 VPBs (1 episode of VT) during reperfusion. The administration of L-NAME before the preconditioning occlusions (Group 3 dogs) did not significantly modify the incidence, or severity, of these arrhythmias. For example, there were 24 ± 6 VPBs during the first preconditioning occlusion and 15 ± 5 VPBs during the second preconditioning occlusion (NS), with a 20% incidence of VT (0.3 ± 0.2 episodes per dog) both during the first preconditioning occlusion and during the second preconditioning occlusion (0.2 ± 0.1 episodes per dog). During reperfusion there were 19 ± 8 and 21 ± 10 VPBs respectively ($P<0.01$ in comparison with those dogs without L-NAME) following release of the first and second occlusions. VT occurred during reperfusion in one animal following the first preconditioning occlusion and in two animals following the second.

Ventricular arrhythmias during the prolonged coronary artery occlusion and during reperfusion; protective effects of preconditioning and the influence of L-NAME

Occluding the LAD coronary artery for 25 min resulted in severe ventricular arrhythmias in the control dogs (Figure 1). The incidence of VF was 47%; no attempt was made to defibrillate. All the animals exhibited VT with a mean of 5.1 ± 1.6 episodes per dog. The number of ventricular premature beats, in those dogs that survived, was 528 ± 140. Of these 212 ± 74 occurred during the first 10 min of occlusion (phase 1a arrhythmias) and 316 ± 27 during the 10–25 min period (phase 1b arrhythmias). All the dogs that survived the 25 min occlusion period fibrillated within 1 min, and usually within 15 s, of reperfusion. There were thus no survivors from the combined ischaemia-reperfusion insult in this control group (Figure 1).

The severity of these arrhythmias was markedly reduced if the 25 min occlusion period was preceded by two 5 min occlusions of the same coronary artery i.e. by preconditioning (Figure 1). Thus, there were only 78 ± 27 VPBs over the 25 min occlusion period ($P<0.01$ compared with controls). Of these, 20 ± 16 occurred during phase 1a and 58 ± 20 during phase 1b (both $P<0.001$ versus controls). There was also a lower incidence of VT (20%; $P<0.05$ versus controls; 0.7 ± 0.3 episodes per dog, $P<0.001$ versus controls), no animal fibrillated during occlusion ($P<0.05$ versus controls) and 4 of the 10 dogs that were reperfused at the end of the occlusion period survived. There was thus a survival from this combined ischaemia-reperfusion insult of 40% ($P<0.05$ versus controls).

The antiarrhythmic effects of preconditioning were less marked in those dogs given L-NAME. For example, there were more VPBs than in dogs preconditioned without L-NAME (220 ± 75 versus 78 ± 27; $P<0.05$), a higher incidence of VT (60% versus 20%) more episodes of VT (9.3 ± 4.3 versus 0.7 ± 0.3; $P<0.001$), a higher incidence of VF on reperfusion (100% versus 60%) and a lower survival from the combined ischaemia-reperfusion insult (0% versus 40%; $P<0.05$). The differences in response between those (Group 3) dogs preconditioned in the presence of L-NAME and those subjected to preconditioning, and to the prolonged coronary artery occlusion, in the absence of L-NAME (Group 2 dogs) are illustrated in Figure 2.

The general conclusion then from these L-NAME studies is that the protective effects of preconditioning are reduced by inhibition of the L-arginine NO pathway during the preconditioning period. However, protection by preconditioning is not completely lost if this pathway is inhibited because even in the presence of L-NAME the number of VPBs (220 ± 75) and the incidence of VF (0%) during occlusion were significantly reduced compared to those in control dogs (528 ± 140 VPBs and 47% incidence of VF; $P<0.01$ and $P<0.05$ respectively).

We also examined, in nine (Group 4) dogs, whether the administration of L-NAME itself modified ventricular arrhythmias during coronary artery occlusion. The haemodynamic changes induced by L-NAME were similar to those already outlined above, e.g. an increase in arterial blood pressure (from 148 ± 10 mmHg systolic and 94 ±

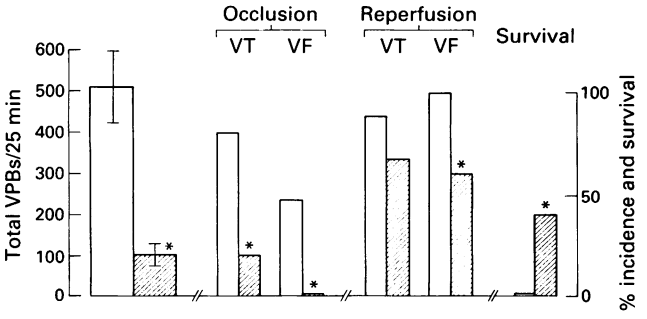

Figure 1 Ventricular arrhythmias (total number of ventricular premature beats (VPBs); incidence of ventricular tachycardia (VT) and ventricular fibrillation (VF)) in control dogs subjected to a 25 min coronary artery occlusion followed by reperfusion (incidence of VT and VF, $n = 15$; open columns) and in dogs preconditioned by two 5 min periods of coronary artery occlusion ($n = 10$, hatched columns). Preconditioning causes a marked reduction in the severity of arrhythmias and a significant increase in survival from the combined ischaemia-reperfusion insult.
*$P<0.05$ versus control.

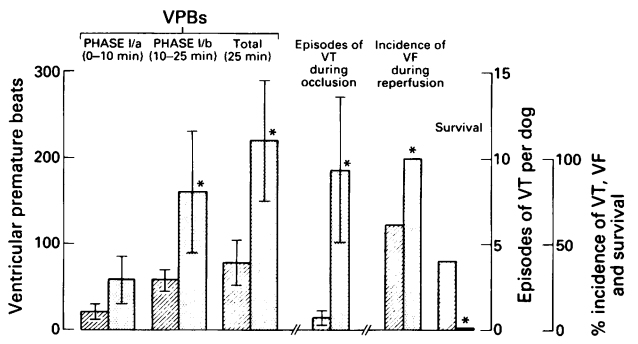

Figure 2 A comparison of the ventricular arrhythmias occurring during a 25 min coronary artery occlusion in dogs subjected to preconditioning ($n = 10$, hatched columns) and in dogs also subjected to preconditioning but in the presence of N^{G}-nitro-L-arginine methyl ester (L-NAME) ($n = 10$, stippled columns). Inhibition of the L-arginine NO pathway attenuates the protective effect of preconditioning (compare Figure 1).
*$P<0.05$ versus preconditioned group.

7 mmHg diastolic to 183 ± 9 and 118 ± 6 mmHg respectively; $P<0.05$), and in LVEDP (from 6 ± 0.6 to 10 ± 1.6 mmHg; $P<0.05$). LV$dP/dt_{max}$ was unchanged (2788 ± 237 and 2786 ± 211 mmHg s^{-1}). There was a small reduction in heart rate (from 160 ± 9 to 144 ± 10 beats min^{-1}; $P<0.05$). Five of these animals (i.e. 55%) fibrillated on occlusion (four between 3 and 10 min of the commencement of occlusion and one between 10 and 15 min); all (except one) had periods of VT. There were more episodes of VT (11.7 ± 4.7) and fewer VPBs (318 ± 104) than in the controls.

Although, in our hands, and with a group of over 40 dogs, there was no significant relationship between area at risk following coronary occlusion (when this was between 30 and 50% of the free left ventricular wall) and the severity of ischaemic arrhythmias (Coker & Parratt, 1985), we did determine risk area in all dogs. There was no significant difference between the two preconditioning groups (Groups 2 and 3); these were 40.4 ± 0.9% and 41.2 ± 3.4%. The risk area in the control Group (1) was somewhat less i.e. 34.2 ± 3.1% ($P<0.05$ compared to the preconditioned groups). In the dogs given an inhibitor of the L-arginine NO pathway (L-NAME) but not preconditioned, it was 39.6 ± 0.8%.

Changes in ST-segment elevation and in the degree of inhomogeneity during coronary artery occlusion: the effect of L-NAME

Preconditioning led to less marked ischaemic epicardial ST-segment changes and there was a significant delay in the development of these changes, especially during the first 5 min of the prolonged occlusion (Figure 3). This slower rate of development was also seen in those dogs that were preconditioned in the presence of L-NAME (Figure 3); only at one time point (15 min into the occlusion period) was the ST-segment elevation significantly different from that in dogs preconditioned without L-NAME.

The modification in the delay of activation by preconditioning was especially pronounced (Figure 4). In those dogs preconditioned in the presence of L-NAME this protective effect of preconditioning was abolished; the inhomogeneity of conduction within the ischaemic area was as pronounced in these dogs in the controls and remained so until reperfusion. This may account for the similar incidence in the severity of arrhythmias on reperfusion; in both these groups all the animals fibrillated on reperfusion (Figures 1 and 2). In contrast, there was a significantly lower incidence of VF in those dogs preconditioned in the absence of L-NAME (Figure 2).

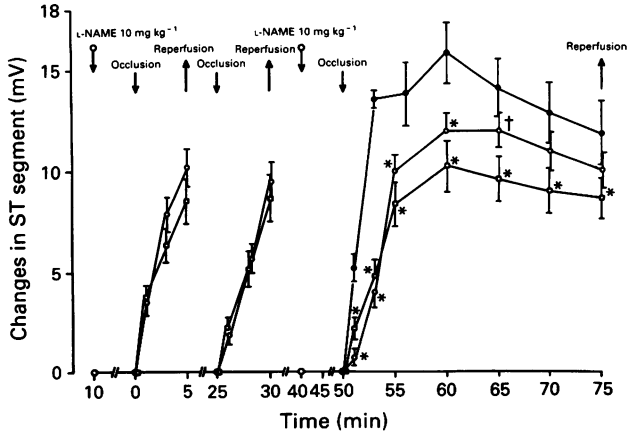

Figure 3 Changes in ST-segment elevation following coronary artery occlusion in control dogs ($n = 15$, ●), in dogs subjected to preconditioning ($n = 10$, □) and in dogs also subjected to preconditioning but in the presence of N^G-nitro-L-arginine methyl ester (L-NAME) given both before the preconditioning occlusion and before the prolonged occlusion ($n = 10$, ○). Values are means from 10–15 experiments; bars = s.e.mean.
*$P<0.05$ versus control; †$P<0.05$ versus preconditioned group.

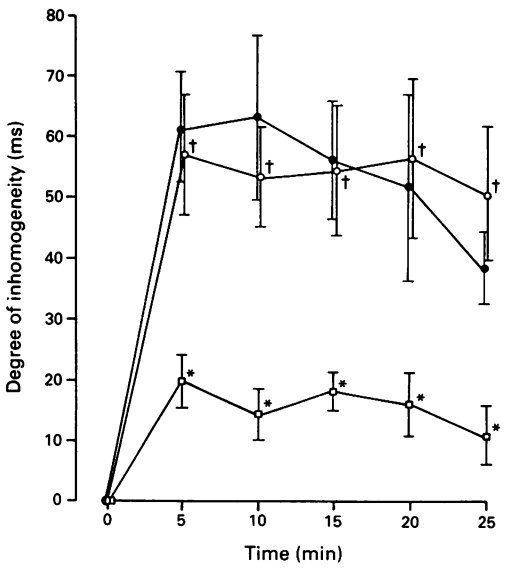

Figure 4 The degree of inhomogeneity of conduction (ms) during a 25 min occlusion period of the left anterior descending coronary artery in control dogs ($n = 15$, ●), in dogs subjected to preconditioning ($n = 10$, □) and in dogs subjected to preconditioning but in the presence of N^G-nitro-L-arginine methyl ester (L-NAME) ($n = 10$, ○). Values are means of up to 15 observations; bars = s.e.mean.
*$P<0.05$ versus control; †$P<0.05$ versus preconditioned group.

Discussion

These studies were concerned with the possibility that NO is involved in the antiischaemic and antiarrhythmic effects of preconditioning. We have previously suggested that brief periods of myocardial ischaemia induced by complete coronary artery occlusion (Vegh *et al.*, 1990) or by rapid ventricular pacing (Vegh *et al.*, 1991a) induce the generation and release of 'endogenous myocardial protective substances' which, in some way, then modify the myocardial response to later, more severe ischaemic episodes. In accord with this hypothesis are the findings that (i) the protective response wanes with time (for example in dogs it is largely lost if the period between the second preconditioning occlusion and the commencement of the prolonged occlusion is increased from 20 to 60 min (Vegh *et al.*, 1992a)) and (ii) that the antiarrhythmic effect of preconditioning is markedly attenuated following inhibition of the cyclo-oxygenase pathway of arachidonic acid metabolism (Vegh *et al.*, 1990).

When an inhibitor of the L-arginine NO pathway was administered before the first of the two preconditioning coronary artery occlusions, some of the marked protective effects of this procedure was lost. This perhaps implies that the generation of NO during the preconditioning period in some way contributes to the protection. Because the profound antiarrhythmic effects of preconditioning are likely to be related to the reduced severity of ischaemia during a subsequent prolonged coronary artery occlusion, as suggested for example by the less pronounced epicardial ST-segment changes (Figure 3), this protective effect of NO could involve dilatation of microvessels or inhibition of platelet adherence to endothelial cells, both of which could contribute to an antiarrhythmic and antiischaemic action of preconditioning. These actions are thought to be largely responsible for the antiischaemic effect of NO 'donors' such as molsidomine and nitroglycerin. It is not possible, at this stage, to eliminate possible extravascular protective effects of NO, for example, on ventricular myocytes which contain a Ca^{2+}-dependent NO synthase enzyme (Schultz *et al.*, 1992). Indeed, the reduction by L-NAME of the beneficial effects of preconditioning on the degree of inhomogeneity of conduction within the

ischaemic area (Figure 4) is probably too great to be accounted for solely by a vascular effect of NO.

How is it possible for NO generation during the preconditioning occlusions to modify the effects of a coronary artery occlusion 20 min later? Although it is possible that brief periods of ischaemic stress might stimulate the generation of NO over such a period (we have no means of evaluating this in the experimental model we have used) it is more likely that the protection involves the resultant stimulation of guanylyl cyclase and the elevation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) within the vascular wall, or in ventricular myocytes. Several years ago Opie (1982) suggested that an elevation of cardiac cyclic GMP could be an antiarrhythmic procedure, a view substantiated by the more recent studies of Billman (1990). In mongrel dogs with a healed myocardial infarction he showed that carbachol and 8-bromo cyclic GMP both substantially reduced the incidence of VF that occurred during a combination of exercise and a brief coronary artery occlusion. It may be no coincidence that those substances which have been suggested as endogenous myocardial protective substances (Parratt, 1987) participating in preconditioning, namely prostanoids (Vegh *et al.*, 1990), adenosine (Van Winkle *et al.*, 1991; Liu *et al.*, 1991) and bradykinin (Vegh *et al.*, 1991b) all stimulate guanylyl cyclase either directly or through NO production. Further evidence for this mechanism comes from studies involving methylene blue, an inhibitor of soluble guanylyl cyclase. The local intracoronary administration of methylene blue reverses completely the antiarrhythmic effect of preconditioning (Vegh *et al.*, 1992d).

The mechanism, whatever it is, deserves investigation because the antiarrhythmic effects of preconditioning, although short lived, are pronounced. Indeed, they are probably as marked in this experimental model as those of pharmacological approaches using standard antiarrhythmic drugs. If, as we suggest, the generation of endogenous myocardial protective substances contributes to the beneficial effects of preconditioning then modification of these substances, by prolonging their action or facilitating their release, could be an alternative approach to the treatment or prevention of ischaemia-induced life-threatening ventricular arrhythmias.

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Effects of bradykinin receptor antagonists on antigen-induced respiratory distress, airway hyperresponsiveness and eosinophilia in guinea-pigs

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1 We examined effects of bradykinin (BK) receptor antagonists on airway hyperresponsiveness and eosinophilia in sensitized guinea-pigs that had been administered single, as well as repeated (chronic) challenges with inhaled ovalbumin. In addition, the effects of BK antagonists on antigen-induced respiratory distress during the chronic study were noted.

2 At 24 h following single antigen challenge, guinea-pigs exhibited airway hyperresponsiveness to the bronchoconstrictor effect of i.v. histamine, characterized by a left shift in the dose-response curve. In addition, responses to the maximum dose of histamine that could be used were significantly increased in hyperresponsive guinea-pigs. The percentages of bronchoalveolar fluid, eosinophil and neutrophils also increased.

3 A BK B_1 receptor antagonist, desArg^9-[Leu^8]-BK, significantly inhibited airway hyperresponsiveness induced by single antigen challenge. A B_2 receptor antagonist, D-Arg-[Hyp^3, Thi^{5,8},D-Phe^7]-BK (NPC 349) had a small, but statistically significant inhibitory effect on responsiveness to the highest histamine dose in challenged animals. DesArg^9-[Leu^8]-BK significantly inhibited the neutrophilia, whereas NPC 349 inhibited infiltration by both cell types.

4 Chronic antigen challenge also caused airway hyperresponsiveness to i.v. acetylcholine (ACh), distinguished by an increase in the slope of the dose-response curve. Thus, the magnitude of the bronchoconstrictor responses to the maximum dose of ACh that could be used was significantly increased. No change in sensitivity to ACh was evident. Marked eosinophilia was also noted in the trachea, bronchi and lung parenchyma.

5 Airway hyperresponsiveness and eosinophilia, induced by chronic antigen challenge, were markedly inhibited by the B_2 antagonists, D-Arg-[Hyp^3,D-Phe^7]-BK (NPC 567) or D-Arg-[Hyp^3,Thi^{5}D-Tic^7,Tic^8]-BK (NPC 16731). NPC 16731 also abolished antigen-induced cyanosis, and delayed the onset of dyspnoea, doubling the time taken for animals to exhibit respiratory distress.

6 The ability of BK receptor antagonists to inhibit antigen-induced airway hyperresponsiveness, in addition to eosinophilia, indicates an important role for endogenous kinins. Moreover, the abrogation of eosinophil infiltration suggests that BK has a significant function in maintaining allergic inflammation of the airways.

Keywords: Bradykinin; B_1 receptor; B_2 receptor; antagonist; airway; allergen challenge; hyperresponsiveness; eosinophil; respiratory distress; asthma

Introduction

Bradykinin (BK) has been implicated as an important mediator in inflammatory diseases of the upper and lower airways (see reviews by Farmer, 1991a,b; Pongracic *et al.*, 1991). For example, inhaled BK is a very potent bronchoconstrictor in asthmatic but not non-asthmatic subjects (Fuller *et al.*, 1987; Polosa & Holgate, 1990). Furthermore, allergic patients are reported to be more responsive to the effect of BK on microvascular leakage, following its topical application in the nasal mucosa, than are non-allergic subjects (Brunnée *et al.*, 1991). Increased levels of immunoreactive kinins are evident in the upper airways of allergic patients, following allergen challenge (Proud *et al.*, 1983). Similarly, in allergic asthmatics, bronchoalveolar lavage (BAL) levels of immunoreactive kinins, as well as kinin-generating enzymes, are significantly elevated following inhalation of antigen (Christiansen *et al.*, 1987). Thus, the kallikrein-kinin system, via generation of inflammatory kinins in the airways, may be involved in the pathogenesis of allergic asthma and rhinitis.

Similar to observations in man (Fuller *et al.*, 1987; Polosa & Holgate, 1990), sheep which are allergic to *Ascaris suum* antigen, are more responsive to the bronchoconstrictor effect of inhaled BK than non-allergic animals (Abraham *et al.*, 1991a). The potential involvement of endogenous kinins in experimental antigen-induced airway hyperresponsiveness and inflammation is supported by observations that aerosol administration of a B_2 receptor antagonist, D-Arg-[Hyp^3,D-Phe^7]-BK (NPC 567), prior to antigen challenge, inhibits both phenomena in allergic sheep (Solér *et al.*, 1990). In addition, NPC 567 abolishes the onset of late bronchial obstruction, as well as increased BAL levels of several inflammatory mediators (peptidoleukotrienes, leukotriene B_4, prostaglandins) in dual responding sheep, 4–8 h following challenge with *A. suum* (Abraham *et al.*, 1991b).

Sensitized guinea-pigs, repeatedly exposed to antigen, exhibit hyperresponsiveness to the bronchoconstrictor action of acetylcholine (ACh), and airway infiltration by eosinophils (Ishida *et al.*, 1989). In addition, we recently reported the induction of airway hyperresponsiveness to histamine 24 h

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after a single challenge with inhaled ovalbumin in sensitized guinea-pigs (Seeds *et al.*, 1991). Also in this species, airway hyperreactivity to ACh and 5-hydroxytryptamine (5-HT) was reported to occur as early as 60 min after single challenge with ovalbumin (Daffonchio *et al.*, 1989). Antigen challenge causes increased circulating levels of kinins and exposure of perfused lungs from sensitized guinea-pigs to antigen results in kallikrein production *in vitro* (Brocklehurst & Lahiri, 1962; Jonasson & Becker, 1966).

We have examined, therefore, the effects of three B_2 receptor antagonists, NPC 567, D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-BK (NPC 349) (Farmer & Burch, 1991), and D-Arg-[Hyp^3,Thi^5,D-Tic^7,Tic^8]-BK (NPC 16731), recently described as a more potent BK antagonist (Farmer *et al.*, 1991a; Kyle *et al.*, 1991), on airway hyperresponsiveness and eosinophilia induced by acute and chronic inhalation of ovalbumin in sensitized guinea-pigs. In addition, the effects of desArg^9-[Leu^8]-BK, a B_1 receptor antagonist, on the acute effects of antigen on airway responsiveness and eosinophil infiltration were examined. Some of these data were presented to the British Pharmacological Society (Farmer *et al.*, 1991c).

Methods

Acute antigen-induced airway hyperresponsiveness

Male Dunkin-Hartley guinea-pigs (250–600 g) from Olac (Bicester, Oxon) were used for this aspect of the study. Each animal was injected i.p. with 40 μg ovalbumin (OA), dissolved in a suspension of $Al(OH)_3$ gel, that had been diluted 1:1 with sterile saline (0.9% w/v NaCl solution). Controls received $Al(OH)_3$ alone; 18–20 days later, all animals were exposed to a mist of OA, generated in a jet-type nebulizer (1 mg ml^{-1}, 8–10 ml h^{-1}), for 1 h in an exposure chamber. The BK receptor antagonists, NPC 349 or desArg^9-[Leu^8]-BK, were administered immediately before antigen inhalation. Doses of each antagonist were 400 μg kg^{-1} i.v., plus 600 μg kg^{-1}, s.c.

Twenty-four hours later, animals were anaesthetized with urethane (7 ml kg^{-1} i.p., 25% w/v solution), and the trachea cannulated and connected to a Harvard ventilator pump. Animals were ventilated (1 ml 100 g^{-1} body weight) with room air at a rate of 70 strokes per min. Pulmonary inflation pressure (PIP), as an index of intrathoracic airway resistance, was measured with a pressure transducer (Druck Ltd.) mounted to a side arm on the tracheal cannula. A jugular vein and carotid artery were also cannulated to allow administration of drugs and the monitoring of arterial blood pressure, respectively. Airway responsiveness was determined from dose-response curves to intravenously administered histamine. Doses of histamine (1–50 μg kg^{-1}) were administered at 5 min intervals.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed as described previously (Seeds *et al.*, 1991). In brief, animals were killed with an overdose of urethane, and 5 ml aliquots of saline were instilled and recovered from the lungs a total of five times. Total cell counts were obtained from the resultant cell suspension and, following adjustment to 5×10^5 cells ml^{-1}, cytospin preparations were prepared, and differential cell counts obtained by use of Lendrum's Stain (Lendrum, 1944) to visualize eosinophils.

Chronic antigen-induced airway hyperresponsiveness

Female Hartley guinea-pigs (200–250 g) (Hazelton, Denver, PA, U.S.A.) were sensitized to OA by a slight modification of the method of Ishida *et al.* (1989). Animals were injected i.p. with 10 μg OA dissolved in 0.5 ml saline containing 10 mg $Al(OH)_3$ in suspension. After two weeks the animals were challenged by inhalation of an aerosolized solution containing OA (0.5% w/v dissolved in normal saline). Aerosols were generated by an ultrasonic nebulizer (DeVilbiss Pulmo-Sonic Nebulizer). Inhalation of antigen was carried out twice a week for five weeks. All animals were given the histamine H_1 receptor antagonist, diphenhydramine (30 mg kg^{-1}, i.p.), 60 min before each antigen challenge.

Animals were exposed to antigen aerosol until the onset of laboured breathing and/or cyanosis was evident. The maximum exposure time was 60 min and, from weeks 3–5, the time taken for each animal to exhibit symptoms was recorded. After an interval of 24–48 h following the final challenge, animals were anaesthetized with urethane, and the trachea and jugular vein were cannulated for measurement of PIP, and i.v. drug administration, in a manner similar to that described for the acute antigen studies. Airway responsiveness was determined from dose-response curves to acetylcholine (ACh, 1–300 μg kg^{-1} i.v.).

Treatment groups

Animals were divided into six treatment groups as follows. Controls consisted of non-sensitized or sensitized guinea-pigs, both groups being challenged with vehicle for drug and OA (saline). As no difference in airway responsiveness between these groups was evident (data not shown), results from these animals were combined. The second group was of sensitized guinea-pigs, chronically challenged with OA. There were four drug treatment groups. NPC 567 or NPC 16731 were administered to controls and also to sensitized, challenged guinea-pigs. Aerosolized NPC 567 (30 mg ml^{-1}) or NPC 16731 (1.5 mg ml^{-1}) were administered by inhalation for 30 min before each antigen challenge. NPC 567 (6 mg ml^{-1}) or NPC 16731 (0.3 mg ml^{-1}) were also co-administered with antigen.

Histology

Airway histological sections were prepared from animals from each group. Anaesthetized guinea-pigs were killed by exsanguination, and the lungs inflated with glutaraldehyde solution (2.5% w/v in saline). The trachea and lungs were removed and placed in glutaraldehyde for at least 48 h. Sections (10 μm) of trachea, right main bronchus, right main lobar bronchus and lung parenchyma were prepared, mounted on slides and stained with haematoxylin and eosin. Eosinophils in 10 random fields were counted in a light microscope. Care was taken to include only airway mucosal and submucosal eosinophils, and not those seen in blood vessels.

Drugs

Urethane, acetylcholine chloride, histamine diphosphate, ovalbumin (Grade V, fatty acid-free) and desArg^9-[Leu^8]-BK were obtained from Sigma Chemical Co. (Poole, Dorset and St. Louis, MO, U.S.A.). $Al(OH)_3$ moist gel was obtained from FSA Laboratory Supplies (Loughborough). D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-BK (NPC 349) was a gift from Dr J.M. Stewart, Department of Biochemistry, University of Colorado School of Medicine, Denver, Colorado, U.S.A. D-Arg-[Hyp^3,D-Phe^7]-BK (NPC 567) was synthesized by Abbott Laboratories (North Chicago, IL, U.S.A.), and D-Arg-[Hyp^3, Thi^5,D-Tic^7,Tic^8]-BK (NPC 16731), at Nova. All drugs were prepared in saline at the time of use.

Data analysis

Dose-response curves were compared by two-tailed *t* tests, and cell counts in different experimental groups were compared by one-way analysis of variance (ANOVA), performed by InStat (GraphPad Software, San Diego, California, U.S.A.). Where the *F* ratio generated a *P* value of <0.05, adjusted *t* tests, with *P* values corrected by the Bonferroni method, were

carried out between appropriate experimental groups. Probability (P) values of <0.05 were considered statistically significant. All data are expressed as mean ± s.e.mean.

Results

Acute studies

Twenty-four hours after single antigen challenge, guinea-pigs exhibited airway hyperresponsiveness to histamine. This was characterized by a leftward shift in the histamine dose-response curve, and a small increase in PIP to the maximum dose of histamine (Figure 1). Associated with the airway hyperresponsiveness, the percentages of BAL fluid eosinophils and neutrophils, but not monocytes, increased significantly 24 h after antigen challenge (Figure 2). The percentage of BAL eosinophils increased from 16.6 ± 3.0 in controls, to 35.0 ± 3.7 24 h after antigen challenge, although no significant alteration in total cell counts was apparent (282,000 ± 54,000 cells ml^{-1} in controls, and 422,000 ± 79,000 cells ml^{-1} following antigen challenge).

In guinea-pigs pretreated with NPC 349, antigen-induced eosinophil infiltration was significantly inhibited, whereas desArg^9-[Leu^8]-BK was without effect on eosinophil numbers (Figure 2). In contrast, the increase in the percentage of BAL neutrophils was inhibited by both antagonists. The small increase in bronchoconstrictor responses, in response to 50 $\mu g\,kg^{-1}$ histamine, was inhibited by NPC 349, while this peptide did not affect the leftward shift in the dose-response curve (Figure 1b). In contrast, desArg^9-[Leu^8]-BK significantly attenuated the antigen-induced leftward shift in the histamine dose-response curve (Figure 1c).

Chronic studies

Each OA challenge caused conspicuous respiratory distress. The animals exhibited laboured breathing, cyanosis and often collapsed. Several animals exhibited these symptoms within a few min and, despite being pretreated with an antihistamine, had to be quickly removed from the exposure chamber to prevent death by asphyxiation. An unexpected observation in the present study was that the time taken for the onset of laboured breathing in all animals increased with each challenge. Times to onset of respiratory distress were subsequently determined on weeks 3, 4 and 5 and, by week 5, they were significantly greater than at the first challenge of week 3 (Table 1).

Although dyspnoea occurred in the animals pretreated with NPC 567 or NPC 16731, cyanosis was rarely observed.

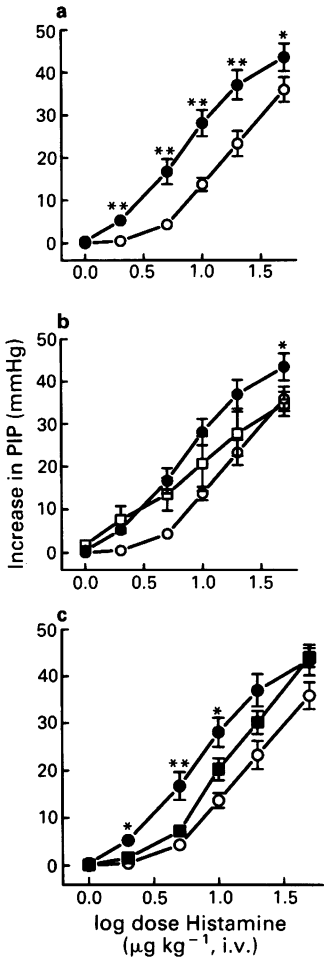

Figure 1 Dose-response curves for the bronchoconstrictor effect of intravenous histamine in anaesthetized guinea-pigs, previously sensitized to ovalbumin. (a) Data show airway hyperresponsiveness to histamine 24 h following acute antigen challenge: (○) non-sensitized controls; (●), antigen-challenged animals (**$P<0.01$ compared with controls). (b) (□), Effect of the bradykinin B_2 receptor antagonist, D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-BK. The antagonist significantly decreased responses of hyperresponsive animals to 50 $\mu g\,kg^{-1}$ histamine (*$P<0.05$). (c) (■) Effects of the B_1 receptor antagonist, desArg^9-[Leu^8]-BK. This drug significantly decreased responses of hyperresponsive animals at 2, 5 and 10 $\mu g\,kg^{-1}$ histamine (*$P<0.05$; **$P<0.01$). Data are expressed as mean ± s.e.mean (vertical bars) of 6–9 experiments.

Table 1 Effect of the bradykinin B_2 receptor antagonists, D-Arg-[Hyp^3, D-Phe^7]-BK (NPC 567) and D-Arg-[Hyp^3,Thi^5,D-Tic^7,Tic^8]-BK (NPC 16731) on time to onset of dyspnoea induced by ovalbumin (OA) inhalation in chronically challenged, sensitized guinea-pigs

	Treatment group		
Challenge No.	*OA controls*	*NPC 567 + OA*	*NCP 16731 + OA*
Week 3, No. 1	2.9 ± 0.3	4.0 ± 0.5[1]	6.6 ± 1.0**
Week 3, No. 2	2.9 ± 0.2	2.9 ± 0.8[1]	6.3 ± 1.2*
Week 4, No. 1	3.3 ± 0.5	6.9 ± 1.1[1]	9.3 ± 1.9**
Week 4, No. 2	4.9 ± 0.8	5.8 ± 1.1[1]	11.4 ± 1.6**
Week 5, No. 1	5.8 ± 1.1	6.1 ± 1.2[1]	10.6 ± 1.2*
Week 5, No. 2	6.2 ± 1.5[2]	8.2 ± 1.4[1,2]	12.0 ± 1.7*[,2]

Values are in min before animals were observed to exhibit laboured breathing. Group data were compared by one-way ANOVA followed, where appropriate, by modified t tests.
*$P<0.05$; **$P<0.01$; [1]not significant, when compared to OA control.
[2]Values are significantly different (t test, $P<0.05$) from those of challenge on Week 3, No. 1.
OA and antagonists were administered by inhalation. See Methods for protocols. Data are expressed as mean ± s.e.mean of 6–8 observations.

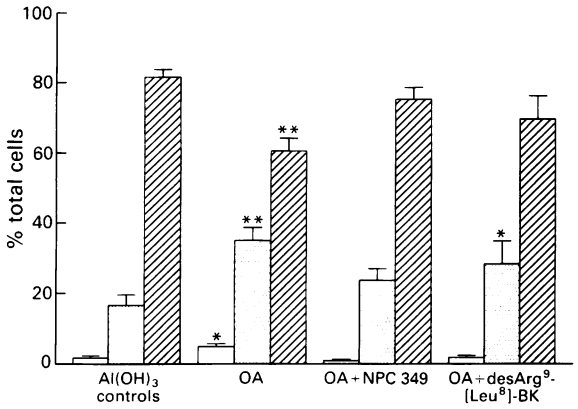

Figure 2 Effects of desArg^9-[Leu^8]-BK and D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-BK on the percentage of neutrophils (open columns), eosinophils (stippled columns) and mononuclear cells (hatched columns) found in bronchoalveolar fluid of sensitized guinea-pigs, 24 h following acute challenge with ovalbumin (OA). $*P<0.05$, and $**P<0.01$ compared with Al(OH)$_3$ controls. Data are expressed as mean ± s.e.mean (vertical bars) of 6–9 experiments.

Moreover, these animals did not collapse, even after 60 min exposure and, upon their removal from the exposure chamber, they recovered relatively quickly. NPC 16731-treated guinea-pigs took approximately twice as long to exhibit signs of laboured breathing when exposed to antigen aerosol (Table 1). This was evident throughout the duration of these studies. NPC 567 had no significant effect on the onset of dyspnoea (Table 1).

Chronic challenges with OA caused airway hyperresponsiveness, characterized by an increase in the slope of the ACh dose-response curve, resulting in an approximately 50% increase (from 33.0 ± 3.8 to 49.6 ± 3.5 cmH_2O, $P<0.01$) in the response to the maximum dose that could be administered (Figure 3). There was no alteration in airway sensitivity to ACh following chronic antigen exposures. Thus the control $-\log ED_{50}$ ($\mu g\,kg^{-1}$) of 1.97 ± 0.16 was not different from the value of 1.81 ± 0.07 in antigen-challenged guinea-pigs. Neither NPC 567 nor NPC 16731 had any effect on airway responsiveness in control animals (Figure 3). Both antagonists abolished the development of airway hyperresponsiveness to ACh (Figure 3b and c).

Chronic exposure of sensitized guinea-pigs to inhaled antigen also resulted in significantly increased numbers of eosinophils in all airway regions examined (Figure 4). For example, the numbers of eosinophils observed in 10 fields rose from 3.2 ± 0.9 in control tracheal sections, to 16.7 ± 2.4 in sections obtained from antigen-challenged animals. Similar degrees of eosinophilia were observed in main and lobar bronchus, as well as in parenchyma (Figure 4).

Airway eosinophilia was attenuated to varying degrees by the BK antagonists. In parenchyma, for example, both NPC 567 and NPC 16731 abolished antigen-induced eosinophilia (Figure 4). In contrast, NPC 16731 had a greater effect on eosinophilia in main bronchus than did NPC 567 (Figure 4). The antagonists had no significant effects on eosinophil numbers in airway tissues from non-sensitized control animals (data not shown). Thus, the B_2 receptor antagonists inhibited chronic antigen-induced airway eosinophilia and hyperresponsiveness.

Discussion

These experiments confirm previous studies demonstrating airway hyperresponsiveness to bronchoconstrictors in sensitized guinea-pigs after a single challenge (Daffonchio *et al.*, 1988; 1989; Seeds *et al.*, 1991), or repeated challenges (Ishida *et al.*, 1989; Schellenberg *et al.*, 1991) with antigen. In addition, airway eosinophil infiltration after acute (Dunn *et al.*,

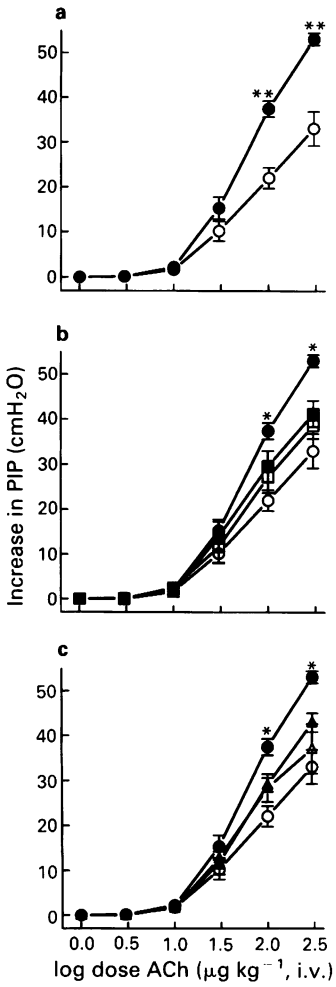

Figure 3 Dose-response curves for the bronchoconstrictor effect of intravenous acetylcholine (ACh) in anaesthetized guinea-pigs. These animals were sensitized to ovalbumin and challenged twice weekly for 4–5 weeks with inhaled ovalbumin. (a) Data show airway hyperresponsiveness to ACh 24–48 h following the final antigen challenge: (○) controls; (●) antigen-challenged animals. Ovalbumin challenges caused a significant increase in airway responsiveness ($**P<0.01$). (b) Effects of the bradykinin B_2 receptor antagonist, D-Arg-[Hyp^3,D-Phe^7]-BK (NPC 567): (□) in control animals, and (■), in animals chronically challenged with antigen. NPC 567 significantly inhibited the effect of ovalbumin challenge ($*P<0.05$). (c) Effects of the bradykinin B_2 receptor antagonist, D-Arg-[Hyp^3,Thi^5,D-Tic^7,Tic^8]-BK (NPC 16731): (△) in control animals, and (▲), in animals chronically challenged with antigen. NPC 16731 significantly inhibited the effect of ovalbumin challenge ($*P<0.05$). Data are expressed as mean ± s.e.mean (vertical bars) of 6–8 experiments.

1988; Hutson *et al.*, 1988; Richards *et al.*, 1989; Seeds *et al.*, 1991) and chronic (Ishida *et al.*, 1989; 1990) exposure to antigen has been reported.

Respiratory distress

As has been described by many investigators, antigen challenge induced acute respiratory distress. Interestingly, guinea-pigs took no longer to respond to each exposure to ovalbumin. Thus, during the third week, they took around 3 min to evince signs of distress, becoming cyanotic and exhibiting physical signs from mild dyspnoea to laboured breathing and anaphylactic collapse. By the final challenges, time to onset of respiratory symptoms had doubled. Although the reasons for this observation are not known, they perhaps involve immunological desensitization. Alternatively, the time to onset of respiratory distress may increase with the age of the animals.

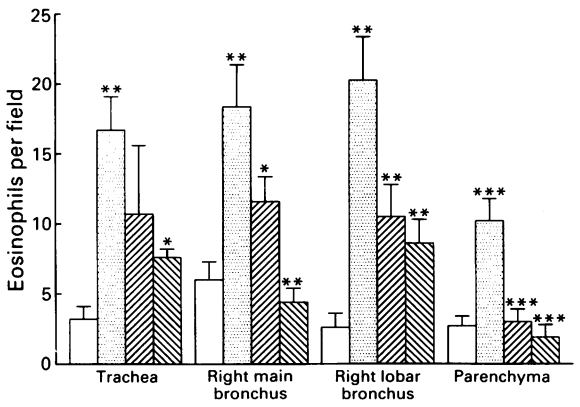

Figure 4 Effects of D-Arg-[Hyp^3,D-Phe^7]-BK (NPC 567) and D-Arg-[Hyp^3,Thi^5,D-Tic^7,Tic^8]-BK (NPC 16731), bradykinin B_2 receptor antagonists, on the numbers of eosinophils present in tissue sections from various airway regions of guinea-pigs. Open columns, control tissues; stippled columns, tissues from animals chronically challenged with ovalbumin (OA); left hatched columns, challenged animals treated with NPC 567; right hatched columns, challenged animals treated with NPC 16731. In all airway regions examined, OA exposures significantly increased tissue eosinophil numbers. NPC 16731 significantly inhibited or abolished OA-induced eosinophilia in all airway regions. NPC 567 had significant effects in main and lobar bronchi, and in parenchyma: $*P<0.05$; $**P<0.01$; $***P<0.001$ (ANOVA). Data are expressed as mean ± s.e.mean (vertical bars) of 6–9 experiments.

Administration of NPC 16731, prior to and simultaneously with inhaled antigen, significantly delayed the onset of respiratory distress in chronically challenged guinea-pigs. While difficult to quantify, these animals rarely exhibited cyanosis and appeared to have a less severe reaction to ovalbumin challenges. Albeit NPC 567 did not significantly affect the onset of antigen-induced dyspnoea, our subjective observations suggested that animals treated with the antagonist also seemed to have a milder reaction to ovalbumin than did untreated controls. The lack of effect of NPC 567 on antigen-induced respiratory distress may be a reflection of its lower potency as a B_2 receptor antagonist than NPC 16731 (Farmer *et al.*, 1991a).

While the mechanisms underlying the ability of NPC 16731 to delay antigen-induced respiratory distress can only be speculated upon, they may reflect the inhibition of airway hyperresponsiveness by B_2 receptor antagonists. As discussed here, chronic challenge with inhaled ovalbumin resulted in airway hyperresponsiveness to ACh. In the single challenge study, guinea-pigs were also hyperresponsive to the bronchoconstrictor action of histamine. Moreover, hyperresponsiveness to ACh and 5-HT has been reported under similar experimental conditions (Daffonchio *et al.*, 1988; 1989). It is feasible then, that the airways were also hyperresponsive to mast cell mediators released endogenously by inhaled antigen. Since BK antagonists inhibited nonspecific airway hyperresponsiveness, the degree of constriction induced by mast cell-derived mediators may have been reduced by NPC 16731, resulting in a delayed and less severe antigen-induced bronchoconstriction.

Antigen-challenge in sensitized guinea-pigs activates the lung kallikrein-kinin system, and releases kinins (Brocklehurst & Lahiri, 1962; Jonasson & Becker, 1966). Similarly, inhalation of allergen in sheep (Abraham *et al.*, 1991b) and allergic asthmatics (Christiansen *et al.*, 1992) dramatically elevates BAL levels of immunoreactive kinins. NPC 567, however, does not inhibit acute antigen-induced bronchoconstriction in sheep (Abraham *et al.*, 1991b) or guinea-pigs (S.G. Farmer, unpublished observation). It is unlikely, therefore, that endogenous kinins, formed in response to antigen challenge, contribute to anaphylactic bronchoconstriction in these species. Rather, our data with NPC 567 and NPC 16731 suggest a role for BK in airway hyperresponsiveness and eosinophilia.

Effects of acute antigen exposure

We have confirmed that, 24 h following single challenge with inhaled ovalbumin, sensitized guinea-pigs exhibited airway hyperresponsiveness, characterized by a left shift in the dose-response curve to i.v. histamine (Seeds *et al.*, 1991). Higher doses could not be employed due to the profound cardiovascular effects of this agonist. Associated with the acute hyperresponsiveness was an increase in the percentages of BAL eosinophil and neutrophil numbers.

Administration of BK antagonists inhibited airway hyperresponsiveness in different ways. Thus, desArg^9-[Leu^8]-BK, a B_1 antagonist, prevented the increase in pulmonary sensitivity (i.e. the left shift of the dose-response curve) to histamine. NPC 349, a B_2 receptor antagonist, prevented the increase in bronchoconstrictor responses to $50\,\mu g\,kg^{-1}$ histamine that occurred after single antigen challenge. However, the magnitude of the latter effect was small, and its 'biological significance' is dubious. The B_2 antagonist prevented antigen-induced increase in both neutrophils and eosinophils, whereas the B_1 antagonist inhibited only the airway neutrophilia. The effect of desArg^9-[Leu^8]-BK on airway supersensitivity and neutrophilia suggest that the two phenomena may be related, and involve endogenous kinins acting on B_1 receptors. Moreover, the small effect of NPC 349 may involve antagonism at B_1 receptors. Although the [D-Phe^7]-substituted analogues of BK, including NPC 349, are usually referred to as 'B_2 antagonists,' they are often nonselective for B_1 and B_2 receptors, probably due to their degradation by carboxypeptidases to their desArg-derivatives (see references in Farmer & Burch, 1991; Ward, 1991).

Although the effects of the BK antagonists on airway granulocyte numbers were statistically significant, they were modest. Moreover, the degree of airway hyperresponsiveness was also modest, being characterized by a less than two fold left shift in the histamine dose-response curve. Although this is considerably less than the magnitude of airway hyperresponsiveness in asthmatic patients (Boushey *et al.*, 1980), it is very similar to the shifts described by other investigators using sensitized guinea-pigs (Daffonchio *et al.*, 1987; 1989). In addition, NPC 567 inhibited airway hyperresponsiveness and neutrophila, induced by inhaled *Ascaris* antigen, in allergic sheep (Solér *et al.*, 1990). Thus, endogenous kinins may play a role in acute airway hyperresponsiveness in these models.

The observation that a B_1 antagonist had effects at all is surprising. DesArg^9-BK, a B_1 agonist, is not a bronchoconstrictor in guinea-pigs (Farmer *et al.*, 1989; Jin *et al.*, 1989), and has no effect on isolated airway smooth muscle (Farmer *et al.*, 1989). In addition, B_1 receptor ligands do not displace [^{3}H]-BK binding in trachea and lung (Farmer *et al.*, 1989; Mak & Barnes, 1991), indicating the absence of B_1 receptors in healthy guinea-pig airways.

However, in rabbit tissues, B_1 receptor expression may be induced by noxious stimuli, possibly as a homeostatic response to inflammation (DeBlois *et al.*, 1991; Farmer *et al.*, 1991b). Inflammatory changes, induced by antigen challenge, may bring about B_1 receptor induction in the airways, but their role in hyperresponsiveness or cell influx remains to be determined.

Effects of chronic antigen exposure on airway responsiveness and eosinophilia

As previously found by Schellenberg's group (Ishida *et al.*, 1989), repeated exposure of sensitized guinea-pigs to a mist of inhaled ovalbumin solution caused airways hyperresponsiveness to i.v. ACh, and eosinophilia. In the present study, administration of the B_2 receptor antagonists, NPC 567 or

NPC 16731, as well as their concomitant inhalation with ovalbumin, abolished hyperresponsiveness and, depending upon the airway region, partially or completely abrogated the infiltration by eosinophils. These data suggest that endogenous BK, formed in response to repeated antigen challenge, is involved in these phenomena. Our results are similar to results with the antiasthmatic drug, nedocromil, which inhibited both airway eosinophilia and hyperresponsiveness, in response to repeated antigen challenge (Schellenberg *et al.*, 1991).

Eosinophils and antigen-induced airway hyperresponsiveness

One interpretation of the data with BK antagonists or nedocromil might be that antigen-induced airway eosinophilia and hyperresponsiveness are interdependent. Nevertheless, other pharmacological interventions have contrasting effects on eosinophilia and hyperresponsiveness. For example, in the same guinea-pig model of repeated antigen challenge a platelet-activating factor (PAF) antagonist, while having no effect on airway eosinophilia, inhibited hyperresponsiveness (Ishida *et al.*, 1990). Similarly, we recently reported that eosinophil infiltration, induced by antigen, can occur at times when bronchial hyperresponsiveness is not evident (Seeds *et al.*, 1991).

Havill and colleagues (1990) also examined the effects of PAF antagonists in guinea-pigs that received three i.p. booster doses of ovalbumin followed, two to three weeks later, by single inhaled challenge. Neither WEB 2086 nor SDZ 64-412 had any effect on airway eosinophil numbers 24 h after antigen challenge, although both drugs abolished hyperresponsiveness. Taken together, these data indicate that PAF may be involved in antigen-induced airway hyperresponsiveness. In contrast, the lack of effect of these agents on eosinophil recruitment suggest that the presence of eosinophils alone is not a prerequisite to the development of allergen-induced airway hyperresponsiveness (Havill *et al.*, 1990; Ishida *et al.*, 1990).

Preteatment of guinea-pigs with capsaicin also differentiates between airway hyperresponsiveness and eosinophilia in response to repeated antigen challenge. Matsuse and co-investigators (1991) recently reported that, in animals depleted of sensory neuropeptides by capsaicin, airway hyperresponsiveness was abolished. However, infiltration of the airways by eosinophils was unaffected. Preliminary data indicate that capsaicin also inhibits airway hyperresponsiveness, but not eosinophilia, after single antigen challenge of sensitized guinea-pigs (Ladenius & Biggs, 1989). Thus, if eosinophils are significant to the development of allergic airway hyperresponsiveness, factors other than simply increasing their number are involved. It is possible that, in the inflammatory microenvironment of the airways, eosinophils trigger the release of neuropeptides, which in turn cause hyperresponsiveness. On the other hand, airway neuropeptides may stimulate the release of an eosinophil-derived factor that induces hyperresponsiveness (Koregel *et al.*, 1990).

Bradykinin and airway hyperresponsiveness

Many physiological effects of BK can be attributed, at least in part, to its ability to release sensory neuropeptides and/or PAF (see Farmer, 1991a,b). Thus, BK stimulates sensory nerve endings, and releases substance P and calcitonin gene-related peptide, in several tissues including those of the airways (Lundberg & Saria, 1983; Saria *et al.*, 1988; Geppetti *et al.*, 1990; Ray *et al.*, 1991). It has also been shown that capsaicin pretreatment markedly reduces the magnitude of bronchoconstriction to tracheal instillation of BK (Ichinose *et al.*, 1990). In addition, in vascular endothelial cells, smooth muscle and fibroblasts, BK and desArg^9-BK both stimulate the synthesis of PAF (Cahill *et al.*, 1988), and BK-induced prostacyclin synthesis is blocked by PAF antagonists (Stewart *et al.*, 1990). Moreover, the prolonged airway microvascular leakage in guinea-pigs, in response to BK, is likewise inhibited by PAF antagonists (Rogers *et al.*, 1990). It is also noteworthy that PAF can release substance P and neurokinin A from guinea-pig isolated, perfused lungs (Martins *et al.*, 1991).

The ability of BK antagonists to inhibit antigen-induced airway hyperresponsiveness in guinea-pigs is in concurrence with other studies. As mentioned, NPC 567 inhibits airway hyperresponsiveness, in addition to generation of several inflammatory mediators, and the late bronchial response following challenge with *Ascaris suum* antigen in sheep (Solér *et al.*, 1990; Abraham *et al.*, 1991b). Furthermore, BK itself was reported to produce airway hyperresponsiveness to ACh in anaesthetized guinea-pigs (Omini *et al.*, 1989) and, also, in perfused cat lungs (Kimura *et al.*, 1989).

In conclusion, our data with BK antagonists in sensitized guinea-pigs, associated with those in allergic sheep (Solér *et al.*, 1989, Abraham *et al.*, 1991b), demonstrate that endogenous kinins may play a pivotal role in the genesis of allergic airway hyperresponsiveness. The additional ability of NPC 567 and NPC 16731 to inhibit antigen-induced eosinophil infiltration also indicates potential antiinflammatory activity of the BK antagonists in guinea-pig airways.

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The pivotal role of tumour necrosis factor α in the development of inflammatory hyperalgesia

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1 The hyperalgesic activities in rats of interleukin-1β (IL-1β), IL-6, IL-8, tumour necrosis factor α (TNFα) and carrageenin were investigated.

2 IL-6 activated the previously delineated IL-1/prostaglandin hyperalgesic pathway but not the IL-8/sympathetic mediated hyperalgesic pathway.

3 TNFα and carrageenin activated both pathways.

4 Antiserum neutralizing endogenous TNFα abolished the response to carrageenin whereas antisera neutralizing endogenous IL-1β, IL-6 and IL-8 each partially inhibited the response.

5 The combination of antisera neutralizing endogenous IL-1β + IL-8 or IL-6 + IL-8 abolished the response to carrageenin.

6 These results show that TNFα has an early and crucial role in the development of inflammatory hyperaglesia.

7 The delineation of the roles of TNFα, IL-1β, IL-6 and IL-8 in the development of inflammatory hyperalgesia taken together with the finding that the production of these cytokines is inhibited by steroidal anti-inflammatory drugs provides a mechanism of action for these drugs in the treatment of inflammatory hyperalgesia.

Keywords: Tumour necrosis factor; interleukin-6; inflammatory hyperalgesia

Introduction

Sensitization of pain receptors causing hyperalgesia is the common denominator of pain of various origins. Two hyperalgesic pathways have been identified in experimental animals and in man; the relative contribution of each pathway may depend upon the characteristics of the injurious stimulus (Ferreira, 1972; Hannington-Kiff, 1974; Coderre *et al.*, 1984; Nakamura & Ferreira, 1987). In both pathways the release of hyperalgesic mediators, e.g. prostaglandins and sympathomimetics, occurs subsequent to the release of the cytokines interleukin-1β (IL-1β) and IL-8, respectively (Cunha *et al.*, 1991). Thus cytokines appear to constitute a link between cellular injury or recognition of non-self and the development of local and systemic manifestations of inflammation, e.g. cell migration, oedema, fever, release of actue-phase proteins and hyperalgesia (Dinarello *et al.*, 1986; Ferreira *et al.*, 1988; Faccioli *et al.*, 1990; Dinarello, 1991; Cunha *et al.*, 1991).

A property of several cytokines is their capacity to induce their own production and that of other cytokines: tumour necrosis factor α (TNFα) induces production of IL-1 (Dinarello *et al.*, 1986) and IL-1 induces production of IL-1 (Dinarello *et al.*, 1987), IL-6 (Van Damme *et al.*, 1987) and IL-8 (Streiter *et al.*, 1989). In the present study we tested TNFα and IL-6 for their capacities to cause sensitization of pain receptors and examined the possibility that TNFα and IL-6 were involved in the IL-1/prostaglandin mediated and the IL-8/sympathetic mediated hyperalgesic pathways activated by the inflammatory agent carrageenin (Ferreira *et al.*, 1988; Cunha *et al.*, 1991).

Methods

Nociceptive test

The intensity of hyperalgesia was assessed by a modification of the Randall-Selitto test (Ferreira *et al.*, 1978). The intensity of hyperalgesia was quantified as the variation in reaction time (delta reaction time) obtained by subtracting the value measured at the time intervals indicated after administration of the hyperalgesic substance from the control reaction time (zero time).

Experimental protocol

Hyperalgesia was measured following injections of IL-6, TNFα, IL-1β and IL-8, and carrageenin into hind paws of rats (intraplantar, i.pl.). The intensity of hyperalgesia was measured before injection, 0.5–6 h and 24 h after injection of IL-6 and TNFα and before and 3 h after injection of IL-1β, IL-8 and carrageenin. Drugs were injected i.pl., 30 min before cytokines. The drugs injected were Lys-D-Pro-Thr (Ferreira *et al.*, 1988), the cyclo-oxygenase inhibitor, indomethacin (Vane, 1971) and the β-adrenoceptor antagonist, atenolol (Robertson *et al.*, 1983). The doses of indomethacin, Lys-D-Pro-Thr and atenolol used have prevously been shown to inhibit hyperalgesia evoked by IL-1β (indomethacin and Lys-D-Pro-Thr; Ferreira *et al.*, 1988; Cunha *et al.*, 1991) and IL-8 (atenolol; Cunha *et al.*, 1991). In other experiments cytokines were incubated with anti-cytokine sera for 15 min before injection of the mixture; anti-cytokine sera were injected (i.pl.) 30 min before carrageenin. Results are presented as means with s.e.mean. Formal statistical tests are not reported since for all the differences discussed, means differed by more than three times the larger s.e.mean.

Materials

Drugs IL-1β, IL-6, IL-8 (72 amino acids) and TNFα were NIBSC preparations coded 86/680, 88/514, 89/520 and 87/650, respectively. Indomethacin (Indo) was a gift from Merck, Sharpe & Dohme Ltd (Hoddesdon, Herts). Carrageenin was a gift from FMC Corporation (Philadelphia, U.S.A.). Atenolol (ATN) was purchased from Sigma (St. Louis, U.S.A.). Lys-D-Pro-Thr was (custom) synthesized by Cambridge Research Biochemicals (Cambridge, England)

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and characterized by the manufacturer by fast atom bombardment mass spectrometry, amino acid analysis and analytical reverse-phase high performance liquid chromatography (h.p.l.c.). The peptide was purified to $\geqslant 95\%$ by preparative h.p.l.c. at NIBSC.

Antisera The following antisera were used: sheep anti-human IL-1β (Poole *et al.*, 1989); sheep anti-rat IL-1β (Bristow *et al.*, 1991); goat anti-human IL-6 (Rafferty *et al.*, 1991); rabbit anti-rat IL-6 (Rothwell *et al.*, 1991), a generous gift from Professor J. Gauldie (McMaster University, Hamilton, Ontario, Canada); sheep anti-human IL-8 (Cunha *et al.*, 1991), kindly provided by Dr R. Thorpe, Division of Immunobiology, NIBSC; goat anti-human TNFα and sheep anti-murine TNFα (Mahadevan *et al.*, 1990), kindly provided by Dr T. Meager, Division of Immunobiology, NIBSC.

Animals Male Wistar rats, 130–180 g, housed in temperature controlled-rooms (23 ± 2°C) with water and food *ad libitum* until use.

Results

Time courses of hyperalgesic responses to IL-6 and TNFα

Injection of IL-6 or TNFα into one hindpaw (i.pl.) evoked a dose-dependent hyperalgesia in both hind paws although the effects of smaller doses were greatest in the injected paws (Figure 1). The intensity of hyperalgesia reached a plateau between 2–3 h after injection of IL-6 or TNFα; the responses to IL-6 were maintained at 6 h whereas responses to TNFα had begun to decline at 6 h after injection. Responses to both cytokines returned to pre-injection values within 24 h.

Inhibition by drugs of the hyperalgesic responses to IL-1β, IL-6, IL-8 and TNFα

Injection of IL-1β (0.5 pg) and IL-8 (100 pg) evoked hyperalgesic responses of similar magnitude to those evoked by IL-6 (1 ng) and TNFα (2.5 pg, Figure 2). Data are shown for injected paws only. Similar responses were obtained in contralateral paws as can be seen from Figure 1 for IL-6 and TNFα and in the study of Cunha *et al.* (1991) for IL-1β and IL-8. Local pretreatment (i.pl.) with indomethacin (Indo, 100 μg) or Lys-D-Pro-Thr (200 μg), 30 min before the cytokine, abolished responses to IL-1β (−91 ± 3% after Indo, −92 ± 1% after Lys-D-Pro-Thr), markedly attenuated responses to IL-6 (−74 ± 1% and −78 ± 3%) and attenuated responses to TNFα (−47 ± 3% and −50 ± 3%) but did not affect responses to IL-8 (+2 ± 4% and 0 ± 1%, Figure 2). In contrast, local pretreatment with atenolol (25 μg) markedly attenuated responses to IL-8 (−68 ± 2%) and TNFα (−66 ± 3%) but not responses to IL-1β (−6 ± 2%) and IL-6 (−3 ± 2%, Figure 2). The inhibitory effects on TNFα evoked hyperalgesia of pretreatment with indomethacin together with Lys-D-Pro-Thr were not additive (−51 ± 4%). In con-

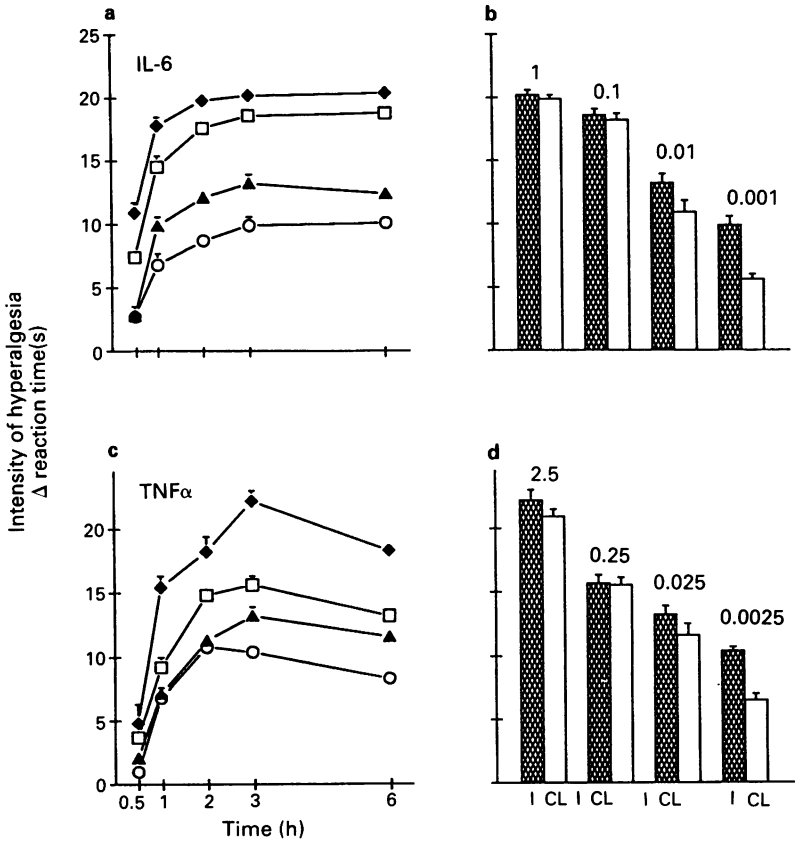

Figure 1 Time course of the development of hyperalgesia to interleukin-6 (IL-6) and tumour necrosis factor α (TNFα) injected into rat paws. Panel (a) shows the intensity of hyperalgesia in injected paws 0.5–6 h after injection of IL-6 (0.001 (○); 0.01 (▲); 0.1 (□) and 1.0 (◆) ng in 100 μl i.pl.). Panel (b) shows the intensity of hyperalgesia in injected paws (I, cross-hatched columns) and in contralateral paws (CL, open columns) 3 h after injection of IL-6 (at the doses indicated above the columns, ng/paw). Panel (c) shows the intensity of hyperalgesia in injected paws 0.5–6 h after injection of TNFα (0.0025 (○); 0.025 (▲); 0.25 (□) and 2.5 (◆) pg in 100 μl i.pl.). Panel (d) shows the intensity of hyperalgesia in injected paws (I, cross-hatched columns) and in contralateral paws (CL, open columns) 3 h after injection of TNFα (at the doses indicated above the columns, pg/paw). Vertical bars are s.e.mean in groups of 5 rats.

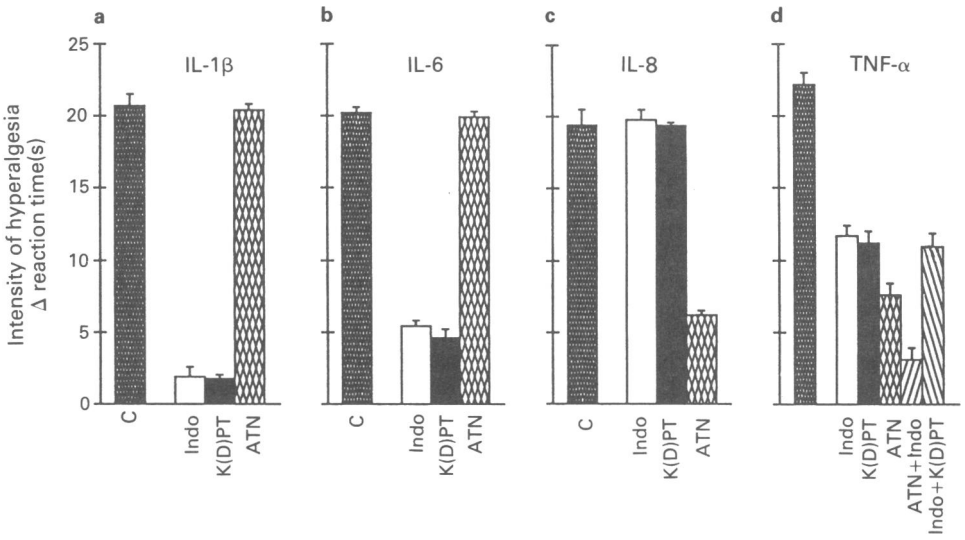

Figure 2 Inhibition of the hyperalgesic effects of (a) interleukin-1β (IL-1β), (b) IL-6, (c) IL-8 and (d) tumour necrosis factor α (TNFα). Responses were measured 3 h after injection (in 100 μl, i.pl.) of (a) IL-1β (0.5 pg); (b) IL-6 (1 ng); (c) IL-8 (100 pg) and (d) TNFα (2.5 pg). Pretreatments were given (100 μl, i.pl.) 30 min before cytokines: C = saline, Indo = indomethacin, 100 μg; K(D)PT = Lys-D-Pro-Thr, 200 μg; ATN = atenolol, 25 μg. Vertical bars are s.e.mean in groups of 5 rats.

trast, the effects of indomethacin together with atenolol were additive: this combination abolished TNFα-evoked hyperalgesia (−86 ± 4%, Figure 2d).

Indomethacin, Lys-D-Pro-Thr and atenolol injected alone were without effect in injected and contralateral paws and had no effect on responses to cytokines measured in contralateral paws: data not shown but published previously (Ferreira *et al.*, 1988; Cunha *et al.*, 1991).

Inhibition by anti-cytokine sera of the hyperalgesic responses to IL-1β, IL-6, IL-8 and TNFα

The hyperalgesic responses to human IL-1β were abolished when it was incubated (for 15 min) and injected with sheep anti-human IL-1β serum but not by incubation and injection with other antisera (Figure 3a). Hyperalgesic responses to human IL-6 were abolished by incubation and injection with goat anti-human IL-6 serum or sheep anti-rat IL-1β serum but not with other antisera (Figure 3b). Hyperalgesic responses to human IL-8 were abolished by incubation with sheep anti-human IL-8 serum but not with other antisera (Figure 3c). Hyperalgesic responses to human TNFα were abolished by incubation and injection with goat anti-human TNFα serum but not with preimmune serum. Antisera neutralising rat IL-1β, IL-6 and IL-8 each attenuated responses to human TNFα (−59 ± 3%, −61 ± 4%, −67 ± 5%). The inhibitory effects on TNFα evoked hyperalgesia of antisera neutralising rat IL-1β and IL-6 were not additive (−58 ± 2%); in contrast, the inhibitory effects of antisera neutralizing rat IL-1β and IL-8 were additive (−89 ± 3%), as were those of antisera neutralizing rat IL-6 and IL-8 (−87 ± 2%, Figure 3d).

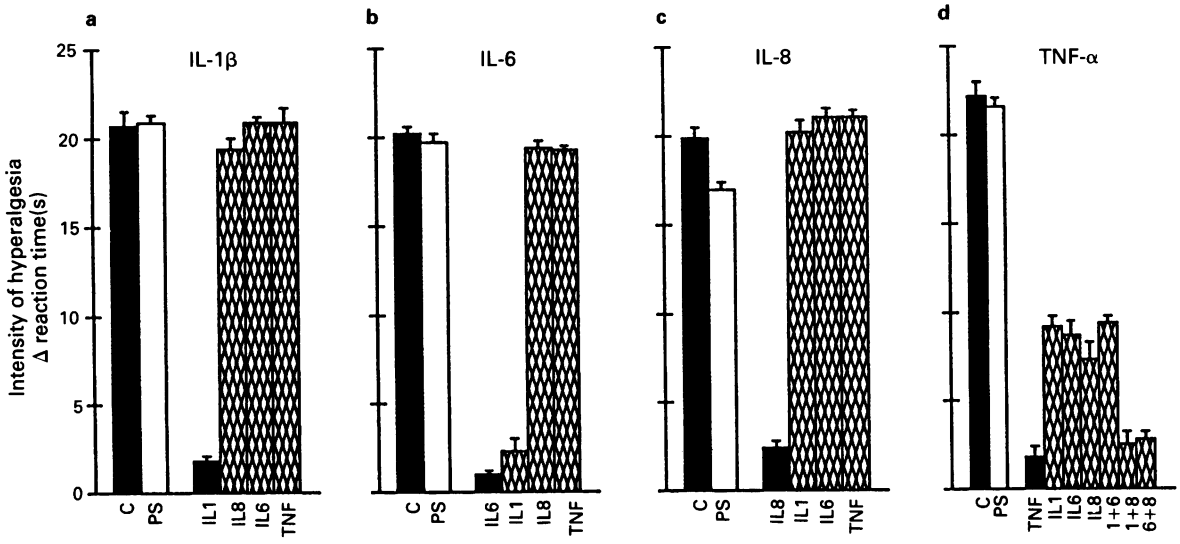

Figure 3 Effects of various antisera on the hyperalgesic effects of (a) interleukin-1β (IL-1β), (b) IL-6, (c) IL-8 and (d) tumour necrosis factor α (TNFα). Responses were measured 3 h after injection (in 100 μl, i.pl.) of (a) IL-1β (0.5 pg); (b) IL-6 (1 ng); (c) IL-8 (100 pg) and (d) TNFα (2.5 pg). Antisera neutralizing the cytokines indicated on the x-axis (100 μl) were incubated for 15 min with the cytokine before injection. Vertical bars are s.e.mean in groups of 5 rats. C = control; PS = pre-immune serum.

Inhibition by anti-cytokine-sera of the hyperalgesic responses to carrageenin

Carrageenin-evoked hyperalgesia was inhibited by pretreatment with sheep anti-rat IL-1β serum (50 μl: −47 ± 5% and 150 μl: −57 ± 3%) and with rabbit anti-rat IL-6 serum (50 μl: −44 ± 4% and 150 μl: −56 ± 2%); the effects of the antisera (50 μl of each: −43 ± 2%) were not additive (Figure 4). Carrageenin-evoked hyperalgesia was also inhibited by a serum neutralizing rat IL-8 (sheep anti-human IL-8 serum, 50 μl: −64 ± 3% and 150 μl: −62 ± 2%). The inhibitory effects of the anti-IL-8 serum (50 μl) were additive with the inhibitory effects of sheep anti-rat IL-1β serum (50 μl: −93 ± 1%) and with rabbit anti-rat IL-6 serum (50 μl: −90 ± 2%, Figure 4). Carrageenin-evoked hyperalgesia was inhibited by a serum neutralising rat TNFα (sheep anti-murine TNFα, 50 μl: −66 ± 3% and 150 μl: −90 ± 2%) and the inhibitory effects of the smaller dose of anti-TNFα serum (50 μl) were additive with the inhibitory effects of each of the three antisera (50 μl) neutralising rat IL-1β (−94 ± 2%), IL-6 (−88 ± 4%) and IL-8 (−84 ± 2%, Figure 4).

Antisera injected alone were without effect in injected and contralateral paws and had no effect on responses to cytokines and carrageenin measured in contralateral paws: data not shown.

Discussion

TNFα and IL-6 have been shown to cause dose-dependent hyperalgesia in rats, like IL-1β (Ferreira *et al.*, 1988) and IL-8 (Cunha *et al.*, 1991). All four cytokines evoked hyperalgesia in both hind paws when injected into one hind paw, suggesting systemic distribution of the injected cytokines. This notion is supported by the findings that smaller doses of cytokines evoked consistently smaller effects in contralateral paws and that intraplantar administration of antagonists and antisera inhibited local hyperalgesia but not hyperalgesia in contralateral paws. Also, the onset of hyperalgesia was slower in contralateral paws and it was possible to restrict hyperalgesic responses to one of the cytokines, IL-1β, to the injected paws by repeated injections of very small doses (Ferreira: unpublished data).

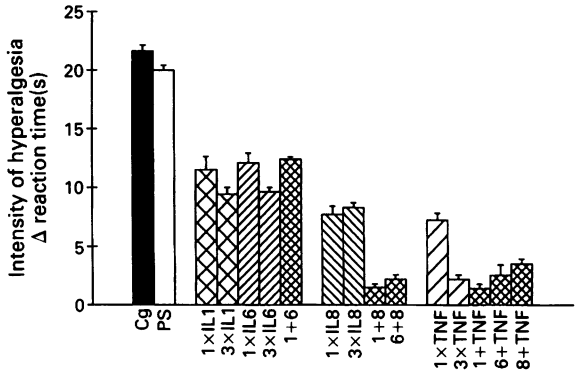

Figure 4 Inhibition of the hyperalgesic effects of carrageenin by antisera neutralising endogenous, i.e. rat, cytokines. Responses were measured 3 h after injection of carrageenin (100 μg in 50 μl, i.pl.). Antisera were injected (50 μl = 1 × and 150 μl = 3 × in a total volume of 150 μl) 30 min before carrageenin. Cg = carrageenin; PS = preimmune serum; 1 × IL1 = 50 μl sheep anti-rat IL-1β serum; 3 × IL1 = 150 μl sheep anti-rat IL-1β serum; 1 × IL6 = 50 μl rabbit anti-rat IL-6 serum; 3 × IL6 = 150 μl rabbit anti-rat IL-6 serum; 1 + 6 = 50 μl sheep anti-rat IL-1β serum + 50 μl rabbit anti-rat IL-6 serum; 1 × IL8 = 50 μl sheep anti-human IL-8 serum; 3 × IL8 = 150 μl sheep anti-human IL-8 serum; 1 + 8 = 50 μl sheep anti-rat IL-1β serum + 50 μl sheep anti-human IL-8 serum; 6 + 8 = 50 μl rabbit anti-rat IL-6 serum + 50 μl sheep anti-human IL-8 serum: 1 × TNF = 50 μl sheep anti-murine TNFα serum; 3 × TNF = 150 μl sheep anti-murine TNFα serum; 1 + TNF = 50 μl sheep anti-rat IL-1β serum + 50 μl sheep anti-murine TNFα serum; 6 + TNF = 50 μl rabbit anti-rat IL-6 serum + 50 μl sheep anti-murine TNFα serum. 8 + TNF = 50 μl sheep anti-human IL-8 serum + 50 μl sheep anti-murine TNFα serum. Vertical bars are s.e.mean in groups of 5 rats.

The doses of the four cytokines that evoked maximum hyperalgesic effects were: IL-1β (0.5 pg), TNFα (2.5 pg), IL-8 (100 pg) and IL-6 (1 ng), giving an order of potency of IL-1β>TNFα>>IL-8>>IL-6 for these human sequence cytokines in rats. Whether this order of potency reflects the order of potency of the endogenous cytokines of the rat is unknown. The rat sequence cytokines were not available for this study and certain cytokines are known to exhibit species preference or specificity (Lumpkin, 1987; Morstyn & Burgess, 1988).

Antagonism of IL-1β-evoked hyperalgesia by indomethacin and Lys-D-Pro-Thr and antagonism of IL-8 evoked hyperalgesia by antenolol confirm previous results that hyperalgesia evoked by IL-1β and IL-8 are effected via prostaglandin-mediated and sympathetic mediated pathways, respectively (Ferreira *et al.*, 1988; Cunha *et al.*, 1991). The sensitivity of IL-6-evoked hyperalgesia to blockade by indomethacin suggests that this response, like that to IL-1β, was mediated by cyclo-oxygenase products, e.g. prostaglandins. The sensitivity of responses to IL-6 to blockade by the IL-1β related peptide Lys-D-Pro-Thr and sheep anti-rat IL-1β serum suggests that IL-6 was causing hyperalgesia via a pathway common with IL-1β. In this pathway it would appear that IL-6 induced production of IL-1β. Although the converse, i.e. IL-1 induced production of IL-6, occurs (Van Damme *et al.*, 1987) we were unable to demonstrate IL-6 induced production of IL-1β *in vitro* in human blood mononuclear cells (Poole: unpublished data); also, IL-6 suppressed endotoxin-induced and TNF-induced IL-1 production in these cells (Schindler *et al.*, 1990). However, it is possible that *in vivo*, in the presence of the full repertoire of cells and mediators involved in inflammatory hyperalgesia, IL-6 has the capacity to induce IL-1 production. This possibilty was suggested recently by Rothwell (1991). Further experiments will be required to elucidate the precise sequence of events in the IL-1β/IL-6/prostaglandins hyperalgesic pathway.

TNFα evoked hyperalgesia by activation of both the IL-1β/IL-6/prostaglandin and IL-8/sympathetic mediated hyperalgesic pathways since local administration of indomethacin, Lys-D-Pro-Thr and atenolol all attenuated responses to TNFα while the combination of indomethacin and atenolol abolished responses to this cytokine. Further evidence for the involvement of both hyperalgesic pathways in the mediation of response to TNFα comes from the finding that antisera neutralizing rat IL-1β, IL-6 and IL-8 each attenuated responses to TNFα. The inhibitory effects of antisera neutralizing rat IL-1β and IL-8 were additive as were those of antisera neutralizing rat IL-6 and IL-8.

Antagonism of carrageenin-evoked hyperalgesia by indomethacin, Lys-D-Pro-Thr, atenolol and antiserum neutralizing rat IL-8 confirms previous results showing that carageenin-evoked hyperalgesia is mediated via the IL-1β/IL-6/prostaglandins and the IL-8/sympathetic pathways (Ferreira *et al.*, 1988; Cunha *et al.*, 1991). The inhibition of responses to carrageenin by antisera neutralizing rat IL-1β and IL-6 and the abolition of responses to carrageenin by antisera neutralizing rat TNFα are new findings, as are the results of experiments in which combinations of antisera were used. The lack of additive inhibitory effects against carrageenin of antisera neutralizing rat IL-1β and IL-6 supports the notion that IL-6 was causing hyperalgesia via a pathway common with IL-1β. The additive effects of antisera neutralizing rat IL-8 with antisera neutralizing rat IL-1β and IL-6 supports previous work indicating the involvement of the IL-1β/prostaglandins and IL-8/sympathetic pathways in carrageenin evoked hyperalgesia (Cunha *et al.*, 1991). The capacity of the larger dose of antiserum neutralising rat TNFα to abolish carrageenin-evoked hyperalgesia and the finding that the smaller dose of this antiserum given together with antisera

neutralizing rat IL-1β, IL-6 or IL-8 also abolished responses to carrageenin suggest that TNFα plays an early and crucial role in the development of hyperalgesia in response to carrageenin. This role is consistent with the early role of TNFα in the development of fever in response to bacterial endotoxin (Dinarello *et al.*, 1986; Rothwell, 1991).

The above experiments show that TNFα activates a cascade of cytokine release. The induction of IL-8 by TNFα leads to the development of sympathetic hyperalgesia. The induction of IL-1β and IL-6 by TNFα leads to the development of hyperalgesia mediated by cyclo-oxygenase products. In inflammatory hyperalgesia evoked by carrageenin, TNFα has a pivotal role since a single injection of this cytokine mimicked the response to carrageenin by inducing production of IL-1β, IL-6 and IL-8 and a single injection of antiserum neutralizing endogenous TNFα abolished the response to carrageenin.

The delineation of the roles of IL-1, IL-6, IL-8 and TNFα in the development of inflammatory hyperalgesia (Ferreira *et al.*, 1988; Cunha *et al.*, 1991; and in the present study) adds to our understanding of the mechanisms underlying the potent anti-inflammatory effects of glucocorticoid drugs. It has been known for many years that this class of drugs inhibit both the early and late changes that contribute to the inflammatory process. A proportion of this anti-inflammatory activity can be accounted for by the inhibition by glucocorticoids of production of prostaglandins, leukotrienes, thromboxanes and related mediators (Blackwell *et al.*, 1980; Hirata *et al.*, 1980). The more recent findings that steroidal anti-inflammatory drugs inhibit production of cytokines (Lew *et al.*, 1988; Waage & Baake, 1988; Barton *et al.*, 1991; Seitz *et al.*, 1991) provide an additional mechanism of action for glucocorticoids in the treatment of inflammatory hyperalgesia.

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Modification by hypoxia, hyperkalaemia and acidosis of the cardiac electrophysiological effects of a range of antiarrhythmic drugs

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1 The electrophysiological effects of a series of drugs with Class I antiarrhythmic activity were examined in sheep Purkinje fibres, superfused *in vitro* with either a normal or hypoxic, hyperkalaemic and acidotic physiological salt solution (PSS).

2 In normal sheep Purkinje fibres, lignocaine, disopyramide, nicainoprol and propranolol all significantly reduced action potential height and the maximum rate of depolarization of phase zero (MRD) and abbreviated the action potential, without modifying resting membrane potential (RMP).

3 Verapamil at the highest concentration studied, 8 μM, significantly reduced MRD with an associated slight membrane depolarization and abbreviated action potential duration measured at 50% repolarization (APD_{50}).

4 Superfusion of sheep Purkinje fibres with a hypoxic, hyperkalaemic and acidotic PSS resulted in marked reductions in resting membrane potential, upstroke and duration of the action potential.

5 In the presence of modified PSS, lignocaine, propranolol and verapamil all reduced MRD to a greater extent than in normal PSS. The effects of nicainoprol on MRD were not affected whereas those of disopyramide were significantly attenuated.

6 Under simulated ischaemic conditions, lignocaine, propranolol and nicainoprol did not produce a concentration-dependent reduction in action potential duration whereas disopyramide and verapamil, respectively, prolonged and abbreviated both APD_{50} and APD_{90}.

7 The Na^+ channel blocking actions of the different subtypes of Class I antiarrhythmic agents studied, as well as their effects on action potential duration, were modified differently by simulated ischaemia.

Keywords: Simulated ischaemia; cardiac electrophysiology; antiarrhythmic drugs

Introduction

Class I antiarrhythmic drugs, i.e. those that act principally by blocking Na^+ channels have been subdivided on the basis of their clinical electrophysiological effects (Harrison, 1985) and their effects *in vitro* on the kinetics of onset and offset interaction with the sodium channel (Campbell, 1983a) into 3 subgroups. Disopyramide (Campbell, 1983b), lignocaine (Bean *et al.*, 1983) and nicainoprol (Kimura *et al.*, 1989), a recently introduced antiarrhythmic drug, are examples of Class Ia, Ib and Ic agents, respectively. The sodium channel blocking activity of lignocaine is markedly potentiated by conditions that simulate the acute phase of myocardial ischaemia, i.e. hypoxia, hyperkalaemia and acidosis (Kimura *et al.*, 1982; Evans *et al.*, 1984). However, no studies have been carried out to compare how simulated ischaemia modifies the effects of each of the subtypes of Class I antiarrhythmic drugs. The aim of this study, therefore, was to compare the electrophysiological effects on sheep Purkinje fibres, of disopyramide, lignocaine, and nicainoprol, as examples of drugs of the Ia, Ib and Ic subgroups, under normal and simulated ischaemic conditions. In addition, the effects of other antiarrhythmic drugs that may in addition to their other pharmacological effects also block Na^+ channels, i.e. propranolol and verapamil, were also investigated. Propranolol was studied because its effects during simulated myocardial ischaemia have not been described and verapamil because conflicting results have been obtained with respect to its action under ischaemic conditions (Dersham & Han, 1981; Northover, 1987).

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Methods

Action potential recording

Sheep hearts were obtained from a local abattoir and delivered in physiological salt solution (PSS) to the laboratory within 30 min of excision. Purkinje fibres were pinned to the silastic base of the recording chamber and superfused at a rate of 5 ml min^{-1} with a normal PSS equilibrated with 95% O_2/5% CO_2. An equilibrium period of about 1 h in normal PSS was allowed. The composition of the control PSS was as follows (mM): NaCl 125, $NaHCO_3$ 25, NaH_2PO_4 1.2, $MgCl_2$ 1.0, KCl 5.4, $CaCl_2$ 1.8 and glucose 5.5. Acute ischaemia was simulated by a hypoxic, hyperkalaemic and acidotic PSS of the following composition (mM): NaCl 141.5, $NaHCO_3$ 8.5, NaH_2PO_4 1.2, $MgCl_2$ 1.0, KCl 8.0, $CaCl_2$ 1.8 and glucose 5.5. This modified PSS was gassed with 95% N_2/5% CO_2 which yielded a PO_2 and a pH in the organ bath of 33.9 ± 1 mmHg and 6.8 ± 0.01 u, respectively.

The preparations were stimulated at a frequency of 1.5 Hz by rectangular pulses, 1 ms in duration and twice threshold voltage, delivered through a bipolar silver electrode. Transmembrane action potentials were recorded by conventional microelectrode techniques. The variables measured were as follows: resting membrane potential (RMP); action potential amplitude (APA); the maximum rate of depolarization of phase 0 (MRD), which was determined by an electronic differentiating circuit; and the action potential duration at 50 and 90% repolarization levels (APD_{50} and APD_{90}).

Experimental protocol

To observe the drug effects on normal preparations, 8–10 consecutive action potentials were recorded before and

30–40 min after cumulative addition of the drug, dissolved in reservoirs of gassed normal PSS to obtain the required final bath concentrations. In a different set of fibres, the effects of the modified PSS alone was measured; action potentials were recorded before and at 30, 60, 90 and 120 min following exposure to the modified PSS. Drug-induced effects in the presence of the modified PSS were examined by recording action potentials before, and 30 min after, superfusion with modified PSS alone and subsequently following the administration of three cumulative concentrations of the drug dissolved in modified PSS. Action potentials were again recorded 30 min after the addition of each concentration of the drug.

Analaysis of data

The measurements from each set of 8 to 10 action potentials were meaned and the mean values used to represent the data from each preparation. Data are given either as mean values ± s.e. mean or mean percentage change from control values ± s.e. mean and are derived from four to eight experiments. Drug effects in the presence of the modified PSS were calculated as a % change from the values at 30 min superfusion with modified PSS alone. Multiple treatment and control mean values were analysed by a one-way analysis of variance for repeated measures and, where the F value permitted further analysis, individual treatment means were compared with respective control values by a modified Student's t test. For all other comparisons a two-tailed Student's t test was carried out. A value of $P<0.05$ was considered to be statistically significant.

In order to assess the degree of potentiation of drug effects by simulated ischaemia on MRD, log concentration vs % reduction in the maximum response curves were drawn in the presence and absence of simulated ischaemia and the EC_{30}, i.e. the molar concentrations that produced a 30% reduction in the maximum response for MRD calculated. The EC_{30} rather than the EC_{50} was used because in some cases the log concentration-response curves were incomplete.

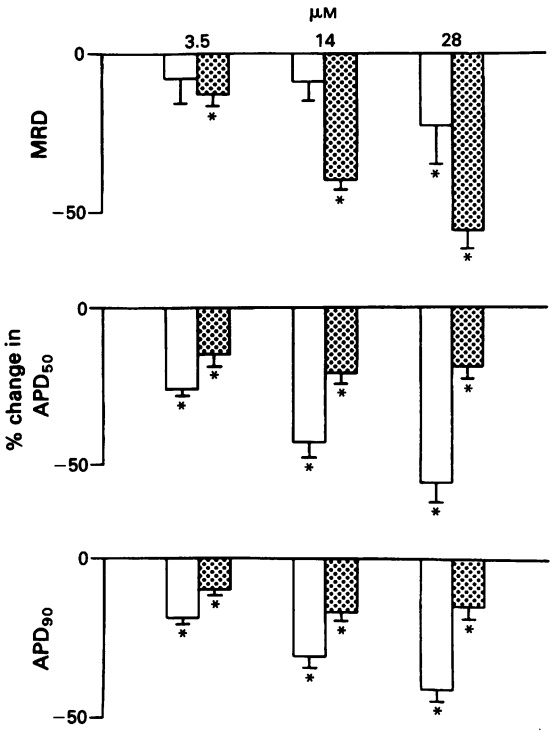

Figure 1 The % reduction in maximum rate of depolarization of the upstroke (MRD), action potential duration measured at 50% (APD_{50}) and 90% of repolarization (APD_{90}) induced by lignocaine (3.5–28 μM) in sheep Purkinje fibres superfused with normal (open columns) and modified (stippled columns) physiological salt solution. Control values for MRD, APD_{50} and APD_{90} were 523 ± 46 Vs^{-1}, 132.6 ± 13.4 and 206.9 ± 11 ms in normal fibres and 383 ± 41 Vs^{-1}, 114.5 ± 8.6 and 182.5 ± 8.5 ms in fibres after 30 min exposure to simulated ischaemia, respectively.
*$P<0.05$ indicates a significant difference from zero. $n = 5–6$.

Results

Effect of hypoxia, hyperkalaemia, and acidosis

The effects of the combination of hypoxia, hyperkalaemia and acidosis on the action potential variables are given in Table 1. Hypoxia, hyperkalaemia and acidosis caused a fall in RMP, MRD and action potential amplitude, and markedly abbreviated the action potential. The changes are stable over the 30–120 min exposure period, i.e. the time period over which the action of the drug was measured.

Effects of lignocaine

Figure 1 illustrates the effects of lignocaine on MRD, APD_{50} and APD_{90} of paced sheep Purkinje fibres under normal and hypoxic, hyperkalaemic and acidotic conditions. On normal Purkinje fibres, only the two higher concentrations of lignocaine, i.e. 28 and 56 μM (data not shown for 56 μM), significantly reduced MRD and the action potential amplitude without modifying the resting membrane potential. In contrast, in the presence of hypoxia, hyperkalaemia and acidosis, lignocaine caused a concentration-dependent reduction in MRD over the full concentration-range studied (3.5 to 28 μM) and this also occurred without modification of the resting membrane potential. This effect of lignocaine on MRD was greater under simulated ischaemia than in normal conditions. The log concentration vs % reduction in the maximum response curve was shifted to the left and the ratio of EC_{30}'s in the absence and the presence of simulated ischaemia was 4.2, i.e. lignocaine was about 4 times as potent in reducing MRD in fibres exposed to hypoxia, hyperkalaemia and acidosis as in normal fibres.

Table 1 The effects of a modified (i.e. hypoxic, hyperkalaemic, and acidotic) salt solution on sheep Purkinje fibre action potential characteristics

Time (min)	*RMP* (mV)	*MRD* (Vs^{-1})	*APA* (mV)	*APD_{50}* (ms)	*APD_{90}* (ms)
0	88.3 ± 1.2	486 ± 27	121.2 ± 2.3	162.1 ± 8.0	264.6 ± 13.7
15	75.2 ± 2.5*	332 ± 36*	96.2 ± 4.1*	119 ± 6.4*	190.8 ± 9.2*
30	74.2 ± 1.0*	290 ± 26*	88 ± 3.3*	95.8 ± 6.2*	166.8 ± 3.9*
60	73.7 ± 1.4*	325 ± 21*	90.4 ± 2.4*	86.3 ± 8.3*	160.0 ± 4.5*
90	73.0 ± 1.5*	336 ± 30*	90.6 ± 2.6*	86.7 ± 8.0*	161.3 ± 5.0*
120	74.6 ± 0.7*	323 ± 30*	90.9 ± 1.7*	86.2 ± 8.6*	163.9 ± 5.2*

$n = 6$; *$P<0.05$ significantly different from values at 0 min. Between 30 and 120 min post simulated ischaemia there were no statistically significant differences in the variables measured.

On normal fibres, lignocaine reduced APD_{50} and APD_{90} at concentrations lower than those required to reduce MRD (Figure 1). In the presence of hypoxia, hyperkalaemia and acidosis, however, although all concentrations of lignocaine studied reduced APD_{50} and APD_{90}, no concentration-dependent effect on action potential duration was observed.

Effects of disopyramide

Figure 2 compares the effects of disopyramide (2.95–23.6 μM) under normal and simulated ischaemic conditions on MRD, APD_{50} and APD_{90}. On normal fibres, these concentrations of disopyramide caused a concentration-dependent reduction in MRD without altering the resting membrane potential. In contrast to lignocaine, this depressant effect on MRD was not potentiated by simulated ischaemia and indeed at the two lower concentrations studied (2.95 and 11.5 μM), the % reduction in MRD was significantly less ($P<0.05$) in the presence of hypoxia, hyperkalaemia and acidosis than that seen in normal fibres.

Disopyramide also caused a concentration-dependent reduction in action potential duration in normal fibres, but under simulated ischaemic conditions the only statistically significant change was a prolongation of APD_{90} observed with the highest concentration studied i.e., 23.6 μM.

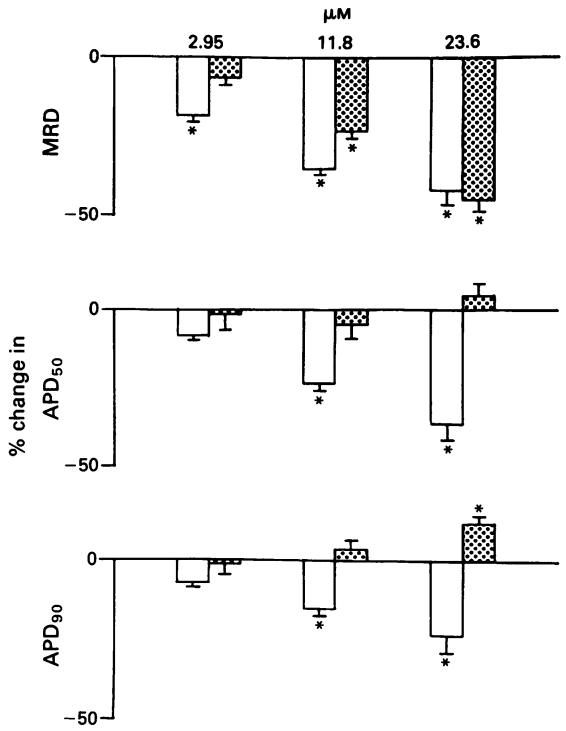

Figure 2 The % reduction in maximum rate of depolarization of the upstroke (MRD), action potential duration measured at 50% (APD_{50}) and 90% of repolarization (APD_{90}) induced by disopyramide (2.95–23.6 μM) in sheep Purkinje fibres superfused with normal (open columns) and modified (stippled columns) physiological salt solution. Control values for MRD, APD_{50} and APD_{90} were 635 ± 21 Vs^{-1}, 213 ± 19 and 320 ± 15 ms in normal fibres and 417 ± 26 Vs^{-1}, 106.8 ± 6.1 and 167.5 ± 7.0 ms in fibres after 30 min exposure to simulated ischaemia, respectively.
*$P<0.05$ indicates a significant difference from zero. $n=4-5$.

Effects of nicainoprol

The effects of nicainoprol on action potential characteristics of normal fibres and those exposed to hypoxia, hyperkalaemia and acidosis are given in Tables 2 and 3, respectively. The % changes in MRD, APD_{50} and APD_{90} induced by the drug under both sets of conditions are compared in Figure 3. Nicainoprol also reduced MRD and action potential amplitude without changing the resting membrane potential both under normal and simulated ischaemic conditions. Although simulated ischaemia tended to increase the depressant effect of nicainoprol on the upstroke velocity of the action potential, the difference between its effects on MRD under normal and simulated ischaemic conditions just failed to reach statistical significance. The dose-ratio for the EC_{30}'s in the presence and absence of simulated ischaemia was 2.2.

Like lignocaine, nicainoprol abbreviated the APD_{50} of normal fibres at a concentration (1 μM) lower than that which depressed MRD. In the presence of hypoxia, hyperkalaemia and acidosis, the reduction in action potential duration was significantly less than that observed in normal fibres, e.g. at a concentration of 5 μM, APD_{50} was reduced from 171.2 ± 13.1 to 88.0 ± 15.3 ms in normal fibres and from 144.7 ± 4.7 to 110.9 ± 5.4 ms in the presence of simulated ischaemia.

Effects of propranolol

Figure 4 illustrates the effects of propranolol (0.35–2.8 μM) on the upstroke velocity and duration of the action potential under normal and hypoxic, hyperkalaemic and acidotic conditions. Like lignocaine, propranolol caused a reduction in MRD and the log concentration vs % maximum response curve was shifted to the left in the presence of simulated ischaemia. The ratio of the EC_{30}'s in the absence and presence of simulated ischaemia was 5.3. Also like lignocaine, propranolol caused an abbreviation of the action potential,

Table 2 Effects of nicainoprol (0.1–10 μM) on the action potential characteristics of normal sheep Purkinje fibres paced at 1.5 Hz

		Nicainoprol (μM)			
	Control	*0.1*	*1*	*5*	*10*
RMP (mV)	− 84.0 ± 1.3	− 84.3 ± 1.3	− 85.5 ± 1.4	− 85.0 ± 0.9	− 84.9 ± 1.2
MRD (Vs^{-1})	476 ± 22	494 ± 36	444 ± 37	359 ± 36*	271 ± 32*
APA (mV)	114.7 ± 1.7	114.1 ± 1.5	112.7 ± 2.5	103.5 ± 2.4*	91.6 ± 2.2*
APD_{50} (ms)	171.2 ± 13.1	162.5 ± 14.4	138.0 ± 14.4*	88.0 ± 5.3*	68.1 ± 12.3*
APD (ms)	252.5 ± 17.4	249.4 ± 19.9	229.3 ± 17.3	180.3 ± 13.3*	156.9 ± 8.3*

$n=5$; *$P<0.05$ significantly different from control value.
Abbreviations are RMP: resting membrane potential; MRD: maximum rate of depolarization of phase 0; APA: action potential amplitude; APD_{50} and APD_{90}: action potential duration measured at 50 and 90% repolarization respectively.

Table 3 Effects of nicainoprol (0.1 ± 5 μM) on sheep Purkinje action potential characteristics during simulated ischaemia (i.e. under conditions of hyperkalaemia, hypoxia and acidosis)

	Normal control	*Ischaemia alone*	*Ischaemia + nicainoprol (μM)* 0.1	1	5
RMP (mV)	− 86.3 ± 0.8	− 78.9 ± 0.4*	− 79.0 ± 0.6*	− 79.9 ± 0.4*	− 78.2 ± 90.5*
MRD (Vs^{-1})	467 ± 19	238 ± 21*	225 ± 22*	185 ± 19*,†	157 ± 21*,†
APA (mV)	117.1 ± 1.5	88.9 ± 2.7*	87.7 ± 2.6*	83.6 ± 3.2*,†	80.0 ± 3.1*,†
APD_{50} (ms)	197.2 ± 8.1	144.7 ± 4.9*	132.6 ± 4.5*,†	123.4 ± 5.4*,†	110.9 ± 5.4*,†
APD_{90} (ms)	279.5 ± 12.2	205.9 ± 5.8*	197.4 ± 6.6*	192.3 ± 8.4*,†	186.5 ± 8.1*,†

$n = 6$; *,† $P < 0.05$ significantly different from normal control value and from ischaemic control value respectively.
Abbreviations are RMP: resting membrane potential; MRD: maximum rate of depolarization of phase 0; APA: action potential amplitude; APD_{50} and APD_{90} - action potential duration measured at 50 and 90% repolarization respectively.

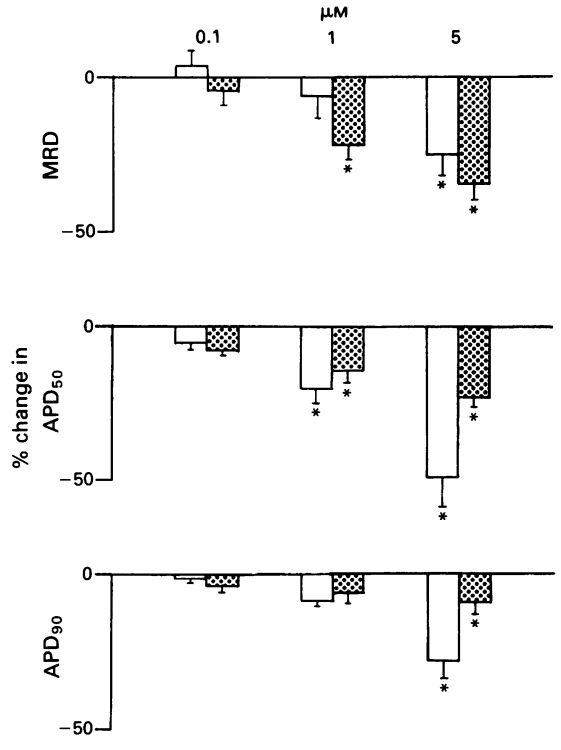

Figure 3 The % reduction in maximum rate of depolarization of the upstroke (MRD), action potential duration measured at 50% (APD_{50}) and 90% of repolarization (APD_{90}) induced by nicainoprol (0.1–5 μM) in sheep Purkinje fibres superfused with normal (open columns) and modified (stippled columns) physiological salt solution. Control values for MRD, APD_{50} and APD_{90} were 476 ± 22 Vs^{-1}, 171.2 ± 13.1 and 252.5 ± 17.4 ms in normal fibres and 238 ± 21 Vs^{-1}, 144.7 ± 4.9 and 205.9 ± 5.8 ms in fibres after 30 min exposure to simulated ischaemia, respectively.
*$P < 0.05$ indicates a significant difference from zero. $n = 5–6$.

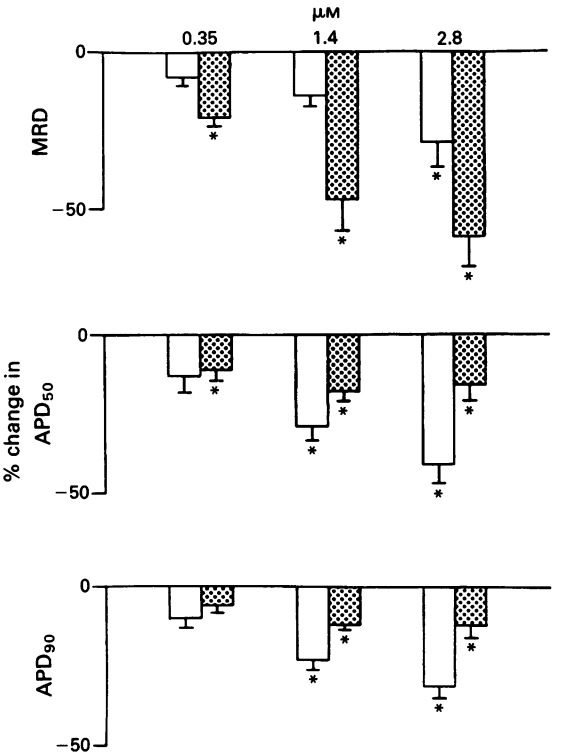

Figure 4 The % reduction in maximum rate of depolarization of the upstroke (MRD), action potential duration measured at 50% (APD_{50}) and 90% of repolarization (APD_{90}) induced by propranolol (0.35–2.8 μM) in sheep Purkinje fibres superfused with normal (open columns) and modified (stippled columns) physiological salt solution. Control values for MRD, APD_{50} and APD_{90} were 657 ± 55 Vs^{-1}, 180 ± 18 and 281.5 ± 30 ms in normal fibres and 435 ± 57 Vs^{-1}, 106.6 ± 9.2 and 170.4 ± 10.1 ms in fibres after 30 min exposure to simulated ischaemia, respectively.
*$P < 0.05$ indicates a significant difference from zero. $n = 4–6$.

under normal conditions, at a concentration that did not significantly reduce MRD. Under simulated ischaemic conditions, propranolol did not abbreviate the action potential in a concentration-dependent manner. Propranolol did not significantly change the resting membrane potential in these experiments.

Effects of verapamil

The effects of verapamil (0.2–8 μM) on MRD and action potential duration in normal and simulated ischaemic fibres are shown in Figure 5. Only the highest concentration studied, i.e. 8 μM, significantly reduced MRD in normal fibres and this was accompanied by a slight fall in resting membrane potential from − 86.5 ± 0.4 to − 81.3 ± 0.3 mV and a reduction in action potential amplitude from 125 ± 3 to 100 ± 4 mV ($P < 0.05$). A concentration-dependent shortening of the action potential duration measured at 50% of repolarization was observed whereas APD_{90} was unaffected (Figure 5).

The effect of verapamil on MRD was potentiated in the presence of hypoxia, hyperkalaemia and acidosis (Figure 5).

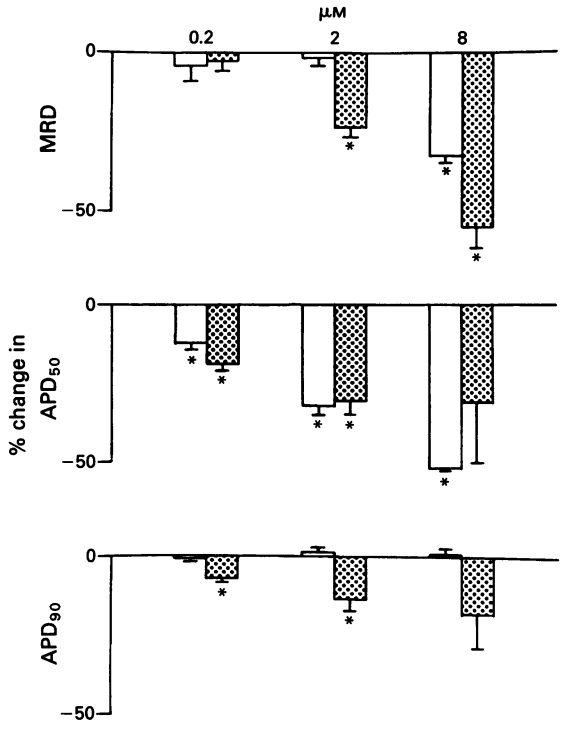

Figure 5 The % reduction in maximum rate of depolarization of the upstroke (MRD), action potential duration measured at 50% (APD_{50}) and 90% of repolarization (APD_{90}) induced by verapamil (0.2–8 μM) in sheep Purkinje fibres superfused with normal (open columns) and modified (stippled columns) physiological salt solution. Control values for MRD, APD_{50} and APD_{90} were 653 ± 28 Vs^{-1}, 171 ± 247 and 320 ± 15 ms in normal fibres and 412 ± 32 Vs^{-1}, 105.8 ± 9.7 and 171.2 ± 12.3 ms in fibres after 30 min exposure to simulated ischaemia respectively.
*$P < 0.05$ indicates a significant difference from zero. $n = 4-8$.

Half (4 out of 8) of those fibres exposed to simulated ischaemia and the highest concentration of verapamil became inexcitable. The log concentration vs % maximum response curve was shifted to the left in fibres exposed to simulated ischaemia and the ratio of the EC_{30}'s was 2.9 in the absence and presence of simulated ischaemia. In contrast to its effect on normal action potential duration, verapamil also abbreviated the APD_{90} of fibres exposed to hypoxia, hyperkalaemia and acidosis. The effect of the highest dose of verapamil was variable, due in part, to the small number of fibres surviving this concentration of the drug.

Discussion

On normal sheep Purkinje fibres, all of the drugs studied reduced the upstroke of the action potential, albeit that with verapamil, for example, such an effect was only observed with the highest concentration studied. This reduction in MRD and action potential amplitude was observed without concomitant changes in resting membrane potential and is indicative of Na^+ channel blocking activity. The action potential duration at both 50 and 90% of repolarization was also reduced by all of the drugs with the exception of verapamil which abbreviated APD_{50} only. Such effects on Purkinje action potential duration have previously been reported for lignocaine (Coraboeuf *et al.*, 1988), nicainoprol (Hamon *et al.*, 1984), propranolol (Davis & Tempte, 1968) and verapamil (Dersham & Han, 1981). Disopyramide has previously been shown to produce complex effects on Purkinje action potential duration which depend upon whether or not a gate region is studied and the external potassium concentration (Kus & Sasyniuk, 1975). The effects of these drugs on action potential duration may be caused by an action on several ionic currents. Lignocaine and disopyramide have been shown to be blockers of the tetrodotoxin-sensitive sodium 'window' current that maintains the plateau in these Purkinje cells (Coraboeuf *et al.*, 1988). Our results with lignocaine, propranolol and nicainoprol suggest that these drugs may block this 'window' current at concentrations lower than those required to block the fast Na^+ current as the plateau phase of the action potential was shortened at lower concentrations than those required to reduce MRD. However, voltage clamp experiments to measure the drug effects on these two currents would be necessary to provide evidence for this suggestion. Verapamil is thought to attenuate the plateau by reducing the inward calcium current (Tytgat *et al.*, 1988) but as it also reduces the delayed outward current the terminal phase of repolarization is not shortened (Kass & Tsien, 1975).

Superfusion of sheep Purkinje fibres with a hyperkalaemic, hypoxic and acidotic PSS caused marked reductions in the resting membrane potential, the upstroke and duration of the action potential. Similar effects of this combination of factors in *in vitro* preparations have been reported previously (Evans *et al.*, 1984) and such changes have also been shown *in vivo* during the early phase of myocardial ischaemia (Downar *et al.*, 1977). The reduction in resting membrane potential and upstroke of the action potential are principally due to the raised extracellular concentration of K^+ whereas both hypoxia and the elevated K^+ concentration contribute to the shortening of the duration in this preparation (Pacini & Kane, 1991). Raising the extracellular concentration of K^+ causes depolarization (due to the reduction in the ratio of intracellular to extracellular K^+ concentrations) which, in turn, would cause partial inactivation of the sodium current and reduce the upstroke velocity of the action potential. Elevation of the extracellular K^+ concentration also induces an increase in the background K^+ current (I_{K1}; Sakmann & Trube, 1984) which would also lead to shortening of the action potential duration, although a depolarization-induced inactivation of the Na^+ window current may also play a role in this observed effect. Although hypoxia has been shown to shorten Purkinje action potential duration under our experimental conditions the ionic basis of this has not been elucidated.

In the presence of simulated ischaemia, the depressant effects on the upstroke velocity of the action potential of lignocaine, propranolol and verapamil were all potentiated with the potentiation being greatest for propranolol and least for verapamil. In the case of lignocaine it has been shown that this potentiation of its Class I effect is mainly due to the marked depolarization produced by hyperkalaemia (Furuta *et al.*, 1982). This depolarization leads to an increase in the proportion of the sodium channel population in the inactivated state and since lignocaine is thought to have a greater affinity for this state of the channel than the activated or rested state (Hondeghem & Katzung, 1977; Wasserstrom & Solata, 1988) this results in potentiation of its blocking activity on the channel. As the depressant effect of propranolol and verapamil on MRD was potentiated in the presence of hypoxia, hyperkalaemia and acidosis this may suggest that these drugs, like lignocaine, may also bind preferentially to the Na channel in its inactivated rather than open or rested state. However, we have no other direct evidence to support this suggestion. In this study we did not observe, as has been previously reported (Kimura *et al.*, 1982; Northover, 1987), that verapamil could attenuate the reduction in MRD produced by simulated ischaemia. The differences in the choice of tissue (cardiac muscle as compared with Purkinje tissue) and in the experimental protocol (the drug being given before rather than during simulated ischaemia) may explain the differences noted in the previous studies compared to our own. Although the effects of nicainoprol on MRD tended to be potentiated in the presence of simulated ischaemia, the difference was not statis-

tically significant. It has, nevertheless, been reported that nicainoprol does preferentially bind to inactivated Na channels (Weirich & Antoni, 1988). The reason why a marked potentiation of its Class I effect was not observed in the present study is not known but it is perhaps of importance that the preferential binding of the drug to inactivated Na channels was noted with concentrations higher than those used in this study. Of the five drugs studied, disopyramide was the only one which exhibited an attenuation rather than a potentiation of its Class I effect during simulated ischaemia. This finding is in line with the observations of Gruber & Carmeliet (1989) who have shown that disopyramide preferentially blocks the Na channel in its open state.

The effects of all the drugs studied on action potential duration were modified in the presence of simulated ischaemia. The abbreviation of the Purkinje action potential (APD_{50} and APD_{90}) induced by lignocaine, propranolol and nicainoprol was attenuated in the presence of hyperkalaemia, hypoxia and acidosis. The ionic mechanism(s) underlying this difference in action is not known. Assuming that these drugs share a common mechanism of action in reducing the Na^+ window current which maintains the plateau in Purkinje fibres, it is possible that if this current is inactivated during simulated ischaemia (as discussed above) the ability of the drugs to reduce action potential duration under simulated ischaemic conditions, would be expected to be attenuated. In the case of disopyramide, a slight prolongation with the highest concentration studied rather than abbreviation of the action potential was noted to occur in the presence of simulated ischaemia. Disopyramide has been shown to reduce both the slowly inactivating Na^+ current and the delayed rectifying outward K^+ current, I_K (Coraboeuf *et al.*, 1988). These two effects would have opposing effects on action potential duration. It is likely that in the presence of simulated ischaemia, disopyramide's effect on the plateau Na^+ current is reduced and consequently the effect on I_K to prolong APD predominates. Verapamil caused a slight but concentration-dependent shortening of the terminal phase of repolarization under simulated ischaemic but not under normal conditions. The ionic basis of this effect of verapamil is not known since the appropriate voltage clamp experiments have not been carried out.

In conclusion, the Class I effects on sheep Purkinje fibres of lignocaine (Subclass Ib) but not disopyramide (1a) nor nicainoprol (1c) were potentiated by a combination of factors mimicking myocardial ischaemia, i.e. hyperkalaemia, hypoxia and acidosis. It would be of interest to examine the effects of other drugs in these subgroups of Class I activity to determine if these observations are general to the subgroups or specific to the drugs selected in this study.

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Characterization of adenosine receptors in brush-border membranes from pig kidney

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1 The adenosine receptors from pig kidney proximal tubules have been studied in membrane vesicle preparations derived from either luminal (brush-border membranes-BBM-) or basolateral (BL) sides. There was a substantial amount of A_2-like NECA binding in both preparations, but the A_1 subtype of adenosine receptors was not found in either BBM or BL membranes. The use of [^{3}H]-CGS21680 which is a more specific ligand for A_{2a} receptors revealed true adenosine receptors in the BBM.

2 The kinetic parameters for [^{3}H]-CGS21680 binding to pig renal BBM were: $B_{max} = 1.48$ pmol mg^{-1} protein and $K_d = 150$ nM. In the presence of Gpp(NH)p the affinity decreased ($K_d = 220$ nM), whereas the addition of Mg^{2+} induced a marked increase in affinity ($K_d = 83$ nM). These equilibrium constants are higher than those found for the A_{2a} adenosine receptors present in pig brain striatal membranes ($K_d = 12$ nM), and are close to those found in rat renal BBM ($K_d = 90$ nM).

3 The order of potency of agonist and antagonists was not consistent with the presence of either A_1 or A_2 receptors, but it was very similar to the agonist order of potency for the A_3 receptor subtype. Furthermore, the blockade of the [^{3}H]-CGS21680 binding by both cholera and pertussis toxin further supports the view that the subtypes present in BBM are neither A_1 nor A_2.

4 Overall the results suggest the presence in BBM of an A_3 receptor, or of a new subtype of adenosine receptor, which is linked to G proteins sensitive to both cholera and pertussis toxins.

Keywords: Adenosine receptors; A_3 receptors; kidney brush-border membranes; kidney basolateral membranes; CGS 21680

Introduction

The beneficial effects of adenosine in kidney are multiple. Adenosine is involved in renal haemodynamics because it reduces renal blood flow (Hall *et al.*, 1985; Arend *et al.*, 1987; Gouyon & Guignard, 1989), in glomerular filtration rate (Churchill, 1982; Hall *et al.*, 1985; Arend *et al.*, 1987; Pawloska *et al.*, 1987), in urine flow (Churchill, 1982; Dillingham & Anderson, 1985; Collis *et al.*, 1986) and in sodium and potassium excretion rate (Churchill, 1982; Collis *et al.*, 1986). Adenosine promotes glomerular constriction related to the entry of calcium into glomerular cells (López-Novoa *et al.*, 1987; Olivera *et al.*, 1989). It has been suggested that the nucleoside may be an endogenous physiological antagonist of the renin-angiotensin system (Hall *et al.*, 1985; Skøtt & Baumback, 1985; Churchill *et al.*, 1987; Ohnishi *et al.*, 1988; Kuan *et al.*, 1989; Deray *et al.*, 1990) but, in some conditions, plasma renin and angiotensin II levels are not necessary determinants of the renal constriction induced by adenosine (Macías-Núñez *et al.*, 1985; Paul *et al.*, 1989). Adenosine is also important in the maintenance of kidney viability before transplantation (Ametani *et al.*, 1990).

Many effects of adenosine in kidney are mediated by its interaction with membrane-bound adenosine receptors. Two adenosine receptor subtypes have been identified on the basis of the ability of adenosine and its analogues to inhibit (subtype A_1) or stimulate (subtype A_2) adenylate cyclase activity (Van Calker *et al.*, 1979; Stiles, 1986; Fredholm & Dunwiddie, 1988). Distinction between the two subtypes of adenosine receptors is usually made on the basis of the rank orders of potency of agonists and antagonists. Recently, the existence of another adenosine receptor subtype (A_3), not related to the adenylate cyclase system, has been described (Ribeiro & Sebastiao, 1985; 1986; Sebastiao & Ribeiro, 1988). The A_3 adenosine receptor subtype is related to calcium channels and has a different order of potency of agonists and antagonists from A_1 or A_2 subtypes.

By use of radioligand binding techniques, A_2 receptors were detected in rat kidney whole membranes (Wu & Churchill, 1985). In more purified tissue fractions, both A_1 and A_2 receptors have been identified in kidney. Thus, in cortical membranes the presence of A_2 receptor was detected by measuring the stimulation of adenylate cyclase activity (Freissmuth *et al.*, 1987b) and no A_1 receptor was found by autoradiography (Weber *et al.*, 1988). In contrast, in the medulla, the presence of A_1 receptors was shown by autoradiography (Weber *et al.*, 1988) and the absence of A_2 receptors was described (Woodcock *et al.*, 1984). The presence of A_2 binding sites in the papillae was detected by changes in adenosine 3'-5'-cyclic-monophosphate (cyclic AMP) levels (Woodcock *et al.*, 1984; 1986). On the other hand, both A_1 and A_2 receptors were detected by binding experiments in purified glomeruli and microvessels and it was suggested that both receptors are involved in the control of renin secretion (Freissmuth *et al.*, 1987b; Palacios *et al.*, 1987). Although the predominant effect of endogenous adenosine on renin release is inhibitory, the kidney has both A_1 and A_2 receptors mediating inhibitory and stimulatory actions on renin release (Kuan *et al.*, 1990). Afferent arterioles possess both A_1 and A_2 receptors but efferent arterioles possess only A_2 receptors, where it seems that both constriction and inhibition of renin release are mediated by an A_1 receptor whereas vasodilatation and renin release stimulation are mediated by an A_2 receptor (Murray & Churchill, 1985). Tubular adenosine receptors are involved in the control of renal electrolyte secretion but the receptor subtypes involved are under discussion. Stimulation of adenylate cyclase via A_2 adenosine receptors in isolated tubules of rabbit renal cortex was found by Freissmuth *et al.* (1987a,b). A_2 also seems to be the principal receptor involved in calcium

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reabsorption in rat kidney (McPhee & Whiting, 1989). In rabbit and guinea-pig collecting tubules, A_1 and A_2 receptors, respectively associated with inhibition and stimulation of adenylate cyclase, were detected (Palacios *et al.*, 1987; Arend *et al.*, 1988) but a pertussis toxin-sensitive receptor system that leads to the mobilization of intracellular calcium was also detected (Arend *et al.*, 1987). In established renal cell lines, the ability of adenosine analogues to modify cyclic AMP levels and intracellular Ca^{2+} levels has been compared (Arend *et al.*, 1989; Weinberg *et al.*, 1989). From the results of such a comparison it is not clear whether there is a single adenosine receptor subtype (e.g. A_1) mediating both effects. Thus, although the physiological effects caused by adenosine have been extensively studied, the biochemical characterization of adenosine receptors in kidney is far from complete.

In this paper we have analyzed the presence of adenosine receptors in membrane-vesicle preparations from the kidney cortical zone. The adenosine receptor present in brush-border membrane vesicles has been fully characterized and the results appear to suggest the presence of the A_3 subtype in this membrane model or, alternatively, point out the presence in BBM of a new subtype of adenosine receptor, which is linked to G proteins sensitive to both cholera and pertussis toxins.

Methods

Preparation of striatal membranes

Fresh pig brains were obtained from the local slaughterhouse immediately after the death of the animals. Striatal tissue was obtained after brain dissection and immediately homogenized in 10 volumes of 0.25 M sucrose, 5 mM Tris-HCl buffer pH 7.4 with a Polytron disruptor (Kinematica, PTA 20TS rotor, setting 4). The homogenate was centrifuged at 105,000 *g* for 30 min and the pellet was resuspended in 10 volumes of 50 mM Tris-HCl buffer pH 7.4 and recentrifuged under the same conditions. The pellet was washed once more as described above and resuspended in the same buffer solution for immediate use.

Preparation of renal membranes

In preliminary binding experiments, simultaneous preparation of luminal (BBM) and antiluminal (basolateral) membranes from pig kidney proximal tubule was performed as described by Kinsella *et al.* (1979). Pig renal cortex was homogenized in 10 volumes of 8% sucrose Tris buffer (pH 7.0). The homogenate was centrifuged for 10 min at 1,000 *g*. The supernatant was centrifuged for 10 min at 9,500 *g* and the soft lighter portion of the pellet and the supernatant were removed, combined and centrifuged for 20 min at 47,000 *g*. The soft lighter portion of the pellet was recovered and resuspended in 25 mM HEPES, 100 mM mannitol, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ buffer (pH 7.0). This suspension was stirred for 1 h and centrifuged for 12 min at 1,400 *g*. The supernatant contained luminal membranes, and the pellet contained crude non-purified antiluminal membranes. The pellet was resuspended, stirred and centrifuged in the same conditions three times. Pooled supernatants were centrifuged for 20 min at 47,000 *g* to obtain BBM. The final pellet was resuspended in 25 mM HEPES, 100 mM mannitol buffer (pH 7.0) and the suspension was layered over a discontinuous sucrose gradient (10 ml sucrose 31% w/w and 12 ml sucrose 8% w/w) and centrifuged for 60 min at 90,000 *g* (SW 25.1 Beckman rotor); antiluminal membranes were collected at the interface. Luminal and antiluminal membranes were dialyzed overnight against Tris 50 mM pH 7.4 buffer to remove the divalent cations, and stored at − 80°C. With respect to the homogenate, the basolateral fraction was enriched 13 fold in Na^+/K^+ ATPase activity.

Isolation of BBM for the characterization of [^{3}H]-CGS21680 binding

Pig BBM were isolated by a calcium or magnesium precipitation method described by Vannier *et al.* (1976) and modified by Lin *et al.* (1981). Briefly, after perfusion, cortex was removed from pig kidneys and placed in 10 mM mannitol, 2 mM Tris-HCl buffer pH 7.1. Homogenization was performed in 5 volumes of the Tris-mannitol buffer using a Polytron disruptor (Kinematica, PTA 20TS rotor, setting 5) for three periods of 10 s separated by intervals of 20 s. After homogenization, 1 M $MgCl_2$ or $CaCl_2$ was added to a final concentration of 10 mM Mg^{2+} or Ca^{2+}. The final mixture was stirred for 15 min and centrifuged for 12 min at 2,000 *g*. The supernatant was removed and centrifuged for 3 h at 20,000 *g*. The pellet was resuspended in 150 ml of 170 mM KCl, and 2 volumes of 200 mM Tris-HCl buffer pH = 7.8 were added to the resuspension. This resuspension was centrifuged for 15 min at 3,000 *g*, and the supernatant was centrifuged for 3 h at 20,000 *g*. The final pellet (BBM) was resuspended in 50 mM Tris-HCl buffer pH 7.4 to a final protein concentration of 20–30 mg ml^{-1}.

Rat BBM were obtained by magnesium precipitation method (Lin *et al.*, 1981) as follows: rat kidney cortex was homogenized in 5 volumes of 2 mM Tris 10 mM mannitol buffer pH 7.1. The homogenate was diluted 1:2 with the same buffer and stirred for 15 min after addition of $MgCl_2$ to a final concentration of 15 mM. This treated homogenate was centrifuged for 12 min at 1,000 *g* and the supernatant was then centrifuged for 20 min at 10,000 *g*. The pellet was resuspended, treated once more with $MgCl_2$ and centrifuged for 12 min at 1,500 *g*. The final supernatant was centrifuged for 30 min at 17,000 *g*. The final pellet (BBM) was resuspended in 50 mM Tris-HCl buffer pH 7.4 to a protein concentration of 20 mg ml^{-1}. All steps were carried out at 4°C. Membrane suspensions were stored at − 80°C.

In all cases, in the final membrane fraction the marker enzyme alkaline phosphatase (E.C. 3.1.3.1.) was enriched 9–11 fold.

Pretreatment of pig BBM with toxins

Incubations of pig BBM with cholera or pertussis toxin were performed in a total volume of 1 ml. BBM (5 mg protein) were incubated with a ribosylation buffer containing (mM) either 300 potassium phosphate (pH 7), 10 thymidine, 1 ATP, 0.1 GTP, 10 $MgCl_2$, 1 EDTA and 5–100 μg of pre-activated cholera toxin (Ribeiro-Neto *et al.*, 1985), or 25 Tris-HCl (pH 8), 10 thymidine, 1 ATP, 0.1 GTP, 1 EDTA and 1,25–5 μg of pre-activated pertussis toxin (Ribeiro-Neto *et al.*, 1985). Reactions were started by addition of NAD^+ to a final concentration of 0.1 mM. Incubations were performed at 30°C for 30 min, they were stopped by dilution with 25 ml of ice cold Tris buffer and centrifugation at 4°C for 30 min at 105,000 *g*. Pellets were resuspended in 50 mM Tris-HCl buffer pH 7.4 for [^{3}H]-CGS21680 binding analysis.

Protein determination

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin used as standard.

Ectophosphodiesterase activity in pig BBM

The AP_4A and AP_5A stability in the presence of pig BBM was evaluated by high performance liquid chromotography (h.p.l.c.). Mixtures of adenosine polyphosphates and BBM were prepared as indicated in Figure 1. At the end of the incubation process, samples of 100 μl were taken and treated as described by Shryock *et al.* (1985) in order to remove proteins and lipids. Nucleotides were quantified on 20 μl aliquot samples by an h.p.l.c. system from LKB consisting of two 2150 h.p.l.c. pumps, a 2152 LC controller, a 2141

variable wavelength monitor, a 2157 autosampler, a 2221 integrator and a Spherisorb ODS2 (5 μm) column (4.2 × 250 mm). Separation was performed in isocratic conditions with 20 mM potassium phosphate buffer pH 7.5 containing 2 mM tetrabutylammonium phosphate and 15% (v/v) acetonitrile. The flow rate was 1 ml min^{-1}. A typical elution profile is given in Figure 1. The metabolism of AP_4A to AMP and ATP, and AP_5A to AMP and AT_4 was observed at 25°C and at 4°C. The degradation time-course is shown in Figure 1.

Radioligand binding assays

Radioligand binding was measured after washing membranes (final protein concentration 0.7–0.8 mg ml^{-1}) with adenosine deaminase (usually 1 u ml^{-1}) at 25°C in 50 mM Tris-HCl buffer pH 7.4, unless otherwise stated. Radioligand, modulators or displacers were then added. After standing at 25°C until the equilibrium was achieved (usually 2 h), free and bound radioligand were separated by rapid filtration of 500 μl aliquots through Watman GF/C filters, which were subsequently washed with 10 ml of ice cold Tris buffer. The filters were presoaked in 0.3% polyethylenimine (2–4 h, pH 10) to improve the performance of the filtration. Non-specific binding was determined in the presence of an excess (100–500 fold) of cold ligand. The filters were placed in standard vials with 10 ml of Formula-989 liquid scintillation cocktail (New England Nuclear Research Products, Boston, MA, U.S.A.). After an interval of at least 12 h, the vials were counted in a Packard 1500 TRICARB liquid scintillation counter with an efficiency of 40%.

Materials

[^{3}H]-CGS21680 ([^{3}H]-2-[4-(2-carboxyethyl)phenethylanimo]-5′-N-ethylcarboxamido adenosine hydrochloride; 47.2 Ci $mmol^{-1}$), [^{3}H]-(**R**)-N^6-phenylisopropyladenosine ([^{3}H]-**R**-PIA 54.0 Ci $mmol^{-1}$), [^{3}H]-N-ethylcarboxamidoadenosine ([^{3}H]-NECA 15.1 Ci $mmol^{-1}$) and [^{3}H]-dipropylcyclopentyl xanthine ([^{3}H]-DPCPX, 109.2 Ci $mmol^{-1}$) were purchased from New England Nuclear Research Products (Boston U.S.A.). Adenosine-(5′)-tetrophosphate-(5′)-adenosine (AP_4A), adenosine-(5′)-pentaphosphate-(5′)-adenosine (AP_5A), α-β-methylene-ADP (α-β-Me-ADP), adenosine tetrophosphate (AT_4), cholera toxin, pertussis toxin, 2-chloroadenosine, cyclopentyladenosine, polyethylenimine, nitrobenzylthioinosine (NBTI), theophylline, 8-phenyltheophylline, isobutylmethylxanthine, thimidine, uridine and inosine from Sigma (St. Louis, U.S.A.). **R**-PIA, S-PIA, NECA, NAD^+, GTP, ATP and 5′-guanylylimidodiphosphate (Gpp(NH)p) from Boehringer Mannheim (Germany). Xanthine Amino Congener and DPCPX were from RBI (Research Biochemicals Incorporated, Natick U.S.A.). Dipyridamole was kindly provided by Dr Cembrano of Boehringer Ingelheim (Barcelona, SPAIN). CGS21680 and CGS 15943 were kindly provided by Dr Lowell from Ciba-Geigy (New Jersey U.S.A.). Stock solutions of 8-phenyl theophylline, DPCPX and NBTI (> 200 μM) or other xanthine derivatives and non water-soluble compounds (10–20 mM) were prepared in different mixtures of 50 mM Tris-HCl buffer and ethanol, or in 0.1 N NaOH. The specific binding of [^{3}H]-CGS21680 to pig BBM, was not modified in the presence of the maximal amount of ethanol in the final incubates (2–3%). Given the poor solubility of many xanthine derivatives, the solutions were used immediately after preparation at room temperature. Precipitation was checked for by monitoring u.v. absorbance spectrum of the most concentrated solution used in the displacement experiments; no changes (or changes < 20% in CGS15943) in the maximal absorbance peak were observed after filtration through 0.45 μm pore size filters.

All other products were the best grade available and were purchased from Merck (Darmstadt, Germany). De-ionized water further purified with a Millipore Milli-Q system was used throughout.

Data analysis

Data from saturation isotherms and displacement curves were analyzed by non-linear regression with the ENZFITTER programme (Elsevier Biosoft) or other available programmes (Canela, 1984; López-Cabrera *et al.*, 1988) as described elsewhere (Casadó *et al.*, 1990a,b; 1991). Three or five replicates of each point were performed. Goodness of fit was tested according to the reduced χ^2 or s.d. values given by the programmes.

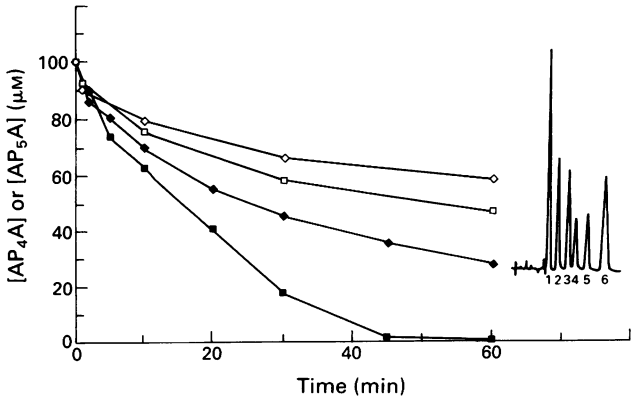

Figure 1 Time-course of AP_4A and AP_5A degradation by pig renal brush border membranes. Membranes (0.8 mg protein ml^{-1}) were incubated in Tris-HCl buffer 50 mM pH 7.4 with 100 μM AP_4A (■,□) or AP_5A (◆,◇) at 25°C (solid symbols) or at 4°C (open symbols). Insert: Chromatogram of a mixture of standards: (a) AMP; (2) ADP; (3) ATP; (4) AT_4; (5) AP_4A and (6) AP_5. For abbreviations, see text.

Results

Binding of adenosine receptor ligands to luminal and basolateral membranes from pig proximal tubules (renal cortex)

Luminal (brush-border membrane BBM) and basolateral (BL) membrane vesicles were prepared as described in Methods and the binding of [^{3}H]-PIA, [^{3}H]-DPCPX, [^{3}H]-NECA and [^{3}H]-CGS21680 was assayed. To differentiate between specific binding to receptors and transport of the ligand, the binding assays were performed in the presence or absence of a cocktail of transport inhibitors (50 μM uridine, 50 μM inosine, 1.5 μM nitrobenzylthioinosine). In the presence of transport inhibitors, neither, 20 nM [^{3}H]-PIA nor 10 nM [^{3}H]-DPCPX showed specific binding to these membranes (0.7 mg protein ml^{-1}) when measured as indicated in Methods. These are clear indications of the absence of A_1 adenosine receptors in these membranes. In contrast, [^{3}H]-NECA and [^{3}H]-CGS21680 binding sites appeared as shown in Figure 2. The [^{3}H]-NECA specific binding was higher in luminal (BBM) than in basolateral (BL) membranes. [^{3}H]-NECA specific binding might correspond to the interaction of the radiolabelled compound with adenosine receptors as well as with non-adenosine receptor A_2-like binding sites. To clarify this, the binding of [^{3}H]-NECA was assayed in the presence of **R**-PIA which does not bind to the A_2-like sites. As shown in Figure 2, the binding of radiolabelled NECA to BL and BBM was not displaced by **R**-PIA, which is indicative of a high content of A_2-like binding sites. The existence of these non-adenosine binding sites means that [^{3}H]-NECA is a poor ligand with which to study the characteristics of adenosine receptors from this procedure. The specific binding of 30 nM [^{3}H]-CGS21680 was higher in BBM

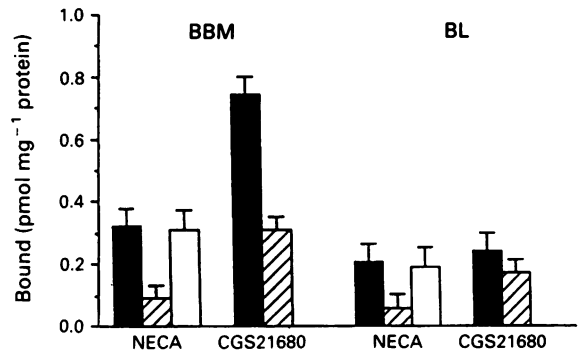

Figure 2 Binding of adenosine receptors specific ligands to pig basolateral (BL) and brush-border (BBM) membrane vesicles. Binding of 20 nM [^{3}H]-NECA and 30 nM [^{3}H]-CGS21680 to the indicated vesicles (0.7 mg prot ml^{-1}) was measured in the presence (hatched columns) or absence (solid columns) of 500 fold excess of the corresponding non-labelled ligand, or 50 μM of **R**-PIA (open columns). The same results were obtained in presence of nucleoside transporter inhibitors cocktail containing 50 μM inosine, 50 μM uridine and 1.5 μM NBTI. For abbreviations, see text.

(0.45 pmol mg^{-1} protein) than in BL membranes, where no significant differences between specific and nonspecific binding were observed. For this reason we used BBM in subsequent studies of [^{3}H]-CGS21680 binding.

Characterization of the [^{3}H]-CGS21680 binding to pig BBM vesicles

Saturation isotherms for BBM (obtained by Ca^{2+} precipitation) with [^{3}H]-CGS21680 (Figure 3) gave the following values at equilibrium: $K_d = 150 \pm 10$ nM, and $B_{max} = 1.48 \pm 0.04$ pmol mg^{-1} protein. As indicated in Table 1, the maximum specific binding in the presence of 100 μM Gpp(NH)p did not change ($B_{max} = 1.52 \pm 0.06$ pmol mg^{-1} protein), although the affinity was markedly reduced ($K_d = 220 \pm 21$ nM). When 10 mM $MgCl_2$ was added to the incubation assay mixture, the affinity for the ligand increased, whereas the maximum binding remained unchanged (See Table 1).

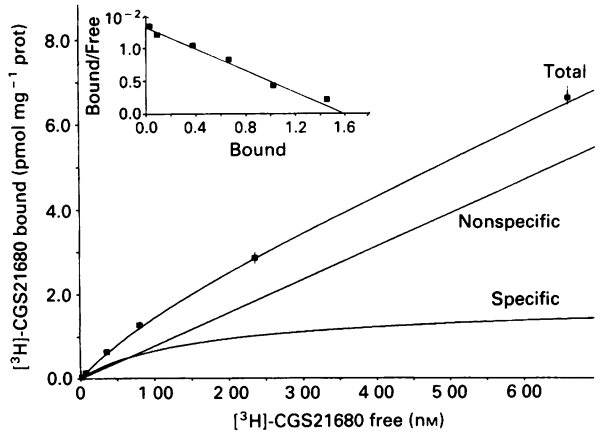

Figure 3 Isotherm curves of [^{3}H]-CGS21680 binding to pig renal brush border membrane. Saturation analysis of [^{3}H]-CGS21680 binding to brush-border membrane vesicles obtained by Ca^{2+} precipitation (0.7 mg prot ml^{-1}) was performed as described in Methods. Nonspecific binding was determined in the presence of 200 fold excess of CGS21680. The same results were obtained when nonspecific binding was determined in presence of the same excess of NECA. All points represent the mean of five replicates; s.e.mean shown by vertical lines. Data were adjusted by a non-linear regression programme to one site model (no significant improvement after considering the two-site model was obtained). Insert: Scatchard plot of the computer-derived specific binding data.

Table 1 Effect of Mg^{2+} and Gpp(NH)p on [^{3}H]-CGS21680 equilibrium binding parameters.

	B_{max} (pmol mg^{-1} protein)	K_d (nM)
BBM Ca^{2+}	1.48 ± 0.04	150 ± 10
BBM Ca^{2+} + 10 mM $MgCl_2$	1.64 ± 0.2	83 ± 21
BBM Ca^{2+} + 10 nM Gpp(NH)p	1.52 ± 0.06	220 ± 21
BBM Mg^{2+}	1.77 ± 0.07	78 ± 9
BBM Mg^{2+} + 10 mM $MgCl_2$	1.87 ± 0.03	86 ± 3
BBM Mg^{2+} + 100 μM Gpp(NH)p	1.83 ± 0.1	199 ± 22

BBM Ca^{2+}: BBM obtained by Ca^{2+} precipitation.
BBM Mg^{2+}: BBM obtained by Mg^{2+} precipitation.

The same set of experiments were performed with BBM obtained by the Mg^{2+} precipitation procedure. Table 1 shows that the affinity for the ligand in the presence of Mg^{2+} is the same, irrespective of whether BBM are obtained with Mg^{2+} or whether the Mg^{2+} is added to BBM obtained by Ca^{2+} precipitation. On the other hand, it should be noted that the effect of Mg^{2+} is abolished by Gpp(NH)p since the presence of this compound in the assays always led to a similar decrease in affinity for [^{3}H]-CGS21680. The content of binding sites (B_{max}) was similar in all established conditions (Table 1).

The equilibrium binding parameters shown in Table 1 are higher than those reported for the [^{3}H]-CGS21680 binding to human and rat brain A_{2a} adenosine receptors (Jarvis *et al.*, 1989; Wan *et al.*, 1990). In order to determine whether the kinetic characteristics reported in this paper correspond to species differences or to an adenosine receptor other than the A_{2a}, the characterization of [^{3}H]-CGS21680 binding to pig A_{2a} receptors was performed. Membranes from pig brain striatum were prepared and the saturation isotherms with [^{3}H]-CGS21680 were determined (Figure 4a). The kinetic parameters at equilibrium were $K_d = 12 \pm 1$ nM and $B_{max} = 0.65 \pm 0.01$ pmol mg^{-1} protein. These equilibrium binding parameters correspond to a pig A_{2a} adenosine receptor, because from the displacement experiments shown in Figure 4b, the order of potency among adenosine receptor agonists was: CGS21680 ($K_i = 12 \pm 4$ nM) = NECA ($K_i = 12 \pm 3$ nM)> CADO ($K_i = 220 \pm 60$ nM)> **R**-PIA, ($K_i = 2200 \pm 400$ nM), which is the same as that found for A_{2a} receptors (Jarvis *et al.*, 1989).

In order to determine the adenosine receptor subtype present in pig BBM, the rank orders of potency of agonist and antagonists were determined by displacement experiments (see Table 2). The radiolabelled compound was 5 nM [^{3}H]-CGS21680 and the displacers were: unlabelled CGS21680, **R**-PIA, S-PIA, cyclopentyladenosine, NECA and chloroadenosine. The order of potency was: CGS21680> cyclopentyladenosine = NECA ≥ S-PIA> chloroadenosine. The displacement curves in presence of 100 μM Gpp(NH)p gave similar results: CGS21680 ≥ cyclopentyladenosine> **R**-PIA> NECA = S-PIA> chloroadenosine (data not shown). The same kind of experiment performed with antagonists (Table 2) led to the following order of potency: 8-phenyltheophylline> xanthine amino congener (XAC) = DPCPX = CGS15943> isobutylmethylxanthine (IBMX)> theophylline. In order to test whether the inhibitors of the nucleoside transporter bind to the same site as CGS21680, displacement experiments were performed with these inhibitors. The order of potency as displacers of [^{3}H]-CGS21680 in the presence (Table 2) or absence (data not shown) of Gpp(NH)p was: nitrobenzylthioinosine (NBTI)> dipyridamole> inosine = uridine. The high K_i values obtained (Table 2) suggest that the [^{3}H]-CGS21680 binding sites are not the adenosine transporter.

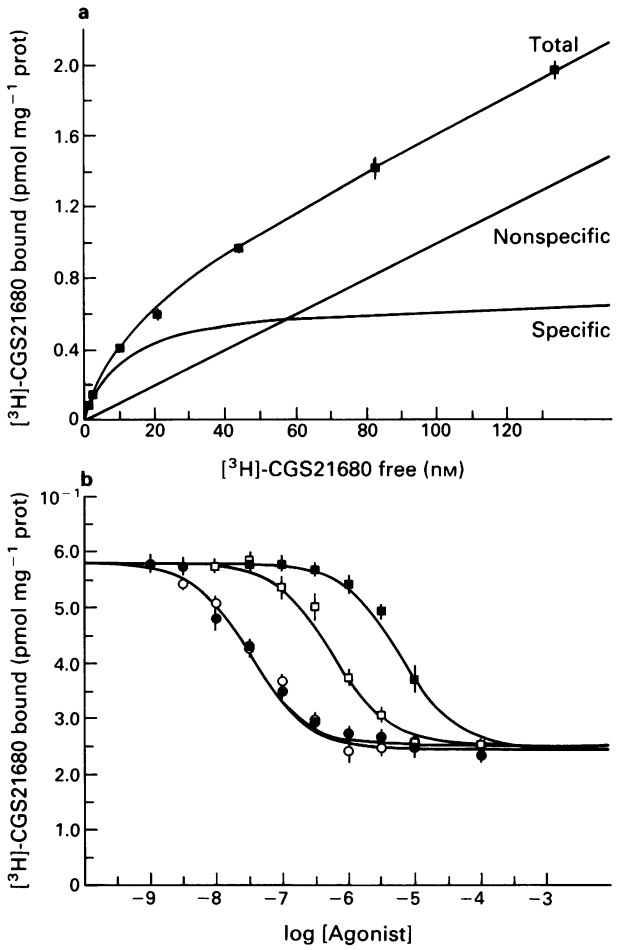

Figure 4 [^{3}H]-CGS21680 binding to pig striatum membranes. Striatum membranes (0.7 mg protein ml^{-1}) were incubated, in the presence of 10 mM $MgCl_2$, with the indicated ligands, as described in Methods. (a) Saturation analysis of [^{3}H]-CGS21680 binding. The nonspecific binding was measured with a 200 fold excess of unlabelled ligand. Data were adjusted by a non-linear regression programme to one site model. (b) Displacement of 20 nM [^{3}H]-CGS21680 binding by CGS21680 (○); NECA (●); CADO (□) and R-PIA (■). All values are mean of three or five replicates; vertical lines show s.e.mean. See text for abbreviations.

Table 2 Potency of different ligands on the displacement of the [^{3}H]-CGS21680 specific binding to BBM.

	K_i (μM)
Agonists	
CGS21680	0.120 ± 0.024
Cyclopentyladenosine	0.552 ± 0.031
NECA	0.877 ± 0.187
R-PIA	1.69 ± 0.29
S-PIA	3.31 ± 0.60
2-Chloroadenosine	34 ± 13.9
Antagonists	
8-Phenyltheophylline	4.4 ± 1.5
CGS15943	12.9 ± 7.2
Xanthine amino congener	13.7 ± 6.3
DPCPX	18.0 ± 4.6
Isobutylmethylxanthine	126. ± 35
Theophylline	485 ± 19
Transport inhibitors	
NBTI	2.9 ± 1.4
Dipyridamole	30 ± 19
Inosine	2350 ± 890
Uridine	2910 ± 1850

Results are taken from data of Figure 5 and fitted using a non-linear regression programme as indicated in Methods. Values ± s.d. given by the programme.

Table 3 Effect of non P_1 purinoreceptors agonists on the [^{3}H]CGS21680 binding.

Displacer	*Total [^{3}H]CGS21680 binding* (pmol mg^{-1} protein)
None	0.47 ± 0.05
AP_4A	0.6 ± 0.1
AP_5A	0.5 ± 0.1
α-β-met-ADP	0.5 ± 0.1
CGS21680	0.29 ± 0.02

BBM (0.8 mg protein ml^{-1}) were incubated with 30 nM [^{3}H]CGS21680 in 50 mM Tris-HCl buffer pH 7.4 at 25°C during 30 min in the absence (none) or in the presence of 100 μM of the indicated displacers. Radioligand binding was measured as described in Methods. Displacement by 100 μM CGS21680 indicates nonspecific binding. Values are mean ± s.d. of ten replicates.

In order to test whether the P_2 purinoreceptor agonist α-β-methylene-ADP or diadenosine polyphosphates bind to the same site as [^{3}H]-CGS21680, the total radioligand binding was measured in the presence and in the absence of an excess of these compounds. Metabolism was monitored at the end of incubation period (see Figure 1); at this time more than 18% of added AP_4A and more than 55% of added AP_5A was not metabolized. As shown in Table 3, α-β-methylene-ADP, AP_4A and AP_5A, in the micromolar range, do not compete with [^{3}H]-CGS21680 for the same binding sites.

As discussed below, our data seem to suggest the existence of A_3 subtype adenosine receptors in pig renal BBM. In order to determine whether this subtype is specific for pig renal BBM or may be present in BBM from other species, rat renal BBM were prepared (0.5 mg protein ml^{-1}) and the saturation isotherms of [^{3}H]-CGS21680 (2–400 nM) were constructed in a similar manner to that used for pig renal BBM. The equilibrium binding parameters were $K_d = 90 \pm 50$ and $B_{max} = 0.7 \pm 0.1$ pmol mg^{-1} protein. These parameters are similar to those found for pig renal BBM. No species differences, i.e. the same binding characteristics, were observed in both, pig and rat BBM.

Since the [^{3}H]-CGS21680 bound to pig renal BBM adenosine receptors, but the order of potency for agonists and antagonists did not correlate well with those reported for either A_1 or A_2 receptors, we were interested in determining whether the adenosine receptor system present in BBM was coupled to G_i or to G_s proteins. To this end, the [^{3}H]-CGS21680 binding was assayed in pig BBM pretreated with either pertussis or cholera toxin. As indicated in Figure 5, both toxins were able to reduce the binding of the ligand and to displace it to values close to those obtained in the presence of Gpp(NH)p.

Discussion

In this paper the presence of the A_2-like NECA binding protein, which is not an adenosine receptor, and the absence of A_1 adenosine receptors in both luminal (BBM) and basolateral (BL) membranes from pig renal cortex was demonstrated. In order to test the presence of A_2 or other adenosine receptors in BBM and BL, the ligand [^{3}H]-CGS21680 was used, which is a very selective agonist of A_2 subtype and does not bind to the NECA-binding protein (Jarvis *et al.*, 1989). The results obtained with [^{3}H]-CGS-21680 demonstrated the existence of adenosine binding sites in pig renal BBM. The K_d value (80 nM in presence of Mg^{2+})

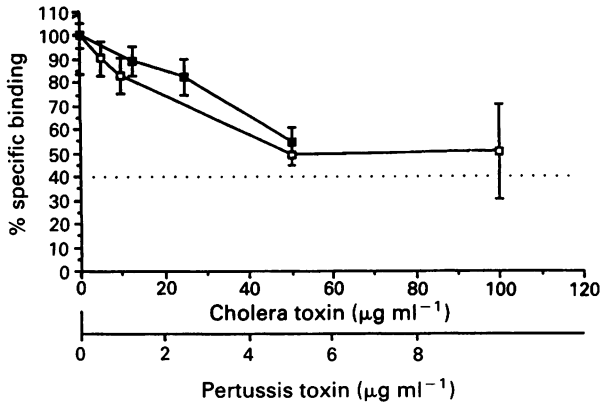

Figure 5 Effect of pertussis and cholera toxins on [^{3}H]-CGS21680 specific binding to brush-border membrane vesicles. Pretreatment of membranes with the indicated amount of pertussis toxin (■) or cholera toxin (□) was carried out as indicated in Methods. Dotted line indicates specific binding in presence of 100 μM Gpp(NH)p.

for the [^{3}H]-CGS21680 binding site is higher than the K_d values described for rat (15 nM, Jarvis *et al.*, 1989) and pig (12 nM, Figure 4) A_{2a} adenosine receptor. Using human brain, both Wan *et al.* (1990) and James *et al.* (1992) have described a heterogeneity in [^{3}H]-CGS21680 binding sites. A large proportion of these sites displayed a pharmacological profile that was consistent with the A_{2a} adenosine receptors subtype, although the K_d value for these binding sites reported by James *et al.* (125 nM) is higher than that found by Wan *et al.* (28 nM), probably due to the different methodological approximations used to calculate the K_d values. As demonstrated by the results shown in Figure 4, pig A_{2a} adenosine receptors have similar characteristics to rat A_{2a} receptors, but are very different from pig renal BBM receptors. The conclusion that pig renal BBM have different adenosine receptors from A_1 and A_2 subtypes is reached by analyzing the rank order of potency of antagonists and agonists in the displacement of [^{3}H]-CGS21680 binding to pig renal BBM. The rank order of potency of antagonists found was: 8-phenyltheophylline > xanthine amino congener (XAC) = DPCPX = CGS15943 > isobutyl methylxanthine (IBMX) >> theophylline. The similar potency of XAC and DPCPX can be compared to that corresponding to A_3 receptors, although the K_i values obtained with BBM in our displacement experiments are higher than those reported from functional studies for the A_3 receptor (Sebastiao & Ribeiro, 1989). What is still evident is that the order for antagonists does not coincide with those which are well established for A_1 (DPCPX > xanthine amino congener > theophylline) and A_2 (CGS15943 > xanthine amino congener > DPCPX > theophylline) receptors. On the other hand, the rank order of potency of agonists found was: CGS21680 > cyclo pentyl adenosine = NECA ≥ **R**-PIA ≥ S-PIA > chloroadenosine, indicating that the adenosine receptor present in BBM was different from A_1 (**R**-PIA > chloroadenosine > S-PIA = NECA) and A_2 (NECA > chloroadenosine > **R**-PIA > S-PIA) (Bruns *et al.*, 1986, and the results from Figure 4b). Interestingly, our results agree with those described for the A_3 receptor in the nervous system. In fact, the order of potency for A_3 receptors described by Ribeiro & Sebastiao (1986) is: **R**-PIA = cyclohexyladenosine = NECA > chloradenosine, with S-PIA usually being less potent than chloroadenosine. From the similar rank order of potency agonists reported here (Table 2), the existence of A_3 receptors in BBM is suggested. The use of [^{3}H]-CGS21680 to label A_3 adenosine receptors must be taken into consideration when heterogeneity of [^{3}H]-CGS21680 binding sites is shown. James *et al.* (1992), in addition to the existence of A_{2a} receptors in human brain, have detected another, ubiquitous, binding site (K_d 125–350 nM); this site might represent a new subtype of adenosine A_2 receptors, but it cannot be ruled out that it represents the A_3 subtype, because it shows an equal potency for the agonists NECA and **R**-PIA, which is one of the most striking characteristics of A_3 receptors described by Ribeiro & Sebastiao (1986).

As is clearly demonstrated by the high K_i for the displacement of the binding found with the inhibitors of the adenosine transporter, this binding of [^{3}H]-CGS21680 is not to the nucleoside transporter of pig renal BBM. Further, these binding sites are not P_2 or other purinoceptors because neither the agonist of the P_2 sites nor the diadenosine polyphosphates were able to displace the binding even in the micromolar range. Although all the results already discussed show the receptor of pig renal BBM as different from A_1 and A_2, further confirmation was obtained from the susceptibility of the receptor to cholera and pertussis toxins. The binding of [^{3}H]-CGS21680 was modified by Gpp(NH)p, which is consistent with the coupling of the BBM receptor with a G protein.

Pertussis toxin ADP-ribosylates the G_i protein which is coupled to A_1 receptor, thus leading to a decreased agonist binding. Besides, as cholera toxin ADP-ribosylates the G_s subunit which is coupled to A_2 receptors, a decreased binding for A_2 agonists is expected in its presence. The behaviour of [^{3}H]-CGS21680 binding was similar in the presence of either toxin. Both toxins were capable of reducing the agonist binding to the values obtained in the presence of Gpp(NH)p, i.e. to the receptor uncoupled from G protein. Ali *et al.* (1990) found in rat tumour (RBL-2H3) cells, that the stimulation of inositol phospholipid hydrolysis by NECA was blocked by pertussis toxin as well as by cholera toxin. The authors suggested the existence of a new type of adenosine receptor (not A_1, A_2 or A_3) in RBL-2H3 cells, although, from the results presented, the existence of A_2-like NECA-binding protein, A_{2b}, or even A_3 sites cannot be ruled out. In rat pancreatic acinar cells, there are G proteins sensitive to both cholera and pertussis toxins (G_i1, G_i2, G_i3) which are linked to cholecystokinin receptors and which may mediate changes in phospholipase C activity (Schnefel *et al.*, 1990). Thus adenosine might mediate changes in phosphatidylinositol metabolism through A_3 receptors and cholera- and pertussis-sensitive G proteins. The modification of phosphatidylinositol metabolism (Arend *et al.*, 1989) as well as intracellular Ca^{2+} accumulation (Arend *et al.*, 1989; Weinberg *et al.*, 1989) induced by exogenous adenosine analogues have been demonstrated in established renal cell lines. However, the conditions of the assays in cells do not permit the identification of the receptor(s) involved.

The existence of A_3 receptors in both pig and rat brush border membranes has been shown, and their link to Ca^{2+} events in the kidney provides an opportunity for increasing our understanding of the effects of adenosine in this organ.

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In vitro effect of oestradiol on thymidine uptake in pulmonary vascular smooth muscle cell: role of the endothelium

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1 The effect of different concentrations of oestradiol-17β (3–300 nM) on [^{3}H]-thymidine uptake was studied in segments from canine pulmonary artery, and cultures of rat pulmonary vascular smooth muscle cells (VSMC).

2 Incubation with oestradiol-17β for 24 h, potentiated in a concentration-dependent manner [^{3}H]-thymidine uptake in VSMC cultures.

3 Oestradiol-17β potentiated thymidine uptake by pulmonary arterial segments but only when the endothelium had been removed. Autoradiography showed dense incorporation of radioactive thymidine in the vascular smooth muscle cells of the media.

4 The non-steroidal oestrogen, stilboestrol (300 nM), also significantly potentiated [^{3}H]-thymidine uptake, in both VSMC cultures and pulmonary artery segments. Testosterone was ineffective at a similar concentration.

5 Pre-incubation of the pulmonary VSMC with the anti-oestrogen tamoxifen (1 μM) antagonized the potentiating effect of oestradiol-17β on [^{3}H]-thymidine incorporation. The effect of tamoxifen was less pronounced in pulmonary arterial segments.

6 These data suggest that oestrogen may promote proliferation of pulmonary VSMC. Endothelial injury or dysfunction may be an important factor in the expression of the oestrogenic effect.

7 We speculate that plasma oestrogen may be a contributing factor to the proliferative lesion observed in certain forms of pulmonary vascular injury in women.

Keywords: Oestrogen; endothelium, pulmonary; vascular smooth muscle; [^{3}H]-thymidine uptake, tamoxifen

Introduction

Primary pulmonary hypertension is reported to be more common in young sexually mature women (Wagenvoort & Wagenvoort, 1970; Rich *et al.*, 1987) than in their male counterparts, and may be exacerbated in conditions associated with high oestrogen levels (Savajiyani *et al.*, 1981). An early morphological study showed that lungs from female rats show a higher incidence of muscularized arteries than those from males, in response to hypoxic challenge (Smith *et al.*, 1974). This is of interest since the pathological lesion in pulmonary hypertension is characterized by medial hypertrophy and myointimal thickening as a result of migration and proliferation of pulmonary vascular smooth muscle cells (Heath *et al.*, 1987). Oestrogen is known to promote growth of many cell lines, including breast cancer cells (Lippman *et al.*, 1976; Chalbos *et al.*, 1982), mouse Leydig cells (Nishizawa *et al.*, 1988) and rat hepatocytes (Francavilla *et al.*, 1989). However, there is no direct evidence to indicate that oestrogen stimulates vascular smooth muscle cell growth. Clinically, oestrogen replacement therapy is associated with decreased incidence of coronary heart disease (Stampfer *et al.*, 1991). Oestradiol-17β also protects against the development of experimental atherosclerosis (Renaud, 1970; Foegh *et al.*, 1987; Clarkson *et al.*, 1990). The effect of oestrogen on pulmonary vascular smooth muscle cell (VSMC) growth and proliferation has not been investigated.

In this study we describe the *in vitro* effect of oestradiol-17β on pulmonary vascular smooth muscle cells using intrapulmonary arterial segments and VSMC cultures and [^{3}H]-thymidine uptake as an index of cell proliferation (Fisher-Dzoga *et al.*, 1983). The specificity of the oestrogenic response was assessed by use of the synthetic oestrogen, stilboestrol, as well as testosterone, and the oestrogen-receptor antagonist, tamoxifen.

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Methods

Pulmonary vascular smooth muscle cell cultures

Rat pulmonary VSMC were obtained from Dr C. Diglio (Detroit, MI, U.S.A). About 10^4 cells, between the 10th and 17th passages, were cultured in 6-well Falcon tissue culture plates (Becton Dickinson, NJ, U.S.A.), and incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2 mM), gentamycin (25 μg ml^{-1}) and 10% foetal bovine serum (FBS). Cells were incubated in a humidified atmosphere at 37°C in the presence of 5% CO_2. At confluence, cell growth was arrested by withdrawing the serum from the culture medium. All experiments were performed in synchronized cells.

The effect of oestrogen on [^{3}H]-thymidine uptake was studied in growth-arrested cells, incubated overnight in DMEM containing 10% FBS, and different concentrations (0, 3, 30 and 300 nM) of oestradiol-17β, and [^{3}H]-thymidine (2 μCi ml^{-1}). The same experiment was repeated in the presence of 300 nM concentrations of testosterone and the synthetic oestrogen stilboestrol. All hormones were dissolved in ethanol and were added to the medium at a final alcohol concentration of 1%.

We also determined the effect of the oestrogen receptor antagonist, tamoxifen, on oestradiol-induced cell growth. VSMC were incubated with tamoxifen (1 μM) for 3 h before treatment with oestradiol. After 3 h, the incubation medium was changed overnight to one containing oestradiol-17β (300 nM) and [^{3}H]-thymidine.

[^{3}H]-thymidine uptake determination

The uptake of radiolabelled thymidine was used as an index of proliferation. Following overnight incubation with [^{3}H]-thymidine, VSMC were incubated for 5 min in phosphate

buffer saline (4°C), containing unlabelled thymidine and then digested overnight in NaOH (0.5 N). Aliquots were then used for determination of both radioactive count (Beckman Scintillation System, model LS 3150T) and protein content by the method of Lowry *et al.* (1951). Results are expressed as counts $min^{-1} mg^{-1}$ protein.

Pulmonary artery segments

Lungs were obtained from anaesthetized male mongrel dogs (17–25 kg) and preserved in ice cold oxygenated Krebs-Heinsleit buffer solution. Intrapulmonary arteries were isolated, cleaned of connective tissue and cut into 4–5 mm segments. In some segments, the endothelium was removed by gentle rubbing of the intima (Furchgott & Zawadzki, 1980). Segments were incubated overnight in DMEM, and 24 h later, transferred to fresh medium with 10% FBS, to which oestradiol-17β (0, 3, 30, 300 nM) and [^{3}H]-thymidine (2 μCi ml^{-1}) were added. As in cell culture studies, the specificity of the oestrogenic response was also tested with testosterone (300 nM), stilboestrol (300 nM), and tamoxifen (1 μM).

Autoradiographic studies

Autoradiographic studies were performed in order to localize the site of uptake of radioactive thymidine by pulmonary arterial segments. Following incubation with [^{3}H]-thymidine, some pulmonary artery segments were washed with PBS and fixed in 10% formalin in phosphate buffer for 3 days. Tissues were then dehydrated, embedded, mounted and stained with Mayer's haematoxylin. Stained sections were then rehydrated overnight and coated with photographic emulsion NTB_2. After 2 weeks of exposure, the sections were developed with Dektol for 45 s and fixed with GBX emulsion for 5 min. The site of [^{3}H]-thymidine incorporation was determined by light microscopy.

Statistical analysis

Statistical analysis was performed by unpaired Student's *t* test, and statistical significance was achieved $P<0.05$. All data are expressed as means ± s.e.

Materials

Oestradiol-17β, testosterone, stilboestrol and tamoxifen were purchased from Sigma; Dektol, GBX and NTB_2 emulsions from Eastman Kodak; Dulbecco's modified essential medium (DMEM), foetal bovine serum (FBS), phosphate buffer saline (PBS) and trypsin from Biofluids.

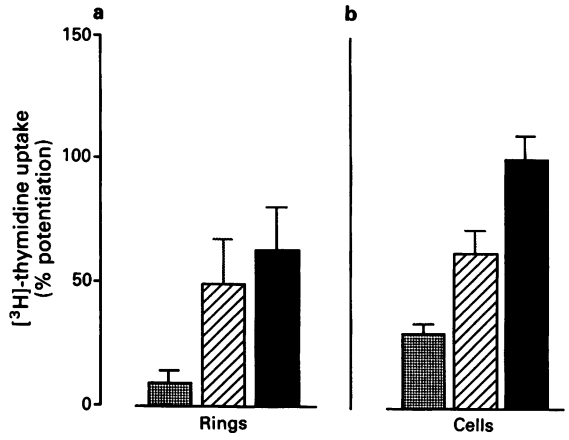

Figure 1 Effect of oestradiol 17-β on [^{3}H]-thymidine uptake in: (a) segments of canine intrapulmonary artery (rings) and (b) cultures of rat pulmonary vascular smooth muscle cells (Cells). Both cells and rings were incubated overnight in DMEM supplemented with 10% FBS, in presence of [^{3}H]-thymidine (2 μCi) and 3 nM (solid columns), 30 nM (hatched columns) or 300 nM (stippled columns) oestradiol-17β. Results are expressed as percentage potentiation of [^{3}H]-thymidine uptake compared to control uptake in presence of vehicle (ethanol). Experiments were triplicated with $n=4-6$.

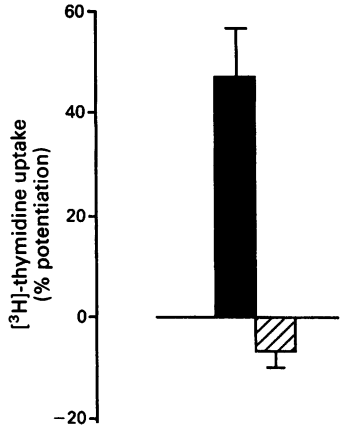

Figure 2 Effect of the endothelium on oestradiol-17β-induced uptake of [^{3}H]-thymidine in canine intrapulmonary artery. Intact (hatched columns) and arterial segments without endothelium (solid column) were incubated overnight in DMEM + 10% FBS, in presence of [^{3}H]-thymidine (2 μCi) and 300 nM oestradiol-17β or vehicle (ethanol). Results are expressed as percentage potentiation of [^{3}H]-thymidine uptake as compared to control uptake in presence of ethanol. Experiments were triplicated with $n=4-6$.

Results

Effect of oestradiol 17β on [^{3}H]-thymidine uptake

Pulmonary VSMC Incubation with 10% foetal bovine serum and oestradiol-17β (3–100 nM) increased in a concentration-dependent manner [^{3}H]-thymidine uptake in cultured pulmonary vascular smooth muscle cells. The potentiating effect was significant ($P<0.01$) at all concentrations, and reached 100 ± 10% at 300 nM oestradiol (Figure 1b).

Pulmonary arterial segments

Oestradiol 17-β also significantly ($P<0.01$) increased the uptake of [^{3}H]-thymidine in segments of canine intrapulmonary artery, in which the endothelium was removed. Oestradiol was ineffective when the endothelium remained intact (Figure 2). The increase in [^{3}H]-thymidine uptake in arterial segments without endothelium was concentration-dependent and ranged from 11 ± 2 to 47 ± 11% between 3 and 300 nM (Figure 1a).

The site of radioactive thymidine incorporation in the vessel segments was determined by autoradiography. The results show dense labelling of the nuclei in the media suggesting heavy uptake of [^{3}H]-thymidine by vascular smooth muscle cells (Figure 3).

Effect of stilboestrol and testosterone

Incubation of pulmonary VSMC with 300 nM of the non-steroid estrogen, stilboestrol, increased [^{3}H]-thymidine incorporation by 70 ± 5% (Figure 4b). Similar results were observed in pulmonary arterial segments, where the same concentration of stilboestrol increased [^{3}H]-thymidine uptake by 30 ± 2% (Figure 4a). Testosterone, at the same concentration, pro-

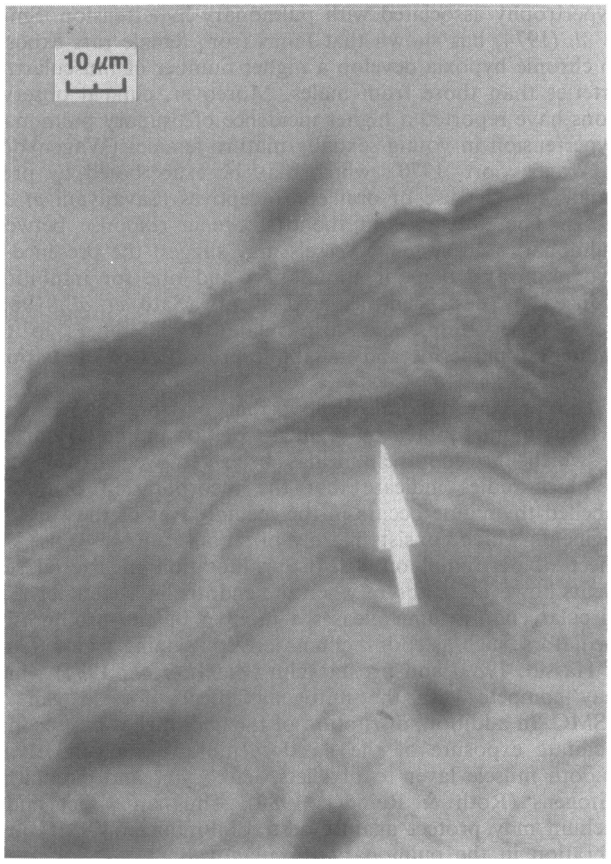

Figure 3 Autoradiograph of segment of canine intrapulmonary artery showing uptake of [^{3}H]-thymidine by nuclei of vascular smooth muscle cells. Arterial segments without endothelium were incubated overnight in DMEM + 10% FBS in presence of [^{3}H]-thymidine and 300 nM oestradiol-17β. The segments were then washed with PBS, fixed in 10% formalin, and processed for autoradiography. The autoradiograph shows dense granulation of nuclei in the media of the vascular wall, suggesting heavy uptake of [^{3}H]-thymidine by vascular smooth muscle cells.

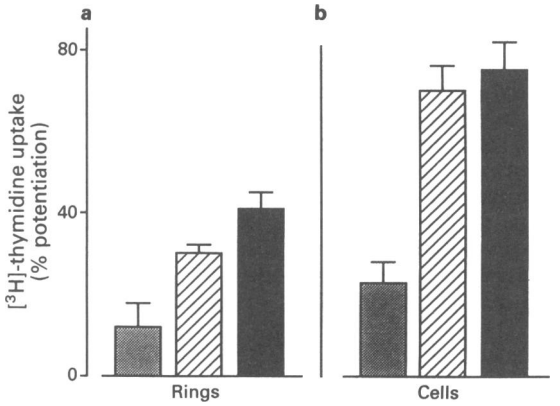

Figure 4 Comparison of the effect of testosterone (stippled columns), stilboestrol (hatched columns) and oestradiol-17β (solid columns) on [^{3}H]-thymidine uptake in (a) segments of canine intrapulmonary artery (rings) and (b) pulmonary vascular smooth muscle cell culture (cells). Cells and rings were incubated overnight in DMEM + 10% FBS in presence of [^{3}H]-thymidine (2 μCi) and ethanol or 300 nM concentrations of testosterone, stilboestrol or oestradiol. Results are expressed as percentage potentiation of [^{3}H]-thymidine uptake as compared to control uptake in presence of vehicle. Experiments were triplicated with $n = 4-6$.

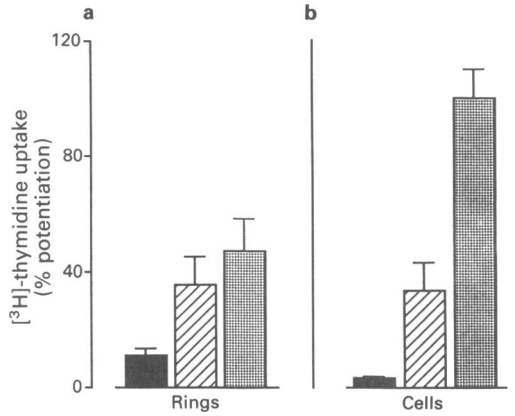

Figure 5 Effect of tamoxifen on basal (stippled columns) and oestradiol-induced (hatched columns) uptake of [^{3}H]-thymidine, compared to the effect of oestradiol-17β alone (solid columns) (a) segments of canine intrapulmonary artery (rings) and (b) cultures of rat pulmonary vascular smooth muscle cells (Cells). Rings and Cells were incubated with tamoxifen (1 μM) for 3 h before addition of ethanol or 300 nM oestradiol-17β to the medium. Results are expressed as percentage potentiation of [^{3}H]-thymidine uptake as compared to control uptake in presence of ethanol. Experiments were triplicated with $n = 4-6$.

duced only a weak and not significant effect in both preparations (Figure 4).

Effect of tamoxifen

Incubation of cell cultures with the anti-oestrogen, tamoxifen (1 μM), a partial agonist, caused a moderate (15%) but significant increase in [^{3}H]-thymidine uptake. However, tamoxifen significantly ($P < 0.01$) antagonized the potentiating effect of oestradiol in this preparation (Figure 5b). A similar qualitative effect of oestradiol was observed in the pulmonary arterial segments, but with values not reaching the level of significance (Figure 5a).

Discussion

Our data show that oestradiol-17β potentiates [^{3}H]-thymidine uptake in cultured pulmonary vascular smooth muscle cells, and in canine isolated pulmonary arterial segments, in which the endothelium has been compromised or removed. In both preparations, potentiation of [^{3}H]-thymidine uptake is also observed with the non-steroidal oestrogen stilboestrol, but not with testosterone. Furthermore the effect of oestradiol is antagonized by the anti-oestrogen, tamoxifen. This drug is a partial agonist (Jordan, 1984), which induces a moderate increase in [^{3}H]-thymidine uptake. Thus this effect of oestradiol appears to be associated with oestrogenic activity and may be mediated by specific oestrogen binding sites in pulmonary vascular smooth muscle cells. Ligand-binding studies have demonstrated the presence of oestrogen binding sites in vascular smooth muscle cells of different animal species (Harder & Coulson, 1979; Lin *et al.*, 1982).

Fisher-Dzoga *et al.* (1983) have studied the effect of oestradiol on [^{3}H]-thymidine incorporation in rabbit aortic medial tissue cultures. They found that oestradiol (20 ng ml^{-1}) does not affect growth of cells incubated in normal rabbit serum, but inhibits hyperlipaemic serum-induced cell proliferation. Our present data show that in cultured pulmonary vascular smooth muscle cells incubated in presence of normal foetal bovine serum, similar concentrations of oestradiol-17β elicited a significant increase in [^{3}H]-thymidine uptake. Our results are in agreement with the observed effects of oestrogen in other cell lines. Francavilla *et al.* (1989) have shown

that oestrogen, in the presence of 5% normal rat serum, stimulated rat hepatocyte DNA synthesis as determined by [^{3}H]-thymidine incorporation and the labelling index. Similarly, oestrogen-responsive mouse Leydig cell tumour shows an oestrogen-dependent enhancement of cell proliferation in medium supplemented with foetal bovine serum (Nishizawa *et al.*, 1988).

Oestrogen may promote cell proliferation by directly altering gene expression (Davidson & Lippman, 1989). Injection of ovariectomized rats with oestradiol-17β induces a rapid and transient increase in c-fos gene transcription in the luminal and glandular cells of the endometrium (Papa *et al.*, 1991). The c-fos oncogene plays a key role in the regulation of normal cell growth, and its transcriptional activation occurs in a number of cell types during the early phases of the proliferative response to hormones (Cohen & Curran, 1989). Oestrogen may also have an autocrine role in promoting the synthesis and/or release of peptide growth factors by growing cells, and their subsequent expression. Oestradiol regulates the synthesis and mRNA coding of mitogenic proteins in MCF-7 and T47D human breast tumour cell line (Adams *et al.*, 1980; Chalbos *et al.*, 1982; Vignon *et al.*, 1986), and endometrial carcinoma biopsies (McGuire *et al.*, 1986). Non-stimulated transformed mouse B1 Leydig cells, grown in serum-free medium also secrete a growth-promoting substance, the levels of which can be enhanced by oestrogen (Nishizawa *et al.*, 1988). Moreover, growth stimulation of many breast carcinomas by oestradiol-17β has been linked to induction of EGF-related polypeptide growth factors (Dickson *et al.*, 1986). Oestrogen may also indirectly promote cell proliferation by inactivating the action of growth-inhibiting factors present in the serum (Soto & Sonnenschein, 1985). Whether any of these mechanisms is involved in the mitogenic effect of oestradiol in pulmonary vascular smooth muscle, remains to be explored.

Epidemiological and clinical studies have supported a protective role of oestrogen against coronary heart disease (Stampfer *et al.*, 1991). Oestradiol also inhibits experimentally induced atherosclerosis in rat and rabbit (Moskowitz *et al.*, 1956; Renaud *et al.*, 1970), as well as myointimal proliferation in aorta and cardiac allograft associated with abdominal aorta transection and surgical re-anastomosis in the rabbit (Foegh *et al.*, 1987; Cheng *et al.*, 1991). Studies in the pulmonary circulation however, suggest a facilitating role of oestrogen on the development of pulmonary vascular wall hypertrophy associated with pulmonary hypertension. Smith *et al.* (1974) has shown that lungs from female rats exposed to chronic hypoxia develop a higher number of muscularized arteries than those from males. Moreover, clinical observations have reported a higher incidence of primary pulmonary hypertension in young sexually mature females (Wagenvoort & Wagenvoort, 1970), which may be exacerbated by pregnancy and the use of oral contraceptives (Savajiyani *et al.*, 1981). The difference in the oestrogenic response between pulmonary and systemic vessels may suggest the presence of two pathways, one for stimulation and one for inhibition, induced by oestrogen-mediated events (Sato *et al.*, 1986). These observations may also relate to differences in the endocrine, paracrine and metabolic properties of the various vascular beds.

The present data also show that oestrogen potentiates [^{3}H]-thymidine uptake by segments of dog pulmonary artery in which the endothelium has been removed. Autoradiographic studies indicate that the incorporation of radiolabelled thymidine occurs in the medial layer of the vascular segments which consists mainly of smooth muscle cells. The effect of oestradiol on VSMC proliferation in arterial segments however, is absent when the endothelium is intact. The vascular endothelium releases a number of anti-proliferative mediators, such as endothelium-derived relaxing factor (Garg & Hassid, 1989) and prostacyclin (Zidek *et al.*, 1987) which may compete with the mitogenic effect of oestrogen on VSMC. In addition, disruption of the endothelial barrier may facilitate exposure of the basal membrane and underlying smooth muscle layer to platelets, leukocytes and circulating mitogens (Roth & Reindel, 1980). Thus an intact endothelium may protect against oestradiol-induced VSMC proliferation in the pulmonary vascular wall.

In conclusion, myointimal hyperplasia is a component of the pathological lesion observed in chronic pulmonary hypertension. Our data suggests that oestradiol-17β enhances the proliferation of pulmonary vascular smooth muscle cells. However, in pulmonary artery segments, oestrogen-induced thymidine uptake was only observed when the endothelium was damaged. We speculate that oestrogen may promote VSMC proliferation in pulmonary vessels in the presence of endothelial injury or dysfunction.

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Novel signal transduction pathway mediating endothelium-dependent β-adrenoceptor vasorelaxation in rat thoracic aorta

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1 Isoprenaline (3×10^{-8}–10^{-5} M), salbutamol (3×10^{-7}–10^{-4} M) and forskolin (3×10^{-9}–3×10^{-7} M) relaxed rat isolated thoracic aortic rings contracted with noradrenaline (10^{-7} M). Removal of the endothelium from the aortic rings abolished the effect of acetylcholine (10^{-6} M) and completely prevented the vascular relaxation induced by isoprenaline, salbutamol or forskolin.

2 The isoprenaline concentration-relaxation curve was shifted in parallel to the right about 10 fold by propranolol (3×10^{-7} M) with no change in the maximum response, showing that the relaxation was mediated by a β-adrenoceptor.

3 The inhibitor of nitric oxide synthesis, N^G-nitro-L-arginine (L-NOARG; 10^{-5} M), shifted the isoprenaline relaxation curve to the right and reduced the maximum response.

4 Isoprenaline (10^{-6} M) relaxed noradrenaline-induced tone by approximately 95% and at the same time increased levels of adenosine 3':5'-cyclic monophosphate (cyclic AMP) 4 fold and guanosine 3':5'-cyclic monophosphate (cyclic GMP) 12 fold in the aortic rings. Sodium nitroprusside (3×10^{-8} M) relaxed noradrenaline-evoked tone by 82% without changing levels of cyclic AMP but raised cyclic GMP 19 fold.

5 Forskolin (10^{-7} M) relaxed noradrenaline-induced tone by approximately 41% and, like isoprenaline, increased levels of cyclic AMP (2.5 fold) and cyclic GMP (12 fold) in the aortic rings.

6 Removal of the endothelium abolished the relaxant effects of isoprenaline (10^{-6} M) and also the associated accumulation of cyclic AMP and cyclic GMP.

7 L-NOARG (10^{-5} M) inhibited the relaxant responses and accumulation of cyclic GMP induced by isoprenaline (10^{-6} M) and forskolin (10^{-7} M) without affecting the associated cyclic AMP accumulation.

8 It is concluded that, in the rat aorta, isoprenaline acts through a β-adrenoceptor on the endothelium to raise cyclic AMP and that this may, directly or indirectly, release nitric oxide to evoke vascular relaxation via the increase in cyclic GMP. The importance of this novel transduction pathway for cardiovascular regulation remains to be determined.

Keywords: Isoprenaline; forskolin; cyclic GMP; cyclic AMP; nitric oxide; endothelium-dependent vascular relaxation; N^G-nitro-L-arginine (L-NOARG); β-adrenoceptor

Introduction

The role of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in vascular relaxation is currently considered to be confined to activation of protein kinase A and subsequent myosin light chain kinase activation within the smooth muscle. Thus, for example, isoprenaline and other β-adrenoceptor agonists are thought to mediate their vascular relaxant effects by activation of adenylate cyclase within the smooth muscle (Kukovetz *et al.*, 1981). Such a mechanism of vascular relaxation is not dependent on the presence of an intact endothelium and would not involve nitric oxide as an endothelium-derived relaxant factor.

However, in the rat aorta the relaxations to isoprenaline are inhibited by methylene blue and haemoglobin (Grace *et al.*, 1988), agents known to affect the action of nitric oxide (Martin *et al.*, 1985). Therefore, it is possible that in this tissue there is a nitric oxide component to the relaxant response to isoprenaline. This could be mediated by endothelial β-adrenoceptors which are known to be present on endothelial cells (Stephenson & Summers, 1987; Molenaar *et al.*, 1988).

In this study we investigated the role of the endothelium and the signal transduction pathway mediating β-adrenoceptor-induced vasorelaxation in the rat thoracic aorta.

A preliminary account of these findings has been presented to the British Pharmacological Society (Gray & Marshall, 1991).

Methods

Male Sprague-Dawley rats (350–450 g) were stunned and killed by cervical dislocation. The thoracic aorta was removed, cleared of fat and connective tissue, and cut into rings of approximately 3 mm length. The endothelium was removed in some experiments by gently abrading the intimal surface of the aortic rings with fine wires. The rings were suspended on tungsten wires (diameter 0.125 mm) under 0.5 g resting tension and allowed to equilibrate for 75 min in Krebs solution containing (mM): Na^+ 143, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128, HCO_3^- 25, HPO_4^{2-} 1.2, SO_4^{2-} 1.2 and glucose 11 at 37°C and oxygenated with 95% O_2/5% CO_2. Tension was recorded with Grass FT.03 isometric transducers connected to a Grass polygraph.

Aortic rings were sub-maximally contracted with noradrenaline (10^{-7} M) and the contraction assessed for stability over a period of 15 min. Then the tissues were contracted and checked for the presence of endothelium by confirming at least 80% relaxation to acetylcholine (10^{-6} M). Tissues showing less than this level of relaxation were discarded as having partially damaged endothelium. The presence of endothelium was confirmed histologically in some tissues by

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en face silver staining (Griffith *et al.*, 1984). This technique was also used to confirm the loss of endothelium from rings which had been rubbed and which showed no relaxation by acetylcholine (10^{-6} M) of noradrenaline (10^{-7} M)–evoked tone. Some tissues were equilibrated in either propranolol or N^G-nitro-L-arginine (L-NOARG, which inhibits nitric oxide production; Ishii *et al.*, 1990; Moore *et al.*, 1990) for 15 min, control tissues receiving no treatment in this period. The aortic rings were contracted again before a cumulative concentration-effect curve to isoprenaline or salbutamol or forskolin was constructed.

For the cyclic nucleotide studies, aortic rings were prepared as described above with either the endothelium remaining intact or being removed. The state of the endothelium in all tissues was checked by use of acetylcholine and, sometimes, additionally by silver staining (see above). A single concentration of isoprenaline (10^{-6} M), or forskolin (10^{-6} M) or sodium nitroprusside (3×10^{-8} M) was added, the tissues then being removed into liquid nitrogen at various time-points for cyclic nucleotide determination. Some rings were incubated for 15 min with L-NOARG (10^{-5} M) before being contracted with noradrenaline and relaxed with a single concentration of either isoprenaline or forskolin. These tissues were removed into liquid nitrogen to determine the effect of L-NOARG on cyclic nucleotide accumulation.

Cyclic nucleotide determination

Frozen tissues were individually ground in 95% ethanol (pH 3.0) in a mortar and pestle and left overnight for extraction of the cyclic nucleotides. The samples were centrifuged to pellet the residual tissue fragments. The supernatant was decanted and evaporated to dryness under nitrogen. The sample was then resuspended in sodium acetate (50 mM at pH 5.0) and split into two aliquots for simultaneous measurement of both cyclic AMP and guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels by scintillation proximity assay (Amersham) using the acetylation protocol.

The tissue residue was dissolved in sodium hydroxide solution (0.5 M) and the protein content determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Chemicals

N^G-nitro-L-arginine (Sigma) was prepared in 1.0 M hydrochloric acid before being neutralized to pH 7.0 and diluted to form a 10^{-3} M stock solution. Forskolin (Sigma) was prepared as a 10^{-2} M stock in 70% ethanol. Salbutamol was obtained from Glaxo and prepared daily in Krebs solution. All other chemicals were obtained from Sigma; noradrenaline bitartrate, acetylcholine chloride, isoprenaline hemisulphate, (±)-propranolol hydrochloride and sodium nitroprusside and prepared daily in Krebs solution.

Statistics

Results are expressed as mean ± s.e.mean. Analysis of variance and Students unpaired *t* test were used where appropriate to assess the significance of differences between means and $P \leqslant 0.05$ was taken as statistically significant.

Results

Noradrenaline (10^{-7} M) evoked an increase in tone of the rat aortic rings with intact endothelium to 1.2 ± 0.1 g ($n = 4$). In endothelium-denuded rings the contractor response to the same concentration of noradrenaline was significantly increased (2.0 ± 0.2 g, $n = 4$). Intact rings relaxed to acetylcholine (10^{-6} M) by greater than 80%, while endothelium-denuded rings displayed no relaxant (or constrictor) response.

Endothelium-dependent relaxation

Isoprenaline (3×10^{-8}–10^{-5} M) concentration-dependent relaxation was only seen in rings with an intact endothelium (Figure 1) and began within 15 s, the effect of a given concentration reaching a maximum within about 180 s after administration. Salbutamol (3×10^{-7}–10^{-4} M) and forskolin (3×10^{-9}–3×10^{-7} M) also elicited concentration-dependent relaxations only in preparations where an intact endothelium was present (Figures 2 and 3). The pD_2 values (the negative log of the molar concentration of the drug giving 50% of the maximal relaxation for that drug) were 6.8 ± 0.1, 5.3 ± 0.3 and 7.6 ± 0.3 for isoprenaline, salbutamol and forskolin, respectively. All the vasodilators gave maximum relaxations of approximately 100% of the contraction induced by noradrenaline (10^{-7} M).

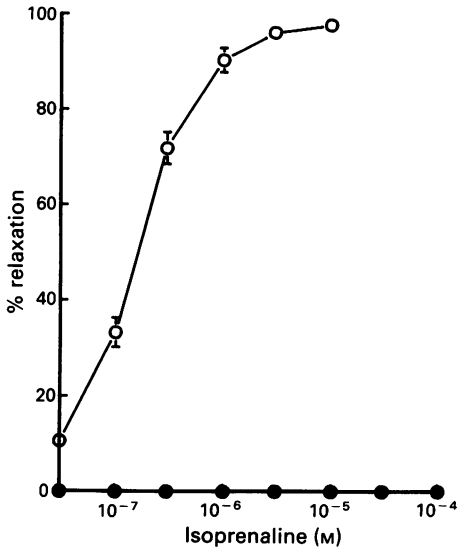

Figure 1 The effect of removal of endothelium on vasorelaxation induced by isoprenaline in rat thoracic aortic rings preconstricted with noradrenaline (10^{-7} M). Results are expressed as percentage relaxation of tone induced by noradrenaline (10^{-7} M): (○) represents the intact rings and (●) represents rings from which the endothelium has been removed. Points represent mean (± s.e.mean, vertical bars) of 4 separate experiments.

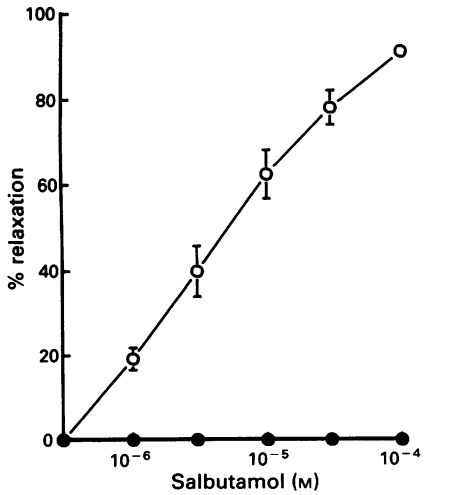

Figure 2 The effect of removal of endothelium on vasorelaxation induced by salbutamol in rat thoracic aortic rings preconstricted with noradrenaline (10^{-7} M). Results are expressed as percentage relaxation of tone induced by noradrenaline (10^{-7} M): (○) represents the intact rings and (●) represents rings from which the endothelium has been removed. Points represent mean (± s.e.mean, vertical bars) of 4 separate experiments.

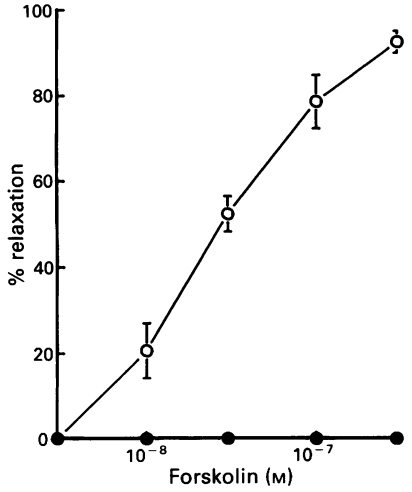

Figure 3 The effect of removal of endothelium on vasorelaxation induced by forskolin in rat thoracic aortic rings preconstricted with noradrenaline (10^{-7} M). Results are expressed as percentage relaxation of tone induced by noradrenaline (10^{-7} M): (○) represents the intact rings and (●) represents rings from which the endothelium has been removed. Points represent mean (± s.e.mean, vertical bars) of 4 separate experiments.

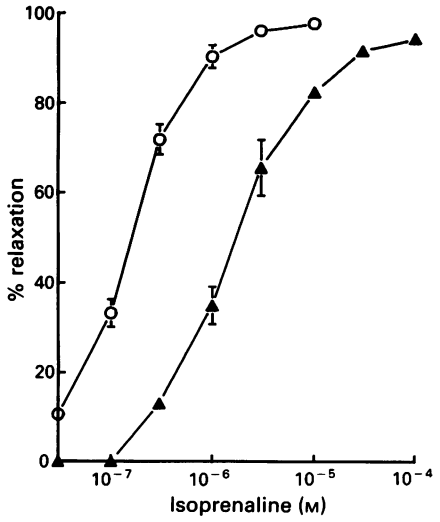

Figure 4 The effect of propranolol (3×10^{-7} M) on isoprenaline-induced vasorelaxation in rat thoracic aortic rings preconstricted with noradrenaline (10^{-7} M). Results are expressed as percentage relaxation of tone induced by noradrenaline (10^{-7} M): (○) represents the control and (▲) represents the propranolol pretreated values. Points represent the mean (± s.e.mean, vertical bars) of 4 separate experiments.

Endothelium-independent relaxation

The nitrovasodilator, sodium nitroprusside (3×10^{-9}–10^{-6} M), relaxed rat aortic rings independently of the presence of endothelium (the pD_2 being 7.1 ± 0.1 and 7.1 ± 0.1 in the presence and absence of the endothelium respectively) with the maximum relaxation being 100% of the noradrenaline (10^{-7} M)-induced contraction.

Effect of propranolol on isoprenaline endothelium-dependent relaxations

Propranolol (3×10^{-7} M) had no significant effect on the noradrenaline (10^{-7} M)-induced contraction. The concentration-effect curve to isoprenaline was shifted by propranolol (3×10^{-7} M) to the right in a parallel fashion (Figure 4). Preliminary experiments with higher concentrations of propranolol gave larger shifts (propranolol, 3×10^{-6} M, giving approximately a 100 fold rightward parallel shift with no decrease in the maximum response), indicating that propranolol is a competitive antagonist at this site (calculated K_B value 3.2×10^{-8} M). The degree of shift is consistent with the known mechanisms of action of both isoprenaline and propranolol, indicating that this endothelium-dependent relaxant effect of isoprenaline is being mediated via a β-adrenoceptor.

Effect of L-NOARG on isoprenaline-induced endothelium-dependent relaxation

The nitric oxide synthase inhibitor, L-NOARG (10^{-5} M), significantly augmented the tone induced by noradrenaline (10^{-7} M) to 1.9 ± 0.2 g ($n = 4$) in endothelium-intact rings of rat aorta. L-NOARG (10^{-5} M) shifted the relaxant response to isoprenaline to the right while decreasing the maximum response from 98% to 48% of the tone induced by noradrenaline (10^{-7} M) (Figure 5) but did not affect the relaxations induced by the endothelium-independent vasodilator, sodium nitroprusside (Gray & Marshall, 1992a).

Time course for cyclic nucleotide accumulation

Cyclic AMP and cyclic GMP control levels in rat thoracic aortic rings with intact endothelium constricted with noradrenaline (10^{-7} M) were 760 ± 114 fmol mg^{-1} protein and 52 ± 9 fmol mg^{-1} protein, respectively. Removal of the endothelium did not significantly alter the levels of cyclic AMP (941 ± 122 fmol mg^{-1} protein), but significantly reduced the levels of cyclic GMP (22 ± 4 fmol mg^{-1} protein).

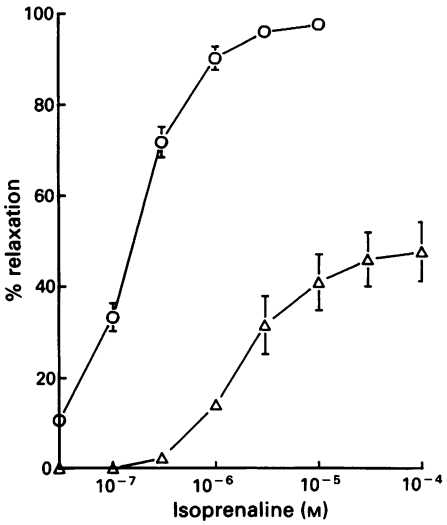

Figure 5 The effect of N^G-nitro-L-arginine (L-NOARG, 10^{-5} M) on relaxation induced by isoprenaline in rat thoracic aortic rings preconstricted with noradrenaline (10^{-7} M). Results are expressed as percentage relaxation of tone induced by noradrenaline (10^{-7} M): (○) represents the control and (▲) represents the L-NOARG pretreated values. Points represent the mean (± s.e.mean, vertical bars) of 4 separate experiments.

The nitrovasodilator, sodium nitroprusside (3×10^{-8} M), which acts within the smooth muscle to increase guanylate cyclase activity directly, caused a maximum relaxation of 82 ± 2% which developed over 180 s. While this vasorelaxation was developing there was no significant increase in levels of cyclic AMP but, as expected, cyclic GMP levels were elevated (19 fold, to 998 ± 209 fmol mg^{-1} protein; Figure 6).

Isoprenaline (10^{-6} M) caused 95 ± 5% relaxation at 60 s in rings with intact endothelium. Cyclic GMP levels were elevated 12 fold (621 ± 105 fmol mg^{-1} protein) above basal levels with almost a 4 fold rise in cyclic AMP (2751 ± 151 fmol mg^{-1} protein) at the optimum time point for cyclic nucleotide accumulations of 30 s (Figure 7).

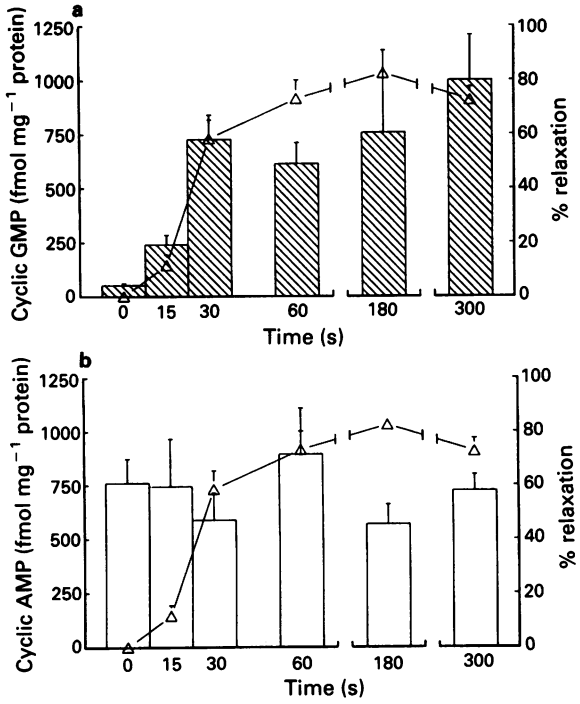

Figure 6 The effect of sodium nitroprusside (3×10^{-8} M) on cyclic GMP and cyclic AMP levels and on vascular tone induced by noradrenaline (10^{-7} M) in rat thoracic aortic rings with intact endothelium. Results are expressed in the form of a time course (s) for accumulation of cyclic GMP (a) and cyclic AMP (b) levels and relaxation. Levels of cyclic nucleotides (columns) are expressed in fmol mg^{-1} protein. Relaxant responses (△) are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7} M) in the same tissues. Columns and triangles represent the mean (± s.e.mean, vertical bars) of between 3 and 10 separate experiments.

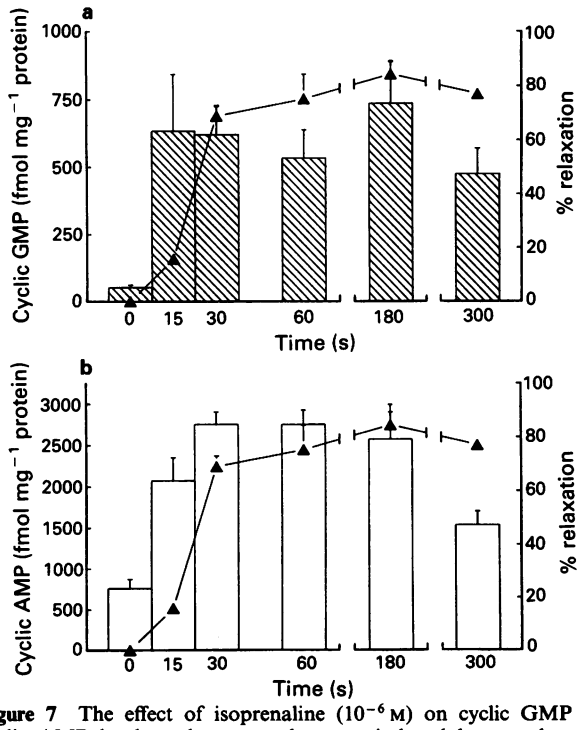

Figure 7 The effect of isoprenaline (10^{-6} M) on cyclic GMP and cyclic AMP levels and on vascular tone induced by noradrenaline (10^{-7} M) in rat thoracic aortic rings with intact endothelium. Results are expressed in the form of a time course (s) for accumulation of cyclic GMP (a) and cyclic AMP (b) levels and relaxation. Levels of cyclic nucleotides (columns) are expressed in fmol mg^{-1} protein. Relaxant responses (▲) are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7} M) in the same tissues. Columns and triangles represent the mean (± s.e.mean, vertical bars) of between 3 and 10 separate experiments.

Effect of endothelium removal on cyclic nucleotide accumulation induced by isoprenaline

In rings denuded of endothelium, isoprenaline (10^{-6} M) evoked no relaxant response in noradrenaline (10^{-7} M)-constricted rings. Further, it elicited no alterations in levels of either cyclic AMP (control, 941 ± 122 fmol mg^{-1} protein; with isoprenaline, 924 ± 78 fmol mg^{-1} protein) or cyclic GMP (control, 22 ± 4 fmol mg^{-1} protein; with isoprenaline, 19 ± 6 fmol mg^{-1} protein; Figure 8).

Effect of L-NOARG on relaxation and second messenger accumulation induced by isoprenaline and forskolin

After 60 s exposure, isoprenaline (10^{-6} M) caused a relaxant response of 69 ± 4% ($n = 10$). Preincubation with L-NOARG (10^{-5} M) significantly reduced this response to 13 ± 6% ($n = 7$). Levels of cyclic GMP were also reduced from 534 ± 105 fmol mg^{-1} protein where isoprenaline (10^{-6} M) alone was present to 38 ± 15 fmol mg^{-1} protein where L-NOARG (10^{-5} M) was also present (Figure 9). Levels of

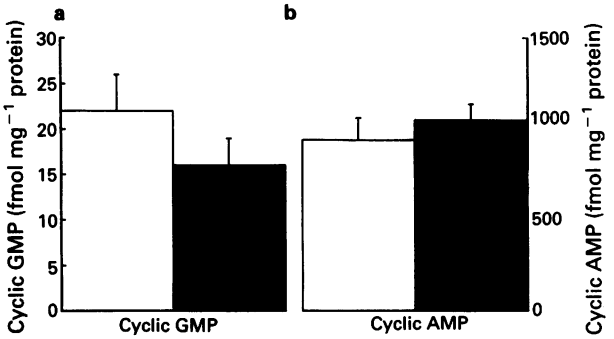

Figure 8 The effect of isoprenaline on cyclic GMP and cyclic AMP levels in rat thoracic aortic rings denuded of endothelium and preconstricted with noradrenaline (10^{-7} M). Open columns represent basal levels of cyclic nucleotides and solid columns represent the levels of cyclic nucleotides after 60 s exposure to isoprenaline (10^{-6} M). Levels of cyclic GMP (a), and cyclic AMP (b) are expressed in fmol mg^{-1} protein. Columns represent the mean (± s.e.mean, vertical bars) of between 3 and 10 separate experiments.

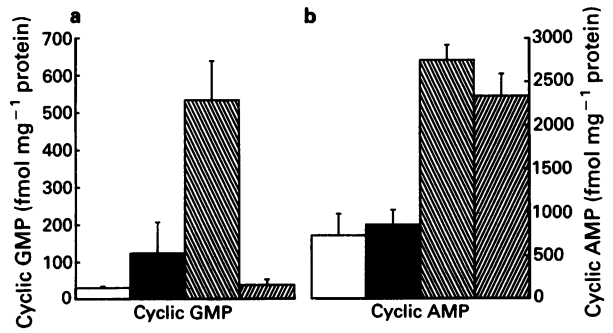

Figure 9 The effect of N^G-nitro-L-arginine (L-NOARG) on cyclic GMP and cyclic AMP levels induced by isoprenaline in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7} M): □ represents basal levels of cyclic nucleotides in rings denuded of endothelium; ■ represents basal levels of cyclic nucleotides in rings with intact endothelium; ▨ represents levels of cyclic nucleotides in endothelium intact rings after 60 s exposure to isoprenaline (10^{-6} M) and ▨ represents levels of cyclic nucleotides after 60 s exposure to isoprenaline (10^{-6} M) in endothelium intact rings preincubated with L-NOARG (10^{-5} M). Levels of cyclic GMP (a) and cyclic AMP (b) are expressed in fmol mg^{-1} protein. Columns represent the mean (± s.e.mean, vertical bars) of between 5 and 10 separate experiments.

cyclic AMP induced by isoprenaline (10^{-6} M) were not significantly affected by pretreatment with L-NOARG (10^{-5} M), the levels being 2750 ± 174 fmol mg^{-1} protein and 2336 ± 253 fmol mg^{-1} protein for control and L-NOARG pretreated tissues respectively.

Forskolin (10^{-7} M, 60 s) caused a relaxant response of $41 \pm 5\%$ which was completely abolished by preincubation with L-NOARG (10^{-5} M). Levels of cyclic GMP were reduced from 775 ± 170 fmol mg^{-1} protein where forskolin alone was present to 52 ± 9 fmol mg^{-1} protein where L-NOARG was also present (Figure 10). The raised levels of cyclic AMP induced by forskolin (10^{-7} M) were not significantly affected by pretreatment with L-NOARG (10^{-5} M), the levels being 1808 ± 140 fmol mg^{-1} protein and 1935 ± 31 fmol mg^{-1} protein for control and L-NOARG pretreated tissues, respectively.

Discussion

There are two main groups of endothelial-independent vasodilators. There are those, like sodium nitroprusside, which act via cyclic GMP and there is another group which are thought to act through cyclic AMP. In this latter group isoprenaline and other β-adrenoceptor agonists have been regarded as 'archetypal' endothelium-independent vasodilators mediating their effects by increasing cyclic AMP in the smooth muscle (Furchgott & Martin, 1985; Furchgott & Vanhoutte, 1989). Recent reports, however, have suggested that at least part of the response to a number of classical endothelium-independent vasodilator drugs known to activate adenylate cylcase is endothelium-dependent. These include:- (1) the inhibition of isoprenaline vasorelaxations in the rat aorta *in vitro* by haemoglobin and methylene blue (Grace *et al.*, 1988); (2) the inhibition of prostacyclin and forskolin vasorelaxations in the pig coronary artery *in vitro* by haemoglobin and methylene blue (Shimokawa *et al.*, 1988); and (3) the inhibition of salbutamol- and adrenaline-induced vasorelaxations *in vivo* in the rat by the nitric oxide synthase inhibitor L-N^G-nitroarginine methyl ester (Gardiner *et al.*, 1991a,b).

In this paper it has been shown that isoprenaline causes relaxations in the rat thoracic aorta which are totally dependent on the presence of an intact endothelium.

The receptors mediating this response appear to be β-adrenoceptors since salbutamol also has an endothelium-dependent mechanism of inducing relaxation, but is between

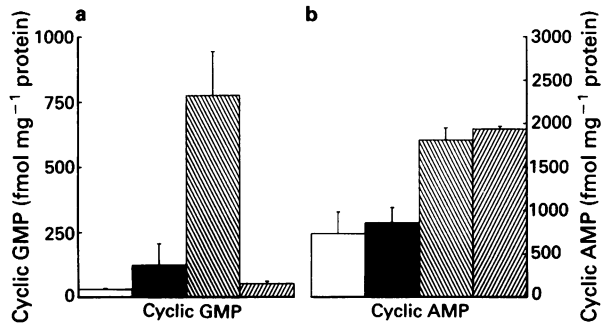

Figure 10 The effect of N^G-nitro-L-arginine (L-NOARG) on cyclic GMP and cyclic AMP levels induced by forskolin in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7} M): ☐ represents basal levels of cyclic nucleotides in rings denuded of endothelium; ■ represents basal levels of cyclic nucleotides in rings with intact endothelium; ▨ represents levels of cyclic nucleotdies in endothelium intact rings after 60 s exposure to forskolin (10^{-7} M) and ▨ represents levels of cyclic nucleotides after 60 s exposure to forskolin (10^{-7} M) in endothelium intact rings preincubated with L-NOARG (10^{-5} M). Levels of cyclic GMP (a) and cyclic AMP (b) are expressed in fmol mg^{-1} protein. Columns represent the mean (± s.e.mean, vertical bars) of between 5 and 10 separate experiments.

10 and 30 fold less potent than isoprenaline. In addition, propranolol competitively inhibits the relaxant response to isprenaline with a potency characteristic of a typical β-adrenoceptor.

Since the β-adrenoceptor is known to be linked to adenylate cyclase (Nahorski *et al.*, 1975) via the guanine nucleotide binding protein, G_s (Gilman, 1986) it appears likely that the first stage in the signal transduction pathway mediating the endothelium-dependent vasorelaxant response to isoprenaline in the rat thoracic aorta is an increase in cyclic AMP within the endothelium. This hypothesis is supported by the lack of vasorelaxation or increase in either cyclic AMP or cyclic GMP in tissues denuded of endothelium when exposed to isoprenaline.

The role of the rise in cyclic AMP appears to be activation of nitric oxide synthase, either directly or indirectly. This is apparent as forskolin, which activates adenylate cyclase directly, causes endothelium-dependent relaxations in this tissue. Further, both isoprenaline and forskolin-induced vasorelaxations and the associated elevations in cyclic GMP levels but not the rises in cyclic AMP levels in the rat aorta can be inhibited by the nitric oxide synthase inhibitor, L-NOARG.

The signal transduction pathway mediating the endothelium-dependent relaxant effects of the β-adrenoceptor agonists and forskolin in the rat thoracic aorta appears to share a number of characteristics with that postulated to mediate human α-calcitonin gene-related peptide (α-CGRP) relaxations in the same tissue (Gray & Marshall, 1992b). Firstly, all these vasodilators require the presence of the endothelium to exert their relaxant effects. Secondly, the vasorelaxations in this tissue induced by the β-adrenoceptor agonists and forskolin are associated with rises in cyclic AMP and cyclic GMP. Thirdly, removal of the endothelium, as well as abolishing vasorelaxations to these agents, also abolishes the increases in cyclic AMP and cyclic GMP. Finally, in endothelium-intact rings of rat thoracic aorta, the relaxant effects of these vasodilators are inhibited by L-NOARG which also selectively inhibits the accumulation of cyclic GMP without altering the increases in cyclic AMP.

In view of these similarities, a common signal transduction mechanism may mediate the endothelium-dependent relaxant responses to the β-adrenoceptor agonists, forskolin and human α-CGRP. This would entail activation of adenylate cyclase either directly or through a receptor on the endothelium, consequent activation of nitric oxide synthase and vasorelaxation by activation of guanylate cyclase within the smooth muscle (Figure 11).

Whether other vasodilators can activate this signal transduction pathway remains open to question. However, there is some circumstantial evidence suggesting that both prostacyclin and vasoactive intestinal polypeptide (VIP) may exert some of their relaxant effects via this mechanism. Both are known to act on receptors that are linked to activation of adenylate cyclase (Tateson *et al.*, 1977; Gorman *et al.*, 1977; Huang & Rorstad, 1983; Itoh *et al.*, 1985) and have at least a component of their relaxant effect which is endothelium-dependent (Davies & Williams, 1983; Thom *et al.*, 1986; Shimokawa *et al.*, 1988).

Although the results presented above appear to indicate that isoprenaline-induced relaxation is endothelium-dependent and mediated by nitric oxide in the rat thoracic aorta, a number of studies have reported that relaxations to this vasodilator occur independently of the endothelium in this tissue (Grace *et al.*, 1988; Kamata *et al.*, 1989; Dainty *et al.*, 1990; Weir *et al.*, 1991). Although different strains of rat have been used in these studies, preliminary experiments indicated that this could not account for the differences observed since, in addition to the Sprague-Dawley rats, aorta from Wistar strain rats (male, 350–450 g) also had endothelium-dependent relaxations to isoprenaline (3×10^{-8}–10^{-5} M) (unpublished observations). Clearly there must be some fundamental variation in the protocol used above and in those from the reported studies.

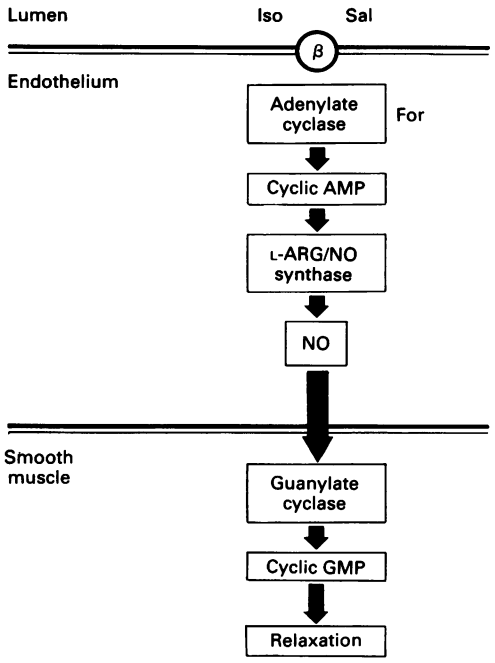

Figure 11 Novel transduction pathway mediating isoprenaline (Iso), salbutamol (Sal) and forskolin (For) endothelium-dependent relaxations in rat aorta. Isoprenaline or salbutamol act on β-adrenoceptors which appear to be present only on the endothelium. These endothelial receptors are linked to the activation of adenylate cyclase, elevating cyclic AMP levels in the endothelium. Forskolin directly activates adenylate cyclase. The cyclic AMP, either directly or indirectly, activates the synthesis of nitric oxide (NO) which results in the relaxant response via guanylate cyclase stimulation in the smooth muscle.

One explanation could be that in previous experiments the endothelium has not been removed sufficiently to abolish the relaxant effects of isoprenaline. There are a number of factors suggesting this may be the case.

Firstly, a number of studies have shown that 'complete' removal of the endothelium (shown by lack of response to muscarinic agonists) alters the relaxant response to isoprenaline in the rat aorta, shifting the concentration-effect curve to the right and decreasing the maximum response (Grace *et al.*, 1988; Kamata *et al.*, 1989; Dainty *et al.*, 1990). Although varying levels of tone were induced in the above studies, there is general agreement that in endothelium-intact rings of rat aorta, isoprenaline induces a relaxant response with EC_{50} and maximum response similar to that found in the present study. However, in supposedly completely endothelium-denuded rings of rat aorta, the maximum relaxation varies widely from approximately 30% (Grace *et al.*, 1988) to 80% of the spasmogen-induced tone (Weir *et al.*, 1991) with virtually every intermediate value being represented (Martin *et al.*, 1986; Kamata *et al.*, 1989; Dainty *et al.*, 1990). This variability of the isoprenaline vasorelaxant response in the rat aorta has also been found in individual studies with maximum responses varying between 30 and 80% of the spasmogen-induced tone in one report (Maurice *et al.*, 1991). This observation is difficult to reconcile with an endothelium-independent mechanism of relaxation but is consistent with an endothelium-dependent relaxant effect where varying proportions of the endothelium have been removed.

Secondly, it has been reported that isoprenaline-induced relaxations in the rat aorta can be inhibited by methylene blue (3×10^{-5} M) and haemoglobin (10^{-5} M) (Grace *et al.*, 1988), compounds known to inhibit the action of nitric oxide. This is in marked contrast to the rabbit aorta (where our preliminary studies indicated that isoprenaline-induced relaxations (3×10^{-8}–10^{-5} M) were endothelium-independent (female, New Zealand White, 1.5–2 kg), unpublished observations), where methylene blue and haemoglobin at similar concentrations were without effect on isoprenaline-induced vasorelaxation (Martin *et al.*, 1985).

The criterion used to confirm absence of endothelium in the literature was lack of relaxation to a muscarinic agonist, normally acetylcholine. The use of a muscarinic agonist to confirm loss of endothelium relies on the assumption that removal of the entire endothelium is required to abolish the endothelium-dependent relaxant response. This has been demonstrated in the rabbit aorta (Furchgott & Zawadzki, 1980) (where isoprenaline induces endothelium-independent vasorelaxation). However, in the rat it has been shown that relaxations to acetylcholine are abolished when some portion of the endothelium is intact, as shown by a doubling of cyclic GMP levels (Grace *et al.*, 1987). The basic assumption i.e. that removal of the entire endothelium is required to abolish the endothelium-dependent relaxant effects of muscarinic agonists, is invalid in the rat aorta where the degree of endothelium removal is best assessed histologically. Therefore, maintenance of vascular relaxation after loss of acetylcholine vasorelaxation does not prove there is an endothelial-independent component in the action of a vasodilator.

In summary, the results presented in this paper support a link between cyclic AMP elevation within the endothelium and the consequent activation of nitric oxide synthase. This represents another endothelium-dependent mechanism by which vasorelaxation can occur. There are some observations *in vivo* with adrenaline and salbutamol which support a potential physiological role for this mechanism (Gardiner *et al.*, 1991a,b). The transduction mechanism might also be found in other situations where nitric oxide is generated as a biological mediator.

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Human α-calcitonin gene-related peptide stimulates adenylate cyclase and guanylate cyclase and relaxes rat thoracic aorta by releasing nitric oxide

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1 The signal transduction pathway for vasorelaxation induced by human α-calcitonin gene-related peptide (human α-CGRP) was studied in rat thoracic aortic rings preconstricted with noradrenaline (10^{-7} M).

2 Vasorelaxation by human α-CGRP was inhibited by haemogobin (10^{-6} M) and methylene blue (10^{-5} M) but was unaffected by ibuprofen (10^{-5} M).

3 Acetylcholine caused a 16 fold increase in levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) with levels of adenosine 3':5'-cyclic monophosphate (cyclic AMP) being unaltered. Human α-CGRP caused a 12 fold increase in levels of cyclic GMP but, in contrast to acetylcholine, evoked a 2.5 fold rise in levels of cyclic AMP. The rises in cyclic nucleotides evoked by human α-CGRP and acetylcholine were dependent on the presence of an intact endothelium.

4 N^G-nitro-L-arginine (L-NOARG: 10^{-5} M), which inhibits nitric oxide synthase, inhibited the relaxant response to human α-CGRP and cyclic GMP accumulation without affecting the cyclic AMP accumulation.

5 The data presented in this paper suggests that human α-CGRP relaxes the rat thoracic aorta by releasing nitric oxide and stimulating guanylate cyclase. The stimulation of adenylate cyclase by human α-CGRP probably precedes the activation of nitric oxide synthase but could be unrelated to the relaxant response.

Keywords: Calcitonin gene-related peptide (CGRP): nitric oxide; cyclic AMP; cyclic GMP; rat thoracic aorta; endothelium-dependent vascular relaxation; N^G-nitro-L-arginine (L-NOARG)

Introduction

Calcitonin gene-related peptide (CGRP) is synthesized as an alternative product of the calcitonin gene (Amara *et al.*, 1982). It contains 37 amino acid residues and has a diverse range of pharmacological activity including being a vasodilator (Brain *et al.*, 1985; Marshall *et al.*, 1986a,b). In many vascular preparations the peptide-induced relaxation occurs in the absence of the endothelium (endothelium-independent) e.g. bovine coronary artery (Greenberg *et al.*, 1987), rat perfused mesentery (Han *et al.*, 1990) and human and cat cerebral arteries (Edvinsson *et al.*, 1985; 1987; Marshall, 1989). However, in the rat thoracic aortic ring preparation, this relaxant effect to CGRP is wholly dependent on the presence of an intact endothelium (Brain *et al.*, 1985).

In the rat aorta we recently demonstrated that human α-CGRP-induced relaxation is inhibited in the presence of analogues of arginine which block the synthesis of nitric oxide e.g. N^G-nitro-L-arginine (L-NOARG) (Gray & Marshall, 1992a). This result implicates nitric oxide as the endothelium-derived relaxant factor (EDRF) released by CGRP. However this conflicts with the reported differences in second messenger accumulation associated with nitric oxide and CGRP-induced relaxation, these being guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Griffith *et al.*, 1985) and adenosine-3':5'-cyclic monophosphate (cyclic AMP) (Kubota *et al.*, 1985; Grace *et al.*, 1987) accumulation respectively.

In this study we show that methylene blue and haemoglobin inhibit the relaxation induced by acetylcholine and human α-CGRP. Further, human α-CGRP stimulates cyclic GMP as well as cyclic AMP accumulation. In view of these results, it seems likely that CGRP relaxes rat thoracic aortic rings by releasing nitric oxide.

A preliminary account of these findings has been presented to the British Pharmacological Society (Gray & Marshall, 1991).

Methods

Rats (males, Sprague-Dawley, 300–400 g) were stunned and killed by cervical dislocation. The thoracic aorta was dissected free, cleared of fat and connective tissue, and cut into rings approximately 3 mm in length. They were mounted on thin wires in Krebs solution containing (mM): Na^+ 143, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128, HCO_3^- 25, HPO_4^{2-} 1.2, SO_4^{2-} 1.2 and glucose 11 at 37°C and oxygenated with 95% O_2/5% CO_2. An initial resting tension of 0.5 g was applied and maintained for a period of 75 min. The rings were then contracted with a submaximal concentration of noradrenaline (10^{-7} M) and the stability of this spasmogen response assessed over 15 min. Then the tissues were contracted and checked for intact endothelium by confirming at least 80% relaxation to acetylcholine (10^{-6} M). Tissues showing less than this level of relaxation were discarded as having partially damaged endothelium. The presence of endothelium was confirmed histologically in some tissues (Griffith *et al.*, 1984). Some tissues were incubated for 15 min in the presence of haemoglobin (10^{-6} M) or methylene blue (10^{-5} M). They were contracted again before a cumulative concentration-effect curve to acetycholine ($3 \times 10^{-9} - 10^{-5}$ M) or human

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α-CGRP ($3 \times 10^{-9} - 3 \times 10^{-7}$ M) was constructed.

For the cyclic nucleotide studies, a single concentration of acetylcholine or human α-CGRP giving relaxation corresponding to 65–80% of the noradrenaline-induced contraction was added, the tissues then being removed into liquid nitrogen at various time-points. The endothelium was removed from some of the tissues by gently abrading the intimal surface with fine wires. In rings of thoracic aortae the failure of acetylcholine (10^{-6} M) to elicit a relaxant response in the presence of tone induced by noradrenaline (10^{-7} M) was taken as an indication of endothelium removal. This was confirmed histologically in some tissues (see above). Some tissues were incubated for 15 min in L-NOARG (10^{-5} M) or for 30 min in ibuprofen (10^{-5} M) before being contracted with noradrenaline and relaxed with a single concentration of human α-CGRP. These tissues were removed into liquid nitrogen to determine the effect of L-NOARG and ibuprofen on second messenger accumulation.

Cyclic nucleotide determination

Frozen tissues were individually ground in 95% ethanol (pH 3.0) in a mortar and pestle and left overnight for extraction of the cyclic nucleotides. The samples were centrifuged to pellet the residual tissue fragments. The supernatant was decanted and evaporated to dryness under nitrogen. The sample was then resuspended in sodium acetate (50 mM at pH 5.0) and divided into two aliquots for simultaneous measurement of both cyclic AMP and cyclic GMP levels by scintillation proximity assay (Amersham) using the acetylation protocol.

The tissue residue was dissolved in sodium hydroxide solution (0.5 M) and the protein content determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Chemicals

Human α-CGRP (Bachem), having been checked for purity and structure by mass spectrometry, was dissolved in distilled water to form a 10^{-3} M stock solution, divided into aliquots and stored at −20°C. N^G-nitro-L-arginine (Sigma) was prepared in 1.0 M hydrochloric acid before being neutralized to pH 7.0 and diluted to form a 10^{-3} M stock solution. Haemoglobin (Sigma) was prepared for use by converting any methaemoglobin to oxyhaemoglobin. This was done by adding a 10 fold molar excess of sodium dithionite to a 10^{-3} M solution of bovine haemoglobin. This was then dialysed for 2 h at 4°C and then stored in aliquots at −20°C. All other chemicals; noradrenaline bitartrate, acetylcholine chloride, and methylene blue were obtained from Sigma and prepared daily in Krebs solution.

Statistics

Results are expressed as mean ± s.e.mean for n separate experiments. Analysis of variance and Student's unpaired t test were used where appropriate to assess the significance of differences between means and $P \leqslant 0.05$ was taken as statistically significant.

Results

Effect of haemoglobin and methylene blue on noradrenaline-induced contraction

Noradrenaline (10^{-7} M) induced a sustained contraction of 1.2 ± 0.1 g ($n = 4$). Haemoglobin (10^{-6} M), reported to bind nitric oxide, and methylene blue (10^{-5} M), a soluble guanylate cyclase inhibitor, significantly increased the tone induced by noradrenaline (10^{-7} M) by 30 ± 14% ($n = 4$) and 36 ± 9% ($n = 4$) respectively. The increase in tone was maintained over a period of 15 min in both cases. These data are consistent with there being a basal release of nitric oxide in this tissue.

Effect of haemoglobin and methylene blue on endothelium-dependent relaxation

Haemoglobin (10^{-6} M) caused approximately a ten fold parallel, rightward shift in the concentration-effect curve to acetylcholine and almost completely abolished the response to human α-CGRP (Figure 1). Methylene blue (10^{-5} M) also shifted the acetylcholine curve to the right in a parallel manner, approximately three fold in this case (Figure 2). The concentration-effect curve to human α-CGRP was shifted to the right by methylene blue with the maximum response being greatly reduced (Figure 2). Neither haemoglobin nor methylene blue caused significant alterations in the speed of onset or equilibration of the relaxant response to either acetylcholine or human α-CGRP.

Time course for cyclic nucleotide accumulation

Cyclic AMP and cyclic GMP control levels in rat thoracic aortic rings with intact endothelium and constricted with noradrenaline 10^{-7} M were 760 ± 114 fmol mg^{-1} protein ($n = 10$) and 52 ± 9 fmol mg^{-1} protein ($n = 10$), respectively. Removal of the endothelium did not significantly alter the levels of cyclic AMP (941 ± 122 fmol mg^{-1} protein, $n = 5$), but significantly reduced the levels of cyclic GMP (22 ± 4 fmol mg^{-1} protein, $n = 5$).

Acetylcholine (3×10^{-8} M) caused a relaxation of 80 ± 7% which developed smoothly over a period of 300 s. While this relaxant response was developing, levels of cyclic AMP remained unaltered but levels of cyclic GMP showed a maximal 16 fold increase (853 ± 224 fmol mg^{-1} protein) at 60 s (Figure 3).

Human α-CGRP (3×10^{-7} M) gave a relaxation of 66 ± 3%. The response did not develop continuously but, in marked contrast to the relaxant response induced by acetylcholine, showed an initial relaxant spike (10–30 s), followed by a recovery of tone (30–60 s) and then a further relaxant response which achieved equilibration at 300 s. Cyclic nucleotide accumulation also showed a different pattern with a maximal 2.5 fold rise in cyclic AMP (2306 ± 206 fmol mg^{-1} protein) and 12 fold rise in cyclic GMP (637 ± 93 fmol mg^{-1} protein) at 30 s (Figure 4).

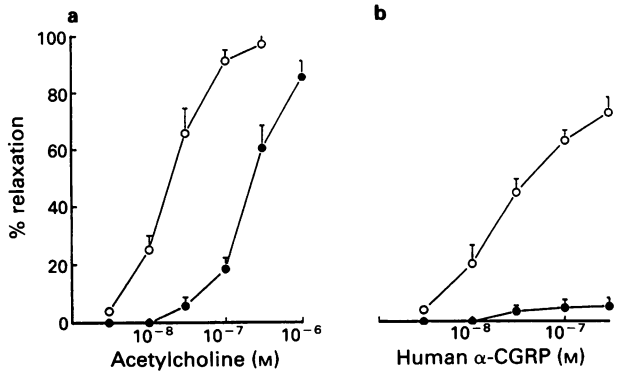

Figure 1 The effect of haemoglobin (10^{-6} M) on relaxations induced by acetylcholine (a) and human α-calcitonin gene-related peptide (α-CGRP) (b) in rat thoracic aorta with intact endothelium and preconstricted with noradrenaline (10^{-7} M). Results are expressed as percentage relaxation of the tone induced by noradrenaline (10^{-7} M): (○) represents the control and (●) represents the haemoglobin pretreated values for each vasodilator. Points represent the mean (± s.e.mean, vertical bars) of 4 separate experiments.

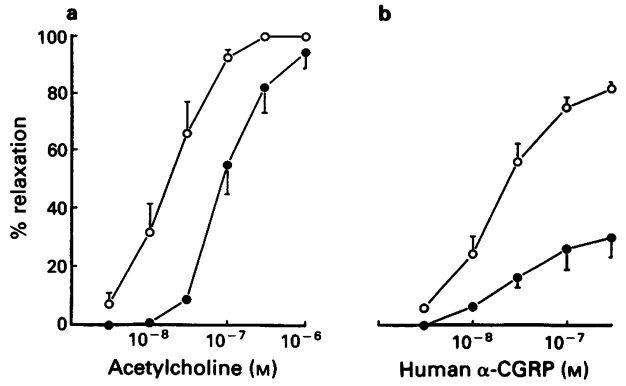

Figure 2 The effect of methylene blue (10^{-5} M) on relaxations induced by acetylcholine (a) and human α-calcitonin gene-related peptide (α-CGRP) (b) in rat thoracic aorta with intact endothelium and preconstricted with noradrenaline (10^{-7} M). Results are expressed as percentage relaxation of the tone induced by noradrenaline (10^{-7} M): (○) represents the control and (●) represents the haemoglobin pretreated values for each vasodilator. Points represent the mean (± s.e.mean, vertical bars) of 4 separate experiments.

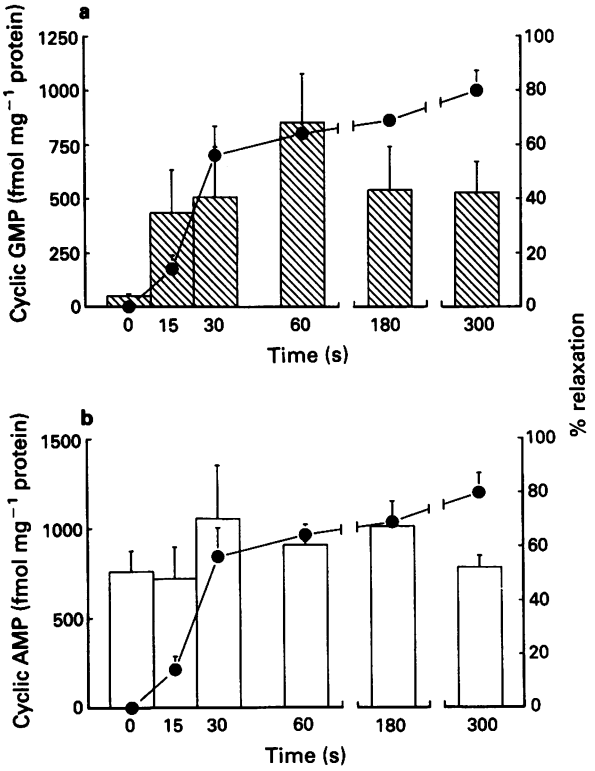

Figure 3 The effect of acetylcholine (3×10^{-8} M) on cyclic GMP and cyclic AMP levels and on vascular tone induced by noradrenaline (10^{-7} M) in rat thoracic aortic rings with intact endothelium. Results are expressed in the form of a time course (s) for accumulation of cyclic GMP (a) and cyclic AMP (b) level and relaxation. Levels of cyclic nucleotides (columns) are expressed in fmol mg^{-1} protein. Relaxant responses (●) are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7} M) in the same tissues. Columns and circles represent the mean (± s.e.mean, vertical bars) of between 3 and 10 separate experiments.

Effect of removal of endothelium on cyclic nucleotide accumulations induced by human α-CGRP

In rings denuded of endothelium, human α-CGRP (3×10^{-7} M) elicited no relaxant responses (data not shown: Gray & Marshall, 1992a). Further, human α-CGRP produced no significant alterations in either cyclic GMP or cyclic AMP after endothelium removal (Figure 5).

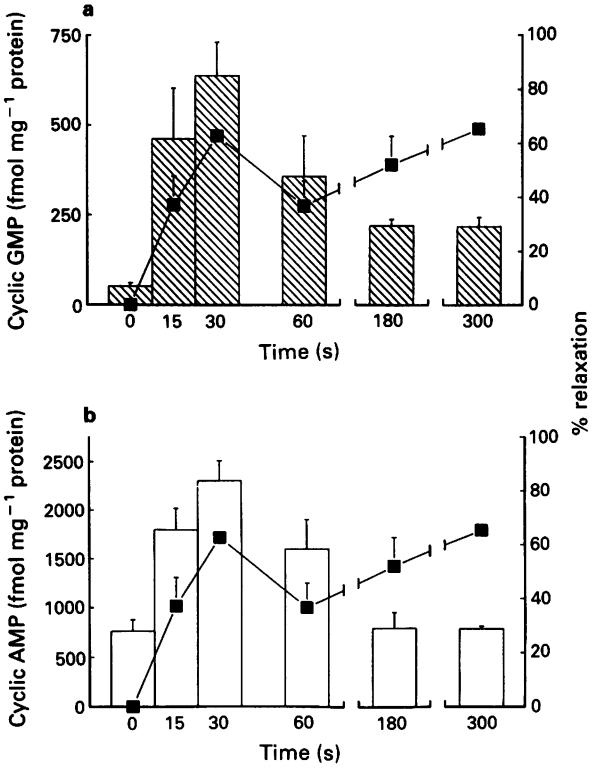

Figure 4 The effect of human α-calcitonin gene-related peptide (α-CGRP, 3×10^{-7} M) on cyclic GMP and cyclic AMP levels and on vascular tone induced by noradrenaline (10^{-7} M) in rat thoracic aorta with intact endothelium. Results are expressed in the form of a time course (s) for accumulation of cyclic GMP (a) and cyclic AMP (b) levels and relaxation. Levels of cyclic nucleotides (columns) are expressed in fmol mg^{-1} protein. Relaxant responses (■) are expressed as a percentage relaxation of the tone induced by noradrenaline (10^{-7} M) in the same tissues. Columns and squares represent the mean (± s.e.mean, vertical bars) of between 3 and 10 separate experiments.

Effect of ibuprofen (10^{-5} M) on relaxation and second messenger accumulation induced by human α-CGRP

At 30 s, human α-CGRP (3×10^{-7} M) caused a relaxant response of noradrenaline-induced tone of 63 ± 3%. Preincubation with ibuprofen (10^{-5} M) had no significant effect on this relaxant response (60 ± 4%). Ibuprofen also had no effect on the accumulations of either cyclic AMP or cyclic GMP induced by human α-CGRP (Figure 6).

Effect of L-NOARG on relaxation and second messenger accumulation induced by human α-CGRP

Preincubation with L-NOARG (10^{-5} M) reduced the relaxant response evoked by human α-CGRP (3×10^{-7} M) at 30 s to 7 ± 3%. Levels of cyclic GMP were also reduced from 667 ± 106 fmol mg^{-1} protein where human α-CGRP alone was present to 49 ± 16 fmol mg^{-1} protein where L-NOARG was also present (Figure 7).

Levels of cyclic AMP were not significantly affected by pretreatment with L-NOARG (10^{-5} M), the levels being 2443 ± 233 fmol mg^{-1} protein and 2376 ± 306 fmol mg^{-1} protein for control and pretreated tissues respectively (Figure 7).

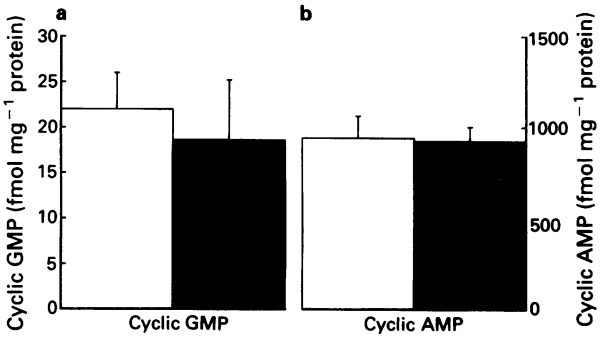

Figure 5 The effect of human α-calcitonin gene-related peptide (α-CGRP) on cyclic GMP and cyclic AMP levels in rat thoracic aortic rings denuded of endothelium and preconstricted with noradrenaline (10^{-7} M). Open columns represent basal levels of cyclic nucleotides and solid columns represent the levels of cyclic nucleotides after 30 s exposure to human α-CGRP (3×10^{-7} M). Levels of cyclic GMP (a) and cyclic AMP (b) are expressed in fmol mg^{-1} protein. Columns represent the mean (± s.e.mean, vertical bars) of between 5 and 10 separate experiments.

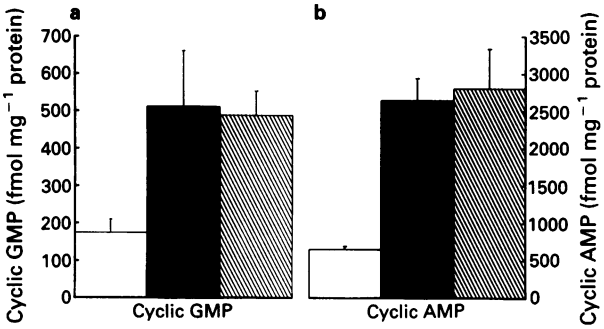

Figure 6 The effect of ibuprofen on cyclic GMP and cyclic AMP levels induced by human α-calcitonin gene-related peptide (α-CGRP) in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7} M). Open column represents basal levels of cyclic nucleotides; solid column represents the levels of cyclic nucleotides after 30 s exposure to human α-CGRP (3×10^{-7} M) and hatched column represents levels of cyclic nucleotides after ibuprofen (10^{-5} M). Levels of cyclic GMP (a) and cyclic AMP (b) are expressed in fmol mg^{-1} protein. Columns represent the mean (± s.e.mean, vertical bars) of between 5 and 10 separate experiments.

Discussion

Acetylcholine and CGRP have been shown to require the presence of an intact endothelium in order to relax rat thoracic aortic rings (Brain *et al.*, 1985; Grace *et al.*, 1987). The endothelium-derived relaxing factor for acetylcholine has been identified as nitric oxide (Palmer *et al.*, 1987). In view of the reported differences in second messenger accumulation for acetylcholine and CGRP, these being cyclic GMP (Griffith *et al.*, 1985) and cyclic AMP (Kubota *et al.*, 1985; Grace *et al.*, 1987) respectively, it appeared possible that another factor and not nitric oxide was being released by CGRP. We recently demonstrated that the relaxations induced by both acetylcholine and human α-CGRP could be inhibited by N^G-monomethyl-L-arginine (L-NMMA) and L-NOARG (Gray & Marshall, 1992a), which are reported to inhibit the synthesis of nitric oxide from the terminal guanidino nitrogen of L-arginine (Rees *et al.*, 1989; Moore *et al.*, 1990). Further this inhibition could be reversed in both cases by the concomitant addition of L- but not D-arginine. This implies a common synthetic pathway for nitric oxide and the EDRF released by CGRP.

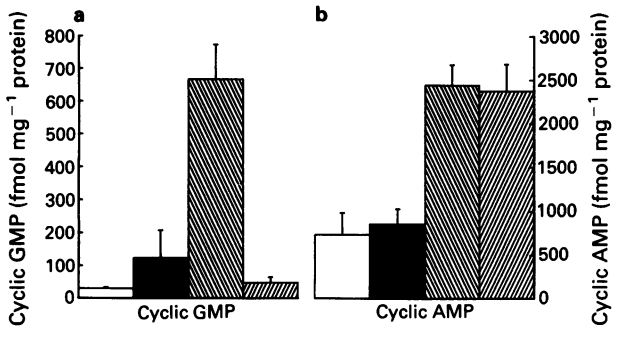

Figure 7 The effect of N^G-nitro-L-arginine (L-NOARG) on cyclic GMP and cyclic AMP levels induced by human α-calcitonin gene-related peptide (α-CGRP) in rat thoracic aortic rings with intact endothelium preconstricted with noradrenaline (10^{-7} M): ☐ represents basal levels of cyclic nucleotides in rings denuded of endothelium; ■ represents basal levels of cyclic nucleotides in rings with intact endothelium; ▨ represents levels of cyclic nucleotides in endothelium intact rings after 30 s exposure to human α-CGRP (3×10^{-7} M); and ▨ represents levels of cyclic nucleotides after 30 s exposure to human α-CGRP (3×10^{-7} M) in endothelium intact rings preincubated with L-NOARG (10^{-5} M). Levels of cyclic GMP (a) and cyclic AMP (b) are expressed in fmol mg^{-1} protein. Columns represent the mean (± s.e.mean, vertical bars) of between 5 and 10 separate experiments.

In this study we show that a compound known to bind nitric oxide, haemoglobin, inhibits the relaxant response to acetylcholine and human α-CGRP. This suggests that the EDRFs released by acetylcholine and CGRP share some chemical properties. However, methylene blue, a soluble guanylate cyclase inhibitor, also inhibited the vasorelaxation induced by acetylcholine and CGRP, implicating elevations in cyclic GMP in the signal transduction pathway for acetylcholine and CGRP. Therefore it is surprising that the published data indicate that CGRP does not evoke a rise in cyclic GMP.

In the present study, the cyclic nucleotide accumulations associated with CGRP endothelium-dependent relaxations were studied. Contrary to some of the findings of Grace *et al.* (1987), it was found that human α-CGRP elevated levels of cyclic GMP, the first time an elevation in this nucleotide has been associated with CGRP-induced vasorelaxation. This result eliminates the only reported difference between the EDRF and nitric oxide.

The discrepancy between the present results and those of Grace *et al.* (1987) over cyclic GMP is only one of the differences in the effects of CGRP. Another difference is the greater inhibition of α-adrenoceptor-induced tone in the present experiments (around 70% compared with 20%). Given the endothelium-dependent response to CGRP in the aorta, it is likely that the greater response may reflect greater integrity of the endothelium. If this were the case, then the greater amount of endothelium would generate more NO and the increase in cyclic GMP production in the vascular smooth muscle would be easier to measure. This may be the explanation for the increase in the cyclic nucleotide detected here but not in the earlier experiments (Grace *et al.*, 1987).

Therefore the EDRF released by CGRP has the following properties: (1) It appears to be synthesized from L-arginine by nitric oxide synthase, since L-NMMA and L-NOARG inhibited the relaxant response to CGRP and this inhibitory effect could be partially reversed by simultaneous administration of L-, but not D-arginine (Gray & Marshall, 1992a). (2) Its action is inhibited by agents known to inhibit nitric oxide action, that is, haemoglobin and methylene blue. (3) Its relaxant effect is associated with rises in cyclic GMP, like nitric oxide. In view of these properties, it seems highly probable that CGRP is releasing nitric oxide as its EDRF.

One difference remains between the endothelium-dependent

relaxations induced by human α-CGRP and nitric oxide, the EDRF released by acetylcholine. While acetylcholine selectivity increases levels of cyclic GMP, human α-CGRP elevates levels of both cyclic GMP and cyclic AMP. If, as seems likely from the data presented above, CGRP is releasing nitric oxide as its EDRF, then the stimulation of guanylate cyclase resulting in increased cyclic GMP levels is readily explained. However, the role of elevations in cyclic AMP levels is unclear.

There are reports that in cultured endothelial cells and cultured vascular smooth muscle cells, CGRP elicits selective increases in cyclic AMP (Kubota *et al.*, 1985; Hirata *et al.*, 1988). In contrast to this, it was shown above that in rings of rat aorta, elevations in both cyclic AMP and cyclic GMP occurred but only when the endothelium was present. This conflicting result between cultured systems and more complex tissues and other disparities including the lack of release of any EDRF and lack of increase in cyclic GMP in cultured endothelial cells highlights the difficulties of extrapolating from cultured systems to tissues.

The observation that no elevations in cyclic AMP occurred when the endothelium was absent, although ruling out the existence of receptors for CGRP mediating the rise in cyclic AMP on the vascular smooth muscle, does not necessarily require the cyclic AMP increases to occur solely within the endothelium. It is possible that a second endothelium-derived factor is being released from the endothelium by human α-CGRP which activates adenylate cyclase within the smooth muscle. This second factor need only mediate the rise in cyclic AMP since the simultaneous release of nitric oxide by CGRP would be sufficient to account for any relaxant response.

Despite the lack of relaxation to prostacyclin in this tissue reported previously (Gray & Marshall, 1992a), it was considered possible that, since CGRP is capable of releasing this prostaglandin from endothelial cells (Crossman *et al.*, 1987) and it activates adenylate cyclase (Gorman *et al.*, 1977; Tateson *et al.*, 1977), prostacyclin was the most likely candidate for the second factor released by CGRP.

However, it was shown that the cyclo-oxygenase inhibitor, ibuprofen, as well as not affecting the endothelium-dependent relaxant response to CGRP, did not affect the cyclic nucleotide accumulations. This result rules out any of the prostaglandins and prostacyclin in particular as mediating the cyclic AMP elevations induced by CGRP.

Although there are a number of mechanisms by which cyclic GMP elevations could subsequently lead to rises in cyclic AMP, for example, activation by phosphorylation of adenylate cyclase by cyclic GMP-dependent protein kinase or inhibition of phosphodiesterase IV by cyclic GMP (Yamamoto *et al.*, 1984), it is highly improbable that any of these mechanisms is responsible for the results presented above for a number of reasons. Firstly, acetylcholine at the concentration used, elevated cyclic GMP levels above those achieved by CGRP but did not cause any alterations in the levels of cyclic AMP. Secondly, an increase in cyclic AMP dependent on an increase in cyclic GMP is incompatible with the results obtained with the nitric oxide synthase inhibitor, L-NOARG. It was shown that this drug inhibits the relaxation and rise in cyclic GMP induced by human α-CGRP without affecting the accumulation of cyclic AMP. This result, while being entirely consistent with the postulated mechanism of action of L-NOARG and with human α-CGRP releasing nitric oxide as its EDRF, requires the elevations in cyclic AMP to precede the activation of nitric oxide synthase. This novel mechanism for the activation of NO synthase is represented diagramatically in Figure 8.

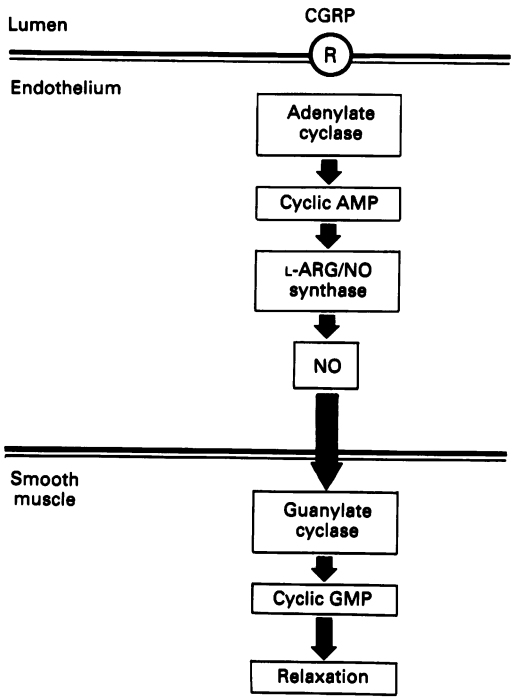

Figure 8 Novel transduction pathway mediating human α-calcitonin gene-related peptide (α-CGRP) endothelium-dependent relaxations in rat aorta. Human α-CGRP has receptors (R) only on the endothelium. The endothelial receptors activate adenylate cyclase, elevating cyclic AMP levels in the endothelium. This, either directly or indirectly, activates the synthesis of nitric oxide (NO) which results in the relaxant response via guanylate cyclase stimulation in the smooth muscle.

There is no evidence in this paper to suggest that the elevations in cyclic AMP levels are linked to the relaxant response. However, there have been a number of reports of vasodilators known to act by increasing levels of cyclic AMP having an endothelium-dependent component to their relaxant responses. These include forskolin and prostacyclin in the pig coronary artery (Shimokawa *et al.*, 1988) and salbutamol and adrenaline *in vivo* in the rat (Gardiner *et al.*, 1991a,b) and isoprenaline, salbutamol and forskolin in the rat thoracic aorta (Gray & Marshall, 1992b). In view of these results, it appears likely that human α-CGRP and a number of other drugs exert their endothelium-dependent relaxant effects through the release of nitric oxide by a novel signal transduction pathway. This involves receptor activation of adenylate cyclase, the rise in cyclic AMP either directly or indirectly activating the synthesis of nitric oxide and a subsequent increase in cyclic GMP resulting from the activation of guanylate cyclase.

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Identification of α_1-adrenoceptor subtypes in the rat vas deferens: binding and functional studies

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1 The α_1-adrenoceptor subtypes of the prostatic and epididymal portion of rat vas deferens were characterized in binding and functional experiments.

2 In saturation experiments, [^{3}H]-prazosin bound to two distinct affinity sites in the epididymal portion of rat vas deferens ($pK_D = 10.1 \pm 0.13$ and 9.01 ± 0.15, $B_{max} = 507$ and 1231 fmol mg^{-1} protein, respectively). In the prostatic portion [^{3}H]-prazosin bound to a single affinity site ($pK_D = 9.82 \pm 0.04$, $B_{max} = 924$ fmol mg^{-1} protein).

3 In the displacement experiments, unlabelled prazosin displaced biphasically the binding of 200 pM [^{3}H]-prazosin to the epididymal portion; the resulting two pK_I values were consistent with the affinity constants obtained in the saturation experiments. WB4101 (2-(2,6-dimethoxy-phenoxyethyl)-aminomethyl-1,4-benzodioxane) and benoxathian also discriminated the two affinity sites in the epididymal portion and the population of low affinity sites for the three antagonists was approximately 40%. On the other hand, the prostatic portion predominantly showed a single affinity site for prazosin, WB4101 and benoxathian, although the presence of a small proportion (less than 10%) of the low affinity site could be detected. HV723 (α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxy)ethyl)-amino)-propyl) benzeneacetonitrile fumarate) displaced the [^{3}H]-prazosin binding monophasically with a low affinity in both halves.

4 Pretreatment with chlorethylclonidine (CEC) at concentrations higher than 1 μM inhibited 700 pM [^{3}H]-prazosin binding to the prostatic portion by approximately 50%. However, the inhibition in the epididymal portion was much less (approximately 21% at 50 μM CEC).

5 In the functional study, the contractile response to noradrenaline was competitively inhibited by prazosin, WB4101, benoxathian and HV723 with similar and low affinities (pK_B value ranging from 8.0 to 9.0) in the epididymal portion of rat vas deferens. In the prostatic portion of rat vas deferens, noradrenaline also produced a contraction, but the maximal amplitude of contraction developed was approximately one-fourth of that in the epididymal portion. Prazosin and WB4101 also inhibited the contractile response of the prostatic portion with the pK_B values similar to those obtained in the epididymal portion. The contractions to noradrenaline in both portions were potently attenuated by 1 μM nifedipine but were not affected by pretreatment with 10 μM CEC.

6 Under conditions where P_{2x}-purinoceptors and prejunctional α_2-adrenoceptors were blocked, electrical transmural stimulation produced a rapidly developing phasic contraction and a subsequent tonic contraction in the epididymal portion of rat vas deferens. The phasic and tonic contractions were inhibited in a concentration-dependent manner by prazosin ($IC_{50} = 25.7$ and 25.9 nM, respectively), WB4101 ($IC_{50} = 7.27$ and 7.58 nM), benoxathian ($IC_{50} = 10.9$ and 8.66 nM) and HV723 ($IC_{50} = 15.9$ and 14.9 nM). Nifedipine selectively attenuated the tonic contraction induced by electrical stimulation, and the residual phasic response was inhibited by the antagonists mentioned above with similar affinities to those in the absence of nifedipine. CEC (10 μM) had little effect on the adrenergic neurogenic contractions.

7 The present results indicate the presence of two distinct α_1-adrenoceptor subtypes in the rat vas deferens, which show respectively high and low affinities for each of prazosin, WB4101 and benoxathian, and presumably correspond to putative α_{1A} and α_{1L} subtypes according to the recent α_1-adrenoceptor subclassifications. The contractions induced by exogenous and endogenous noradrenaline seem to be predominantly mediated through the α_{1L} subtype. The heterogeneous distribution of the low affinity sites (α_{1L} subtype) may well explain differences in functional responsiveness between the two portions of rat vas deferens.

Keywords: α_1-Adrenoceptor subtype; noradrenaline-induced contraction; rat vas deferens; α_1-adrenoceptor subclassification

Introduction

The α_1-adrenoceptors are not homogeneous in all tissues and it has been suggested that their heterogeneity may be related, in part, to the presence of different α-adrenoceptor subtypes (Bülbring & Tomita, 1987; McGrath & Wilson, 1988; Minneman, 1988; Wilson *et al.*, 1991). Recent radioligand binding studies with [^{3}H]-prazosin or [^{125}I]-BE2254 (2-[β-4-hydroxy-3-[^{125}I]-iodophenyl)-ethylaminoethyl]-tetralone) demonstrated two separate populations of α_1-adrenoceptors in the rat brain and the rat vas deferens which were designated α_{1A} (or α_{1a}) and α_{1B} (or α_{1b}), respectively (Morrow & Creese, 1986; Han *et al.*, 1987a). The α_{1A} (or α_{1a}) subtype has high affinity for (2-(2,6-dimethoxy-phenoxyethyl-amino methyl-1,4-benzodioxane (WB4101), benoxathian and phentolamine, while the α_{1B} (α_{1b}) subtype has lower affinity for the competitive antagonists and is potently inactivated by chlorethylclonidine (CEC) (Han *et al.*, 1987b). However, the subtypes cannot be discriminated by prazosin and yohimbine (Hanft & Gross, 1989). In contrast, results of functional studies with blood vessels have suggested another subclassification, where α_1-adrenoceptors can be classified into two (α_{1H}, α_{1L}) or three (α_{1H}, α_{1L} and α_{1N}) subtypes by their different affinities for

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prazosin and α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxy)ethyl)-amino)-propyl benzeneacetonitrile fumarate (HV723) (Flavahan & Vanhoutte, 1986; Muramatsu *et al.*, 1990b). We have recently demonstrated that the α_{1A} and α_{1B} subtypes can be identified as a single site with high affinity for prazosin, suggesting that α_{1A} and α_{1B} subtypes may be included in the α_{1H} subtype defined in the α_{1H}, α_{1L}, α_{1N} subclassification (Oshita *et al.*, 1991; Muramatsu *et al.*, 1991). Further, we demonstrated the low selectivity of CEC for α_1-adrenoceptor subtypes and proposed a possible conciliation of the two distinct α_1-adrenoceptor subclassifications as shown in Table 1.

The α_1-adrenoceptors of rat vas deferens have so far been studied according to the α_{1A} and α_{1B} subclassification and the presence of both the subtypes has been suggested (Han *et al.*, 1987a; Hanft & Gross, 1989). However, the occurrence of only α_{1A} but not α_{1B} subtype in the rat vas deferens was recently confirmed by molecular biological approaches (Lomasney *et al.*, 1991b). The contractile response to noradrenaline has been reported to be mediated through the α_{1A} subtype which is closely coupled to voltage-dependent Ca channels (Han *et al.*, 1987a; Minneman, 1988), whereas the adrenergic neurogenic contraction induced by electrical stimulation with a single pulse is completely resistant to nifedipine (Blakeley *et al.*, 1981; McGrath & Wilson, 1988; Spriggs *et al.*, 1991). Further, prostatic and epididymal portions of rat vas deferens differ not only in their neuro-effector transmission (McGrath, 1978; Brown *et al.*, 1983) but also in their postjunctional responses to various α-adrenoceptor agonists (Kasuya & Suzuki, 1979; MacDonald & McGrath, 1980; Moore & Griffiths, 1982; Badia & Salles, 1989). Such evidence suggests complexity of the adrenergic transmission in rat vas deferens which should be resolved. In the present paper, we characterized the α_1-adrenoceptor subtypes in the epididymal and prostatic portions of rat vas deferens on the basis of the criteria defined in the α_1-adrenoceptor subclassifications mentioned above.

Methods

Binding study

Vasa deferentia were isolated from male Wistar rats (260–450 g) and cut into two (prostatic and epididymal) portions. Each portion was separately homogenized in 100 vol of buffer (Tris HCl 50 mM, NaCl 100 mM, EDTA 2 mM, pH 7.4) with a polytron (setting 8, 15 s × 2). The homogenates were filtered through 4 layers of gauze and centrifuged at 80,000 *g* for 20 min at 4°C. The pellets were resuspended in the same volume of assay buffer (Tris HCl 50 mM, EDTA 1 mM, pH 7.4), incubated for 20 min at 37°C, and centrifuged again as described above. All procedures to prepare membranes were conducted at 4°C except preincubation of the membranes, and ice cold buffers were used. The final pellet was resuspended in assay buffer and used for the binding assay. The membranes were incubated with [^{3}H]-prazosin for 45 min at 30°C. Incubation volume was 1 ml in all experiments. Reactions were terminated by rapid filtration using a Brandel cell harvester on to Whatman GF/C filters. The filters were then washed 4 times with 4 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and dried and the filter-bound radioactivity determined. Non-specific binding was defined as binding in the presence of 1 or 10 μM prazosin. Assays were conducted in duplicate.

Table 1 Putative α_1-adrenoceptor subtypes and relative affinities for representative competitive antagonists

	Relative affinity		
α_1-subtype	*Prazosin*	*WB4101*	*HV723*
α_{1H}—α_{1A}	High	High	Medium or Low
α_{1H}—α_{1B}	High	Low	Low
α_{1L}	Low	Low	Low
α_{1N}	Low	Low	High

Chlorethylclonidine treatment

Membrane preparations were incubated for 30 min at 37°C with 1, 10 and 50 μM CEC and centrifuged at 80,000 *g* for 20 min. The pellets were washed once with assay buffer before the binding experiment.

Binding data were analysed by the weighted least-squares iterative curve fitting programme LIGAND (Munson & Rodbard, 1980). The data were first fitted to a one- and then a two-site model, and if the residual sums of squares was statistically less for a two-site fit of the data than for a one-site, as determined by *F*-test comparison, then the two-site model was accepted. *P* values less than 0.05 were considered significant.

Proteins were assayed according to the method of Bradford with bovine serum albumin used as standard (Bradford, 1976).

Functional experiments

Vas deferens of male Wistar rats (260–350 g) was isolated and cut into two (prostatic and epididymal) portions. Each portion was mounted vertically in an organ bath containing 20 ml Krebs-Henseleit solution of the following composition (mM): NaCl 112, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2, $NaHCO_3$ 25, $NaHPO_4$ 1.2 and glucose 11.5. The medium was maintained at 37°C, pH 7.4 and was equilibrated with a gas mixture consisting of 95% O_2 and 5% CO_2. A resting tension of 0.5 g was applied and the responses were recorded isometrically through a force-displacement transducer. The preparations were equilibrated for 90 min before starting the experiments.

Concentration-response curves for noradrenaline were obtained by adding the drug directly to the bathing media in a non-cumulative fashion. Desmethylimipramine (0.1 μM), deoxycorticosterone acetate (5 μM) and propranolol (3 μM) were present throughout this series of experiments in order to block neuronal and extraneuronal uptake of noradrenaline and β-adrenoceptors, respectively. α-Adrenoceptor antagonists were present for 30 min or 60 min before and during the contractile-response to noradrenaline. With CEC treatment, the preparations were treated once for 20 min with 10 μM CEC and then washed repeatedly with drug-free solution.

The pK_B value was estimated according to Arunlakshana & Schild (1959). Briefly, the concentration of noradrenaline necessary to give a half-maximal response in the presence of α-adrenoceptor antagonist was divided by the concentration giving a half-maximal response in the control to determine the agonist concentration-ratio (CR). Data were plotted as the −log antagonist concentration (M) vs the log (CR-1) and pA_2 values were calculated from Schild plots. The mean slope and 95% confidence limits (95% CL) were obtained from straight lines drawn by least square linear regression. When the straight line yielded a slope not significantly different from unit, the pA_2 value estimated was represented as pK_B (Arunlakshana & Schild, 1959). In the prostatic portion of rat vas deferens, the pK_B value for α-adrenoceptor antagonist was determined for single concentrations of antagonist (10 or 100 nM) by the concentration-ratio method (Furchgott, 1972).

Electrical transmural stimulation was applied through a pair of platinum-wire electrodes at 10–15 min intervals (Muramatsu *et al.*, 1989). The preparation was placed in parallel between electrodes. The distance between the electrodes was approximately 3 mm. Stimulus parameters were 0.1 ms duration, frequencies of 5 Hz and supramaximal voltage (13 V) for 10 s, unless stated otherwise. In this series of experiments, DG-5128 (10 μM) and propranolol (1 μM) were added to the bath medium to block prejunctional α_2-adreno-

ceptors and β-adrenoceptors, respectively (Muramatsu *et al.*, 1983; 1989). DG-5128 (10 μM) had no effect on the contractile response to noradrenaline in each preparation. α,β-Methylene ATP (10 μM) was also present throughout the experiments in order to block the purinergic component (Brown *et al.*, 1983; Sneddon & Burnstock, 1984). No effect of α,β-methylene ATP on noradrenaline-induced contraction had been established in preliminary experiments.

Statistical analyses

Experimental values are given as a mean ± s.e.mean. Results were analyzed by Student's *t* test and a probability of less than 0.05 was considered significant.

Drugs

The following drugs were used: [^{3}H]-prazosin (specific activity 76.6 Ci mmol^{-1}, NEN, Boston, U.S.A.), prazosin hydrochloride (Taito-Pfizer, Tokyo, Japan), phentolamine mesylate (Ciba, Basal, Switzerland), WB4101 hydrochloride (2-(2,6-dimethoxy-phenoxyethyl)-aminomethyl-1,4-benzodioxane hydrochloride), benoxathian hydrochloride, chlorethylclonidine dihydrochloride (CEC) (Funakoshi, Tokyo, Japan) and HV723 (α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxy-phenoxy)ethyl)-amino)-propyl)benzeneacetonitrile fumarate, Hokuriku Seiyaku, Katsuyama, Fukui, Japan), nifedipine, desmethylimipramine hydrochloride (Sigma, St. Louis, U.S.A.), (−)-noradrenaline bitartrate, deoxycorticosterone acetate, (±)-propranolol hydrochloride (Nacalai, Kyoto, Japan), tetrodotoxin (Sankyo, Tokyo, Japan) and DG-5128 (2-(2-(4,5-dihydro-1H-imidazol-2-yl)-1-phenylethyl) pyridine dihydrochloride sesquihydrate, Daiichi Seiyaku, Tokyo, Japan).

Results

Saturation experiments with [^{3}H]-prazosin

[^{3}H]-prazosin at concentrations ranging from 20–3000 pM was used to label α_1-adrenoceptors of rat vas deferens. The specific binding was approximately 90% of the total binding at 200 pM [^{3}H]-prazosin and showed a saturable tendency at the concentrations of 2000–3000 pM. However, Scatchard plots of the binding data in the epididymal portion were curvilinear, suggesting more than a single class of binding site (Figure 1a). LIGAND analysis fitted the data to a two site model. The pK_D value of high and low affinity sites were 10.1 ± 0.13 and 9.01 ± 0.15, and the B_{max} values for both sites were 507 ± 79 and 1231 ± 563 fmol mg^{-1} protein, respectively ($n = 4$).

On the other hand, Scatchard plots of the data obtained from the prostatic portion were apparently linear, resulting in a better fitting to a one-site model in computerized analysis (Figure 1b). The pK_D value estimated (9.82 ± 0.04, $n = 5$) was close to the value for the high affinity site in the epididymal portion, whereas the B_{max} value (924 ± 175, $n = 5$) was slightly but not significantly greater than that in the epididymal portion. Close inspection of Figure 1b also revealed that the binding of high concentrations of [^{3}H]-prazosin deviates slightly from a straight line, suggesting the possible existence of low affinity sites in a minor proportion.

Effects of competitive antagonists on [^{3}H]-prazosin binding

The pharmacological profile of high and low affinity sites for [^{3}H]-prazosin was further examined in displacement experiments.

Epididymal portion When 200 pM [^{3}H]-prazosin was used, unlabelled prazosin, WB4101 and benoxathian showed shallow displacement curves. However, HV723 displaced the binding in a monophasic manner (Figure 2a). Computerized analysis revealed that prazosin, WB4101 and benoxathian bound to two distinct sites. The high and low pK_I values for prazosin were respectively the same as the pK_D values obtained in the saturation experiments with [^{3}H]-prazosin. The pK_I values at high and low affinity sites for WB4101 or benoxathian were also not significantly different from the values at the corresponding sites for prazosin. The proportion of the low affinity sites for each antagonist was approximately 40% of the total binding sites (Table 2).

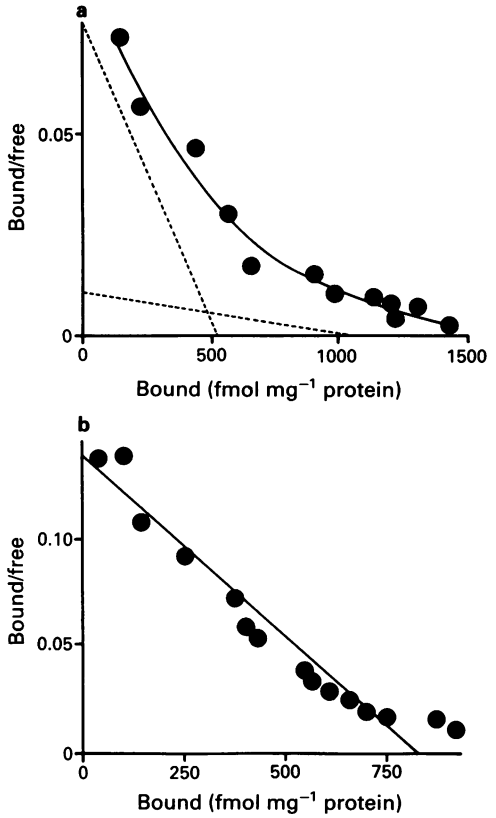

Figure 1 Scatchard plots for specific [^{3}H]-prazosin binding to rat vas deferens membranes in saturation experiments. [^{3}H]-prazosin 20–3000 pM. The figures are obtained from a single experiment where each point is the mean of duplicate determinations: (a) epididymal and (b) prostatic portions of rat vas deferens.

Prostatic portion In three out of four experiments, unlabelled prazosin displaced the binding of 200 pM [^{3}H]-prazosin in a monophasic manner and the pK_I value obtained was consistent with high pK_I value in the epididymal portion (Figure 2b and Table 2). In a remaining experiment, prazosin detected two distinct sites although the proportion of low affinity site was small (less than 10%). WB4101 also produced similar results to those for prazosin. On the other hand, benoxathian and HV723 displaced the binding of [^{3}H]-prazosin in a monophasic manner (Table 2).

Effects of pretreatment with chlorethylclonidine on [^{3}H]-prazosin binding

Since at least two distinct affinity sites for prazosin were detected in the rat vas deferens membranes, we examined the effects of pretreatment with various concentrations of CEC. In this series of experiments, 700 pM [^{3}H]-prazosin was used to label a greater number of binding sites. Therefore, the proportion of the prazosin-low affinity sites in the membranes of epididymal portion increased to approximately 80%, whereas the sites in the prostatic portion were not

clearly detected ($n = 3$ in each portion). Pretreatment of the epididymal portion with CEC at concentrations of 1 and 10 μM did not reduce the number of total specific binding sites, but pretreatment with 50 μM CEC decreased the specific binding by 21 ± 6% ($n = 3$) (Figure 3). On the other hand, [^{3}H]-prazosin binding to the membranes of prostatic portion was significantly reduced by CEC at concentrations higher than 1 μM, but a complete inhibition was not produced even at 50 μM CEC.

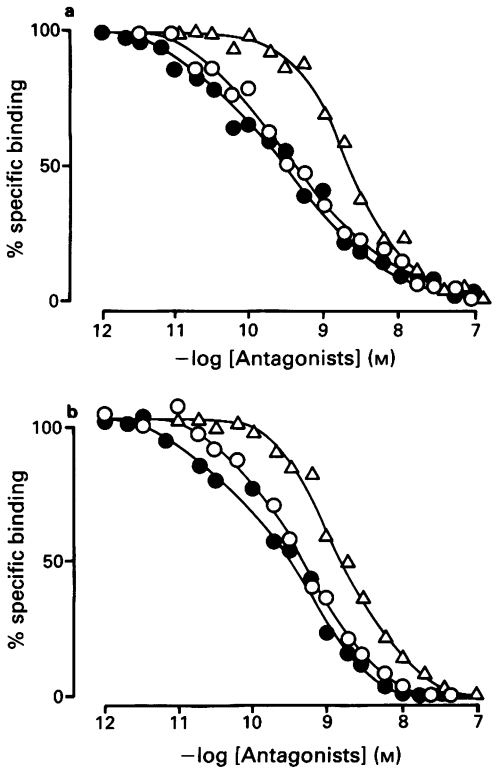

Figure 2 Displacement of [^{3}H]-prazosin binding from (a) epididymal and (b) prostatic portions of rat vas deferens membranes by prazosin (●), WB4101 (○) and HV723 (△). [^{3}H]-prazosin (200 pM) was incubated with various concentrations of unlabelled drugs under the assay conditions described in Methods. The figure represents a single experiment for each drug, where each point is the mean of duplicate determinations.

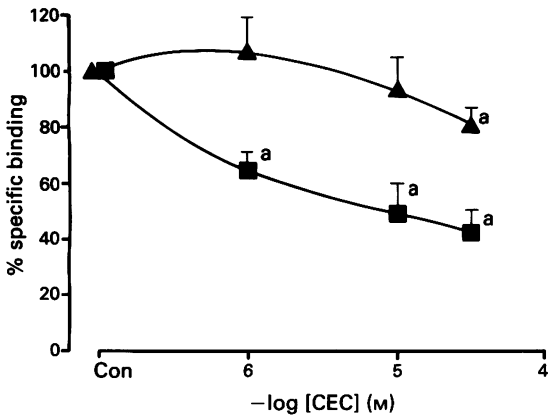

Figure 3 Effects of pretreatment with chlorethylclonidine (CEC) on the specific binding of [^{3}H]-prazosin (700 pM) to epididymal (▲) and prostatic (■) portions of rat vas deferens. The ordinate scale represents relative values of specific binding in CEC-treated membranes to that in CEC-untreated membranes (Con). Each value is the mean of 3 experiments with s.e.mean shown by vertical lines. [a]Significantly different from the value in CEC-untreated membranes ($P < 0.05$).

Effects of nifedipine and chlorethylclonidine on the contractile responses to noradrenaline

Noradrenaline at concentrations in excess of 100 nM produced concentration-dependent contractions both in the epididymal and prostatic portions of rat vas deferens (Figure 4). The pD_2 value (6.01 ± 0.10) in the epididymal portion was significantly higher than that (5.14 ± 0.17) in the prostatic portion ($P < 0.05$, $n = 5$ in each portion). The maximum amplitude of contractions in the epididymal portion was also approximately four times greater than that in the prostatic portion. Pretreatment with 10 μM CEC failed to affect the contractions induced by noradrenaline in both portions. However, the contractions were potently attenuated by 1 μM nifedipine (Figures 4 and 5a).

Effects of prazosin, WB4101, benoxathian and HV723 on noradrenaline-induced contractions in the epididymal and prostatic portions of rat vas deferens

The contractile-responses to noradrenaline in the epididymal portion were attenuated by prazosin, HV723, WB4101 and benoxathian. The slopes of Schild plots were close to unity for all the antagonists tested, indicating that the four anta-

Table 2 Inhibition of 200 pM [^{3}H]-prazosin binding to α_1-adrenoceptor of rat vas deferens

Portion	*Antagonist*	n	*Slope factor*	$pK_{I\ high}$	$pK_{I\ low}$	*% low*
Epididymal	Prazosin	4	0.50 ± 0.04[a]	10.50 ± 0.19	8.47 ± 0.42	37.9
	WB4101	4	0.75 ± 0.03[a]	10.18 ± 0.47	8.96 ± 0.35	42.1
	Benoxathian	4	0.78 ± 0.04[a]	10.10 ± 0.11	8.80 ± 0.12	48.6
	HV723	3	0.98 ± 0.02	8.90 ± 0.71	–	–
Prostatic	Prazosin	1	0.70	9.98	8.29	7.6
		3	0.87 ± 0.09	10.28 ± 0.31	–	–
	WB4101	1	0.78	9.71	8.14	7.7
		3	0.90 ± 0.03	9.47 ± 0.07	–	–
	Benoxathian	4	0.92 ± 0.07	9.58 ± 0.29	–	–
	HV723	4	0.95 ± 0.02	8.89 ± 0.04	–	–

Data shown are mean ± s.e.mean., n = number of experiments
Displacement experiments were done with 200 pM [^{3}H]-prazosin.
$pK_{I\ high}$ and $pK_{I\ low}$: negative log of the equilibrium dissociation constants (−log M) at prazosin-high and low affinity sites for antagonists tested.
% low: population binding at the low affinity site compared to the total specific binding sites.
[a]Significantly different from unity ($P < 0.05$).

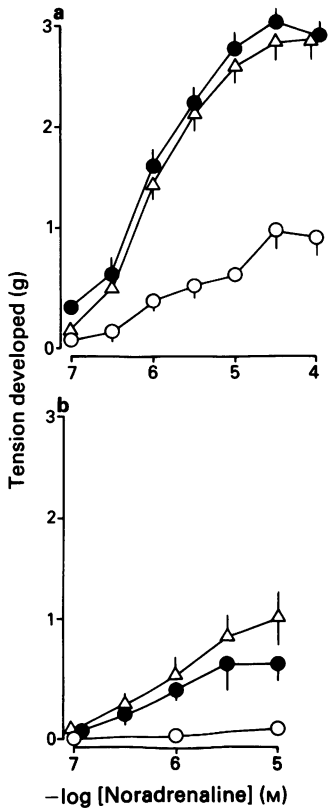

Figure 4 Effects of 10 μM chlorethylclonidine (CEC) and 1 μM nifedipine on the concentration-response curves for noradrenaline in the (a) epididymal and (b) prostatic portions of rat vas deferens. Control response (●); in the responses after pretreatment with CEC (△); or in the presence of nifedipine (○). Each point is the mean of data from 4 to 6 experiments and vertical line shows s.e.mean.

gonists competitively inhibited the contractile responses to noradrenaline. The estimated pK_B values were less than 9.0 for the antagonists tested (Table 3). There was no significant difference in the pK_B values for prazosin or WB4101 between the antagonist equilibration times of 30 and 60 min (Table 3). In the prostatic portion of rat vas deferens also, prazosin and WB4101 inhibited the contractile responses induced by noradrenaline and the pK_B values were similar to the values obtained in epididymal portion of rat vas deferens (Table 3).

Effects of various treatments on adrenergic nerve-mediated contractions in the epididymal portion of rat vas deferens

In the presence of propranolol (1 μM), DG-5128 (10 μM) and α,β-methylene ATP (10 μM), electrical transmural stimulation produced a contraction that consisted of a rapidly developing phasic component and a tonic component lasting during the stimulation (Figure 5b). These responses were completely inhibited by tetrodotoxin (0.5 μM) ($n = 5$). Nifedipine (1 μM) markedly attenuated the tonic component without affecting the phasic response (Figure 5b). Pretreatment with CEC (10 μM) for 20 min slightly attenuated the phasic contractions (15 ± 6% inhibition, $n = 7$) without affecting the tonic responses (Figure 5c). Figure 6 shows the relationship between the stimulus frequency and the contractile amplitude of each phase in the absence or presence of 1 μM nifedipine. The contractile amplitudes were dependent on the stimulus frequencies, resulting in a submaximum value at 5 Hz in each of phasic and tonic responses. Therefore, we examined the effects of α-adrenoceptor antagonists on the neurogenic responses at 5 Hz.

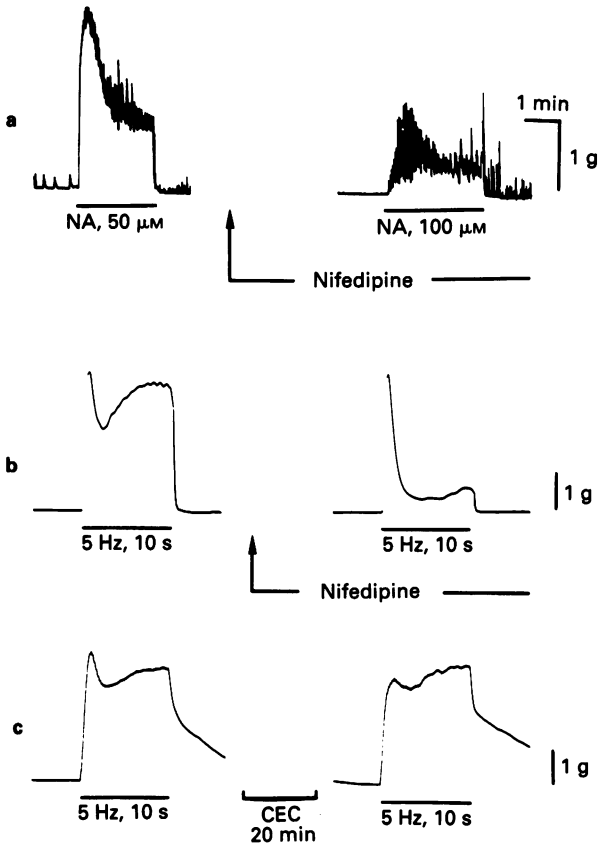

Figure 5 Effects of nifedipine or chlorethylclonidine (CEC)-pretreatment on the adrenergic contractions induced by (a) noradrenaline (100 μM) and (b and c) electrical stimulation (5 Hz, 10 s) in the epididymal portions of rat vas deferens. Left: control, right: response in the presence of 1 μM nifedipine or after pretratment with 10 μM chlorethylclonidine (CEC). In (b) and (c), 1 μM propranolol, 10 μM DG5128 and 10 μM α,β-methylene ATP were present throughout the experiments.

Table 3 α_1-Adrenoceptor affinities for prazosin, HV723, WB4101 and benoxathian in the epididymal and prostatic portions of rat vas deferens

	Epididymal portion		*Prostatic portion*
Antagonist	pK_B	*Slope* (95% CL)	pK_B
Prazosin	8.32 ± 0.05	1.066 (0.999–1.133)	8.37 ± 0.24[b]
	8.39 ± 0.10[a]	1.070 (0.914–1.224)	
HV723	8.22 ± 0.05	1.068 (0.993–1.141)	–[c]
WB4101	8.52 ± 0.08	1.032 (0.932–1.132)	8.75 ± 0.16[b]
	8.70 ± 0.06[a]	1.078 (0.955–1.162)	
Benoxathian	8.41 ± 0.07	1.052 (0.945–1.157)	–[c]

[a]The values were estimated from 60 min equilibration experiments; other values were obtained from 30 min equilibration experiments.
[b]The pK_B values were estimated from the inhibitory effects of 10 and 100 nM prazosin or WB4101.
[c]Not determined.

Prazosin, HV723, WB4101 and benoxathian inhibited concentration-dependently and eventually abolished the contractile responses to electrical transmural stimulation. Figure 7 shows the concentration-inhibition curves for the four antagonists, where the phasic and tonic components in the absence or presence of 1 μM nifedipine were measured separately. The inhibition by WB4101 and benoxathian was slightly more potent than that by prazosin or HV723, but the ratios between the IC_{50} values were in a range less than 4 times (Table 4). Nifedipine (1 μM) did not affect the inhibitory potencies of the antagonists.

Discussion

The present study clearly demonstrates that prazosin binds to two distinct populations of binding sites in the rat vas deferens. However, the density of two sites varied between the epididymal and prostatic portions. The prazosin-high affinity sites were present in almost equal density in both halves, whereas prazosin-low affinity sites predominantly occurred in the epididymal portion and the density was approximately twice that of high affinity sites in the epididymal portion. The proportion of low affinity sites in the prostatic portion was markedly low (less than 10% of total binding sites), so that the low sites could not be consistently detected. This may reflect a limitation of computer analysis (Molinoff *et al.*, 1981; De Lean *et al.*, 1982).

The finding of two distinct binding sites for prazosin in the rat vas deferens is new, only a single affinity site having been demonstrated for prazosin in previous studies (Hanft & Gross, 1989; Salles & Badia, 1991). This discrepancy may be in part associated with the radioligand concentrations used, as the low affinity sites may be overlooked at low concentrations of radioligand. Use of the membrane fraction prepared from whole vas deferens may also mask the prazosin-low affinity sites, because these sites mainly occur in the epididymal portion which corresponds to approximately 35% of the whole vas deferens in protein content, resulting in an apparent reduction of the relative proportion of low affinity sites in total membrane fractions. However, Salles & Badia (1991) failed to detect the low affinity sites even though they used high concentrations of [^{3}H]-prazosin (up to 4 nM) and membranes prepared separately from the epididymal and prostatic portions. One of the reasons for this conflicting result may be a difference in the methods used to determine specific binding, as 10 μM phentolamine may be insufficient to inhibit completely the specific binding of high concentra-

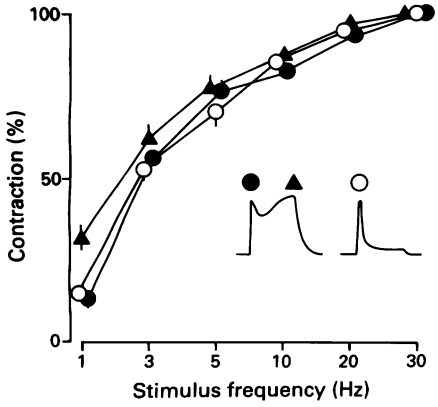

Figure 6 Relation between the contractile amplitudes and stimulus frequencies in the epididymal portion of rat vas deferens. The phasic and tonic contractions induced by stimulation with various frequencies for 10 s were measured as shown in the inset. Closed and opened symbols show the responses in the absence and presence of 1 μM nifedipine, respectively. Other experimental conditions were the same as those in Figure 5b.

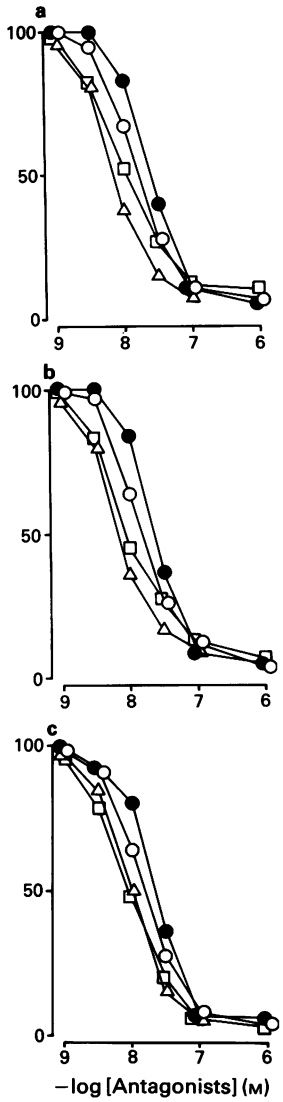

Figure 7 Concentration-response curves for prazosin (●), WB4101 (△), benoxathian (□) and HV723 (○) in inhibiting the adrenergic neurogenic contractions in the epididymal portion of rat vas deferens. Adrenergic neurogenic contraction was elicited by the application of electrical transmural stimulation (5 Hz, 10 s). The other experimental conditions are the same as those in Figure 5b. The contractile amplitudes before addition of prazosin, WB4101, benoxathian and HV723 were taken as 100%. Each value is the mean of 4–5 experiments. (a) and (b) Phasic and tonic contractions in the absence of nifedipine (see the inset of Figure 6); (c) phasic contraction in the presence of 1 μM nifedipine. For IC_{50} values see Table 4.

Table 4 IC_{50} values for prazosin, HV723, WB4101 and benoxathian in inhibiting the adrenergic contractions induced by electrical stimulation in the epididymal portion of rat vas deferens

	IC_{50} (nM)		
	− *Nifedipine*		+ *Nifedipine*
Antagonist	*Phasic*	*Tonic*	*Phasic*
Prazosin	25.7 ± 5.1	25.9 ± 6.0	22.4 ± 3.3
HV723	15.9 ± 2.2	14.9 ± 1.9	15.2 ± 1.6
WB4101	7.27 ± 0.54	7.58 ± 1.39	10.2 ± 0.6
Benoxathian	10.9 ± 1.6	8.66 ± 0.55	9.26 ± 0.76

Electrical transmural stimulation (5 Hz, for 5 s) was applied in the absence or presence of 1 μM nifedipine, and the phasic and tonic components of contraction evoked were measured.
Mean ± s.e. of 4 to 5 experiments.

tions of [^{3}H]-prazosin. In the present study, we used 1 or 10 μM prazosin in the saturation experiments.

In the displacement experiments, the prazosin-high affinity sites were characterized as WB4101- or benoxathian-high affinity sites, while the prazosin-low affinity sites showed a low affinity for WB4101 or benoxathian. HV723, an α_{1N}-selective drug (Oshita *et al.*, 1988; Muramatsu *et al.*, 1990a), did not discriminate between the sites, resulting in low affinity constant.

As mentioned in the Introduction, the α_1-adrenoceptor was originally subdivided into two classes (α_{1A} and α_{1B}) in the binding studies (Morrow & Creese, 1986; Han *et al.*, 1987a) and into three subtypes (α_{1H}, α_{1L} and α_{1N}) in functional studies (Muramatsu *et al.*, 1990a,b). Subsequently, a possible conciliation has been proposed (Oshita *et al.*, 1991; Muramatsu *et al.*, 1991). According to the criteria proposed (Table 1), the characteristics of α_1-adrenoceptor of rat vas deferens observed in the present study show that the prazosin-high and -low affinity sites correspond to the putative α_{1A} and α_{1L} subtypes, respectively. Recently, the existence of mRNA for α_{1A} in the rat vas deferens was confirmed by Northern blotting analysis (Lomasney *et al.*, 1991b).

The functional study reveals that prazosin, HV723, WB4101 and benoxathian competitively antagonize the contractile response to noradrenaline with relatively low affinities ranging from 8.0 to 9.0. Such low affinities for the antagonists have been reported previously (prazosin: Kenakin, 1984; Beckeringh & Brodde, 1990; Salles & Badia, 1991; benoxathian: Han *et al.*, 1987a). However, a higher (approximately 9.5: Han *et al.*, 1987a) or intermediate value (9.01: Hanft & Gross, 1989) was reported for WB4101. At present, we cannot account for this discrepancy because low affinity constants for WB4101 and prazosin were still obtained even after a longer equilibration (60 min) with the antagonist. All of these studies, however, clearly show that the contractile response to noradrenaline of the rat vas deferens is predominantly mediated through a single α_1-adrenoceptor subtype, even though two distinct subtypes co-exist in the vas deferens. Good correlation between the pK_B values for antagonists in the present functional study and the $pK_{I\text{low}}$ values in the binding study strongly suggests that the contractile response to noradrenaline in the rat vas deferens is predominantly mediated through the α_{1L} subtype, although a minor contribution of the α_{1A} subtype cannot be ruled out completely.

It is well known that the epididymal portion produces larger contractions in response to various α-adrenoceptor agonists than the prostatic portion (present study; Vardolv & Pennefather, 1976; Kasuya & Suzuki, 1979; MacDonald & McGrath, 1980; Moore & Griffiths, 1982; Badia & Salles, 1989). The existence of spare receptors has been demonstrated in the response to noradrenaline in the epididymal but not prostatic portion (Salles & Badia, 1991). The heterogeneous distribution of the α_{1L} but not the α_{1A} subtype observed in the present study may well account for such differences in functional responsiveness between the two portions of rat vas deferens.

CEC was originally reported to produce a selective and complete inactivation of α_{1B} subtype (Han *et al.*, 1987b). However, a partial inactivation of other subtypes by CEC was recently demonstrated (Schwinn *et al.*, 1990; Oshita *et al.*, 1991; Lomansney *et al.*, 1991b). In the present study also, [^{3}H]-prazosin binding to prazosin-high and -low affinity sites (α_{1A} and α_{1L} subtypes) was slightly but significantly inhibited by CEC. The lack of potent inhibitory effect by 10 μM CEC on the contractile responses to exogenous noradrenaline and to adrenergic nerve stimulation (in the presence of the prejunctional α_2-adrenoceptor antagonist, DG-5128; Muramatsu *et al.*, 1989) may be related to low susceptibility to CEC of the α_{1L} subtype which would be predominantly involved in the adrenergic contractile responses as mentioned above.

The contractile response to exogenous noradrenaline and the tonic contraction evoked by electrical transmural stimulation (in the presence of α,β-methylene ATP) were both potently inhibited by nifedipine, whereas the phasic adrenergic response to nerve stimulation was resistant to nifedipine. Such nifedipine-resistance has been observed in the adrenergic but not purinergic contractions induced by single pulse stimulation (Blakeley *et al.*, 1991; Brown *et al.*, 1983; McGrath & Wilson, 1988). This indicates that endogenous noradrenaline may produce contractions through at least two different effector pathways. Since α_{1A}-adrenoceptors were originally suggested to be selectively coupled to Ca channels (Han *et al.*, 1987a), we compared the inhibitory potencies of α_1-adrenoceptor antagonists on the phasic and tonic contractions. Both responses were equipotently inhibited by the antagonists used in the binding study, and the order of inhibitory potencies was consistent with that for inhibition of exogenous noradrenaline-responses or the order of affinity for the α_{1L}-subtype estimated from the binding study. These results indicate that α_1-adrenoceptors involved in both phasic and tonic contractions cannot be discriminated by the competitive antagonists, suggesting that both the response may be caused through the same subtype as that in the response to exogenous noradrenaline (presumably α_{1L}) even though the effector pathways may differ. It seems that α_1-adrenoceptor subtypes cannot be classified strictly by difference in signal transduction mechanisms (Muramatsu *et al.*, 1990b; Lomasney *et al.*, 1991a).

In conclusion, the present study clearly indicates the occurrence of two distinct α_1-adrenoceptor subtypes in the rat vas deferens (presumably α_{1A} and α_{1L}), and suggests that the α_{1L} subtype is predominantly involved in the adrenergic contractions induced by exogenous and endogenous noradrenaline. Variation in adrenergic responsiveness between the epididymal and prostatic portions seems to be related to the heterogeneous distribution of α_{1L} subtypes.

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Effects of the PAF antagonists BN50726 and BN50739 on arrhythmogenesis and extent of necrosis during myocardial ischaemia/reperfusion in rabbits

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1 The effects of two novel platelet activating factor (PAF) antagonists BN50726 and BN50739 on arrhythmias, haemodynamics and extent of necrosis during myocardial ischaemia and reperfusion were investigated in anaesthetized rabbits subjected to coronary artery ligation.

2 BN50739 reduced heart rate prior to coronary artery occlusion ($P < 0.005$) but had no other significant haemodynamic effects at this time. BN50739 and BN50726 did not significantly alter heart rate or blood pressure during 30 min of ischaemia or 30 min of reperfusion, compared to control hearts.

3 BN50739 and BN50726 had no effect on the incidence of arrhythmias during ischaemia. BN50726 significantly reduced the incidence of reperfusion ventricular fibrillation compared to controls (0% v 40%, $P < 0.05$), and improved survival (80% v 39%, $P < 0.05$). Similar trends were observed with BN50739.

4 BN50726 reduced the extent of necrosis compared to control hearts ($18 \pm 2\%$ v $30 \pm 3\%$, $P < 0.01$). A similar trend was observed with BN50739.

5 These results demonstrate that PAF antagonism with BN50726 attenuates reperfusion-induced arrhythmias and preserves myocardium in the early phase of ischaemia, independently of haemodynamic effects.

Keywords: Platelet activating factor; ischaemia; reperfusion; haemodynamics; arrhythmias; necrosis; infarction

Introduction

Clinical and experimental studies suggest that platelet activation contributes to myocardial necrosis during ischaemia and reperfusion (Mehta & Mehta, 1979; Mikhailidis *et al.*, 1987; Rösen *et al.*, 1987; Wainwright *et al.*, 1989; Chakrabarty *et al.*, 1991a). Release of platelet activating factor (PAF) from the ischaemic myocardium has been demonstrated experimentally and in man (Lotner *et al.*, 1980; Annable *et al.*, 1985; Zimmerman *et al.*, 1985; Montrucchio *et al.*, 1986; 1989; Sisson *et al.*, 1987). Experimental studies have shown that PAF increases myocardial necrosis and arrhythmogenesis (Mickelson *et al.*, 1988; Chakrabarty *et al.*, 1991a) during myocardial ischaemia and that it has direct myocardial cellular electrophysiological and arrhythmogenic effects (Flores & Sheridan, 1990). Recent studies have shown that PAF antagonists have opposite actions, reducing the incidence of arrhythmias and preventing necrosis during ischaemia and reperfusion (Wainwright *et al.*, 1989; Chakrabarty *et al.*, 1991a; Koltai *et al.*, 1991a). Such findings suggest that PAF could play a significant role in the progression and complications of myocardial ischaemia. While PAF antagonism has been shown to be effective during experimental myocardial ischaemia it is not clear whether it will be beneficial clinically at improving survival and reducing morbidity.

Although several PAF antagonists are available, relatively high concentrations are required *in vivo* and *in vitro* to antagonize the effects of PAF. BN50739 (6-(2-chlorophenyl)-9-[2- (3,4-dimethoxyphenyl) thio]-1-thioxoethyl) -7,8,9,10-tetrahydro-1-methyl]-4H-pyrido [4'3':4,5] thieno [3,2-f] [1'2'4] triazolo [4,3-a] [1,4] diazepine) and BN50726 (6-(2-chlorophenyl)-9- (hexadecylsulphonyl) -7,8,9,10-tetrahydro-1-methyl-4H-pyrido [4'3':4,5] thieno [3,2-f] [1'2'4] triazolo [4,3-a] [1,4] diazepine) are two novel synthetic PAF antagonists which have been developed recently and shown to be more potent than other compounds in antagonizing the effects of PAF (Yue *et al.*, 1990). Koltai *et al.* (1991a) recently reported that BN50739 reduced the incidence of ventricular arrhythmias during myocardial ischaemia in rat isolated hearts in the absence of platelets, but relatively little is known about the effects of BN50739 and BN50726 *in vivo*. To gain further information, we performed experiments designed to examine the haemodynamic, antiarrhythmic and anti-necrotic effects of these PAF antagonists during regional myocardial ischaemia and reperfusion in anaesthetized open chest rabbits.

Methods

New Zealand White rabbits (2.5–3.0 kg) were maintained at 21–22°C, $50 \pm 5\%$ humidity and 12/12 hourly light/dark cycles for 3 to 5 days prior to study. Animals were fed on R14 'high fibre without grass' meal and water *ad libitum*. All experiments were carried out in a single laboratory maintained at 21–22°C throughout the year between the hours of 9 am and 6 pm.

Surgical procedures

Anaesthesia was induced with alphaxalone (9 mg kg^{-1}, i.v.) and maintained with pentobarbitone (25 mg kg^{-1}). Tracheostomy and tracheal intubation were performed and animals were ventilated with room air by means of a mechanical pump (CF Palmer (London) Ltd., Model No. 16/24) at a fixed rate of 45 strokes min^{-1} and a tidal volume of 25–30 ml producing an arterial P_{O_2} of 96 mmHg, P_{CO_2} of 36 mmHg and pH of 7.36. Body temperature was monitored with an oesophageal thermistor and maintained at a constant level by an overhead heating lamp. The right carotid artery was cannulated to monitor the arterial pressure using a Lec-

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tromed transducer (No. 3552; Lectromed Ltd., Letchworth). ECG leads were connected to the four limbs and the signals amplified using a Lectromed ECG amplifier. Arterial pressure and ECG signals were continuously recorded on a Lectromed Multitrace 4 chart recorder.

A thoracotomy was performed via a left parasternal incision between the fourth and fifth ribs. The heart was exposed by incising the pericardium and was then supported in a pericardial cradle. The left ventricular branch of the circumflex artery was identified (Flores *et al.*, 1984) and a loose 2/0 polyester ligature was placed around it adjacent to its origin. Each preparation was allowed to stabilize for 45 min before coronary artery occlusion. Regional ischaemia was produced with a snare made of polyethylene tubing 2.5 cm in length and 3 mm in diameter which was threaded over the suture and clamped firmly in place for 30 min. Reperfusion was induced by releasing the clamp. Following 30 min of reperfusion the animals were killed with an overdose of anaesthetic. The hearts were removed for measurement of the extent of myocardial damage.

Drug administration

BN50739 and BN50726 were obtained from Institut Henri Beaufour, Le Plessis Robinson, France. Both compounds, at a dose of 5 mg kg^{-1}, were dissolved in 0.5 ml of dimethylsulphoxide (DMSO, Sigma Chemicals Co., Poole, Dorset) and made up to 5 ml with distilled water and given intravenously through the marginal ear vein 10 min before coronary occlusion. Ten animals received BN50739, with ten receiving BN50726. Eighteen animals received vehicle alone and served as controls. Unequal numbers were studied so as to offset the greater survival in the treated group and provide similar numbers of hearts for infarct size measurement.

Arrhythmia analysis

The ECG recordings were analysed according to the guidelines of the Lambeth Conventions (Walker *et al.*, 1988). The incidence of ventricular tachycardia (VT) and ventricular fibrillation (VF) were noted during ischaemia and reperfusion. Only those animals that remained in sinus rhythm throughout ischaemia, or had spontaneously reverted from a ventricular arrhythmia to sinus rhythm by the end of the ischaemic period, were reperfused. Animals which remained in sinus rhythm until the end of reperfusion, or spontaneously reverted so that they were in sinus rhythm at the end of reperfusion, were regarded as survivors. VT was defined as a run of four or more consecutive ventricular premature beats.

Infarct size measurement

Infarct size measurements were made only on survivors. Following the 30 min of reperfusion each heart was removed and immediately placed in a 10% KCl solution to induce rapid asystole. Thereafter, the heart was cut into four sections from base to apex, each approximately 2 mm thick. The sections were washed in cold normal saline and placed in freshly prepared 0.5% w/v nitrobluetetrazolium (Sigma Chemical Co., Poole, Dorset) dissolved in phosphate buffer at pH 7.4 at 37°C for 10 min. They were then washed in water and placed in formaldehyde solution for fixation overnight. The sections were photographed with a reference grid on 35 mm colour slides and their projected images were used to measure the areas of infarction (absence of blue staining) and non-infarction (blue staining) by computerized planimetry, as previously described (Chakrabarty *et al.*, 1991a). The volumes of each section were calculated as the product of the planimetered areas and the section thickness. The volume of infarction was calculated as a percentage of each section and the total left ventricular volume.

Statistical analysis

Haemodynamic parameters are presented as means ± standard error of the mean. Results were compared by Student's paired *t* test within groups and Student's unpaired *t* test between groups. Arrhythmia analysis was performed by Chi-squared analysis and infarct size analysis was performed by Student's unpaired *t* test. Statistical significance was accepted if $P < 0.05$.

Results

Haemodynamic changes

Figures 1 and 2 show the haemodynamic changes that occurred in the three groups. No significant differences in blood pressure were observed prior to coronary artery occlusion. Coronary artery occlusion significantly reduced blood pressure in both the control and the BN50739 groups (from 98 ± 2 mmHg to 86 ± 3 mmHg after 5 min of ischaemia, $P < 0.001$ in control hearts, and from 95 ± 3 mmHg to 83 ± 4 mmHg, $P < 0.01$, after 5 min in BN50739 treated hearts). A similar trend was observed in the BN50726 group but this failed to reach statistical significance (from 99 ± 3 mmHg to 88 ± 5 mmHg after 5 min of ischaemia). No significant differences in blood pressure were observed between the groups during ischaemia or reperfusion. BN50739 reduced heart rate from 260 ± 9 beats min^{-1} to 237 ± 8 beats min^{-1} ($P < 0.01$) prior to coronary artery occlusion, but BN50726 was without effect. No significant differences in heart rate were observed between the groups during ischaemia or reperfusion.

Arrhythmias

Figure 3 illustrates the percentage incidence of VF during ischaemia and reperfusion for the control and treated groups. No significant differences in the incidence of VT during ischaemia were observed between the control group and either of the treated groups: 7/18 (39%) of control rabbits, 1/10 (10%) of rabbits treated with BN50739 and 1/10 (10%)

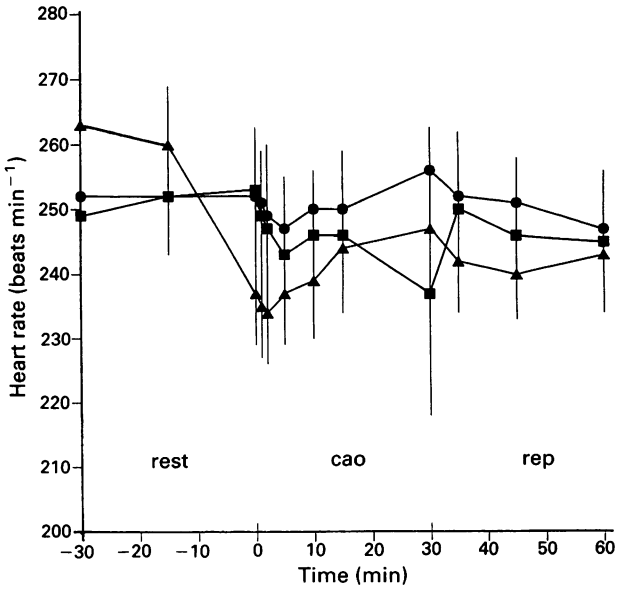

Figure 1 Changes in heart rate in control (●), BN50739 (▲), and BN50726 (■)-treated rabbits before coronary occlusion (rest), during occlusion (cao) and during reperfusion (rep). BN50739 reduced heart rate prior to coronary occlusion. No significant differences were observed between the groups during ischaemia or reperfusion.

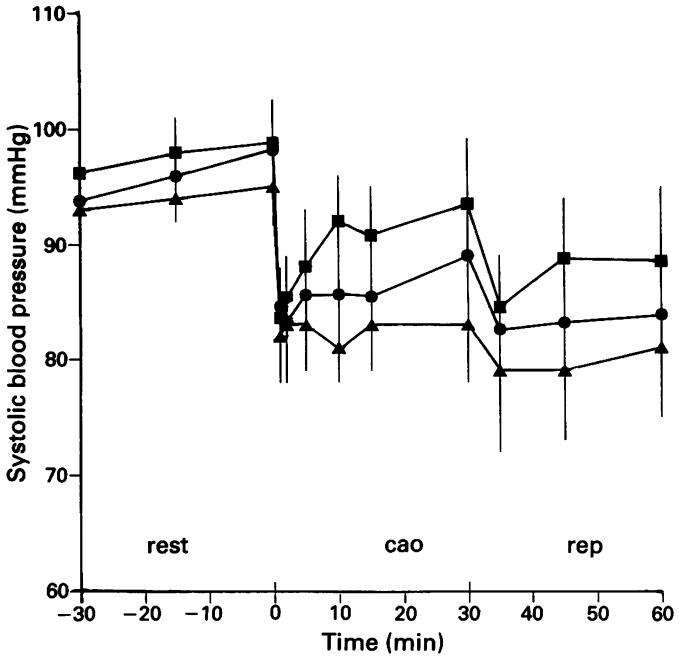

Figure 2 Changes in systolic blood pressure in control (●), BN50739 (▲) and BN50726 (■)-treated rabbits before coronary artery occlusion (rest), during occlusion (cao) and during reperfusion (rep). Blood pressure fell significantly following ligation in the control and BN50739 treated groups, a similar trend was seen in the BN50726-treated group but this did not reach significance. No significant differences were observed between the groups prior to, during or following ischaemia.

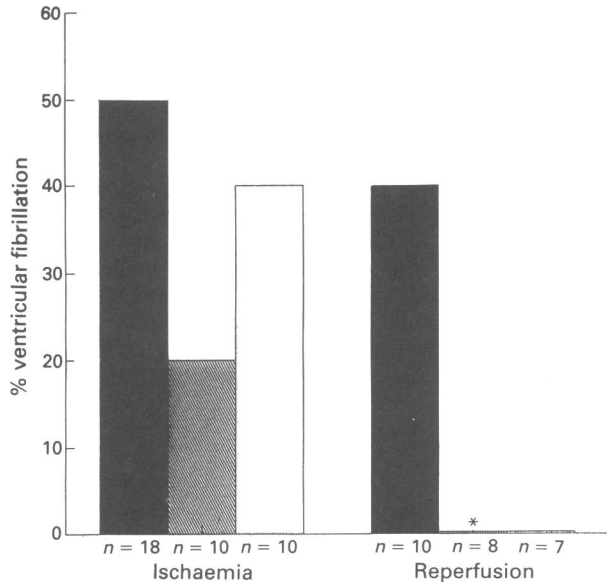

Figure 3 Effects of BN50726 (hatched column) and BN50739 (open column) on the incidence of ventricular fibrillation (VF) during ischaemia and reperfusion. BN50726 significantly reduced the incidence of VF during reperfusion compared to control hearts (solid column).
*$P < 0.05$ v the control group.

of rabbits treated with BN50726. Similarly, no significant differences in the incidence of VF during ischaemia were observed between control and treated groups: 9/18 (50%) of control rabbits, 4/10 (40%) for the BN50739-treated group and 2/10 (20%) for the BN50726-treated group. Although 9/18 control hearts developed VF during ischaemia, one of these recovered spontaneously so that 10 control rabbits survived ischaemia. Of the 10 rabbits treated with BN50739,

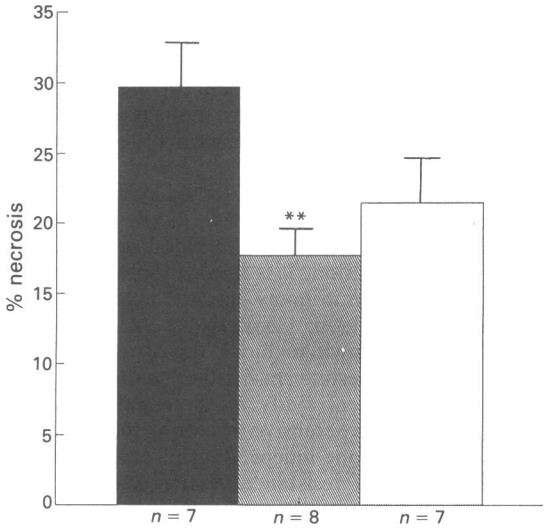

Figure 4 Effects of BN50726 (hatched column) and BN50739 (open column) on the extent of myocardial necrosis expressed as a percentage of total left ventricular volume. BN50726 significantly reduced the extent of necrosis compared to control hearts.
**$P < 0.01$ v control group.

7 survived ischaemia and 8 of the 10 rabbits treated with BN50726 survived ischaemia and went on to reperfusion. Death during ischaemia was due to terminal VF. No significant differences in the incidence of VT during reperfusion were observed between the control group and either of the treated groups: 4/10 (40%) of controls, 1/7 (14%) of the BN50739-treated group and 2/8 (25%) of the BN50726-treated group. BN50726 significantly reduced the incidence of VF occurring during reperfusion compared to the control group (0/8 [0%] v 4/10 [40%], $P < 0.05$). A similar trend was observed in the BN50739 treated group, but this just failed to reach significance (0/7 [0%] v 4/10 [40%]).

Administration of BN50726 significantly increased the number of rabbits surviving to the end of reperfusion compared to untreated rabbits (8/10 [80%] BN50726 v 7/18 [39%] controls, $P < 0.05$). BN50739 tended to improve survival but this just failed to reach statistical significance (7/10 [70%] BN50739 v 7/18 [39%] controls).

Infarct size

Figure 4 illustrates the extent of myocardial necrosis, as a percentage of total left ventricular volume, observed in control and treated groups. Pretreatment with BN50726 reduced the extent of necrosis compared to the control group from $29.61 \pm 3.17\%$ to $17.85 \pm 1.92\%$ ($P < 0.01$). A similar trend was observed for BN50739 but failed to reach statistical significance ($29.61 \pm 3.17\%$ v $21.41 \pm 3.10\%$).

Discussion

This study demonstrated that PAF antagonism with BN50726 significantly reduced the incidence of ventricular fibrillation during reperfusion, compared to control hearts. This was associated with a reduction in the extent of necrosis and a reduction in mortality. BN50739 produced similar trends but these just failed to reach statistical significance. These antiarrhythmic and anti-necrotic effects appeared to be independent of haemodynamic changes.

We have previously used this anaesthetized, open chest rabbit model to investigate the effects, on arrhythmogenesis and necrosis, of modulating PAF activity during myocardial ischaemia (Chakrabarty *et al.*, 1991a). Following coronary artery ligation in the rabbit, there is a significant fall in blood

pressure, high incidence of ventricular arrhythmias and a substantial volume of myocardial necrosis. In this model the volume of necrosis tends to be greatest at the apex, which is due to the coronary artery chosen for ligation. The left ventricular branch of the circumflex supplies a consistent and large portion of the left ventricle with little collateral flow (Flores et al., 1984).

Despite the ability of BN50726 to reduce the incidence of arrhythmias and reduce necrosis, both BN50726 and BN50739 failed to prevent the fall in blood pressure observed following coronary ligation. It is known that exogenous PAF can induce hypotension in many species, including the rabbit and rat (Yue et al., 1990; Chakrabarty et al., 1991a) and BN50739 has been shown to block this in rats (Yue et al., 1990). Although the mechanism for the hypotensive action of PAF is unclear, a recent study by Yamanaka et al. (1992) revealed a biphasic hypotensive response mediated by two different mechanisms, one independent of prostaglandins and involving dilatation of resistance vessels, and the other involving venodilatation and mediated by prostaglandins. Previous studies have shown that PAF antagonists can attenuate the hypotension following coronary occlusion (BN52021, SDZ63-675 in the rabbit; Montrucchio et al., 1990; Chakrabarty et al., 1991a), however, other studies have failed to show such an effect (BN52021, SRI63441 and CV3988 in the dog; Wainwright et al., 1989; Maruyama et al., 1990). This may indicate that other mechanisms, e.g. loss of regional contractility and cardiac output following an abrupt reduction in flow, are involved. Platelets from different species are however known to respond to PAF differently (McManus et al., 1981; Cargill et al., 1983) and it appears that there may be different PAF receptors on different cells in the same species (Stewart & Dusting, 1988).

The fall in heart rate with injection of BN50739 is difficult to explain and has not been reported before with other antagonists (Wainwright et al., 1989; Montrucchio et al., 1990; Chakrabarty et al., 1991a). PAF is known to cause a bradycardia as well as other haemodynamic effects such as vasoconstriction, hypotension, decreased contractility and decreased cardiac output (Chakrabarty et al., 1991a) and it may be that BN50739 has partial agonist activity although there were no other haemodynamic changes, with its injection, to support this. The structure of BN50739 also allows for potential free radical scavenging activity (Koltai et al., 1991b).

The antiarrhythmic properties of BN50726 that were observed in this study and the similar trend seen with BN50739 give further evidence for a role of PAF in mediating arrhythmogenesis during myocardial ischaemia, and correlate well with other reports using PAF antagonists (Wainwright et al., 1989; Yue et al., 1990; Koltai et al., 1991a; Chakrabarty et al., 1991a). The exact mechanism through which PAF mediates these effects is not clear. Direct myocardial actions, which are known to be arrhythmogenic (Tamargo et al., 1985; Stahl et al., 1988; Flores & Sheridan, 1990) may be important, together with the activation of platelets and/or white cells (Wainwright et al., 1989; Chakrabarty et al., 1991a). Koltai et al. (1991a) recently commented on the complexity of the actions of PAF in the whole animal. Interactions between PAF and cytokines may promote cellular necrosis, and PAF may also alter calcium influx and enhance sodium/hydrogen exchange (Koltai et al., 1991a) producing cellular electrophysiological effects which alter the susceptibility to arrhythmogenesis. The relative ability of PAF antagonists to prevent these effects may be important in determining their efficacy in preventing arrhythmogenesis and reducing or delaying the extent of necrosis.

Comparison of results obtained in this study with our earlier investigation of the effects of PAF antagonism using BN52021 at a dose of 10 mg kg^{-1} (Chakrabarty et al., 1991a) reveals that BN50726 and BN50739 reduced the extent of myocardial necrosis following coronary ligation to a similar extent, but with a dose of 5 mg kg^{-1}, compared to that seen with BN52021 and pretreatment with platelet antiserum. Whereas the antiarrhythmic potency of BN50739 has been shown to be six times that of BN52021 (Koltai et al., 1991b), no comparable data are available for BN50726 (M. Koltai, personal communication). Yue et al. (1990) compared the ability of BN50739, BN50726 and BN52021 to antagonize PAF-induced 5-hydroxytryptamine release from rabbit platelets and reported IC_{50} values of 3.67 ± 0.20 nM, 5.40 ± 1.68 nM and $14\,900 \pm 1\,600$ nM respectively. Little information is available about the effects of these compounds *in vivo*, although a preliminary report by Schaer et al. (1990) described beneficial effects of BN50739 against reperfusion injury at a dose of 5 mg kg^{-1} in dogs. Although we did not measure risk zone in these experiments, we have previously shown that the risk zone represents a consistent and highly reproducible percentage of the left ventricular volume when the left ventricular branch of the circumflex artery is occluded (Chakrabarty et al., 1991b).

In conclusion, these results provide further evidence that modification of the effects of platelet activating factor has important benefits in the early stages of acute myocardial infarction. Further studies to investigate the mode of action of these agents, and to investigate their potential role in clinical practice should be useful in providing a clearer understanding of the importance of PAF in mediating the effects observed.

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Chronic antihypertensive treatment with captopril plus hydrochlorothiazide improves aortic distensibility in the spontaneously hypertensive rat

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1 Adult male spontaneously hypertensive rats (SHR) were given captopril plus hydrochlorothiazide mixed in the diet for 10 weeks. Calculated daily doses were 44 mg kg^{-1} per day for captopril, and 22 mg kg^{-1} per day for hydrochlorothiazide. Separate groups received captopril or hydrochlorothiazide alone, at similar doses, or no treatment. A final group of WKY normotensive rats received no drug.

2 Systolic arterial blood pressure, measured at regular intervals throughout the 10 weeks' period was lowered but not normalized, in groups receiving either captopril plus hydrochlorothiazide, or captopril alone, but not in the group receiving hydrochlorothiazide alone.

3 Following pentobarbitone anaesthesia, systolic arterial blood pressure, measured in the femoral artery, was found to be lower in all treated groups, but the greatest effect was observed in SHR previously treated with captopril plus hydrochlorothiazide. Aortic pulse wave velocity was also lower in treated SHR, and once again the greatest decrease was observed in the group previously treated with captopril plus hydrochlorothiazide.

4 Following pithing, systolic arterial blood pressures were similar in all SHR groups. Aortic pulse wave velocity was lower in pithed rats previously treated with captopril and hydrochlorothiazide.

5 In conclusion, antihypertensive treatment of SHR produces falls in blood pressure and pulse wave velocity, an indicator of aortic distensibility. Results in pithed rats suggest that treatment with the combination of captopril plus hydrochlorothiazide may increase aortic distensibility independently of blood pressure.

Keywords: Rat blood pressure; pulse wave velocity; captopril; hydrochlorothiazide

Introduction

Hypertension is associated not only with structural and functional changes in small arteries and arterioles (leading to an increase in peripheral resistance), but also with changes in large arteries (leading to an increase in rigidity; Milnor, 1989; Nichols & O'Rourke, 1990). This decrease in large artery distensibility is an important factor in target organ damage such as cardiac hypertrophy (Safar *et al.*, 1987; Pannier *et al.*, 1989). Improvement in arterial compliance may require a specific type of antihypertensive therapy (Safar, 1989). Amongst the drugs available, angiotensin I converting enzyme inhibitors may possess a crucial mechanism of action at the level of the large arteries, possibly through inhibition of the local renin-angiotensin system (Dzau & Safar, 1988). Chronic treatment with angiotensin I converting enzyme inhibitors has been shown to increase arterial compliance in both man (Simon *et al.*, 1985), and animals (Levy *et al.*, 1988).

A therapeutic combination of an angiotensin I converting enzyme inhibitor with a diuretic appears to offer potent antihypertensive efficacy and good tolerability (Hansson, 1987). As the possible impact of chronic treatment with such combinations on large artery distensibility has not to our knowledge been investigated, we studied the effect of chronic treatment with the combination of the angiotensin I converting enzyme inhibitor, captopril, and the diuretic, hydrochlorothiazide, on blood pressure and aortic distensibility, in the adult spontaneously hypertensive rat (SHR). Changes in aortic distensibility were estimated from changes in aortic pulse wave velocity (Messerli *et al.*, 1985; Pannier *et al.*, 1989; Nichols & O'Rourke, 1990). Aortic pulse wave velocity was measured in anaesthetized and pithed rats. In the latter preparation, measurement of pulse wave velocity, in the absence of an intact autonomic nervous system, and at a low arterial blood pressure, was taken as an indication of changes in distensibility arising from structural changes of the aortic wall.

Some of these results were presented at the Seventh Scientific Meeting of the American Society of Hypertension, New York, May 1992 (Chillon *et al.*, 1992).

Methods

Animals and chronic drug treatment

Forty-four male SHR and ten male WKY rats were purchased from Iffa-Credo, L'Arbresle, France. On arrival they were 3 months old and weighed 250–270 g. Following measurement of systolic arterial blood pressure (see below), SHR were randomly assigned to groups receiving the following treatments: standard rat chow (UAR, Villemoisson sur Orge, France), chow plus captopril and/or hydrochlorothiazide (Table 1). WKY rats were given standard rat chow. Treatment was continued for 10 weeks during which time food intake and body weight were measured weekly. Food and water were available *ad libitum*.

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Table 1 Chronic treatment of spontaneously hypertensive rats (SHR) for 10 weeks with captopril and hydrochlorothiazide (Cap + HCZ): experimental groups

Group	n	*Rat*	*Treatment*
SHR	12	SHR	Standard rat chow
Cap + HCZ	12	SHR	Captopril (44) + hydrochlorothiazide (22)
Cap	11	SHR	Captopril (41)
HCZ	9	SHR	Hydrochlorothiazide (21)
WKY	10	WKY	Standard rat chow

The average daily doses (mg kg^{-1}) are given in parentheses and were calculated from the quantity of drug in the rat chow and the food intake (determined weekly).

Systolic arterial blood pressure and heart rate measurements in awake rats

Rats were placed in restraining cages in a specially constructed incubator (40°C) for 15 min. A cuff was put around the base of the tail and a microphone was placed over the ventral tail artery, distal to the cuff. The cuff was inflated and systolic arterial blood pressure (mmHg) was taken as being equivalent to the cuff pressure at which the pulse of the tail artery could no longer be detected. Heart rate (beats min^{-1}) was obtained from a fast speed chart recording. Measurements were repeated at least 3 times and an average taken. Systolic arterial blood pressure and heart rate were recorded before the start of treatment, and at 28, 42 and 70 days after the start of treatment.

Arterial blood pressure and pulse wave velocity recording in anaesthetized and pithed rats

Twenty-four h following the final measurement of systolic arterial blood pressure, rats were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.), then given atropine (1 mg kg^{-1}, i.p.). Nylon cannulae (1.02 mm external, 0.58 mm internal diameter; Portex-LSA, Fontenay-sous-Bois, France) were introduced into the right common carotid and left femoral arteries up to their aortic ostia. Cannulae were connected to low volume pressure transducers that were in turn connected to a MacLab/MacBridge system (AD Instruments Ltd., Hampstead, UK) for on-line recording of the two arterial blood pressure signals. The frequency response of the cannula plus pressure transducer, filled with 0.15 M NaCl, was flat within ± 5% up to 25 Hz. At 25 Hz, the phase lag was −7°. From 25 to 50 Hz the cannula plus pressure transducer system was underdamped.

Aortic pulse wave velocity (cm s^{-1}) was determined by first calculating the foot of each systolic arterial blood pressure wave, following digital conversion of the original analogue pulse signals. The foot was defined as the point obtained by extrapolating the wave front downward to the point of intersection with the exponential decay of the diastolic arterial blood pressure. The distance (cm) between the 2 cannulae tips was determined by direct measurement following *post mortem* dissection of the aorta.

Carotid arterial blood pressure dP/dt was estimated by electronic differentiation of the blood pressure signal in the carotid artery, before data storage in the computer. As any frequency distortion up to the 20th harmonic of the input signal could affect a rapid phenomenon such as dP/dt, our values are not absolute. Our underdamped system presumably overestimates values for dP/dt. As heart rates were similar in all SHR groups, it can be argued that overestimation of dP/dt would be similar in all groups.

Pulse wave amplification was calculated by dividing the femoral pulse arterial blood pressure by the carotid pulse arterial blood pressure. Pulse wave velocity and amplification, carotid arterial dP/dt, systolic, diastolic, mean and pulse arterial blood pressures, and heart rate were determined over a respiratory cycle and the values of 5 respiratory cycles averaged.

Pressure and heart rate were measured 45 min following induction of anaesthesia. Rats were then pithed and artificially ventilated (50 strokes min^{-1}, 10 ml kg^{-1}) with air. Forty-five min following pithing, a blood sample was taken to check blood gas values (PCO_2, PO_2, and pH) and pressure and heart rate were again recorded.

A final blood sample (5 ml) was taken from the carotid artery cannula for the determination of calcium, phosphate, creatinine, urea, cholesterol, triglycerides, glucose, protein, albumin, and bilirubin levels, and alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase activities. This analysis was performed by use of standard clinical chemistry techniques modified for rat plasma by J.P. Nicolas (INSERM U308, CHRU Brabois, Nancy, France). The first 0.5 ml of blood removed was used for the determination of plasma renin activity (Peters-Haefeli, 1971) and plasma angiotensin I converting enzyme activity (Ryan *et al.*, 1977). The heart was removed, the ventricles dissected out and weighed.

Statistics

One- or two-way analysis of variance followed by the Scheffé test was used. Linear and multiple regression analyses were performed by use of standard parametric methods (a = intercept, b = slope, r = correlation coefficient). Results are given as means ± s.e.mean.

Drugs

Captopril, hydrochlorothiazide and atropine were purchased from Sigma Chemical Co., St Louis, MO, U.S.A. and sodium pentobarbitone from Sanofi SA Paris, France.

Results

Body weight, food intake and clinical chemistry

Body weight increased progressively throughout the 10 weeks' treatment period, but this progression was slower in SHR receiving diets containing hydrochlorothiazide (Table 2). The slower growth rate in these groups was not due to a decrease in foot intake (due to adverse taste, for example) as food intake was significantly higher in the group receiving captopril and hydrochlorothiazide (Table 2).

Furthermore, clinical chemistry analysis revealed that drug treatment had no effect apart from a fall in blood glucose in groups treated with diets containing captopril (Table 3).

Plasma renin activity (SHR: 25 ± 3 g AI ml^{-1} h^{-1}), and angiotensin I converting enzyme activity (SHR: 28 ± 4 nmol ml^{-1} min^{-1}) were similar in all groups. Although these values do not represent basal values because both pithing and pentobarbitone anaesthesia stimulate renin release, they suggest that drug treatment did not chronically stimulate renin release.

The heart weight was reduced in groups treated with captopril and captopril plus hydrochlorothiazide (Table 2). Regression analysis revealed significant correlations between heart weight and pulse wave velocity ($r = 0.485$, $P < 0.05$, d.f. 51), and mean arterial blood pressure ($r = 0.486$, $P < 0.05$, d.f. 51), determined in anaesthetized rats. Multiple regression analysis revealed no significant independent correlation between either arterial blood pressure, or pulse wave velocity, and heart weight (results not shown).

Table 2 Body weight, heart weight and food consumption in rats used in these experiments

	SHR	*Cap + HCZ*	*Cap*	*HCZ*	*WKY*
Body weight (g)					
Day 1	261 ± 5	259 ± 5	257 ± 6	251 ± 3	256 ± 5
Day 70	363 ± 8	311 ± 5*	352 ± 8	325 ± 6*	360 ± 6
Food comsumption (g kg^{-1} per day)					
Day 1	71 ± 1	75 ± 1	71 ± 2	69 ± 2	75 ± 3
Day 70	54 ± 2	69 ± 2*	60 ± 1	62 ± 1	61 ± 2
Heart weight (mg kg^{-1} body weight)	377 ± 11	344 ± 7*	347 ± 9*	381 ± 11	288 ± 8*

*$P < 0.05$ compared to group SHR.
SHR = untreated spontaneously hypertensive rats.
Cap + HCZ = spontaneously hypertensive rats treated with captopril and hydrochlorothiazide.
Cap = spontaneously hypertensive rats treated with captopril.
HCZ = spontaneously hypertensive rats treated with hydrochlorothiazide.
WKY = untreated Wistar-Kyoto normotensive rats.

Table 3 Clinical chemistry of plasma samples from groups of rats used in this study

	SHR	*Cap + HCZ*	*Cap*	*HCZ*	*WKY*
Calcium (mM)	2.54 ± 0.12	2.62 ± 0.06	2.71 ± 0.05	2.68 ± 0.04	2.62 ± 0.08
Phosphate (mM)	2.20 ± 0.34	2.67 ± 0.35	2.59 ± 0.35	2.15 ± 0.23	2.97 ± 0.26
Creatinine (μM)	112 ± 10	107 ± 3	94 ± 13	98 ± 10	94 ± 10
Urea (mM)	8.1 ± 0.6	10.4 ± 0.9	6.0 ± 1.6	7.8 ± 1.2	8.0 ± 0.9
Cholesterol (mM)	0.8 ± 0.1	0.7 ± 0.03	0.9 ± 0.1	0.8 ± 0.1	1.2 ± 0.1*
Triglycerides (mM)	0.43 ± 0.07	0.51 ± 0.08	0.42 ± 0.09	0.48 ± 0.12	0.61 ± 0.09
Glucose (mM)	8.9 ± 1.8	5.3 ± 0.5*	5.5 ± 1.0*	7.2 ± 0.7	4.5 ± 0.9*
Protein (g l^{-1})	47 ± 1	46 ± 1	44 ± 5	46 ± 1	46 ± 2
Albumin (g l^{-1})	26 ± 1	28 ± 1	26 ± 3	26 ± 1	27 ± 1
Bilirubin (μM)	1.08 ± 0.22	1.86 ± 0.91	0.90 ± 0.25	1.33 ± 0.31	1.29 ± 0.40
Alkaline phosphatase (iu l^{-1})	77 ± 6	97 ± 8	92 ± 3	75 ± 11	103 ± 9
Aspartate amino-transferase (iu l^{-1})	282 ± 109	256 ± 82	246 ± 38	204 ± 14	202 ± 20
Alanine amino-transferase (iu l^{-1})	149 ± 86	148 ± 89	97 ± 29	71 ± 8	61 ± 10

*$P < 0.05$ compared to group SHR.
SHR = untreated spontaneously hypertensive rats.
Cap + HCZ = spontaneously hypertensive rats treated with captopril and hydrochlorothiazide.
Cap = spontaneously hypertensive rats treated with captopril.
HCZ = spontaneously hypertensive rats treated with hydrochlorothiazide.
WKY = untreated Wistar-Kyoto normotensive rats.

Systolic arterial blood pressure and heart rate in awake rats

Systolic arterial blood pressure in control SHR increased up to the 42nd day. Diets containing captopril produced an initial fall in systolic arterial blood pressure up to the 28th day (Figure 1).

In SHR given captopril alone, the antihypertensive response flattened out, whereas if captopril was given in combination with hydrochlorothiazide, systolic arterial blood pressure continued to fall. The latter treatment did not, however, completely normalize systolic arterial blood pressure (comparison with WKY rats). Hydrochlorothiazide alone had no effect on systolic arterial blood pressure. Systolic arterial blood pressure in WKY rats was stable throughout the study (day 70: 143 ± 2 mmHg).

There was no significant effect of drug or time on heart rate; values on day 70 were 431 ± 16 beats min^{-1} for SHR and 360 ± 9 beats min^{-1} for WKY.

Arterial blood pressure and pulse wave velocity in anaesthetized and pithed rats

Arterial blood pressure measured under pentobarbitone anaesthesia revealed that all drug treatments lowered mean and diastolic arterial blood pressures but did not normalize these pressures (Table 4). Only diets containing hydrochlorothiazide lowered systolic and pulse arterial blood pressures. Pulse wave velocity was diminished by all treatments, the greatest fall being observed in the group of SHR previously treated with captopril and hydrochlorothiazide. In WKY rats, pulse wave velocity (Y) was significantly correlated to arterial blood pressure (X). Values for mean arterial blood pressure were $a = -21$, $b = 5.6$, $r = 0.77$, $P < 0.05$, $n = 10$. Values for systolic arterial blood pressure were $a = -32$, $b = 4.8$, $r = 0.76$, $P < 0.05$, $n = 10$. In the different SHR groups there were no significant correlations between pulse wave velocity and arterial blood pressure.

Pulse wave amplification was not significantly different from 1 in SHR. In SHR treated with captopril plus hydrochlorothiazide, pulse wave amplification was 1.23 ± 0.06 and was similar to the value for WKY (1.32 ± 0.05).

In pithed rats, there was no significant correlation between pulse wave velocity and arterial blood pressure in any group. Drug treatment did not lower arterial blood pressure. The combination of captopril and hydrochlorothiazide produced a significant fall in pulse wave velocity. Drug treatment had no effect on heart rate (Table 4) or carotid arterial dP/dt (anaesthetized SHR 1750 ± 231 mmHg s^{-1}, and pithed SHR 1174 ± 111 mmHg s^{-1}, $n = 12$), in either anaesthetized or pithed rats.

Discussion

The main result of this experiment is that antihypertensive treatment of adult SHR with a combination of captopril and

hydrochlorothiazide improves aortic distensibility as shown by an increase in pulse wave amplification in anaesthetized SHR, and a decrease in pulse wave velocity, in the absence of any significant effect on arterial blood pressure, in pithed SHR. The latter result suggests that the combination of captopril and hydrochlorothiazide may have a specific effect on aortic rigidity.

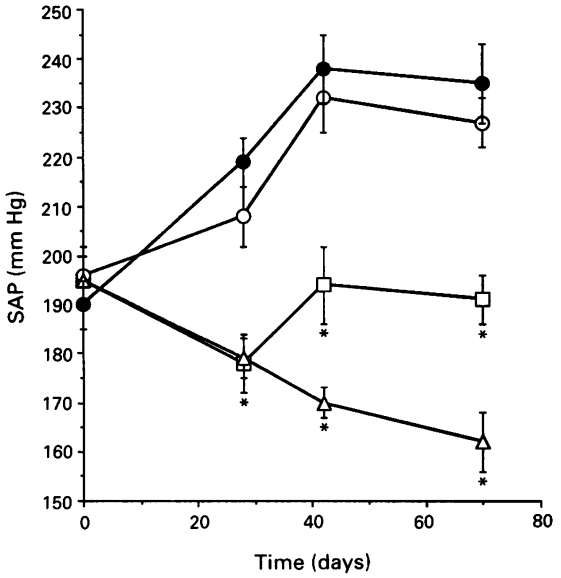

Figure 1 Change in systolic arterial pressure (SAP) (mmHg) in spontaneously hypertensive rats (SHR) treated (open symbols) with captopril plus hydrochlorothiazide (triangles), captopril (squares) or hydrochlorothiazide (circles). A control group of SHR (solid circles) received standard rat chow. *$P<0.05$ compared to SHR group.

Our observation that in the group treated with captopril plus hydrochlorothiazide, the antihypertensive effects of the representatives of two separate classes of drugs are additive is interesting but not original (Rubin *et al.*, 1978; Oster & Epstein, 1987). The explanation generally given for this synergistic effect is stimulation by the diuretic of the renin-angiotensin-aldosterone axis, possibly following diuretic-induced salt and water loss (Miyamoto *et al.*, 1983). Other factors such as direct vascular effects may be involved. Hydrochlorothiazide and captopril are synergistic in SHR in which urinary loss is prevented by bilateral ureteral ligation (Chiu *et al.*, 1985), although this has been contested (Chan *et al.*, 1982). One argument in favour of an effect on vascular function and structure in our experiment can be based on the fact that the synergism between the two drugs was seen after 1 month of treatment only. The authors cited previously reported an immediate synergistic effect using protocols in which the drugs were given by gavage, and arterial blood pressure was measured shortly afterwards. Our protocol in which the drugs were mixed into the diet and arterial blood pressure was measured during the day, presumably a long time after the drug was ingested during the night, probably investigates longer term effects of the antihypertensive treatment on vascular structure.

It should be noted that the evidence for the synergism between captopril and hydrochlorothiazide comes from the recordings of systolic arterial blood pressure in awake SHR. Systolic arterial blood arterial recordings obtained in pentobarbitone anaesthetized SHR were similar to those obtained in awake SHR except in the case of SHR previously treated with hydrochlorothiazide.

An effect of antihypertensive treatment with captopril plus

Table 4 Arterial blood pressure (mmHg), heart rate (beats min^{-1}) and pulse wave velocity (PWV, cm s^{-1}) in SHR chronically treated with captopril (Cap) and/or hydrochlorothiazide (HCZ) and in WKY, measured in the anaesthetized and pithed states

	SHR	*Cap + HCZ*	*Cap*	*HCZ*	*WKY*
Pentobarbitone anaesthesia					
Arterial blood pressure (carotid)					
systolic	209 ± 11	149 ± 7*	188 ± 7	179 ± 13*	112 ± 5*
diastolic	158 ± 10	107 ± 7*	135 ± 7*	136 ± 9*	84 ± 4*
mean	175 ± 10	121 ± 7*	153 ± 7*	149 ± 10*	94 ± 4*
pulse	51 ± 3	42 ± 3*	53 ± 4	43 ± 5*	28 ± 1*
Arterial blood pressure (femoral)					
systolic	215 ± 9	155 ± 8*	184 ± 8	177 ± 11*	118 ± 5*
diastolic	155 ± 9	102 ± 8*	127 ± 7*	130 ± 9*	81 ± 4*
mean	175 ± 10	120 ± 7*	149 ± 7*	146 ± 9*	93 ± 4*
pulse	60 ± 3	53 ± 4	67 ± 5	47 ± 2*	37 ± 1*
PWV	1005 ± 43	587 ± 47*	708 ± 35*	674 ± 72*	490 ± 25*
Heart rate	336 ± 9	343 ± 7	352 ± 12	332 ± 7	276 ± 7*
Pithed					
Arterial blood pressure (carotid)					
systolic	67 ± 2	64 ± 3	64 ± 4	71 ± 4	60 ± 2
diastolic	38 ± 2	34 ± 2	38 ± 3	42 ± 2	31 ± 2*
mean	46 ± 2	44 ± 2	47 ± 3	52 ± 3	40 ± 2
pulse	29 ± 2	30 ± 1	27 ± 2	29 ± 3	29 ± 2
Arterial blood pressure (femoral)					
systolic	61 ± 3	53 ± 3	55 ± 3	64 ± 3	49 ± 2*
diastolic	35 ± 2	33 ± 2	31 ± 2	41 ± 2	28 ± 1*
mean	44 ± 2	40 ± 2	39 ± 2	49 ± 2	35 ± 2*
pulse	25 ± 2	20 ± 1	24 ± 1	23 ± 2	21 ± 2
PWV	434 ± 12	368 ± 22*	392 ± 20	379 ± 29	368 ± 19*
Heart rate	247 ± 9	253 ± 5	251 ± 5	268 ± 12	242 ± 8

*$P<0.05$ compared to group SHR.
SHR = untreated spontaneously hypertensive rats.
Cap + HCZ = spontaneously hypertensive rats treated with captopril and hydrochlorothiazide.
Cap = spontaneously hypertensive rats treated with captopril.
HCZ = spontaneously hypertensive rats treated with hydrochlorothiazide.
WKY = untreated Wistar-Kyoto normotensive rats.

hydrochlorothiazide on vascular structure could explain the decrease in pulse wave velocity seen in pithed rats. The values for pulse wave velocity in normotensive WKY rats are similar to those published for man and the dog (for review see Milnor, 1989). Pulse wave velocity was increased in anaesthetized or pithed SHR, and the increase seen in anaesthetized SHR corresponds to the increase reported to occur in hypertensive patients by Messerli *et al.* (1985). Pulse wave velocity also increases with age in man (Messerli *et al.*, 1985; Milnor, 1989) and we have recently shown that the same phenomenon occurs in rats (Chillon *et al.*, 1992).

Angiotensin I converting enzyme-inhibitors have been previously reported to improve large artery compliance in rats (Levy *et al.*, 1988; 1989), and in man (Simon *et al.*, 1985). To our knowledge this is the first description of the effect of a combination of an angiotensin I converting enzyme inhibitor and a diuretic on arterial distensibility. Levy and coworkers reported that the decrease in arterial distensibility (as reflected by carotid artery compliance) in hypertensive rats, and the increase produced by antihypertensive drugs, were maximal when measured at pressures near the operating blood pressure of the animal. A similar phenomenon is observed in our results. Changes in pulse wave velocity induced by hypertension or antihypertensive treatment are much greater in anaesthetized rats than in pithed rats in which arterial blood pressure is much lower. Measurement of pulse wave velocity at the lower arterial blood pressure of the pithed rat presumably reflects elastic stiffness relatively unaffected by smooth muscle contractility.

Pulse wave velocity and arterial blood pressure were both higher in SHR than in WKY, and in anaesthetized than in pithed rats. The observation that pulse wave velocity is linked to arterial blood pressure is not original (Milnor, 1989). The fact that in pithed rats, captopril plus hydrochlorothiazide significantly lowered pulse wave velocity, but not arterial blood pressure, argues against our observing a lower pulse wave velocity in this group simply because they had a lower arterial blood pressure. A chronic effect of arterial blood pressure cannot be excluded. The more efficient chronic control of blood pressure obtained with the captopril plus hydrochlorothiazide combination may have produced a more pronounced decrease in transmural pressure leading to a greater regression of the structural changes of the vessel wall.

Drug-induced changes in the arterial blood pressure of the anaesthetized rats suggest that treatment with diets containing hydrochlorothiazide has a specific effect on arterial rigidity. In both the hydrochlorothiazide and captopril plus hydrochlorothiazide groups, the decrease of systolic arterial blood pressure was greater than that of diastolic arterial blood pressure, and both treatments decreased pulse arterial blood pressure. Systolic arterial blood pressure depends upon ventricular ejection and aortic rigidity (Safar *et al.*, 1983). Although we have no measurement of possible drug effects on ventricular hypertrophy, simple regression analysis revealed a significant correlation between pulse wave velocity and cardiac hypertrophy. Further experiments with larger groups and longer treatment may permit a definite answer to be given.

In conclusion, antihypertensive treatment with captopril plus hydrochlorothiazide improved aortic distensibility, as judged from the decrease in aortic pulse wave velocity in SHR. We suggest that this effect may be related to an effect of this combination of drugs on the vascular wall.

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An investigation of some S-nitrosothiols, and of hydroxy-arginine, on the mouse anococcygeus

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1 The effect of five S-nitrosothiols, and of the stereoisomers of N^G-hydroxy-arginine (HOARG), were investigated on the mouse anococcygeus.

2 All five S-nitrosothiols produced concentration-related (0.1–100 μM) relaxations of carbachol (50 μM)-induced tone; the order of potency was S-nitroso-L-cysteine (CYSNO) > S-nitroso-N-acetyl-D,L-penicillamine (SNAP) > S-nitrosoglutathione (GSNO) > S-nitrosocoenzyme A (CoASNO) > S-nitroso-N-acetyl-L-cysteine (NACNO). The relaxations were unaffected by the nitric oxide synthase (NOS) inhibitor, L-N^G-nitro-arginine (10 μM) (L-NOARG).

3 Cold-storage of the tissue for 72 h resulted in loss of sympathetic and non-adrenergic, non-cholinergic (NANC) nerve function. NOS activity in the tissue was reduced by 97%. Despite this, relaxations induced by the S-nitrosothiols were unaffected.

4 Haemoglobin (50 μM) attenuated relaxations induced by NO and the S-nitrosothiols, although responses to 3-isobutyl-1-methyl-xanthine were unaffected. N-methyl-hydroxylamine (2 mM) which has been shown previously to produce selective inhibition of NANC and nitrovasodilator responses in this tissue, also reduced responses to all S-nitrosothiols.

5 Hydroquinone (100 μM) greatly reduced relaxations to CYSNO (by 88%) but had no effect on those to SNAP, GSNO, CoASNO or NACNO. Since hydroquinone does not reduce responses to NANC stimulation, CYSNO is unlikely to be the NANC transmitter.

6 L-HOARG by itself (up to 100 μM) had no significant effect on carbachol-induced tone or on NANC (10 Hz; 10 s train every 100 s) relaxations. However, it produced reversal of the inhibitory effects of L-NOARG (10 μM), being only slightly less potent than L-arginine. D-HOARG was without effect. L-HOARG had no effect on relaxations induced by 1.5 μM NO.

7 The results show that S-nitrosothiols are potent relaxants of the mouse anococcygeus; they act directly on the smooth muscle with a mechanism similar to NO and other nitrovasodilators. In addition, the results are consistent with L-HOARG being an intermediate in the biosynthesis of NO from L-arginine, although there is no evidence for it acting to stabilize NO extracellularly.

Keywords: Cold-storage; haemoglobin; hydroquinone; hydroxy-arginine; mouse anococcygeus; nitric oxide synthase; nitrosothiol; non-adrenergic, non-cholinergic

Introduction

Recent observations from several laboratories have established that inhibitors of nitric oxide synthase (NOS) reduce non-adrenergic, non-cholinergic (NANC) relaxations of the anococcygeus muscle (Gillespie *et al.*, 1989; Li & Rand, 1989; Ramagopal & Leighton, 1989; Gibson *et al.*, 1990; Hobbs & Gibson, 1990); the effects of the NOS inhibitors are stereoselective and reversed by L-, but not D-arginine. These results provide strong evidence that NANC relaxations in this tissue, as in several others (Rand, 1992), involve the L-arginine: nitric oxide (NO) pathway, and that the neurotransmission system resembles that responsible for the production and release of the endothelium-derived relaxing factor (EDRF; Moncada *et al.*, 1991). However, in both the endothelial (McCall & Vallance, 1992) and NANC (Gillespie & Sheng, 1989) systems there is still considerable debate over the nature of the substance actually released from the synthesizing cell, since several observations are difficult to reconcile with the release of free NO. Indeed, in the mouse anococcygeus we have demonstrated that hydroquinone, acting as a free radical scavenger, greatly reduces relaxations induced by NO, but has no effect on those to NANC nerve stimulation or several other nitrovasodilators (Hobbs *et al.*, 1991). It has been proposed that, rather than free NO, both endothelial cells and NANC nerves may release a NO-containing molecule, from which NO is subsequently liberated by the target cell; a favoured candidate is some form of S-nitrosothiol, particularly S-nitroso-L-cysteine (CYSNO; Myers *et al.*, 1990; Rubanyi *et al.*, 1990; Thornbury *et al.*, 1991). In addition, Zembowicz *et al.* (1991) have shown that L-N^G-hydroxy-arginine (L-HOARG), an intermediate in the synthesis of NO from L-arginine (Stuehr *et al.*, 1991; Wallace & Fukuto, 1991), can interact with NO to form a relatively stable nitroso-adduct which exhibits marked vasodilator potency. Neither the S-nitrosothiols nor L-HOARG have been widely studied in non-vascular smooth muscle and, therefore, the object of the present study was to investigate the effects of several S-nitrosothiols (Kowaluk & Fung, 1990), and of the stereoisomers of HOARG, on the mouse anococcygeus.

Methods

Mouse anococcygeus

Male mice (LACA; 25–35 g) were killed by stunning and exsanguination. The paired anococcygeus muscles were dissected, joined by the ventral bar, and set up in series in 2 ml glass organ baths containing Krebs bicarbonate buffer (mM: NaCl 118.1, KCl 4.7, $MgSO_4$ 1.0, KH_2PO_4 1.0, $CaCl_2$

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2.5, $NaHCO_3$ 25.0, glucose 11.1) which was maintained at 37°C and gassed continuously with 95% O_2: 5% CO_2. A resting tension of 200–400 mg was placed on the tissue and changes in tension recorded with a Grass FTO3 force-displacement transducer attached to a Graphtec pen-recorder (WR3101). Muscles were allowed to equilibrate for 45 min before the experiment was started. Field stimulation was applied via two parallel platinum electrodes running down either side of the tissue; these were attached to a Grass S48 stimulator (0.5 ms pulse width; 70 V). To observe relaxations to field stimulation in the anococcygeus it is necessary to raise muscle tone and to negate contractions due to release of noradrenaline (NA) from the sympathetic nerves. Tone was raised with 50 μM carbachol in all cases; sympathetic responses were prevented by including 1 μM phentolamine in the Krebs solution and by pre-incubating each muscle with 30 μM guanethidine for 10 min during the 45 min equilibration period. Field stimulation (10 Hz; 10 s trains every 100 s), under these conditions, produces NANC relaxations which are reduced by 95% in the presence of the NOS inhibitor L-N^G-nitro-arginine (L-NOARG; 50 μM; Gibson *et al.*, 1990).

To record relaxations to drugs, tone was first raised with 50 μM carbachol. When carbachol-tone had reached a plateau (usually within 3 min), the relaxant drug was added to the organ bath. The response was calculated as the peak % reduction of carbachol-induced tone occurring within 4 min of addition of the relaxant; if no peak occurred by 4 min the response at that time was used. Both carbachol and the relaxant were then washed out of the bath and the muscle allowed to rest for 20 min before tone was raised again.

In the cold-storage experiments, anococcygeus muscles were dissected and placed in 500 ml Krebs solution, pre-cooled to 4°C; up to 12 muscle preparations were stored together. The Krebs container was then transferred to a refrigerator (4°C). After the appropriate period of cold storage, muscles were removed from the refrigerator and mounted in organ baths as described above.

Nitric oxide synthase assays

These were carried out on cytosolic fractions obtained from muscles of 12–16 mice per experiment; the procedure used was that outlined by Dwyer *et al.* (1991) for the assay of NOS in brain cytosol. Muscles were weighed and then homogenized (Ultra-Turrax type 18/2N; 30 s) in 10 vol Tris/EDTA buffer (Tris-HCl 20 mM; EDTA 2 mM; pH 7.4). After centrifugation (10000 *g*; 15 min; 4°C), aliquots of supernatant (25 μl) were incubated with [^{3}H]-arginine (50 nM; 0.5 μCi; Amersham), $CaCl_2$ (0.75 mM) and NADPH (0.5 mM) in a total volume of 105 μl. After incubation (15 min; 37°C), the reaction was stopped by addition of 3 ml ice-cold HEPES/EDTA buffer (HEPES 20 mM; EDTA 2 mM; pH 5.5). [^{3}H]-arginine was separated from [^{3}H]-citrulline by anion exchange chromatography on 0.5 ml columns of Dowex AG 50 WX8 (Na^+ form). [^{3}H]-citrulline was eluted with 1 ml distilled water and estimated by liquid scintillation spectroscopy. Protein was measured by the method of Lowry *et al.* (1951).

Synthesis of S-nitrosothiols

S-nitrosothiols were synthesized according to the methods outlined by Kowaluk & Fung (1990), which involve reacting the appropriate thiol with sodium nitrite under acidic conditions.

S-nitrosoglutathione (GSNO; Hart, 1985)

The reaction mixture (8 ml; pH 2; 4°C) contained 5 mmol glutathione and 5 mmol sodium nitrite. After 40 min, during which time the mixture was continuously stirred, the resultant red solution was treated with acetone (10 ml; 4°C). This produced a fine pink precipitate which was filtered off and then washed successively with water (5 ml), acetone (3 ml) and ether (3 × 10 ml). Solutions of the dried powder (GSNO) were prepared daily for experimentation.

S-nitroso-N-acetyl-D,L-penicillamine (SNAP; Field *et al.*, 1978).

The reaction mixture (60 ml; pH 2; 20°C) contained 10 mmol N-acetyl-D,L-penicillamine (dissolved in 20 ml methanol and 20 ml 1 N NaOH) and 20 mmol sodium nitrite. After 35 min, during which time the mixture was continuously stirred, the suspension was filtered off to yield deep green crystals. Solutions of the dried crystals (SNAP) were prepared daily for experimentation.

S-nitroso-L-cysteine (CYSNO) and S-nitroso-N-acetyl-L-cysteine (NACNO; Hart, 1985)

The reaction mixture (4 ml; pH 2; 4°C) contained 200 μmol L-cysteine (for CYSNO) or 200 μmol N-acetyl-L-cysteine (for NACNO) together with 200 μmol sodium nitrite. After 30 min, the pH of the solution was adjusted to 7.0 (1 N NaOH). Aliquots of the neutralized solutions (CYSNO or NACNO) were added directly to the organ baths, fresh solutions being prepared each day.

S-nitrosocoenzyme A (CoASNO; Tu *et al.*, 1984)

The reaction mixture (4 ml; pH 2; 20°C) contained 20 μmol coenzyme A and 20 μmol sodium nitrite. After 30 min, the pH of the solution was adjusted to 7.0 (1 N NaOH). Aliquots of the neutralized solution (CoASNO) were added directly to the organ baths, fresh solutions being prepared each day.

All S-nitrosothiol solutions exhibited a visible absorption maximum at 544–547 nm, which is characteristic of the red colour of primary S-nitrosothiols (Oae *et al.*, 1978), and were protected from light until added to the organ bath. The concentrations given were calculated on the assumption that all of the parent thiol was converted to its S-nitrosothiol (Kowaluk & Fung, 1990).

Haemoglobin and NO

Reduced haemoglobin was prepared by adding a 10 fold molar excess of sodium hydrosulphite to a 10 mM solution of commercially available haemoglobin (Sigma; Type I) and stirred for 30 min at 4°C (Martin *et al.*, 1985). The sodium hydrosulphite was then removed by dialysis through cellulose tubing, against 100 volumes of distilled water for 12 h (4°C). The resulting solution was stored as 1 ml aliquots at −20°C for no longer than 14 days. Reduced haemoglobin exhibited characteristic absorption maxima at 577 and 540 nm.

NO solutions were prepared as described previously (Gibson & Mirzazadeh, 1989).

Statistics

Results are expressed as mean ± s.e.mean. Statistical analysis was by Student's *t* test (unpaired); a probability (*P*) value of <0.05 was taken to indicate significance.

Drugs

The following were used: acetone (BDH), N-acetyl-L-cysteine (Sigma), N-acetyl-D,L-penicillamine (Sigma), L-arginine hydrochloride (Sigma), 8-bromo-cyclic guanosine monophosphate (Sigma), carbachol (Aldrich), coenzyme A (Sigma), L-cysteine (Sigma), ether (BDH), glutathione (Sigma), guanethidine sulphate (Ciba), haemoglobin (Sigma), D- and L-N^G-hydroxy-arginine (kindly donated by Dr P. Feldman, Glaxo, USA), hydroquinone (Sigma), hydroxylamine hydrochloride (Sigma), 3-isobutyl-1-methyl xanthine (Aldrich), methanol (BDH), N-methyl-hydroxylamine hydrochloride (Sigma), nitric oxide (99%; BDH), L-N^G-nitro-arginine

(Sigma), noradrenaline bitartrate (Sigma), phentolamine mesylate (Ciba), sodium nitrite (Sigma), sodium nitroprusside (Sigma), tyramine hydrochloride (BDH).

Results

S-nitrosothiols

All five S-nitrosothiols produced concentration-related relaxations of carbachol (50 μM)-induced tone; the order of potency was CYSNO> SNAP> GSNO> CoASNO> NACNO (Figure 1a); concentrations in excess of 100 μM were not studied to avoid effects of unreacted nitrite or native thiol.

In order to determine whether these relaxations were dependent upon the generation of NO via NOS, or on the presence of intact NANC nerves within the tissue, two series of experiments were attempted. In the first, the effects of the NOS inhibitor L-NOARG were evaluated; 10 μM L-NOARG, which reduced NANC relaxations of the mouse anococcygeus by 75% (Gibson *et al.*, 1990), had no effect on responses to submaximal concentrations of CYSNO (2 μM), SNAP (2 μM), GSNO (20 μM), NACNO (64 μM) or CoASNO (32 μM; Figure 1b). In the second series of experiments, cold-storage was used to produce denervation of the tissue. In order to determine the time course of denervation, contractile response to field stimulation of the sympathetic nerves were first observed (obtained in the absence of phentolamine, guanethidine and carbachol). Field stimulation induced contractions (10 Hz; 10 s train every 100 s) were progressively depressed with increasing periods of cold-storage, and by 72 h were completely lost (Figure 2a). Contractile responses to tyramine were also abolished in muscles cold-stored for 72 h, although those to carbachol were unchanged (data not shown); the concentration-response curve for NA-induced contractions was displaced to the left (control pD_2, 6.50 ± 0.14, $n = 6$; 72 h cold-stored pD_2, 7.66 ± 0.13, $n = 8$, $P < 0.05$), with no change in maximum response (control, 526 ± 103 mg tension; 72 h cold-stored, 373 ± 49 mg tension, $P > 0.05$). These results suggested that 72 h of cold-storage results in loss of sympathetic nerves within the mouse anococcygeus, with little change in sensitivity of the smooth muscle to carbachol. The time-course of the loss of NANC relaxations with cold-storage mirrored that of sympathetic contractions, such that by 72 h no NANC relaxations were observed (Figure 2b). In addition, in cytosol from cold-stored (72 h) muscles NOS activity, as measured by the conversion of [^{3}H]-arginine to [^{3}H]-citrulline, was greatly reduced (3.3 ± 0.9 d.p.m. ($\times 10^3$) mg^{-1} protein, compared with that from control tissues (99.9 ± 12.4 d.p.m. ($\times 10^3$) mg^{-1} protein; $n > 6$ in both cases; $P < 0.05$), supporting the view that by 72 h of cold-storage the NANC nerves were non-functional. However, in such tissues the sensitivity to relaxations induced by NO, sodium nitroprusside, 8-Br-cyclic GMP, CYSNO, GSNO and CoASNO was unaltered, while responses to hydroxylamine were slightly potentiated (Table 1).

Since the above results suggested that the relaxant effects of the S-nitrosothiols were not due to an indirect action via the NANC nerves, we next investigated the influence of three drugs known to modify the direct relaxant response to exogenous nitrovasodilators and/or NO; haemoglobin, N-

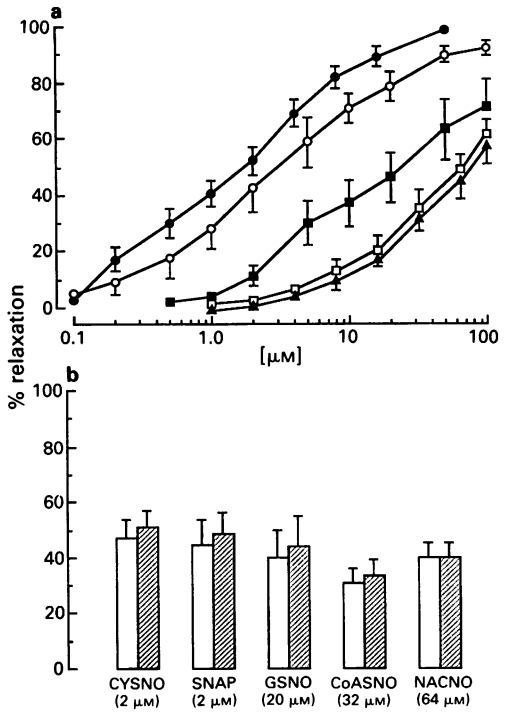

Figure 1 (a) Concentration-response curves for relaxations of the mouse anococcygeus in response to S-nitroso-L-cysteine (CYSNO; ●), S-nitroso-N-acetyl-D,L-penicillamine (SNAP; ○), S-nitroso-glutathione (GSNO; ■), S-nitrosocoenzyme A (CoASNO; □) and S-nitroso-N-acetyl-L-cysteine (NACNO; ▲). (b) Histogram showing the relaxations produced by submaximal concentrations of the above agents in the absence (open columns) or presence (hatched columns) of 10 μM L-N^G-nitro-arginine (L-NOARG). In both (a) and (b), results are shown as mean (± s.e.mean, vertical bars) with a minimum of 6 observations per group. Muscle tone was raised with 50 μM carbachol. Each S-nitrosothiol produced concentration-related relaxations that were unaffected by L-NOARG.

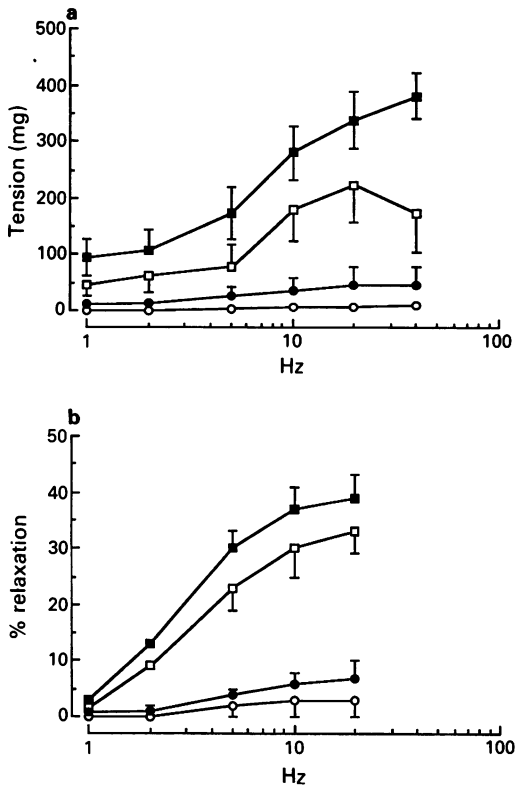

Figure 2 Frequency-response curves for sympathetic contractions (a) and non-adrenergic, non-cholinergic (NANC) relaxations (b) induced by field stimulation (10 Hz; 10 s train every 100 s) of the mouse anococcygeus. Responses (mean ± s.e.mean, vertical bars; $n \geq 6$) are from control tissues (■) or muscles stored at 4°C for 24 h (□), 48 h (●) or 72 h (○). In (b), muscle tone was raised with 50 μM carbachol and the tissues were treated with guanethidine and phentolamine to negate sympathetic responses (see methods). Cold-storage produced a progressive decline in both sympathetic and NANC responses; by 72 h both responses were absent.

Table 1 Potency of relaxant drugs in control and cold-stored muscles

Drug	$-\log_{10}IC_{50}$(M) 0 h	72 h
Nitric oxide	5.20 ± 0.09 (6)	5.32 ± 0.09 (6)
Sodium nitroprusside	6.19 ± 0.12 (9)	6.34 ± 0.15 (8)
Hydroxylamine	4.71 ± 0.08 (14)	5.17 ± 0.08* (12)
8-Br-cyclic GMP	4.35 ± 0.15 (6)	4.17 ± 0.14 (6)
S-nitroso-L-cysteine	5.75 ± 0.12 (8)	6.03 ± 0.09 (8)
S-nitrosoglutathione	4.09 ± 0.11 (6)	4.10 ± 0.08 (6)
S-nitrosocoenzyme A	4.56 ± 0.12 (6)	4.31 ± 0.22 (6)

Results show mean ± s.e.mean for control tissues (0 h) and those cold-stored for 72 h. Values in parentheses give numbers of muscles studied in each group. Muscle tone was raised with 50 μM carbachol. *Value significantly different from control.

methyl-hydroxylamine and hydroquinone. In these experiments, each drug was in contact with the tissue for 10 min before the relaxing stimulus was applied.

In order to determine an effective concentration of haemoglobin in the mouse anococcygeus, various concentrations were first tested against relaxations induced by 15 μM NO (Figure 3a). Haemoglobin (1–100 μM) produced a concentration-related inhibition of these relaxations; 50 μM haemoglobin reduced the response to NO by 87% and was used in further studies; 50 μM haemoglobin also reduced relaxations induced by NANC stimulation and by all five S-nitrosothiols, however, relaxations induced by a submaximal concentration of 3-isobutyl-1-methyl xanthine (IBMX; 10 μM; Gibson & Mirzazadeh, 1989) were unaffected (Figure 3b). N-methyl-hydroxylamine (2 mM), which has been shown to reduce NANC- and NO-induced relaxations of the mouse anococcygeus by about 50% (Gibson & Mirzazadeh, 1989) without affecting those to IBMX, also reduced the relaxations induced by each S-nitrosothiol (Figure 4a).

Finally, the effects of hydroquinone were investigated: 100 μM hydroquinone has been shown to reduce relaxations to NO by 90% without affecting those to NANC stimulation (Hobbs *et al.*, 1991); responses to CYSNO were also significantly inhibited by 100 μM hydroquinone, while those to SNAP, GSNO, NACNO and CoASNO were unaffected (Figure 4b).

Hydroxy-arginine

By itself, L-HOARG (10–200 μM) produced little effect on carbachol-induced tone; 200 μM L-HOARG reduced tone by only 9.3 ± 1.7% ($n = 6$); however, 200 μM D-HOARG produced a similar effect (13.3 ± 2.9%; $n = 6$; $P > 0.05$). In addition, neither L-, nor D-, HOARG by themselves affected NANC relaxations (10 Hz; 10 s trains every 100 s). However, as shown in Figure 5a, 50 μM L-HOARG could reverse the inhibitory effect of 10 μM L-NOARG on NANC relaxations, although no reversal was apparent with D-HOARG. When compared to L-arginine, L-HOARG was about 3 times less potent in reversing inhibition by L-NOARG (Figure 5b). To determine whether L-HOARG might form a complex with NO under the conditions of our experiment (Zembowicz *et*

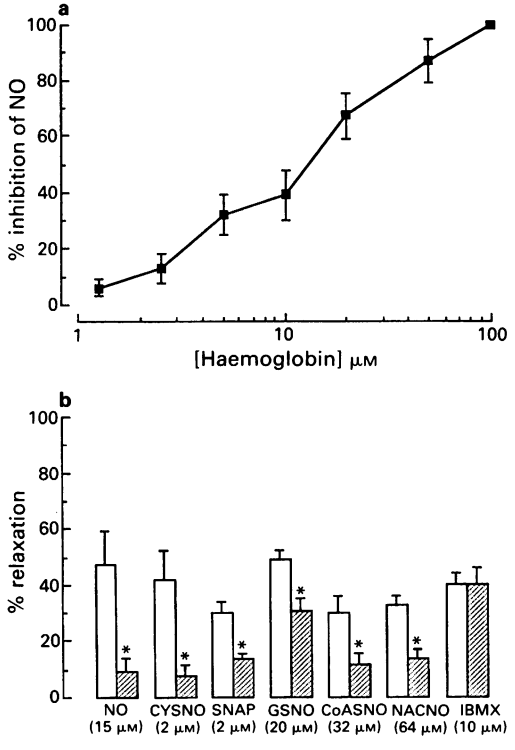

Figure 3 (a) Concentration-response curve for haemoglobin causing inhibition of nitric oxide (NO; 15 μM)-induced relaxations of the mouse anococcygeus muscle. (b) Histogram showing relaxations induced by submaximal concentrations of NO, S-nitroso-L-cysteine (CYSNO), S-nitroso-N-acetyl-D,L-penicillamine (SNAP), S-nitrosoglutathione (GSNO), S-nitrosocoenzyme A (CoASNO), S-nitroso-N-acetyl-L-cysteine (NACNO) and 3-isobutyl-1-methyl-xanthine (IBMX) in the absence (open columns) or presence (hatched columns) of 50 μM haemoglobin. Results are calculated as mean (± s.e.mean, vertical bars) ($n \geq 6$). In both (a) and (b) muscle tone was raised with 50 μM carbachol. Haemoglobin reduced responses to NO and the S-nitrosothiols but had no effect on IBMX. *$P < 0.05$ value significantly different from adjoining column.

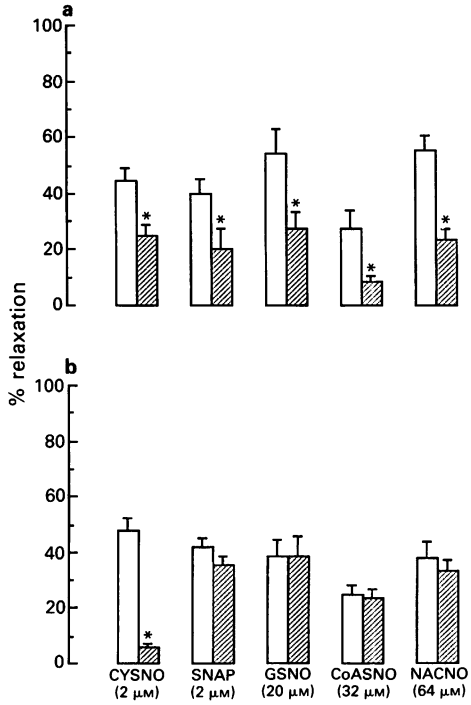

Figure 4 Histogram showing relaxations of the mouse anococcygeus induced by submaximal concentrations of S-nitroso-L-cysteine (CYSNO), S-nitroso-N-acetyl-D,L-penicillamine (SNAP), S-nitrosoglutathione (GSNO), S-nitrosocoenzyme A (CoASNO) and S-nitroso-N-acetyl-L-cysteine (NACNO) in the absence (open columns) or presence (hatched columns) of 2 mM N-methyl-hydroxylamine (a) or 100 μM hydroquinone (b). Responses are calculated as mean (± s.e.mean, vertical bars) ($n \geq 6$). Muscle tone was raised with 50 μM carbachol. N-methyl-hydroxylamine reduced responses to all five S-nitrosothiols, while hydroquinone selectively reduced those to CYSNO. *$P < 0.05$ value significantly different from adjoining column.

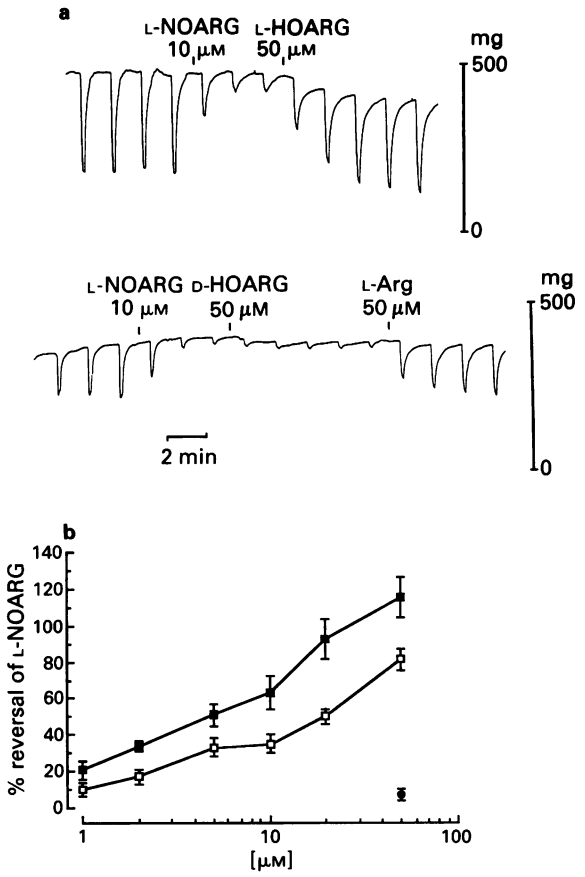

Figure 5 (a) Traces showing the effect of 50 μM L-N^G-hydroxy-arginine (L-HOARG; upper panel) and 50 μM D-HOARG (lower panel) on the inhibition of non-adrenergic, non-cholinergic (NANC; 10 Hz; 10 s trains every 100 s) relaxations of the mouse anococcygeus produced by 10 μM L-N^G-nitro-arginine (L-NOARG). Muscle tone was raised with 50 μM carbachol. L-HOARG completely reversed the inhibitory effect of L-NOARG; D-HOARG, however, could not, although 50 μM L-arginine (L-Arg) produced complete reversal. (b) Concentration-response curves for the reversal of L-NOARG (10 μM) inhibition of NANC relaxations of the mouse anococcygeus (protocol similar to that in (a)) by L-arginine (■) and L-HOARG (□). Results are mean ± s.e.mean ($n \geq 6$). For comparison, the effect of 50 μM D-HOARG (●) is also shown.

al., 1991), we investigated its effect on relaxations induced by 1.5 μM NO. By itself, NO produced relaxations of 41.0 ± 4.3% ($n = 9$) of carbachol-induced tone; these were unchanged ($P > 0.05$) in the presence of 20 μM L-HOARG (40.2 ± 4.4%; $n = 9$) or 20 μM D-HOARG (38.6 ± 4.9%; $n = 9$).

Discussion

This study has shown that five S-nitrosothiols produce concentration-related relaxations of the mouse anococcygeus, with an order of potency of CYSNO > SNAP > GSNO > CoASNO > NACNO; the range of effective concentrations (0.1–100 μM) is similar to that already found for NO and other nitrovasodilators in this tissue (Gibson & Mirzazadeh, 1989). These results confirm a recent report on guinea-pig airway smooth muscle (Jansen *et al.*, 1992) that S-nitrosothiols are potent relaxants of non-vascular smooth muscle, in addition to their well-established actions on vascular tissue (Ignarro *et al.*, 1981; Ignarro, 1989).

Two series of experiments indicate that the S-nitrosothiols act directly on smooth muscle, and not indirectly via release of the endogenous nitrate transmitter. First, the relaxations were unaffected by the NOS inhibitor L-NOARG, at a concentration that almost abolishes NANC responses (Gibson *et al.*, 1990). Secondly, the potency of the S-nitrosothiols was undiminished following cold-storage of the tissue for 72 h. Cold-storage has been used frequently as a method to induce autonomic denervation in tissues, including the anococcygeus, in which surgical denervation would be difficult (Holman & Hughes, 1965; Burnstock *et al.*, 1966; Hattori *et al.*, 1972; Gillespie & McGrath, 1974). Observation of the motor sympathetic responses of the mouse anococcygeus revealed that 72 h of cold-storage resulted in loss of sympathetic nerve function, which is similar to the time course required in other tissues (Burnstock *et al.*, 1966; Hattori *et al.*, 1972). This was confirmed by lack of responses to the indirect sympathomimetic tyramine and potentiation of response to NA. The latter effect would be due to removal of the neuronal uptake 'sites of loss' within the tissue; indeed, the degree of potentiation observed is similar to that obtained with cocaine in this muscle (Gibson & Wedmore, 1981). Importantly, however, responses to carbachol were unaffected by 72 h of cold-storage allowing it to be used to raise tone to observe NANC relaxations. These were also lost after 72 h of cold-storage and the NOS activity of the tissue, measured biochemically, was reduced by 97%. Since NOS appears to be located mainly within nerves in peripheral organs (apart from the vascular endothelium; Bredt *et al.*, 1990; Mitchell *et al.*, 1991; Grozdanovic *et al.*, 1992) this provides confirmatory evidence that 72 h of cold-storage results in loss of the NANC nerves. It should be noted that the sympathetic and NANC nerves in the anococcygeus are distinct entities, since destruction of the sympathetic nerves with 6-hydroxydopamine leaves the NANC nerves intact (Gibson & Gillespie, 1973; Gibson & Wedmore, 1981). Responses to the S-nitrosothiols were unaffected by 72 h of cold-storage, indicating that the presence of the NANC nerves in the tissue is not required for these drugs to exert their effects. Cold-storage did, however, result in enhanced responses to hydroxylamine. Since the sensitivity to sodium nitroprusside, NO and 8-Br-cyclic GMP was unaltered, the increased response to hydroxylamine is unlikely to be due to enhanced guanylate cyclase/cyclic GMP function. Hydroxylamine requires enzymic conversion by the smooth muscle cell to generate NO (Waldman & Murad, 1987) and therefore one explanation maybe that the activity of this enzyme is increased following cold storage.

Since the S-nitrosothiols appeared to act directly on the smooth muscle to produce relaxation, it was important to investigate the effect of drugs known to influence the direct effects of nitrovasodilators. N-methyl-hydroxylamine, a guanylate cyclase inhibitor which has been shown to reduce relaxations of the mouse anococcygeus to sodium nitroprusside, hydroxylamine and NO, but not those to vasoactive intestinal peptide (VIP), papaverine or IBMX (Gibson & Mirzazadeh, 1989), also reduced the responses to all five S-nitrosothiols. Haemoglobin reduced the responses to NO and the S-nitrosothiols, although it did not affect responses to IBMX. Previously, it has been shown that haemoglobin (in the form of haemolysed blood) inhibited NANC relaxations of the mouse anococcygeus without affecting those to VIP or ATP (Gibson & Tucker, 1982). The results with N-methyl-hydroxylamine and haemoglobin confirm that the S-nitrosothiols produce relaxations via a similar mechanism to NO and other nitrovasodilators. The results also show that blockade of a relaxation by haemoglobin is indicative of nitrovasodilator actions in general, rather than NO in particular.

It has been suggested that an S-nitrosothiol, possibly CYSNO, might be stored within synaptic vesicles in the NANC nerve and be liberated during nerve stimulation (Thornbury *et al.*, 1991). The experiment with hydroquinone was designed to test this possibility since we have shown that hydroquinone greatly reduces responses to NO in concentrations which leave NANC responses unaffected (Hobbs *et al.*, 1991). This and other observations (Gillespie & Sheng, 1989)

have led to the proposal that NO may be released from the nerve attached to a carrier molecule, which protects it within the synaptic cleft. Hydroquinone, however, greatly reduced responses to CYSNO; this could be explained by the fact that, of the S-nitrosothiols studied, CYSNO most readily decomposes to NO and native thiol in solution (Kowaluk & Fung, 1990). This observation makes it unlikely that CYSNO is the substance released from the NANC nerves, but clearly other physiologically relevant S-nitrosothiols could be involved. However, as pointed out by Li & Rand (1989), the suggestion that there is some kind of store of transmitter (Thornbury *et al.*, 1991) seems very unlikely given the rapid onset of action of the NOS inhibitors such as L-NOARG. Interestingly, our results do not rule out the possibility that CYSNO may be an important component of EDRF (Myers *et al.*, 1990; McCall & Vallance, 1992), since hydroquinone does reduce relaxations to EDRF, although not those to the endogenous NANC transmitter (Hobbs *et al.*, 1991).

Our results with L-HOARG are entirely consistent with it being a precursor in the formation of NO from L-arginine (Stuehr *et al.*, 1991; Wallace & Fukuto, 1991), since it could reverse the inhibitory effects of L-NOARG on NANC relaxations, while D-HOARG was ineffective. In agreement with the findings of Zembowicz *et al.* (1991) we found that L-HOARG had no direct effect on muscle tone; however, unlike Zembowicz *et al.* (1991), we found no evidence for an interaction between HOARG and NO, since L-HOARG had no effect on relaxations to exogenous NO. It may be that the conditions of our experiment (temperature and oxygenation) caused rapid degradation of any adduct formed before it could produce a pharmacological effect (Zembowicz *et al.*, 1991).

In conclusion, the main finding of this study is that S-nitrosothiols are potent, direct relaxants of the mouse anococcygeus and, therefore, that thiols could be potential carriers of NO across the synapse; however, the involvement of CYSNO seems unlikely.

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Contribution of NO and cytochrome P450 to the vasodilator effect of bradykinin in the rat kidney

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1 Inhibition of nitric oxide generation with N_w-nitro-L-arginine (nitroarginine) reduced vasodilator responses to bradykinin and acetylcholine and enhanced those to nitroprusside in the rat isolated perfused kidney, preconstricted with phenylephrine.

2 Inhibition of cyclo-oxygenase with indomethacin, decreased the vasodilator responses to bradykinin by ~25% without affecting those to acetylcholine or nitroprusside.

3 BW755c, a dual inhibitor of cyclo-oxygenase and lipoxygenase, reduced renal vasodilator responses to bradykinin, comparable to the effect of indomethacin suggesting an effect related to inhibition of cyclo-oxygenase rather than lipoxygenase.

4 ETYA, an inhibitor of all arachidonic acid metabolic pathways, markedly reduced vasodilator responses to bradykinin but was without effect on the renal vasodilatation induced by acetylcholine or nitroprusside.

5 Clotrimazole and 7-ethoxyresorufin, inhibitors of cytochrome P450, greatly attenuated vasodilator responses to bradykinin without affecting those to acetylcholine or nitroprusside.

6 These data suggest that the renal vasodilator response to bradykinin is subserved by arachidonic acid metabolites as well as nitric oxide, the former accounting for up to 70% of the vasodilator effect of bradykinin.

Keywords: Rat perfused kidney; bradykinin-induced vasodilatation; NO synthesis inhibition; inhibition of arachidonate metabolism; cytochrome P450 inhibitors

Introduction

Bradykinin is a vasoactive peptide that produces endothelium-dependent vasodilatation by stimulation of vascular B_2 receptors (Baydoun & Woodward, 1991) which results in the release of one or more of several vasorelaxant factors that are elaborated by the endothelium (Vane *et al.*, 1990), depending on species and the vascular bed. Thus, in rat skeletal muscle the vasodilator effect of bradykinin appears to be mediated primarily by prostaglandins (Messina *et al.* 1975) while in the canine kidney, the vasodilator effect of bradykinin is associated with the release of prostaglandins (McGiff *et al.*, 1972). Further, in the cat cerebral vasculature, bradykinin stimulates the release of oxygen-derived free radicals (Kontos *et al.*, 1984), generated as a result of cyclo-oxygenase metabolism of arachidonic acid which is released consequent to stimulation of phospholipases. However, the vasodilator effect of bradykinin has been most commonly attributed to the release of endothelium derived relaxing factor, now identified as nitric oxide (NO), although there is also evidence for the release of an as yet to be defined, hyperpolarizing factor (Vanhoutte, 1987). Based upon the results of studies utilizing inhibitors of NO synthesis or action, it is apparent that NO cannot fully account for the relaxant activity of bradykinin. Thus, in the rat kidney, Cachofeiro & Nasjletti (1991) showed that inhibition of NO synthesis with N_w-nitro-L-arginine (nitroarginine) reduced the response by only 50% yet abolished the associated increase in guanosine 3':5'-cyclic monophosphate (cyclic GMP) release. In the perfused heart of the rat, Baydoun & Woodward (1991) showed that inhibition of NO synthesis was without effect on the magnitude of the vasodilator response to bradykinin. These studies clearly implicate an additional component to the bradykinin vasodilator response.

We studied several components of the renal vascular action of bradykinin by using inhibitors of NO generation and arachidonic acid metabolism. For the latter we attempted to separate cyclo-oxygenase and lipoxygenase-related effects from those dependent on arachidonic acid products generated by the cytochrome P450 pathway. Thus, the sequential effects of indomethacin, BW755c and ETYA in inhibiting-cyclo-oxygenase, lipoxygenase and all oxygenases, respectively, on the vasodilator response to bradykinin indicated a role for cytochrome P450 arachidonic acid metabolites. The subsequent use of clotrimazole and 7-ethoxyresorufin to block cytochrome P450 provided further evidence for the involvement of this pathway in the vasodilator action of bradykinin in the rat kidney.

Methods

Male Wistar rats, 12–15 weeks of age, were anaesthetized with pentobarbitone (65 mg kg^{-1}, i.p.). Following a mid-line laparotomy, the right kidney was cannulated via the mesenteric artery and perfused at constant flow with oxygenated Krebs buffer at 37°C. The vena cava was ligated above and below the renal vein and cut to allow exit of the perfusate. The ureter was also cut and the rat killed with an intracardiac injection of pentobarbitone. Flow rate was adjusted to obtain a basal perfusion pressure of 70–90 mmHg. To amplify vasodilator responses, vascular tone was elevated with phenylephrine (7.5×10^{-7} M, except for experiments with nitroarginine) which increased perfusion pressure to approx. 200 mmHg. Subsequently, dose-response curves to bradykinin (30–300 ng) were obtained using a randomized dosing schedule in the absence and presence of inhibitors of arachidonic acid metabolism, cytochrome P450 and NO synthesis. Cyclo-oxygenase was inhibited with indomethacin (2.8 μM), cyclo-oxygenase/lipoxygenase with BW755c (10 μM) and all pathways of arachidonic acid metabolism with ETYA

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(2 μM). Cytochrome P450-dependent enzymes were inhibited with either clotrimazole (1 μM) or 7-ethoxyresorufin (1 μM). We have previously shown that these concentrations of clotrimazole and 7-ethoxyresorufin are specific for cytochrome P450 and do not influence renal vasodilator responses to acetylcholine or nitroprusside (Oyekan *et al.*, 1991). Nitroarginine (50 or 100 μM) was used to inhibit NO synthesis. These agents were added to the buffer prior to perfusion of the kidney to avoid any changes in elevated perfusion pressure subsequent to their addition. Thus, in preliminary experiments in which perfusion pressure was elevated, nitroarginine caused a further increase in pressure. Consequently, in the presence of nitroarginine, the concentration of phenylephrine required to raise perfusion pressure to 200 mmHg was reduced 7 fold to 10^{-7} M. Acetylcholine (100 ng) and nitroprusside (1000 ng) were used as reference vasodilators to determine any non-specific effects of inhibitors on the synthesis and action of NO.

Data from the various treatment groups and the control were compared by analysis of variance and individual data points were compared by Fisher's test using the Stat View statistical programme (Brain Power Inc., California). $P<0.05$ was considered statistically significant.

Results

Mean body weights in the experimental groups were not different from that of the control group, 393 ± 16 g. Renal perfusate flow rates in the experimental groups were not different from the control group except for kidneys treated with the higher concentration of nitroarginine (100 μM) where flow rate was slightly reduced, 10.0 ± 0.7 ml min^{-1} compared to 12.1 ± 0.5 ml min^{-1}, for the control group ($P<0.05$). Neither basal nor elevated renal perfusion pressures in the experimental groups differed significantly from those obtained in the control group, i.e. 80 ± 1 mmHg and 205 ± 7 mmHg, respectively.

Bradykinin (30–300 ng) produced dose-dependent reductions in perfusion pressure, the maximum decrease being 95 mmHg. We first addressed the contribution of NO to the renal vascular action of bradykinin using nitroarginine, an inhibitor of NO synthesis. Nitroarginine (50 μM) significantly reduced the vasodilator response to bradykinin (Figure 1), the effect being more pronounced at the lower dose of bradykinin (30 ng) where the fall in perfusion pressure was 27 ± 2 mmHg versus 61 ± 6 mmHg for the control. At the higher doses of bradykinin, 100 ng and 300 ng, nitroarginine reduced the vasodilator responses from 90 ± 5 mmHg and 95 ± 3 mmHg to 60 ± 4 mmHg and 69 ± 7 mmHg, respectively. Nitroarginine (50 μM) also significantly reduced the response to acetylcholine, 85 ± 4 mmHg versus 111 ± 3 mmHg for the control (Figure 2) but not to the degree anticipated, suggesting incomplete inhibition of NO synthesis. However, increasing the concentration of nitroarginine from 50 to 100 μM did not significantly decrease further the response to acetylcholine (70 ± 14 mmHg) and also failed to reduce further the vasodilator effect of 300 ng bradykinin, 63 ± 9 mmHg versus 69 ± 7 mmHg for 50 μM nitroarginine. Nitroarginine at 50 and 100 μM significantly increased the vasodilator response to nitroprusside from a control value of 92 ± 6 mmHg to 116 ± 3 and 122 ± 5 mmHg, respectively (Figure 2).

We next addressed the possible contribution of arachidonate metabolites to the renal vasodilator action of bradykinin using selective inhibitors of the several oxygenases that generate arachidonate derived vasoactive metabolites. Figure 3 shows the effects of inhibitors of arachidonic acid metabolism on the vasodilator responses to bradykinin. Inhibition of cyclo-oxygenase with indomethacin (2.8 μM) reduced responses to 100 and 300 ng bradykinin from 90 ± 5 mmHg and 95 ± 3 mmHg, respectively, to 66 ± 6 mmHg and 79 ± 4 mmHg, respectively ($P<0.05$) but did not affect the response to the lowest dose. Similar results were obtained with

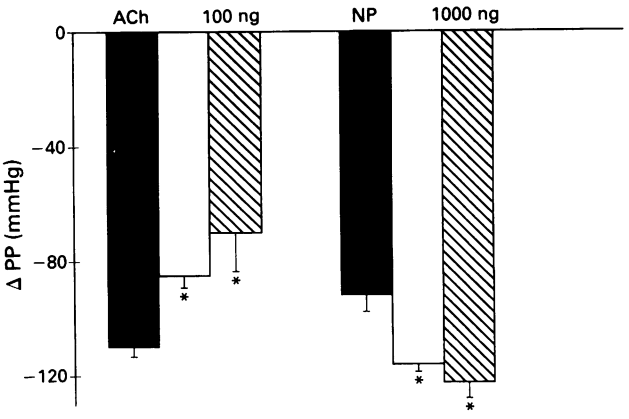

Figure 2 Decreases in perfusion pressure (PP) in response to acetylcholine (ACh) and nitroprusside (NP) in control rat kidneys (solid columns; $n = 12$) and kidneys treated with 50 μM (open columns; $n = 5$) and 100 μM nitroarginine (hatched columns; $n = 5$). *$P<0.05$

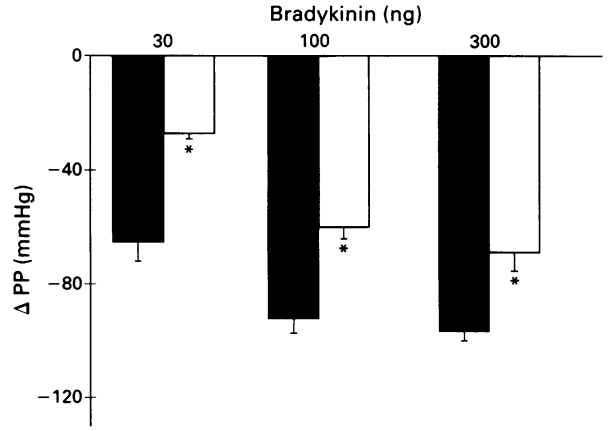

Figure 1 Dose-dependent effects of bradykinin on perfusion pressure (PP) of control rat kidneys (solid columns; $n = 12$) and those treated with 50 μM nitroarginine (open columns; $n = 5$). *$P<0.05$

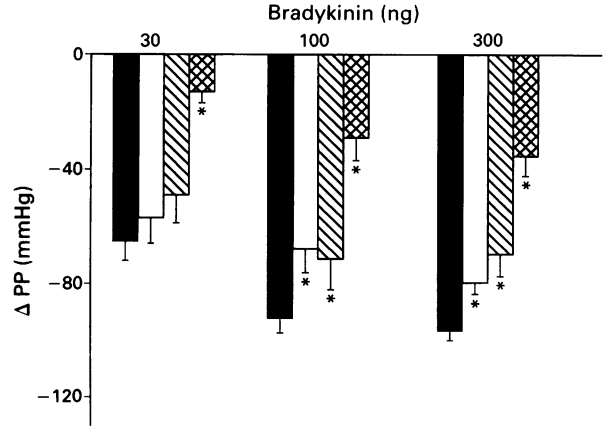

Figure 3 Decrease in perfusion pressure (PP) in response to bradykinin in control rat kidneys (solid columns; $n = 12$) and those treated with 2.8 μM indomethacin (open columns; $n = 6$), 10 μM BW755c (hatched columns; $n = 6$) and 2 μM ETYA (cross-hatched columns; $n = 5$). *$P<0.05$

BW755c, a dual inhibitor of cyclo-oxygenase and lipoxygenase which also reduced responses to 100 and 300 ng bradykinin from 90 ± 5 mmHg and 95 ± 3 mmHg, respectively, to 72 ± 11 mmHg and 70 ± 8 mmHg, respectively. Thus, the effects of BW755c could not be attributed to inhibition of the lipoxygenase pathway but related only to effects on cyclo-oxygenase. In contrast, ETYA, an inhibitor of all pathways of arachidonic acid metabolism, markedly reduced the renal vascular responses to all doses of bradykinin. Thus, in control rat kidneys 30, 100 and 300 ng bradykinin reduced perfusion pressures by 61 ± 6, 90 ± 5 and 95 ± 3 mmHg, respectively, whereas in the presence of ETYA the decreases in perfusion pressure were 13 ± 4, 29 ± 8 and 35 ± 7 mmHg, respectively. Neither indomethacin nor ETYA altered the vasodilator responses to acetylcholine or nitroprusside whereas BW755c reduced the responses to both agonists; the response to acetylcholine was reduced to 88 ± 11 mmHg from 111 ± 3 mmHg and that of nitroprusside to 63 ± 9 mmHg from 92 ± 6 mmHg. When viewed collectively, the results of the experiments using inhibitors of arachidonic acid metabolism implicate the cytochrome P450 pathway in the vasodilator response to bradykinin in the kidney. Consequently, we addressed the role of cytochrome P450. Figure 4 shows the effects of two inhibitors of cytochrome P450, clotrimazole and 7-ethoxyresorufin, on the perfusion pressure responses to bradykinin. Both agents produced similar substantial reductions in the vasodilator responses to all doses of bradykinin without affecting the responses to acetylcholine and nitroprusside. For example, clotrimazole reduced the responses to 30, 100 and 300 ng bradykinin from 61 ± 6, 90 ± 5 and 95 ± 3 mmHg, respectively, to 20 ± 5, 41 ± 5 and 43 ± 5 mmHg, respectively.

Discussion

The results of this study demonstrate that, in the isolated perfused kidney of the rat, vasodilator responses to both bradykinin and acetylcholine exhibit NO-dependent and -independent components. Thus, inhibition of NO synthesis with nitroarginine reduced vasodilator responses to bradykinin (30–300 ng) by 28–56% depending on the dose of bradykinin while responses to a fixed dose of acetylcholine were reduced by only 37%. Further inhibition of the vasodilator responses to acetylcholine and bradykinin could not be effected by doubling the concentration of nitroarginine suggesting that 50 μM nitroarginine was maximally effective in inhibiting NO synthesis. This conclusion is supported by the results obtained with nitroprusside whereby nitroarginine enhanced the vasodilator response, suggesting that impaired basal release of NO results in increased sensitivity to NO. Doubling the concentration of nitroarginine did not further enhance the vasodilator effect of nitroprusside.

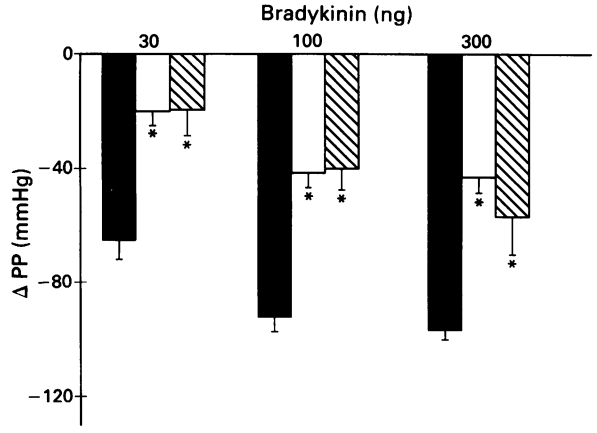

Figure 4 Dose-dependent decreases in perfusion pressure (PP) to bradykinin in control rat kidneys (solid columns; $n = 12$) and those treated with 1 μM clotrimazole (open columns; $n = 5$) and 1 μM 7-ethoxyresorufin (hatched columns; $n = 5$)

There are now many studies to support the contention that NO is not the sole, or even primary, mediator of endothelium-dependent responses to acetylcholine and bradykinin depending on the species and vascular preparation examined. For example, in porcine and canine isolated coronary arteries the vasorelaxant responses to bradykinin were only slightly reduced or not affected by interventions aimed at preventing the synthesis or action of NO (Richard *et al.*, 1991; Cocks & Angus, 1991; Tschudi *et al.*, 1991). Similarly, in the perfused heart of the rat, inhibition of NO synthesis failed to influence the magnitude of the vasodilator response to bradykinin although its duration was reduced (Baydoun & Woodward, 1991). Moreover, in the perfused kidney of the spontaneously hypertensive rat, nitroarginine only reduced the vasodilator response to bradykinin by ~50% although it completely abolished the associated increase in cyclic GMP release (Cachofeiro & Nasjletti, 1991). Consequently, it is necessary to invoke mediators other than NO to account for the full vasorelaxant activity of bradykinin. The results of the present study implicate a role of cytochrome P450-dependent metabolites of arachidonic acid in the vasodilator response to bradykinin in the isolated perfused kidney of the rat based upon the following observations: (1) indomethacin resulted in inhibition of the vasodilator response to higher doses of bradykinin (~25%) suggesting a minor role of cyclo-oxygenase products; (2) BW755c reduced the vasodilator response to the higher doses of bradykinin also suggesting a potential role of cyclo-oxygenase/lipoxygenase products of arachidonic acid. However, BW755c also reduced vasodilator responses to acetylcholine and nitroprusside, effects consistent with the ability of BW755c to accelerate the inactivation of NO (Moncada *et al.*, 1986); (3) Inhibition of all pathways of arachidonic acid metabolism with ETYA markedly reduced the renal vasodilator response to bradykinin (65–84%) without affecting responses to acetylcholine or nitroprusside suggesting an effect of ETYA independent of change in NO synthesis or action. By exclusion, a role for the third pathway of arachidonic acid metabolism, via cytochrome P450, is indicated; (4) Clotrimazole and 7-ethyoxyresorufin, inhibitors of cytochrome P450, also reduced responses to bradykinin (45–76%) without affecting those to acetylcholine and nitroprusside, results consistent with a role for cytochrome P450-dependent arachidonic acid metabolites in the vasodilator response to bradykinin. The greater inhibitory effect of ETYA versus clotrimazole or 7-ethoxyresorufin probably reflects its ability to inhibit cyclo-oxygenase as well as cytochrome P450, a property not shared by clotrimazole or 7-ethoxyresorufin. Indeed, the sum of the inhibitory effects of indomethacin and either clotrimazole or 7-ethoxyresorufin on bradykinin-induced renal vasodilatation are similar to those of ETYA alone which is in accordance with the interpretation that cyclo-oxygenase and cytochrome P450 account for the arachidonate-mediated components of the vasodilator response to bradykinin. Further, a lipoxygenase component under these experimental conditions is negligible or absent as indomethacin and BW755c have similar effects on the renal vasodilator action of bradykinin. The results of this study are qualitatively similar to those reported for canine coronary arteries (Pinto *et al.*, 1987). Thus, based on the use of indomethacin and SKF525A it was suggested that both cyclo-oxygenase and cytochrome P450-derived arachidonic acid metabolites contributed to the vasorelaxant activity of bradykinin.

In summary, we have provided evidence that bradykinin, in addition to releasing NO, stimulates metabolism of arachidonic acid which contributes to the vasodilator effect of this peptide in the isolated perfused kidney of the rat via cyclo-oxygenase and cytochrome P450 mono-oxygenase pathways. These results are consistent with the well-established ability

of bradykinin to stimulate the release of arachidonic acid which can be converted by vascular cytochrome P450, the highest levels of which are localized to the endothelium (Abraham *et al.*, 1985), to vasodilator products. Although there also appear to be at least two components to the renal vasodilator response to acetylcholine, it is also apparent that the NO-independent component is not the same as that for the bradykinin, i.e., it is unaffected by ETYA, clotrimazole or 7-ethoxyresorufin.

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Multiple σ binding sites in guinea-pig and rat brain membranes: G-protein interactions

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1 Evidence is accumulating for multiple sigma (σ) sites in the mammalian CNS.

2 We have addressed this problem and have examined σ site - G-protein coupling in guinea-pig and rat brain membranes.

3 Ditolylorthoguanidine (DTG), (+)-3-(3-hydroxyphenyl)-N-1-(propyl)piperidine (3PPP) and dextromethorphan displaced [^{3}H]-DTG (3.4 nM) with low Hill slopes of 0.5, 0.6 and 0.6, respectively in guinea-pig brain membranes.

4 In the presence of 5'-guanylylimidodiphosphate (Gpp(NH)p; 100 μM), the specific binding of [^{3}H]-DTG was reduced by 36.7%, the Hill slope of 3PPP was increased to near unity, the ability of dextromethorphan to displace DTG was virtually abolished and the Hill slope for DTG remained low (0.7), indicating the presence of at least two binding sites. These data indicate that although Gpp(NH)p removes a dextromethorphan high affinity site, two DTG selective sites remain in the presence of Gpp(NH)p.

5 The present study suggests that DTG binds to at least three sites in guinea-pig brain membranes, at least one of which is G-protein linked.

6 In rat brain membranes, DTG displaced itself (3.4 nM) with a Hill slope near 1. 3PPP displacement of [^{3}H]-DTG was comparable with the guinea-pig (Hill slope 0.5) and displaced from more than 1 site. Dextromethorphan did not displace [^{3}H]-DTG at concentrations below 10 μM.

7 The heterogeneity of σ sites appears to be less in rat than in guinea-pig brain membranes.

Keywords: σ binding sites; ditolyorthoguanidine (DTG); dextromethorphan; guanine nucleotide binding proteins; antipsychotics

Introduction

The sigma (σ) binding site in the mammalian central nervous system has been intensively investigated by use of ligand binding methodology. A unique pattern of sensitivity of the σ site to a wide variety of different compounds, (Walker *et al.*, 1990) together with their anatomical distribution in brain (Gundlach *et al.*, 1986), distinguishes this site from any known extracellular receptor sensitive to neurotransmitters or neuromodulators. However, since it has not proved possible to associate unequivocally modulation of physiological function with the interaction of ligands at the σ site, it remains unknown whether this site is a biologically active receptor. Furthermore, assuming the σ site is a functional receptor it remains unknown whether ligands act as agonists or antagonists.

Amongst the most commonly used and selective ligands used to characterize σ binding are ditolylorthoguanidine (DTG) and (+)-3-(3-hydroxyphenyl)-N-1-(propyl)piperidine (3PPP). DTG was originally reported to bind with high affinity to a single class of binding sites in guinea-pig brain membranes (Weber *et al.*, 1986) and to be a specific σ ligand (Weber *et al.*, 1986). Similarly, 3PPP selectively binds to σ binding sites (Weber *et al.*, 1986). Subsequent investigations of the binding profiles of these ligands have led investigators to suggest that there are two σ sites; σ_1 and σ_2 (Hellewell & Bowen, 1990; Quirion *et al.*, 1992). Other groups have suggested that as many as four binding sites (R_1–R_4) are identifiable (Zhou & Musacchio, 1991).

Multiple affinity states of a single receptor have also been proposed in explanation of modulation of [^{3}H]-3PPP binding in rat brain membranes by GTP binding protein modifying agents (Itzhak, 1989; Beart *et al.*, 1989). These studies, which indicate an interaction with G-proteins, are major pieces of evidence for a receptor role for σ sites.

The aim of the present study was to investigate further the heterogeneity of σ sites and affinity states in guinea-pig brain membranes by use of [^{3}H]-DTG and [^{3}H]-3PPP as ligands. Experiments were also conducted to examine the potential interaction of σ sites with G-proteins. Additional experiments were performed with rat brain membranes to examine the possibility that species differences may exist in the heterogeneity of sites.

Preliminary results of this investigation have been presented previously (Connick *et al.*, 1991).

Methods

Brains from male Dunkin-Hartley guinea-pigs (approximately 350 g) or male Wistar rats (approximately 250 g) were homogenized in ice-cold sucrose (0.32 M) with 8 strokes of a Potter-'S' homogenizer at 850 r.p.m. and centrifuged at 900 *g* for 10 min at 4°C. The resultant pellet was resuspended in 10 volumes (w/v) of 50 mM Tris-HCl buffer (pH 7.4). This was subsequently centrifuged twice (20,000 *g* for 20 min) before final resuspension in 10 volumes of 50 mM Tris-HCl buffer (pH 7.4) for experiments with [^{3}H]-DTG or 5 mM Tris-HCl (pH 7.7) for experiments with [^{3}H]-3PPP and 5 ml aliquots frozen at − 70°C.

For radioreceptor assays, aliquots of the frozen membrane suspension were thawed on ice, diluted and 250 μl (i.e. 1 mg of protein) added to either of the following: (i) 3.4 nM [^{3}H]-DTG (53 Ci mmol^{-1}), or (ii) 0.34 nM [^{3}H]-DTG, or (iii) 1.1 nM [^{3}H]-3PPP (115 Ci mmol^{-1}) and unlabelled drugs (up to 22 data points between 10^{-5} M and 10^{-12} M) in a final volume of 1 ml made up with buffer. Non-specific binding was defined

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as that remaining in the presence of the drugs used at 10^{-5} M. After 90 min incubation at room temperature, the membrane suspension was rapidly filtered under vacuum through Whatman GF/B filters using a Brandel 24-well cell harvester. The filters were washed 5 times with 1 ml ice-cold buffer, oven dried and placed in 3.5 ml ultima gold scintillation fluid in mini vials and counted.

Data analysis

Results from 3–10 separate experiments were pooled and data were analysed by use of a computerised iterative curve fitting programme (Graphpad Inplot v 3.1). Initially, sigmoidal curves were fitted to the data to obtain estimates of Hill coefficients (nH). Where Hill coefficients were significantly less than one, displacement curves were constructed for both a one and two site model to obtain pIC_{50} values and percentages at each site. Models were compared with respect to the sum of squares value and where applicable an *F*-test (Graphpad Inplot v 4.0) was employed to select the best fit. Where $P<0.05$, results are presented for the more complex model.

Materials

DTG was obtained from Aldrich Chemical Co. (Poole, U.K.) and 3PPP from RBI Inc. (+)- and (−)-SKF 10047 were generous gifts from the NIDA, Bethesda, M.D. Radiochemicals were obtained from NEN du Pont. All other chemicals were obtained from the Sigma Chemical Co. (Poole, U.K.).

Results

Guinea-pig brain membranes

Displacement curves together with Hill coefficients and pIC_{50} values and percentage occupation estimates from two component curves derived from competition studies, using a range of σ ligands, against [^{3}H]-DTG, binding are presented in Figure 1. Displacement of [^{3}H]-DTG by 3PPP, (+)-SKF 10047 and dextromethorphan gave Hill coefficients of less than 1 and corresponded to displacement of DTG from at least two sites (Figure 1a). No enantiomeric selectivity between (+)- and (−)-SKF 10047 was observed (pIC_{50} values 6.6 ± 0.1 and 6.4 ± 0.1, respectively) but the (−)-enantiomer displaced [^{3}H]-DTG with a Hill slope near 1 (0.93 ± 0.13). DTG and haloperidol also displaced [^{3}H]-DTG with low Hill coefficients suggesting that these ligands also interact with multiple binding sites (Figure 1b). Total specific binding of [^{3}H]-DTG remained unchanged in the presence of either 5′-guanosine monophosphate or adenosine triphosphate but was reduced to $63.3 \pm 3.1\%$ of control ($n = 6$) in the presence of 100 μM Gpp(NH)p. The displacement curves produced by 3PPP (Figure 2a) were also shifted to the right in the presence of Gpp(NH)p and the Hill coefficients were increased to values approaching unity. The ability of dextromethorphan (10^{-12}–10^{-5} M) to displace [^{3}H]-DTG in the presence of Gpp(NH)p was essentially abolished (Figure 2b).

Gpp(NH)p had no effect on the Hill slope of the concentration-displacement curve of [^{3}H]-DTG by DTG itself or haloperidol (Figures 2c,d). Indeed, despite a reduction of total binding in the presence of Gpp(NH)p, suggesting a G protein modulation of a proportion of [^{3}H]-DTG binding, the pIC_{50} values for haloperidol were essentially unchanged.

An attempt was also made to reduce the high affinity, Gpp(NH)p-sensitive component of 3.4 nM [^{3}H]-DTG binding by conducting the incubation in the presence of 100 nM dextromethorphan. Under such conditions, [^{3}H]-DTG binding was reduced to $68.2 \pm 2.9\%$ ($n = 4$) of that in the absence of dextromethorphan (i.e. an equivalent reduction to that produced by 100 μM Gpp(NH)p). [^{3}H]-DTG apparently labelled only a single population of sites (Figure 3). 3PPP also appeared to label predominantly a low affinity site ($pIC_{50} = 7.1 \pm 0.01$, 71%) and in addition, occupation of a very low affinity site (pIC_{50} 5.6 ± 0.5, 29%) was revealed (Figure 3). Since no selective agents are so far available which might mask the low affinity component of DTG binding, we attempted to look at the high affinity component by reducing the concentration of [^{3}H]-DTG to 0.34 nM. Under these conditions, DTG displaced [^{3}H]-DTG from essentially one site and yielded an IC_{50} value for complete displacement which was essentially identical to that obtained using 3.4 nM DTG (Table 1). The Hill slopes for haloperidol and 3PPP displacement of [^{3}H]-DTG were less than unity.

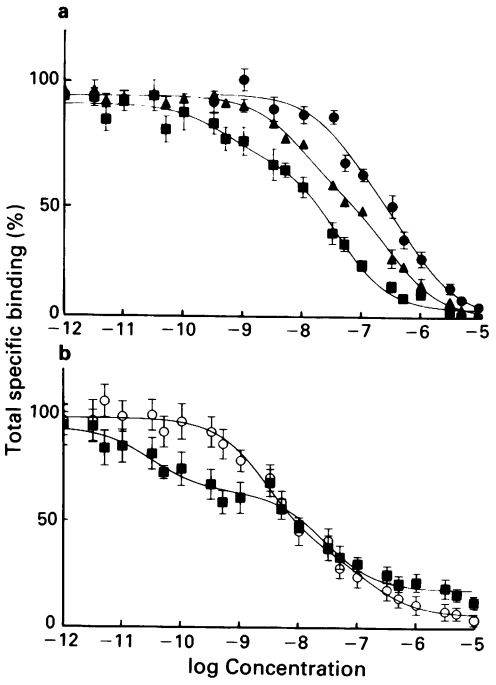

Figure 1 Displacement curves of [^{3}H]-ditolylorthoguanidine ([^{3}H]-DTG, 3.4 nM) binding in guinea-pig brain membranes in the absence of Gpp(NH)p. Data (± s.e.mean, vertical bars) from 3–10 separate experiments were analysed (see text for details). Where Hill coefficients (nH) were significantly less than one, displacement curves were constructed for a two site model to obtain $pIC_{50} \pm$ s.e.mean values at site 1 and site 2 together with percentages at each site given in parentheses. (a) (■) (+)-3-(3-hydroxyphenyl)-N-1-(propyl) piperdine (3PPP)-nH = 0.56 ± 0.06, site 1: pIC_{50} 9.3 ± 0.3 (26%); site 2: pIC_{50} 7.4 ± 0.1 (74%). (●) (+)-SKF 10047-nH = 0.75 ± 0.15, site 1: pIC_{50} 7.1 ± 0.5 (54%); site 2: pIC_{50} 6.0 ± 0.8 (46%) or (▲) dextromethorphan-nH = 0.58 ± 0.03, site 1: pIC_{50} 8.0 ± 0.1 (46%); site 2: pIC_{50} 6.4 ± 0.1 (54%). (b) (○) DTG-nH = 0.50 ± 0.07, site 1: pIC_{50} 9.1 ± 0.2 (42%); site 2: pIC_{50} 7.3 ± 0.2 (58%). (■) Haloperidol-nH = 0.36 ± 0.10, site 1: pIC_{50} 10.5 ± 0.4 (33%); site 2: pIC_{50} 7.8 ± 0.2 (67%).

[^{3}H]-3PPP binding in guinea-pig brain membranes

3PPP apparently displaced itself from more than one site yielding a Hill coefficient of 0.7 and occupation of primarily a high affinity site pIC_{50} 9.2 (67%) in addition to a lower affinity site ($pIC_{50} = 7.7$; 33%) (Figure 4). DTG also displaced [^{3}H]-3PPP from more than one site (nH = 0.5), again occupying primarily a high affinity site (pIC_{50} 8.8, 60%) in addition to a lower affinity site ($pIC_{50} = 7.0$, 40%) (Figure 4). Dextromethorphan displaced 3PPP from essentially a single site (nH = 0.8 pIC_{50} 7.4) (Figure 4). (+)-SKF 10047 also displaced from more than one site (nH) = 0.34 ± 0.11), occupying some high affinity sites (site 1 $pIC_{50} = 10.4 \pm 0.5$, 27%), although mostly occupying a lower affinity site (site

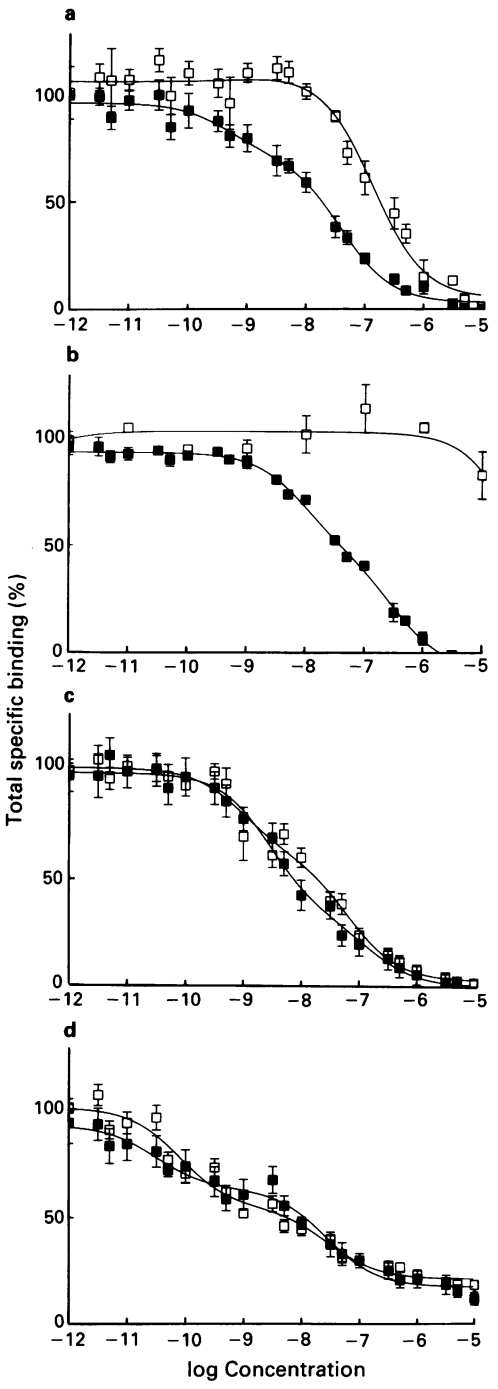

Figure 2 Displacement curves of [^{3}H]-ditolylorthoguanidine ([^{3}H]-DTG, 3.4 nM) binding in guinea-pig brain membranes in the absence of Gpp(NH)p. Data (± s.e.mean, vertical bars) from 3–10 separate experiments were analysed (see text for details). Where Hill coefficients (nH) were significantly less than one, displacement curves were constructed for a two site model to obtain pIC_{50} ± s.e.mean values at site 1 and site 2 together with percentages at each site given in parentheses. All pIC_{50} values refer to affinities in the presence of Gpp(NH)p. (a) (+)-3-(3-hydroxyphenyl)-N-1-(propyl)piperdine (3PPP) displacement of [^{3}H]-DTG in the absence (■) (see Figure 1 for details) or presence (□) of Gpp(NH)p. The specific binding was reduced to 41.0 ± 1.0%; nH = 0.94 ± 0.15, pIC_{50} = 6.5 ± 0.1. (b) Dextromethorphan displacement of [^{3}H]-DTG in the absence (■) (see Figure 1 for details) or presence (□) of Gpp(NH)p. The specific binding was reduced to 78.7 ± 5.4%; no effective displacement of [^{3}H]-DTG was observed at concentrations below 10^{-5} M. (c) DTG displacement of [^{3}H]-DTG in the absence (■) (see Figure 1 for details) or presence (□) of Gpp(NH)p. The specific binding was reduced to 55.5 ± 1.3%; nH = 0.68 ± 0.06, site 1: pIC_{50} 8.3 ± 0.1 (79%); site 2: pIC_{50} 6.3 ± 0.3 (21%). (d) Haloperidol displacement of [^{3}H]-DTG in the absence (■) (see Figure 1 for details) or presence (□) of Gpp(NH)p. The specific binding was reduced to 69.1 ± 3.0% nH = 0.54 ± 0.10, site 1: pIC_{50} 10.3 ± 0.5 (29%); site 2: pIC_{50} 6.3 ± 0.3 (71%).

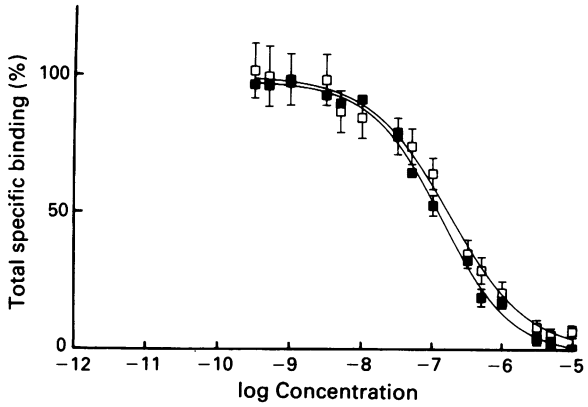

Figure 3 Displacement curves of [^{3}H]-ditolylorthoguanidine ([^{3}H]-DTG) in the presence of 100 nM dextromorphan in guinea-pig brain membranes. Data (± s.e.mean, vertical bars) from 3–10 separate experiments were analysed (see text for details). Where Hill coefficients (nH) were significantly less than one, displacement curves were constructed for a two site model to obtain pIC_{50} ± s.e.mean values at site 1 and site 2 together with percentages at each site given in parentheses: (■) DTG nH = 0.82 ± 0.06, pIC_{50} = 7.3 ± 0.1; or (▲) (+)-3-(3-hydroxyphenyl)-N-1-(propyl)piperdine nH = 0.72 ± 0.10, site 1: pIC_{50} 7.1 ± 0.01 (71%); site 2: pIC_{50} 5.6 ± 0.5 (29%).

2 = 7.1 ± 0.2, 73%). (−)-SKF 10047 displaced 3PPP from essentially a single site (nH) = 0.92 ± 0.08) of low affinity (pIC_{50} 6.3 ± 0.2).

Rat brain membranes

In rat brain membranes DTG has apparently equal affinity for all site(s) present since the Hill coefficient increased (nH = 0.8 ± 0.1; pIC_{50} 7.0) in comparison with results from guinea-pig brain membranes (Figure 5a). 3PPP displacement of [^{3}H]-DTG is essentially comparable with the guinea-pig, revealing displacement from more than 1 site (Figure 5b). In the presence of Gpp(NH)p, the Hill slope for 3PPP displacement increased to near 1 (nH = 0.87 ± 0.18). No decrease in total specific binding was observed (97.3 ± 3.4%) in the presence of Gpp(NH)p. Haloperidol also appears to displace from predominantly 1 site (nH = 0.8, pIC_{50} 8.1) (Figure 5a). Dextromethorphan did not displace [^{3}H]-DTG in rat brain at concentrations below 10 μM, suggesting that the dextromethorphan site observed in guinea-pig brain membranes is either absent or present in very low density in rat brain membranes. No enantiomeric selectivity was observed

Table 1 Displacement of [^{3}H]-ditolylorthoguanidine ([^{3}H]-DTG, 0.34 nM) in guinea-pig brain membranes

	pIC_{50}[1]	nH
3PPP	7.2 ± 0.2	0.66 ± 0.10
DTG	7.7 ± 0.2	0.90 ± 0.10
Haloperidol	8.6 ± 0.3	0.75 ± 0.03

Values are mean ± s.e.mean. $n = 4$. 3PPP = (+)-3-(3-hydroxyphenyl)-N-1-(propyl)piperidine.
[1]Data are derived from 5 point displacement curves and no attempt has therefore been made to impose one or two site models on these results.

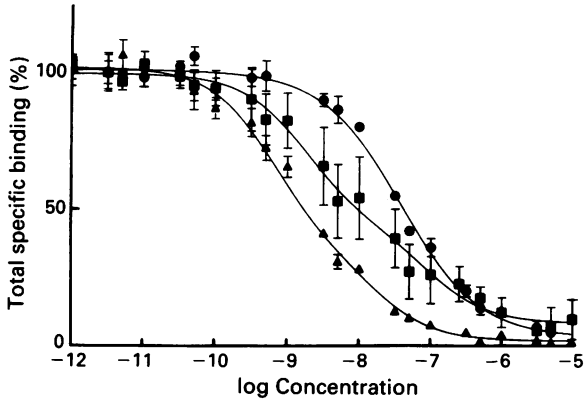

Figure 4 Displacement curves of [^{3}H]-(+)-3-(3-hydroxyphenyl)-N-1-(propyl)piperdine ([^{3}H]-3PPP) binding in guinea-pig brain membranes. Data (± s.e.mean, vertical bars) from 3–10 separate experiments were analysed (see text for details). Where Hill coefficients (nH) were significantly less than one, displacement curves were constructed for a two site model to obtain pIC_{50} ± s.e.mean values at site 1 and site 2, together with percentages at each site given in parentheses: (▲) 3PPP n(H) = 0.70 ± 0.10 site 1: pIC_{50} 9.2 ± 0.1 (67%); site 2: pIC_{50} 7.88 ± 0.2 (33%), (■) DTG nH = 0.53 ± 0.04, site 1: pIC_{50} 8.8 ± 0.2 (60%); site 2: pIC_{50} 7.0 ± 0.2 (40%) or (●) dextromethorphan nH = 0.79 ± 0.07 pIC_{50} = 7.4 ± 0.1.

between (+)- or (−)-SKF 10047, although the Hill slope of (+)-SKF 10047 was lower than the (−)-enantiomer.

Discussion

Although σ binding sites were originally thought to be a single entity, the current consensus is that two subtypes exist, σ_1 and σ_2 (Bowen & Hellewell, 1988; Hellewell & Bowen, 1990; Knight *et al.*, 1991a,b; Rothman *et al.*, 1991; Quirion *et al.*, 1992). Indeed, sophisticated computer assisted models of self and cross-displacement σ ligand binding experiments have identified up to four binding sites (R_1–R_4) for σ ligands (Zhou & Musacchio, 1991; Klein & Musacchio, 1992). Computerized analysis of the binding data from the present study has been restricted to determining whether one or more sites best fits the data. Discussion of data is restricted to an indication of the number of sites and whether ligands have relatively higher or lower affinity. The initial observation which suggested the feasibility of such an approach was that under the conditions used in our experiments, and in contrast to the literature at the time (Weber *et al.*, 1986), DTG and haloperidol displaced [^{3}H]-DTG (3.4 nM) from guinea-pig brain membranes with a Hill-slope considerably less than 1. This is in accord with recent reports demonstrating [^{3}H]-DTG to label at least two binding sites in guinea-pig brain membranes (Karbon *et al.*, 1991; Rothman *et al.*, 1991; De-Haven-Hudkins & Fleissner, 1992). A further study showed two high affinity sites and at least one low affinity site to be labelled by [^{3}H]-DTG in guinea-pig brain membranes (Knight *et al.*, 1991a). However, no investigation has been made of the sensitivity of these sites to guanine nucleotide binding protein modifying agents.

The complexity of [^{3}H]-DTG binding observed in guinea-pig brain membranes, in the present study, most closely parallels the four site model (R_1–R_4) of σ ligand interaction (Zhou & Musacchio, 1991; Klein & Musacchio, 1992). According to this classification, R_1 and R_3 are sites at which σ ligands have a high affinity, R_2 is a dextromethorphan selective site and R_4 is a low affinity site for all examined σ ligands. R_2 would not have been examined in the present study as the radiolabelled ligands used, [^{3}H]-DTG and [^{3}H]-3PPP, have a very low affinity for this site. DTG does not discriminate between R_1 and R_3, whilst haloperidol, 3PPP and particularly dextromethorphan have a higher affinity for

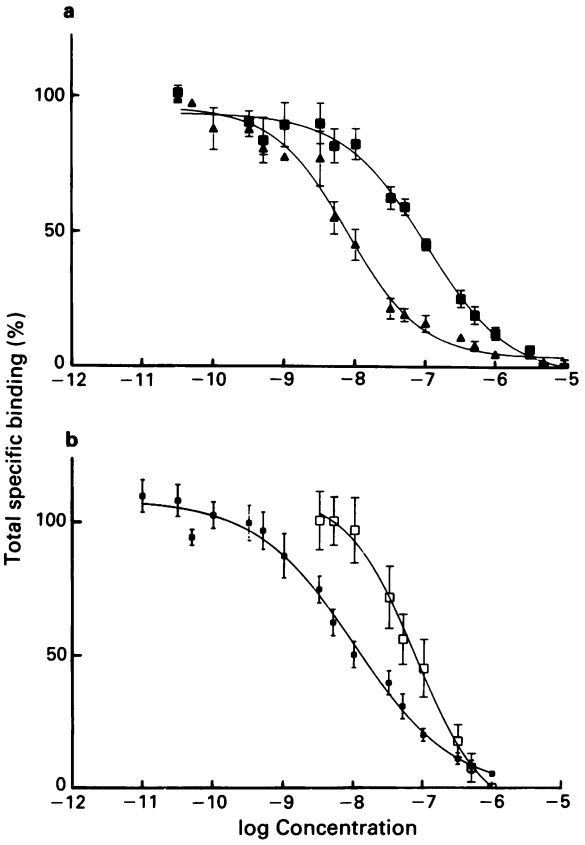

Figure 5 Displacement of [^{3}H]-ditolylorthoguanidine ([^{3}H]-DTG, 3.4 nM) in rat brain membranes. Data (± s.e.mean, vertical bars) from 3–10 experiments were analysed (see text for details). Where Hill coefficients (nH) were significantly less than one, displacement curves were constructed for a two site model to obtain pIC_{50} ± s.e.mean values at site 1 and site 2 or at one site where nH = 1, together with percentages at each site given in parentheses. (a) (■) DTG nH = 0.79 ± 0.11 pIC_{50} = 7.0 ± 0.1 or (▲) haloperidol nH = 0.84 ± 0.09 pIC_{50} = 8.1 ± 0.1 and (b) (■) (+)-3-(3-hydroxyphenyl)-N-1-(propyl)piperdine in the absence (nH = 0.51 ± 0.06, site 1: 8.3 ± 0.1 (68%), site 2: pIC_{50} 6.4 ± 0.3 (32%)) or (□) presence (nH = 0.87 ± 0.18 pIC_{50} = 7.1 ± 0.1) of Gpp(NH)p. No change in specific binding was observed.

R_1 (Zhou & Musacchio, 1991).

According to this classification, [^{3}H]-DTG can be anticipated to bind to R_1, R_3 and R_4 sites in guinea-pig brain membranes. The reduction in the total specific binding of [^{3}H]-DTG, which was induced by Gpp(NH)p in the present study, without a change in the Hill slope of the DTG displacement curve, is explicable if one of the high affinity sites (R_1 and R_3) is G-protein linked. The reduction in total [^{3}H]-DTG binding representing a dramatic reduction in DTG binding to this site. The inability of dextromethorphan to displace [^{3}H]-DTG in the presence of Gpp(NH)p, indicates that it is the dextromethorphan high affinity site R_1 that is no longer labelled. The Gpp(NH)p-induced reduction in [^{3}H]-DTG binding suggests that DTG is an agonist at this G-protein linked site, as has previously been suggested for 3PPP, SKF 10047 and dextromethorphan in rat brain (Itzhak, 1989; Beart *et al.*, 1989). Reducing the concentration of [^{3}H]-DTG from 3.4 nM to 0.34 nM resulted in a reduction in labelling of the low affinity site, R_4. DTG, which does not distinguish between R_1 or R_3 (Zhou & Musacchio, 1991), has a Hill slope close to one. Haloperidol and 3PPP which have higher affinities for R_1 (Zhou & Musacchio, 1991) have lower Hill slopes. To avoid labelling the low affinity site it may be advisable to use low concentrations of [^{3}H]-DTG, at least in assays with guinea-pig brain membranes.

The presence of Gpp(NH)p or dextromethorphan (100 nM) dramatically reduces [^{3}H]-DTG binding to R_1; the principle site labelled under these conditions is R_3. The DTG binding revealed by [^{3}H]-DTG in the presence of 100 nM dextromethorphan showed no enantiomeric selectivity for SKF 10047 displacement, suggesting that this site (R_3) may be equivalent to the σ_2 site of Hellewell & Bowen (1990). A concentration of 100 nM dextromethorphan may not be sufficient to occupy all R_1 sites since 3PPP still displaced DTG with a Hill-slope of less than 1.

Results obtained with [^{3}H]-3PPP in guinea-pig brain membranes are consistent with the above discussion. Dextromethorphan completely displaced [^{3}H]-3PPP from essentially one site, indicating that 3PPP primarily labels R_1 under the conditions used in the present study. SKF 10047 showed enantiomeric selectivity for [^{3}H]-3PPP displacement, indicating that R_1 is equivalent to the σ_1 site of Hellewell & Bowen (1990). DTG and 3PPP itself displaced [^{3}H]-3PPP from more than one site or affinity state.

The complexity of the analysis when using guinea-pig brain membranes may be reduced by conducting similar experiments with rat membranes, where interaction with the R_4 binding site is not anticipated (Klein & Musacchio, 1991). It has been suggested that rat brain membranes contain predominantly R_3 (Klein & Musacchio, 1991), R_1, the other affinity site, being found in much lower density (10%) than in guinea-pig brain membranes. Nevertheless, the use of [^{3}H]-(+)-pentazocine has allowed the presence of σ_1 and σ_2 sites in rat brain to be confirmed (Vilner & Bowen, 1992). In contrast to the guinea-pig, no reduction of total specific [^{3}H]-DTG binding was shown in the rat in response to Gpp(NH)p; this can be interpreted as being due to the small amount of the G-protein linked high affinity site (σ_1/R_1) and confirms that the other DTG high affinity site (σ_2/R_3) is unlikely to be G-protein linked. However, Gpp(NH)p was still able to increase the Hill-slope of 3PPP displacement of [^{3}H]-DTG. The results obtained with Gpp(NH)p are comparable with those obtained previously in the rat when [^{3}H]-3PPP was used as the σ ligand (Beart *et al.*, 1989; Itzhak, 1989). Since no change in total specific [^{3}H]-DTG binding was observed in the presence of Gpp(NH)p, this result might not have been expected. However, similar findings have been made previously. Itzhak & Stein (1992) also found [^{3}H]-DTG binding in rat brain membranes not to be modulated by guanine nucleotides, whilst in the same preparation [^{3}H]-3PPP binding demonstrated sensitivity to guanine nucleotides. Several aspects of the inhibition of σ ligand binding by guanine nucleotides are parallelled by the enhancement of σ binding by phenytoin (McCann & Su, 1992). The contribution of interactions at this site have not been addressed by our work and require further study.

In conclusion, the present study illustrates the complexity of the use of DTG as a radiolabel for σ receptors. Clearly this compound labels several affinity states/sites. The problem is compounded if guinea-pig brain membranes are used; more selective labels for the different sites are required to aid our understanding of the affinity of compounds for the individual sites. At least one site appears to be coupled to G-proteins and thus, may be linked to signal transduction mechanisms. No unequivocal evidence exists to identify the specific second messenger pathway but the existence of linkage to a G-protein strongly suggests a functional consequence of σ binding and thus a receptor function. Such evidence is, however, circumstantial and it remains possible, though unlikely, that guanine nucleotides could interact directly with a proportion of σ binding sites. Given the widespread distribution of σ binding sites and their presence in a variety of subcellular fractions, evidence for a potential σ receptor may be strengthened by investigating the guanine nucleotide sensitivity of such fractions. G-protein linkage should be restricted to the plasma membrane fraction and would not be expected in the microsomal fraction (subject to the purity of the respective fractions). To date, no differences in the pharmacology of σ binding has been observed between the various fractions (Knight *et al.*, 1991a).

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The pharmacology of recombinant $GABA_A$ receptors containing bovine α_1, β_1, γ_{2L} sub-units stably transfected into mouse fibroblast L-cells

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1 Responses to γ-aminobutyric acid (GABA) were evoked in mouse fibroblast L-cells stably transfected with bovine, α_1, β_1, γ_{2L} sub-units of the $GABA_A$ receptor. Expression was stimulated via a steroid-inducible promoter system.

2 In near symmetrical intracellular and extracellular chloride concentrations, GABA evoked inward currents at negative holding potentials that reversed at +5 mV and displayed slight outward rectification. Concentration-response curves were fitted well by the logistic equation. GABA had a $pEC_{50} = 5.1 \pm 0.1$ and the curves had a slope of 1.9 ± 0.1

3 Responses to GABA were antagonized by bicuculline, picrotoxin and penicillin. The action of bicuculline was competitive ($pA_2 = 6.4$) whilst the block by picrotoxin was uncompetitive and strongly agonist-dependent.

4 Benzodiazepine receptor agonists potentiated responses to 3 μM GABA. The rank order of potency was FG 8205 > flunitrazepam > zolpidem > C1218872. FG 8205 and C1218872 produced markedly lower maximal potentiations with efficacies 0.4 and 0.6 × that of flunitrazepam, respectively. The potencies of zolpidem and C1218872 observed are in agreement with the BZ_1 type pharmacology of this sub-unit combination. The potentiation of GABA by flunitrazepam was antagonized by flumazenil with a K_i of 3.8 nM.

5 GABA responses were potentiated in the presence of pentobarbitone and alphaxalone. The response was also noticeably broadened by these compounds due to a decrease in the response decay rate. Concentrations of pentobarbitone of 100 μM and above evoked an inward current in the absence of GABA. Alphaxalone up to 10 μM did not evoke a direct response.

6 This expression system produced functional receptors that behaved in a fashion analogous to those found endogenously in other preparations. Thus, this system appears to provide a useful and versatile preparation for the analysis of sub-unit regulation of $GABA_A$ receptor pharmacology.

Keywords: Recombinant receptor; $GABA_A$ receptor; benzodiazepine; pentobarbitone; alphaxalone; bicuculline; picrotoxin; penicillin

Introduction

The physiology and pharmacology of γ-aminobutyric $acid_A$ ($GABA_A$) receptors have been studied extensively over the past few decades (see reviews by Krnjevic 1974; Nicoll & Alger, 1979). Recently, the structure of the receptor was reported to be a complex of sub-units that were classified as α or β based on sequence homology (Schofield *et al.*, 1987). Subsequent studies have revealed the existence of variants of both α and β sub-units which have been termed α_1, α_2, etc. (e.g. Levitan *et al.*, 1988) and β_1, β_2, etc. (Ymer *et al.*, 1989). The description of a γ_2 sub-unit that was a requisite for benzodiazepine modulation (Pritchett *et al.*, 1989b) appeared to provide an adequate structural characterization of many $GABA_A$ receptors.

The component sub-units of each $GABA_A$ receptor are not likely to be identical across the CNS but will probably vary both inter- and intra-regionally. This, potentially, allows for subtle variations in the pharmacology of $GABA_A$ receptors dependent upon sub-unit composition. Thus, in addition to the requirement for a γ_2 sub-unit, the α sub-unit bestows additional constraints on benzodiazepine modulation (Pritchett *et al.*, 1989a). On the basis of ligand binding studies, it was reported that the presence of the α_1 sub-unit resulted in a BZ_1 type pharmacology, with a BZ_2 pharmacology conferred with α_2 or α_3. Further pharmacological variations have been reported for α_5 (Puia *et al.*, 1991) and α_6 (Luddens *et al.*, 1990) containing receptor complexes.

A previous publication reported on a steroid-inducible promoter expression system for the production of a stably transfected clonal cell line expressing $GABA_A$ receptors of predetermined sub-unit composition (Hadingham *et al.*, 1992). The expressed GABA receptors were shown to be modulated by benzodiazepines, alphaxalone and pentobarbitone. In this paper we extend the functional pharmacological characterization of this cell line transfected with α_1, β_1, γ_{2L} sub-units of the $GABA_A$ receptor. Some of these results have appeared in a preliminary form (Kemp *et al.*, 1992).

Methods

Transfections were performed by use of the expression vector pMSGneo containing the mouse mammary tumour virus promoter as described previously (Whiting *et al.*, 1991; Hadingham *et al.*, 1992). Bovine cDNA's encoding for α_1, β_1 (Schofield *et al.*, 1987) and γ_{2L} (Whiting *et al.*, 1990) sub-units were used for transfection of mouse fibroblasts. A geneticin resistant cell line was selected and maintained in EMEM supplemented with foetal calf serum (10%), L-glutamine (2 mM), penicillin (100 units ml^{-1}), streptomycin

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(100 μg ml^{-1}) and geneticin (2 mg ml^{-1}). Five days before electrophysiological recording, cells were plated onto cover slips coated with poly-L-lysine and expression was induced with dexamethasone (0.1–1 μM).

During recording, cells were superfused with a solution containing (mM): NaCl 124, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 5 and D-glucose 11. The pH was adjusted to 7.2 with NaOH. Patch pipettes contained (mM): CsCl 130, $MgCl_2$ 1, HEPES 10 and EGTA 11. The pH was adjusted to 7.3 with HCl (final concentration = 5 mM). Cells were patch clamped in whole cell mode with pipettes connected to a List LM-EPC 7 patch clamp amplifier. Series resistance was estimated at 4–6 MΩ and was not compensated for. In order to investigate whether significant clamp errors were introduced, concentration-response curves were constructed at two different holding potentials (− 5 mV and − 20 mV) on some cells. Apart from this and the construction of I-V curves, the holding potential was maintained at the same level for each experiment (− 20 mV or − 60 mV). In some cases equivalent data were obtained at each holding potential and, as they were found to be near identical, were combined. All drugs were applied rapidly via a double barrelled pipette assembly, with each pipette of approximately 500 μm internal diameter (Kemp & Priestley, 1990). The pipette assembly could be moved rapidly from side to side by a stepping motor attached to a Leitz manipulator and the flow of solutions was controlled by three-way solenoid valves and driven by gravity. The rate of solution exchange around the cells produced in this way was estimated by switching between solutions with different chloride concentrations in the continued presence of a low concentration of GABA. The mean time constant of the resulting current response was 23 ± 3 ms (n = 6 cells).

Apart from the construction of GABA concentration-response curves, the bulk of the pharmacological investigations of modulators of $GABA_A$ receptors were performed with single concentrations of GABA. Stable responses were obtained to GABA (2–3 s pulse) and the compound under test added. Effects were assessed as %-reductions or %-potentiations as appropriate. Individual values were plotted against the log concentration of the test compound and, using an iterative least squares regression analysis, fitted to the function:

$$y = R_{max}/(1 + (EC_{50}/x)^H)$$

where R_{max} = maximal response, EC_{50} = concentration producing half maximal response and H = slope factor.

From such fits the EC_{50} and maximal effect values were obtained for each compound. In addition, a Schild plot (Arunlakshana & Schild, 1959) for bicuculline was constructed. In this case, 2-point log concentration-response curves to 5 μM and 10 μM GABA were obtained initially. Following addition of bicuculline, concentration-response curves were re-determined so as to allow concentration ratios to be calculated. Log concentration ratio − 1 was plotted against − log bicuculline concentration and a straight line was fitted by least squares linear regression. All values are shown as mean ± s.e.mean.

All bulk chemicals were purchased from FSA and tissue culture reagents from Gibco. (+)-Bicuculline, picrotoxin, penicillin Na, GABA, flunitrazepam, pentobarbitone Na, EGTA and HEPES were purchased from Sigma. Zolpidem was obtained from Synthelabo and C1218872 (3-methyl-6-(3-trifluoromethyl-phenyl)-1,2,4-triazolo(4,3-b)pyridazine) from Lederle. FG8205 (7-chloro-5,6-dihydro-5-methyl-6-oxo-3(5-isopropyl-1,2,4-oxadiazol-3-yl)-4H-imidazo-[1,5a][1,4]benzodiazepine; Tricklebank *et al.*, 1990) was synthesized at Merck Sharp and Dohme Ltd. Hoddesdon. (+)-Bicuculline was initially dissolved in 13 mM HCl to a concentration of 10 mM; flunitrazepam, zolpidem, FG8205, alphaxalone and flumazenil (Ro15-1788) were dissolved in ethanol to 10 mM, and C1218872 was dissolved in 50% ethanol to 10 mM. EGTA was dissolved in the minimum required CsOH. All other compounds were sufficiently water soluble to be added direct.

Results

Responses to GABA

Cells were whole-cell patch clamped and GABA applied rapidly from a locally positioned pipette. The evoked currents were inward at negative holding potentials and reversed at a potential of + 5.5 ± 0.55 mV (n = 5). Slight outward rectification of the I-V relationship was observed (Figure 1) with the mean ratio of chord conductances at − 40 mV compared to + 40 mV = 0.74 ± 0.08 (n = 5).

At a given holding potential, response size was dependent upon the concentration of GABA applied (Figure 2a). Construction of log. concentration-response curves produced a mean pEC_{50} = 5.1 ± 0.1 and a slope factor = 1.9 ± 0.1, n = 9 (Figure 2b). On 4 of these cells (Vhold − 20 mV) the concentration-response curves were repeated at a holding potential of − 5 mV (Table 1). There was little difference between the concentration-response curves at either holding potential which suggests that any clamp errors produced by current flow through the series resistance were not large enough to affect significantly the results produced, at least over these current amplitudes (Table 1).

GABA antagonists

To examine the antagonists bicuculline and picrotoxin, stable responses were obtained to 100 μM GABA, a near maximal concentration. For experiments involving penicillin, stable

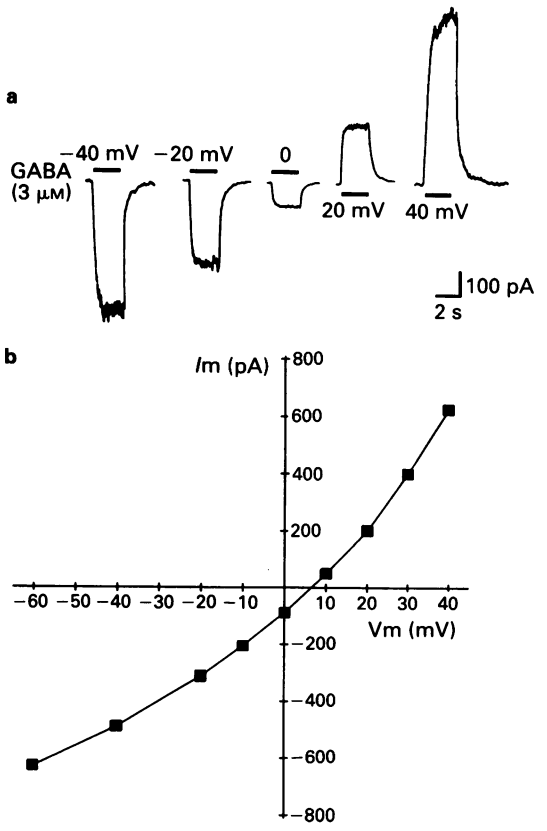

Figure 1 Voltage-dependence of responses to 3 μM γ-aminobutyric acid (GABA). (a) Individual responses are shown at selected holding potentials. The amplitude of all responses from this experiment are plotted against holding potential in (b). The slight outward rectification illustrated was typical of that seen in 4 other cells.

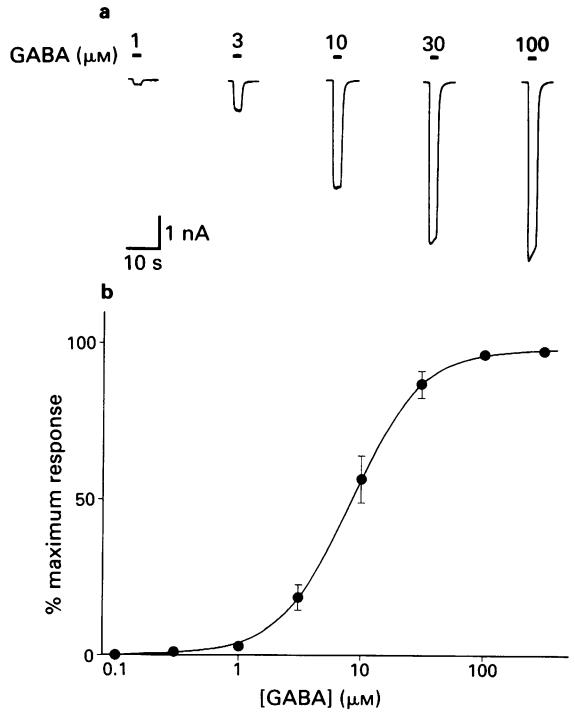

Figure 2 Concentration-response curves to γ-aminobutyric acid (GABA). (a) Individual responses to incremental concentrations of GABA obtained at a holding potential of − 20 mV. The results of similar experiments from 9 cells were combined to produce the cumulative curve shown in (b). Responses were normalized to the maximum of their respective concentration-response curves. Mean ± s.e.mean values are plotted with each value representing 3–9 observations.

Table 1 Comparison of concentration-response curves at two different holding potentials: the curves were constructed at both holding potentials on each of 4 different cells

Holding potential	*Slope factor*	*pEC_{50} (M)*	*Maximal response (nA)*
-5 mV	1.79 ± 0.06	4.98 ± 0.15	0.94 ± 0.26
-20 mV	1.80 ± 0.09	4.93 ± 0.14	2.49 ± 0.84

responses to 3 μM GABA were obtained. Antagonists were then added and %-reductions of the control response calculated for a given antagonist concentration. Picrotoxin showed a marked agonist dependency of action (Figure 3a), only antagonizing GABA when applied concurrently. Picrotoxin antagonized GABA with an $IC_{50} = 8.4$ μM (Figure 3b) which is compared to the IC_{50} of the other antagonists tested, in Table 2. Penicillin was less potent than picrotoxin and, by contrast, displayed no agonist dependency. The nature of bicuculline's antagonist action allowed the construction of a Schild plot (Figure 4) from which a $pA_2 = 6.4$ and a slope of 0.94 ± 0.01 was calculated.

Benzodiazepine receptor ligands

Stable responses were obtained to 3 μM GABA, a concentration found to fall on the lower part of the linear portion of the log. concentration-response curve. Benzodiazepine receptor agonists were added and responses measured as %-potentiations (Figure 5a). EC_{50} values and maximal effects for flunitrazepam, zolpidem, C1218872 and FG 8205 were taken from the data in Figure 5b and are given in Table 3. Compared with the other agonists tested, FG 8205 appeared to take a long time to reach equilibrium and several minutes

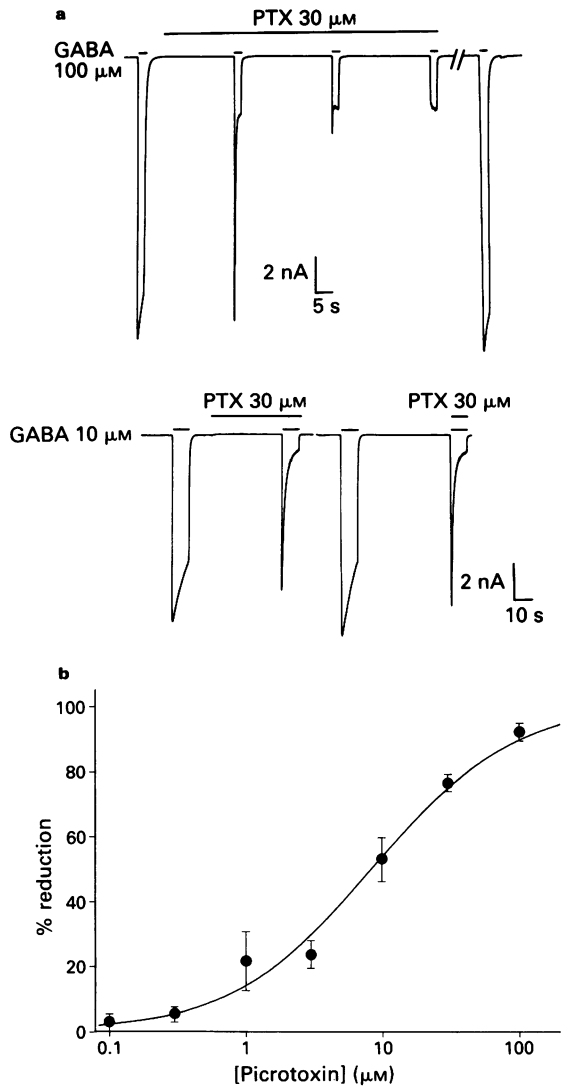

Figure 3 Antagonism of γ-aminobutyric acid (GABA) by picrotoxin. (a) Agonist dependency of picrotoxin action. Top trace: Control response to 100 μM GABA (Vhold = − 60 mV) was rapidly attenuated in the presence of 30 μM picrotoxin. The antagonism of GABA commenced only during the application of GABA resulting in the rapid fade. Recovery was obtained following a period of ~5 min washout. Bottom trace: Responses to 10 μM GABA are shown in the absence and presence of 30 μM pictrotoxin. Little difference in the nature of the antagonism was obtained whether picrotoxin was allowed a period of preincubation (left) or only applied concurrently with GABA (right). (b) Concentration-effect curve to picrotoxin. The %-reduction of responses to 100 μM GABA were calculated and plotted against picrotoxin concentration. Each point represents the mean (± s.e.mean shown by vertical lines) of 4 observations.

Table 2 IC_{50} values for antagonists of γ-aminobutyric acid (GABA)

Antagonist	*IC_{50}*	*pK_i*	*pA_2*	n
Picrotoxin	8.4 ± 1.1 μM	–	–	28
Penicillin	1.2 ± 0.1 mM	–	–	15
Bicuculline	8.7 ± 3.0 μM	6.2	6.4	20

The logistic equation was fitted to individual %-reduction values to obtain IC_{50} estimates. For the competitive antagonist, bicuculline, the IC_{50} is compared with the pA_2 obtained from Schild analysis. The pK_i value for bicuculline was calculated from the IC_{50} value (Cheng & Prussoff, 1973).

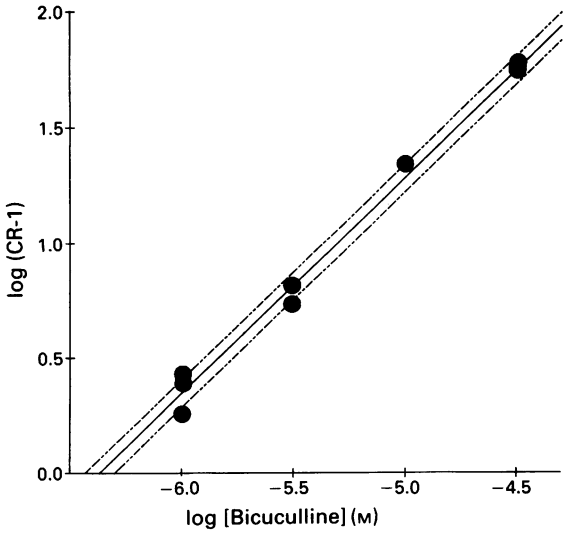

Figure 4 Schild plot for bicuculline antagonism of γ-aminobutyric acid (GABA). Concentration ratios (CR) were calculated from the parallel rightward shift of a control concentration-response line produced from 5 μM and 10 μM GABA. log CR − 1 was plotted against the corresponding log bicuculline concentration. From the fitted line a pA_2 of 6.4 was obtained. Dashed lines indicate 95% confidence intervals.

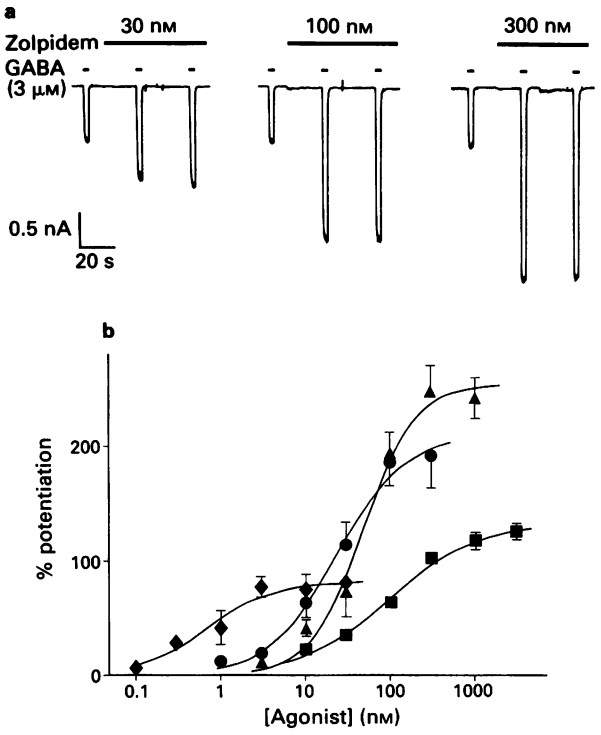

Figure 5 Benzodiazepine modulation of γ-aminobutyric acid (GABA) responses. (a) Potentiation of GABA responses by zolpidem. Reproducible inward currents produced from 3 s applications of 3 μM GABA every 30 s were obtained (Vhold = − 20 mV) prior to the addition of zolpidem (30–300 nM). A concentration-dependent potentiation of the response was produced in the presence of zolpidem that fully reversed upon washout during the 1 min intervals between the excerpts shown. (b) Concentration-response curves for benzodiazepine agonist potentiation of responses to 3 μM GABA. %-potentiations were calculated from experiments essentially identical to that shown in (a). Mean ± s.e.mean values are plotted for zolpidem (▲), flunitrazepam (●), C1218872 (■) and FG 8205 (◆). The curves resulting from fitting the logistic equation to the data points are also shown to illustrate the differences in potency and efficacy found between each of the agonists.

were required for this to occur at low concentrations.

The potentiation of 3 μM GABA by 100 nM flunitrazepam was examined in the presence of various concentrations of the benzodiazepine receptor antagonist, flumazenil. Flumazenil antagonized this potentiation with an IC_{50} = 20.2 nM, which resulted in a K_i value (Cheng & Prussoff, 1973) of 3.8 nM.

Pentobarbitone and alphaxalone

Responses to 3 μM GABA were potentiated by both pentobarbitone and alphaxalone. EC_{50} values are given in Table 3. GABA responses were also prolonged in the presence of these modulators. Concentrations of pentobarbitone of 100 μM or above produced small inward currents in the absence of applied GABA. No evidence of direct effects of alphaxalone were obtained with concentrations up to 10 μM.

Table 3 EC_{50} values for positive allosteric modulators of γ-aminobutyric acid (GABA)

Modulator	*EC_{50}* (nM)	*Maximum potentiation* (%)	n
Flunitrazepam	23.1 ± 9.1	208 ± 27	48
Zolpidem	47.8 ± 8.9	252 ± 15	42
C1218872	101.4 ± 28.2	134 ± 10	44
FG8205	0.68 ± 0.42	81 ± 13	24
Alphaxalone	290 ± 310	198 ± 60	24
Pentobarbitone	25000 ± 7000	318 ± 40	24

Individual %-potentiation values were plotted against modulator concentration and the logistic equation fitted.

Discussion and conclusions

This functional characterization of α_1, β_1, γ_{2L} containing GABA$_A$ receptors stably transfected into mouse fibroblast L-cells is an extension of a previous study on this clonal cell line (Hadingham *et al.*, 1992). The I-V characteristics were consistent with GABA having activated a Cl^- channel. The reversal potential obtained was close to that predicted by substitution of the intracellular (137 mM) and extracellular (135 mM) chloride concentrations into the Nernst equation. The small deviation may be attributable to differing activity coefficients between the intra and extracellular solutions (e.g. Robertson, 1989). The outward rectification, although not as marked as reported with native receptors (Robertson, 1989) nor those constructed from similar combinations of recombinant sub-units in *Xenopus* oocytes (Malherbe *et al.*, 1990), indicates that the opening of the channel following occupation of the recombinant receptor was sensitive to the charge separation across the plasma membrane. The concentration-response curves appeared to be accurately described by the logistic equation with no systematic deviation from the experimental data points. Thus, the steepness of the curve may indicate cooperativity of binding, particularly as the curves were not better fitted by modelling up to 5 independent binding sites (Colquhoun & Ogden, 1988; Bean, 1990). As far as can be compared, the concentration-response curves in these cells appear similar to those reported in patch clamp studies on native GABA receptors (Inoue & Akaike, 1988; Suzuki *et al.*, 1990) and on transiently transfected cells containing similar combinations of sub-units (Verdoorn *et al.*, 1990).

The antagonism produced by bicuculline was perhaps not unexpected as bicuculline has been shown to act as a competitive antagonist at GABA$_A$ receptors (Simmonds, 1980). The Schild plot slope of ~1 is consistent with such a com-

petitive interaction and the pA_2 value of 6.4 is also in good agreement with previous studies on native GABA receptors in the cuneate nucleus (Simmonds, 1980) and hippocampus (Kemp *et al.*, 1986) of the rat. An IC_{50} for bicuculline was also obtained from the percentage reduction of responses to a single, high concentration of GABA (100 μM) at a holding potential of − 60 mV. The large currents resulting from this procedure would theoretically result in a substantial voltage error which was not compensated for. In the presence of antagonists, when the currents were smaller, these errors would also presumably be reduced thus affecting the values obtained from the inhibition curves to bicuculline and picrotoxin. However, transformation of the IC_{50} obtained for bicuculline into a pK_i (Cheng & Prusoff, 1973) produced a value of 6.2, which was very similar to the pA_2 obtained, indicating that any possible errors did not substantially compromise the data obtained. Overall, these results indicate that the pharmacology of the agonist recognition site produced by this combination of sub-units was essentially identical to that of native $GABA_A$ receptors.

Penicillin is believed to be an open channel blocker of $GABA_A$ Cl^- channels (Hochner *et al.*, 1976; Smart & Constanti, 1986; Pickles & Simmonds, 1980) and picrotoxin has an apparently different channel-activated preferring mode of action (Smart & Constanti, 1986; Newland & Cull-Candy, 1992). The strong agonist dependency of the antagonism by picrotoxin seen in these experiments exemplified the requirement for receptor activation. The lack of any obvious agonist dependency seen with penicillin presumably reflects its fast kinetics of channel block. That these two non-competitive antagonists were effective blockers of the GABA responses in these experiments is therefore evidence that the sub-units formed channels that closely resembled the native situation. Analysis of single channels would be of further use in this respect.

Allosteric modulation has long been known to be a feature of $GABA_A$ receptors. Benzodiazepine receptor agonists potentiate responses to GABA (Choi *et al.*, 1977), probably by increasing the frequency of channel openings (Study & Barker, 1981). The range of benzodiazepines found to be effective in this study, the BZ_1 preferring compounds zolpidem and C1218872 (Pritchett *et al.*, 1989a), flunitrazepam and the imidazobenzodiazepine FG 8205 (Tricklebank *et al.*, 1990) suggests that this site of action is also accurately assembled. The benzodiazepine antagonist flumazenil was found to have a $K_i = 3.8$nM in these experiments, a value in close agreement with previously reported affinities of flumazenil (Kemp *et al.*, 1987).

Collation of the curves in Figure 5b allows the relative potencies and efficacies of the benzodiazepine agonists to be differentiated. Thus, FG 8205 was the most potent but its low maximal effect suggests it had an efficacy ~0.4 times that of flunitrazepam (cf. Tricklebank *et al.*, 1990). Similar partial agonism was demonstrated by C1218872 (0.6 times the efficacy of flunitrazepam) which was the least potent benzodiazepine agonist tested. The potencies of zolpidem and C1218872 observed are in agreement with the BZ_1 type pharmacology of this sub-unit combination. Barbiturates (Nicoll, 1972; 1975; Barker & Ransom, 1978) and steroids (Scholfield, 1980; Harrison & Simmonds, 1984) have been shown to potentiate GABA by increasing the channel mean open time (Study & Barker, 1981; Callachan *et al.*, 1987). The potentiation of GABA responses from these recombinant receptors by both pentobarbitone and alphaxalone is, again, in good agreement with the actions of these agents on native $GABA_A$ receptors. The broadening of the GABA response evoked in the presence of pentobarbitone and alphaxalone was due to a marked decrease in the response decay rate following the step back into GABA-free solution. The inward currents produced in the presence of higher concentrations of pentobarbitone presumably resulted from direct activation of the receptor complex (Barker & Ransom, 1978).

Therefore, mouse fibroblast L-cells, transfected with α_1, β_1, γ_{2L} sub-units of the $GABA_A$ receptor, exhibit the pharmacological range reported for native receptors. Results so far obtained from these types of investigation suggest that such approaches will allow detailed investigations into the effects of sub-unit composition on the physiology (cf. Verdoorn *et al.*, 1990) and pharmacology of $GABA_A$ receptors. These results are likely to provide valuable information in the continuing study of receptor structure and consequent function, and its implications to fundamental biological and applied pharmaceutical research.

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Protection by Ca^{2+} channel blockers (nifedipine, diltiazem and verapamil) against the toxicity of oxidized low density lipoprotein to cultured lymphoid cells

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1 Ca^{2+} channel blockers from 3 different chemical classes (diltiazem, verapamil and nifedipine) were compared in their ability to inhibit low density lipoprotein (LDL) oxidation and to protect cells directly against the cytotoxicity of oxidized LDL.

2 LDL oxidation promoted either by u.v. radiations or by copper ions was inhibited by nifedipine (IC_{50} of 10 ± 2 and $4 \pm 0.5\,\mu mol\,l^{-1}$, respectively) whereas diltiazem and verapamil were only poorly active or completely ineffective. As expected, LDL protected from oxidation by nifedipine (nifedipine/oxidized LDL) were much less cytotoxic than (unprotected) oxidized LDL (or than LDL oxidized in the presence of diltiazem or verapamil). The cytotoxic effect correlated well with the lipid peroxidation derivatives measured as the thiobarbituric acid reactive substances (TBARS) content of LDL oxidized in the presence of Ca^{2+} channel blockers, which suggests that the antioxidant effect of Ca^{2+} channel blockers protected cells indirectly from the cytotoxic effect of oxidized LDL.

3 Nifedipine also exhibited a direct cytoprotective effect against the cytotoxicity of oxidized LDL as demonstrated by incubating cells in the presence of unprotected oxidized LDL and nifedipine (IC_{50} of $1 \pm 0.2\,\mu mol\,l^{-1}$), whereas diltiazem and verapamil did not exhibit any significant protective effect. At the concentrations used, the protective effect of nifedipine was not due to inhibition of LDL uptake by Ca^{2+} channel blockers.

4 The direct protective activity of nifedipine is probably unrelated to its antioxidant properties since it did not inhibit the cellular TBARS evoked by oxidized LDL taken up by the cells, in contrast to vitamin E.

5 Nifedipine (and diltiazem to a lesser extent) inhibited the slow $[Ca^{2+}]_i$ rise induced by oxidized LDL and the subsequent cytotoxicity.

6 It is proposed that relatively high concentrations of nifedipine (unlike diltiazem and verapamil) protect cultured lymphoid cells against the cytotoxicity of oxidized LDL by two different mechanisms: (i) an antioxidant effect inhibiting LDL oxidation (outside the cell); (ii) a direct cytoprotective effect (at lower concentrations), the mechanism of which is unknown. These data could explain in part the mechanism of action of nifedipine in the prevention of cellular damage potentially involved in atherogenesis.

Keywords: Low density lipoproteins; oxidized LDL; lipid peroxidation; cytotoxicity; cytoprotection; nifedipine, diltiazem; verapamil; atherosclerosis

Introduction

Oxidized low density lipoproteins (LDL) are involved in the formation of lipid laden foam cells, an early event in atherogenesis (Brown & Goldstein, 1983; Haberland & Fogelman, 1987; Steinberg *et al.*, 1989). During oxidation by transition metals and/or by cultured cells, LDL undergo lipid peroxidation, loss of endogenous antioxidants and apolipoprotein B (apoB) modifications (Jürgens *et al.*, 1987; Steinbrecher *et al.*, 1990; Esterbauer *et al.*, 1990). Subsequent to apoB alterations, the metabolism of oxidized LDL is deviated towards the scavenger-receptor pathway of macrophagic cells, finally leading to foam cell formation (Brown & Goldstein, 1983; Steinberg *et al.*, 1989). Lipid peroxidation derivatives contained in oxidized LDL have been shown to affect recruitment and retention of monocytes (Steinberg *et al.*, 1989) and to be cytotoxic for cultured cells (Henricksen *et al.*, 1982; Hessler *et al.*, 1979). We have recently developed a new experimental model system consisting of LDL irradiated by u.v.-C that promote lipid peroxidation without major alteration of apoB-100 (Dousset *et al.*, 1990). In cultured lymphoid cells, we recently reported that u.v.-treated LDL are taken up through the apoB/E receptor-mediated pathway, and are cytotoxic to cultured cells in a dose-dependent manner and after a lag phase period (Nègre-Salvayre *et al.*, 1990). Moreover, after their internalization, oxidized LDL induce a peroxidation of cellular lipids (Nègre-Salvayre *et al.*, 1991a) and a sustained $[Ca^{2+}]_i$ rise (Nègre-Salvayre & Salvayre, 1992). Antioxidants are able to protect cells against the cytotoxic effect of oxidized LDL (Nègre-Salvayre *et al.*, 1991a,b) by at least two mechanisms: (i) indirect extracellular inhibition of LDL oxidation; (ii) 'direct' cellular protection against the cytotoxicity of lipid peroxidation products contained in oxidized LDL (Nègre-Salvayre *et al.*, 1991a).

Ca^{2+} channel blockers (particularly nifedipine), in addition to their well characterized inhibitory effect on voltage-dependent L-type Ca^{2+} channels (Godfraind *et al.*, 1986; Triggle & Janis, 1987), have been tentatively used in the prevention of ischaemic myocardial injury (Nayler *et al.*, 1990) and atherosclerosis (Henry, 1990). Moreover, antioxidant effects of dihydropyridine Ca^{2+} channel blockers have recently been described (Janero & Burghardt 1989). Therefore, it was of interest to test the protective properties of Ca^{2+} channel blockers against LDL oxidation, and against the cytotoxic

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effect of oxidized LDL (potentially involved in several steps of atherogenesis).

In this work, using our well characterized and sensitive experimental model system (Nègre-Salvayre *et al.*, 1990; 1991a,b), we have compared the protective effect of Ca^{2+} channel blockers from three different chemical classes (verapamil, diltiazem and nifedipine). We conclude that relatively high concentrations of nifedipine could constitute (at least *in vitro*) two lines of defence by inhibiting LDL oxidation and/or by increasing the resistance of living cells to the cytotoxic effect of lipid peroxidation products contained in oxidized LDL.

Methods

Cell culture

Lymphoid cell lines were established by Epstein-Barr Virus transformation and grown in standard medium, i.e. RPMI 1640 supplemented with 10% foetal calf serum, then 48 h before experiments, the standard medium was replaced by RPMI 1640 containing 2% Ultroser HY (a serum substitute without lipoprotein) as previously described (Nègre-Salvayre *et al.*, 1990).

LDL isolation, labelling with cholesteryl oleyl ether and cellular uptake

Human LDL (d 1.01–1.063) were isolated by sequential ultracentrifugation according to Havel *et al.* (1955), dialyzed, sterilized on 0.2 μm millipore filters and kept at + 4°C under nitrogen till use, as previously described (Nègre-Salvayre *et al.*, 1990). LDL were labelled with [^{3}H]-cholesteryl oleyl ether (around 10^5 d.p.m. of [^{3}H]-cholesteryl oleyl ether per mg apoB) by a procedure derived from that of Roberts *et al.* (1985), isolated again by ultracentrifugation, dialyzed and sterilized on 0.2 μm millipore filters and kept at + 4°C under nitrogen as previously described (Nègre-Salvayre *et al.*, 1990). Radiolabelled LDL were added to the culture medium for 12 h, then cells were isolated by centrifugation (700 *g* for 10 min), carefully washed twice in phosphate buffered saline, homogenized by sonication in 1 ml of distilled water and the cell-associated radioactivity was determined by liquid scintillation counting (Packard counter model Tricarb 4530).

LDL oxidation and antioxidant activity of Ca^{2+} channel blockers

LDL (2 mg apoB ml^{-1}), mixed with various concentrations of Ca^{2+} channel blockers (0–100 μmol^{-1}) dissolved in 10 μl dimethylsulphoxide, were submitted to mild oxidation promoted either by u.v.-C treatment (254 nm, 0.5 mW cm^{-2} for 2 h), as under the standard conditions previously reported (Dousset *et al.*, 1990; Nègre-Salvayre *et al.*, 1990) or by copper ions (1 $\mu mol\ l^{-1}$ $CuSO_4$ at 37°C for 2 h, see details in legend of the figures). Lipid peroxidation of LDL was evaluated by determining thiobarbituric acid reactive substances (TBARS) and tocopherol content as previously indicated (Nègre-Salvayre *et al.*, 1990; 1991a). Non-irradiated LDL were used as controls.

Tests of cytotoxicity and cytoprotective effect of Ca^{2+} channel blockers

LDL, treated by u.v. in the presence of Ca^{2+} channel blockers (as indicated above), were added to the culture medium: briefly, cells distributed in 24 microwell tissue culture plates (around 5×10^5 cells in 1 ml of culture medium per microwell) were incubated with 200 $\mu g\ ml^{-1}$ LDL (expressed as μg apoB ml^{-1}). After 48 h incubation, cell viability was determined by trypan blue test according to Morel *et al.* (1983), CFDA test according to McGinnes *et al.* (1986), and release of lactate dehydrogenase in the culture medium (LDH was determined with a Roche assay kit, MA kit 10) as previously used (Nègre-Salvayre *et al.*, 1990).

To determine the 'direct' cytoprotective effects, Ca^{2+} channel blockers (dissolved in 10 μl dimethylsulphoxide and used at various concentrations) were added to the culture medium simultaneously with oxidized LDL (u.v.-treatment under the standard conditions, in the absence of Ca^{2+} channel blockers or any additive). After 48 h incubation, cell viability was determined as indicated above. The potential cytotoxicity of Ca^{2+} channel blockers *per se* was evaluated by the same procedure, but this time omitting oxidized LDL from the culture medium. Cell viability was determined after 48 h incubation as indicated above and IC_{50} concentrations were determined as under previously described conditions (Nègre-Salvayre *et al.*, 1991b).

Determination of $[Ca^{2+}]_i$

Free cytosolic calcium concentration $[Ca^{2+}]_i$ was determined by using Quin-2/AM (Quin-2/AM is hydrolyzed by cellular carboxylesterases and liberates the intracellular trappable fluorescent calcium indicator Quin-2). Briefly, lymphoid cells were incubated in 25 mmol l^{-1} HEPES buffered RPMI 1640 medium containing BSA 0.5% and Quin-2/AM (15 $\mu mol\ l^{-1}$) for 1 h just before $[Ca^{2+}]_i$ determination. After this incubation period, cells were washed (once with RPMI 1640 and twice with phosphate buffered saline containing 25 mmol l^{-1} HEPES) and were used to determine the fluorescence F and the calibration of the dye response (by measuring F_{min} and F_{max}) as described by Arslan *et al.* (1985).

Proteins were determined by the method of Lowry *et al.* (1951).

Chemicals

3- [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), trypan blue dye, 2,4,6-trinitrobenzenesulfonic acid (TNBS), Ca^{2+} channel blockers nifedipine, diltiazem, verapamil, were purchased from Sigma (St Louis, MO, U.S.A.), [^{3}H]-cholesteryl oleyl ether (38 Ci $mmol^{-1}$) from Amersham (Paris, France), carboxyfluorescein diacetate (CFDA) from Molecular Probe (Eugene, OR, U.S.A.), cell culture reagents, RPMI 1640, foetal calf serum, penicillin, streptomycin, sterile phosphate buffered saline (PBS) from Seromed (Paris), Ultroser HY from IBF (Villeneuve-la-Garenne, France), Picofluor from Packard Instrument (Paris, France) and the other chemicals from Merck (Darmstadt, Germany) or Prolabo (Paris).

Presentation of data and statistical analysis

Data are given as means ± s.e.mean. Estimates of statistical significance were made by Student's *t* test (Schwartz, 1981).

Results

Nifedipine inhibits lipid peroxidation of LDL and their subsequent cytotoxicity

When LDL was treated by u.v. in the presence of Ca^{2+} channel blockers, the formation of lipid peroxidation derivatives (measured as TBARS) was inhibited by nifedipine (under these experimental conditions, the IC_{50} was 10 ± 2 $\mu mol\ l^{-1}$) whereas under the same experimental conditions diltiazem was much less efficient (estimated IC_{50} 200 ± 50 $\mu mol\ l^{-1}$) and verapamil was nearly ineffective. Concurrently, the loss of vitamin E was significantly reduced by nifedipine, but only poorly by diltiazem and not at all by verapamil (Figure 1a,b). In order to exclude an inhibitory effect of lipid peroxidation specific to the u.v. system (e.g. absorption of u.v. light by nifedipine, independent of any antioxidant

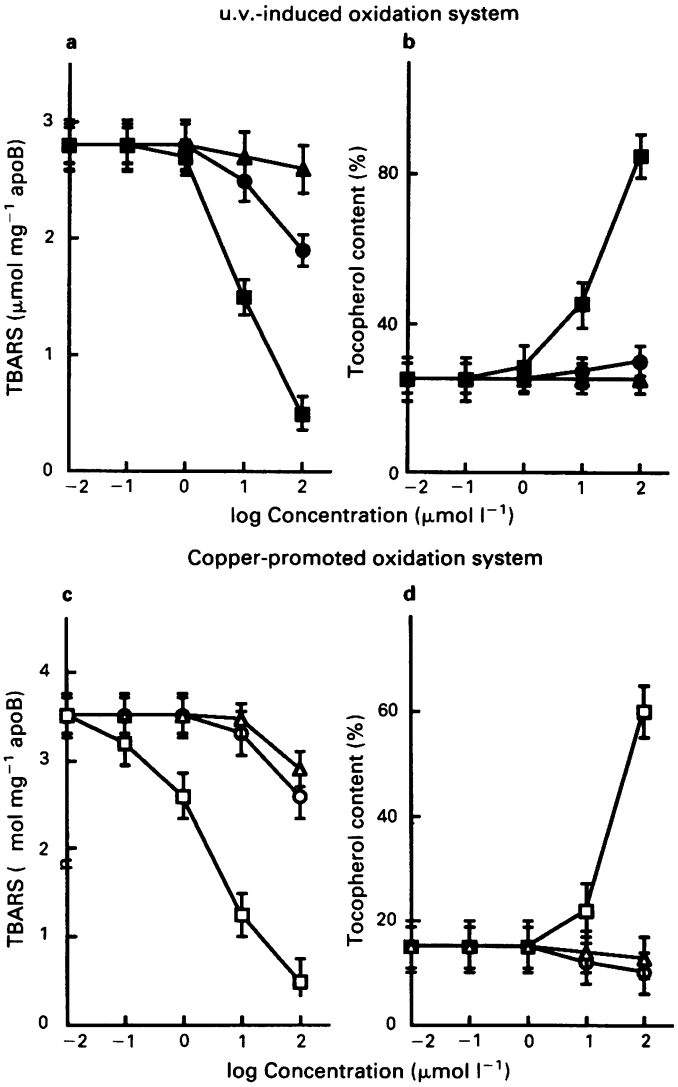

Figure 1 Test of inhibition of LDL lipid peroxidation by increasing concentrations of Ca^{2+} channel blockers, verapamil (▲), diltiazem (●) or nifedipine (■). LDL were submitted to oxidation either by u.v.-C radiations (254 nm, 0.5 mW cm^{-1} for 2 h) (a and b; solid symbols) or by copper (1 μmol l^{-1} for 2 h at 37°C) (c and d; open symbols), in the presence of increasing concentrations of antioxidants (0 to 100 μmol l^{-1}). Lipid peroxidation indexes were determined as described in the Methods section. In (a) and (c), thiobarbituric acid reactive substances (TBARS); in non-oxidized LDL, the TBARS levels were 0.3 nmol mg^{-1} apoB. In (b) and (d), tocopherol content expressed as % of the level in non-oxidized LDL (initial level in non-oxidized LDL: 2.3 ± 0.3 nmol mg^{-1} apoB). Means (± s.e.mean, vertical bars) of 3 separate experiments.

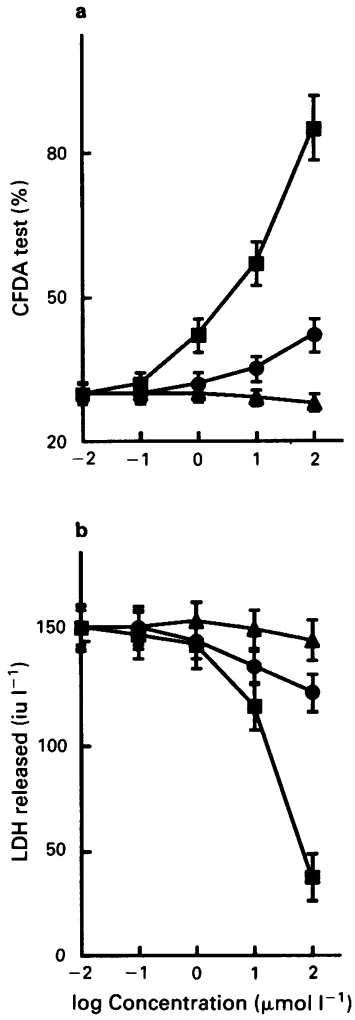

Figure 2 Cytotoxicity to lymphoid cells of the LDL of Figure 1. LDL were irradiated by u.v.-C in the presence of Ca^{2+} channel blockers (under conditions of Figure 1) verapamil (▲), diltiazem (●), or nifedipine (■). A fixed concentration (200 μg apoB ml^{-1}) of these LDL was incorporated in the culture medium (RPMI 1640 containing 2% Ultroser) and the lymphoid cells were cultured for 48 h in this medium. At the end of the 48 h pulse period, cell viability was assessed by (a) the CFDA test (expressed as % of control cells, i.e. cells grown in the absence of any oxidized LDL or drug) and (b) the leakage of LDH in the culture medium. Means (± s.e.mean, vertical bars) of 3 separate experiments.

activity) we have compared the inhibitory effect of these drugs in a copper-induced oxidation system. Under these experimental conditions, IC_{50} values were 4 ± 0.5 and 400 ± 200 (estimated) μmol l^{-1} for nifedipine and diltiazem respectively (these values being in the same range as in the u.v. system) and verapamil was less effective (Figure 1c). These results demonstrate that nifedipine is effective in preventing lipid peroxidation of LDL, whereas the other Ca^{2+} channel blockers tested are either poorly active or completely inefficient as antioxidants, under our experimental conditions (Figure 1).

In order to investigate the biological relevance of the protective effect of Ca^{2+} channel blockers against LDL oxidation, we have tested the cytotoxicity of LDL treated by u.v. in the presence of Ca^{2+} channel blockers. As shown in Figure 2, unprotected u.v.-treated LDL (u.v. irradiation in the absence of any additional compound) were severely cytotoxic (around 80% dead cells, as assessed by trypan blue uptake, after 48 h of cell culture in the presence of oxidized LDL at a concentration of 200 μg apoB ml^{-1}), the basal level of cell death being around 15%. In comparison to unprotected u.v.-treated LDL, LDL irradiated by u.v. in the presence of protective concentrations of nifedipine (nifedipine/u.v.-treated LDL) were much less cytotoxic. In contrast, diltiazem/ or verapamil/u.v.-treated LDL exhibited a cytotoxicity similar to unprotected u.v.-treated LDL (Figure 2). Note that the cytotoxicity of LDL irradiated in the presence of variable concentrations of Ca^{2+} channel blockers was relatively well correlated with the levels of lipid peroxidation markers (TBARS) in u.v.-treated LDL.

Nifedipine protects directly lymphoid cells against the cytotoxicity of oxidized LDL

We have recently found that several antioxidants (e.g. vitamin E or catechin) were able to prevent the cytotoxic effects

Table 1 Cytotoxicity of Ca^{2+} channel blockers on lymphoid cells grown in the standard culture medium and evaluated by the CFDA test

	Ca^{2+} channel blocker concentration (μmol l^{-1})				
Ca^{2+} channel blockers	0	0.1	1	10	100
Nifedipine	100 ± 12	102 ± 15	99 ± 10	90 ± 10	35 ± 7
Diltiazem	100 ± 12	100 ± 10	99 ± 14	100 ± 11	95 ± 9
Verapamil	100 ± 12	101 ± 11	104 ± 12	99 ± 14	88 ± 9

The fluorometric CFDA test was performed under previously described conditions (Nègre-Salvayre *et al.*, 1991a) and expressed as % of control cells grown in the absence of any of the Ca^{2+} channel blocker. The results are the mean ± s.e.mean of 3 separate experiments.

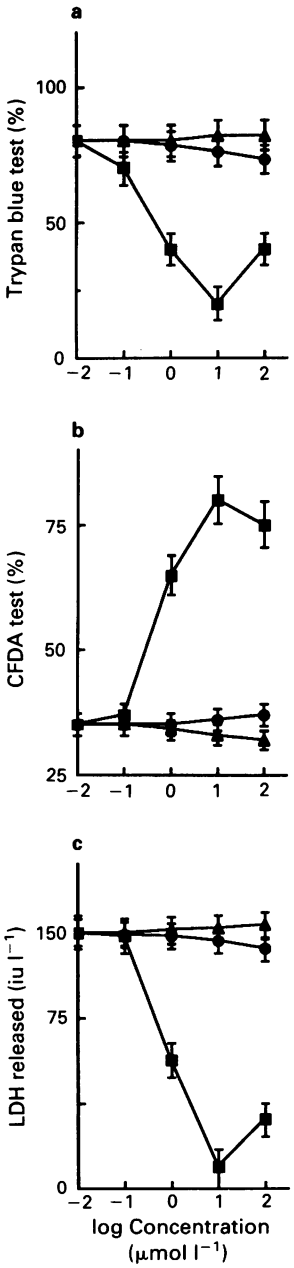

Figure 3 Direct cytoprotective effect of Ca^{2+} channel blockers against the cytotoxicity of u.v.-treated LDL. A fixed concentration of oxidized LDL (200 μg apoB ml^{-1} of LDL oxidized by u.v. under the standard conditions used as in Figure 1, but in the absence of Ca^{2+} channel blockers) was added to the culture medium simultaneously with increasing concentrations of Ca^{2+} channel blockers, (up to 100 μmol l^{-1}) verapamil (▲), diltiazem (●) or nifedipine (■). The cell viability was determined after 48 h pulse by (a) trypan blue test (expressed as stained cells %), (b) CFDA test (expressed as % of control cells grown in the absence of any oxidized LDL or drug) and (c) LDH leakage (expressed as iu l^{-1}). Means (± s.e.mean vertical bars) of 4 separate experiments.

of oxidized LDL by a mechanism completely independent of the inhibition of the LDL oxidation (Nègre-Salvayre *et al.*, 1991a). This prompted us to examine the ability of Ca^{2+} channel blockers to protect directly the cells against the cytotoxicity of previously oxidized LDL (LDL oxidized in the absence of any additive), Ca^{2+} channel blockers being incorporated in the culture medium simultaneously with oxidized LDL. Nifedipine was found to be highly protective (IC_{50} of 1 ± 0.2 μmol l^{-1}; 10 μmol l^{-1} nifedipine protected cells almost completely against the cytotoxic effects of oxidized LDL). Note that the protective effect of nifedipine was not affected by preincubation of nifedipine with oxidized LDL (for 24 h before incorporation in the culture medium): thus we can conclude that (1) nifedipine is not inactivated by the preincubation with oxidized LDL; (2) oxidized LDL are not inactivated by preincubation with nifedipine. The decrease of the protective effect of a high concentration of nifedipine was due to cytotoxicity of nifedipine *per se* at concentrations higher than 10 μmol l^{-1} (Table 1). Diltiazem and verapamil were devoid of any protective effect over the range of concentrations tested (i.e. up to 100 μmol l^{-1}) (Figure 3).

As shown in Figure 4, the protective effect of Ca^{2+} channel blockers (at the concentrations used) was not due to an inhibition of uptake of LDL.

Effect of Ca^{2+} channel blockers on cellular lipid peroxidation and $[Ca^{2+}]_i$ rise induced by oxidized LDL

We have recently demonstrated that oxidized LDL induced two intracellular events possibly involved in the genesis of the cytotoxicity in lymphoid cells: (1) a significant rise of cellular TBARS occuring at around 12 h following the beginning of the pulse with oxidized LDL and being much higher than the levels of TBARS contained in oxidized LDL internalized by the cells; (2) a delayed and sustained $[Ca^{2+}]_i$ rise (Nègre-Salvayre & Salvayre, 1992). In order to begin to investigate the mechanism of the cytoprotective effect of nifedipine, we have studied its effect on the two above parameters which are potentially involved in the genesis of the cellular lesions leading to cytotoxic events. As shown in Figure 5, the cellular TBARS formation was not inhibited by relatively high concentrations of Ca^{2+} channel blockers. It is noteworthy that a cytoprotective concentration of nifedipine (10 μmol l^{-1}) was ineffective against TBARS formation.

A relatively high concentration (10 μmol l^{-1}) of nifedipine (which exhibits a nearly complete cytoprotective effect) effectively inhibited the $[Ca^{2+}]_i$ rise (Figure 6b) elicited in lymphoid cells by oxidized LDL (Figure 6a), whereas diltiazem (100 μmol l^{-1}) induced only a slight inhibition of the $[Ca^{2+}]_i$ peak and verapamil (100 μmol l^{-1}) had no significant effect (Figure 6b).

Discussion

The results described in this paper point out clear differences between the cytoprotective effect of Ca^{2+} channel blockers from the phenylalkylamine (verapamil), benzothiazepine (dil-

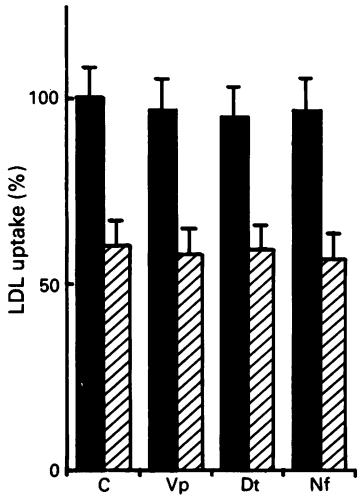

Figure 4 Uptake of LDL (30 or 100 μg apoB ml^{-1}, hatched and solid bars, respectively) in the presence or absence of Ca^{2+} channel blockers. LDL, previously labelled with [^{3}H]-cholesteryl oleyl ether, were incorporated in the culture medium of lymphoid cells in the absence (controls C) or presence of Ca^{2+} channel blockers (verapamil, Vp, diltiazem, Dt or nifedipine, Nf, at a fixed concentration 10 μmol l^{-1}). After 12 h, the radioactivity of the cell-associated [^{3}H]-cholesteryl oleyl ether was measured as described in the Methods section. Means (± s.e.mean, vertical bars) of 3 separate experiments.

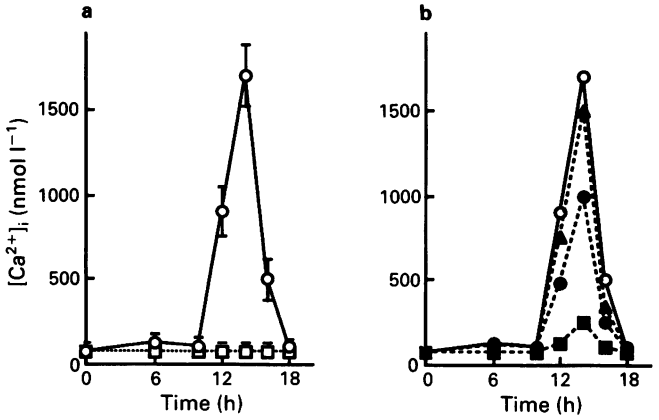

Figure 6 Time course of $[Ca^{2+}]_i$ determined as indicated in Methods using quin-2/AM) in lymphoid cells pulsed continuously with a fixed concentration of non-oxidized LDL (□) or oxidized LDL (200 μg apoB ml^{-1} prepared under the conditions of Figure 3) in the absence (a and b, ○) or in the presence (b, dotted line) of a fixed concentration of Ca^{2+} channel blockers, 100 μmol l^{-1} verapamil (▲), 100 μmol l^{-1} diltiazem (●) or 10 μmol l^{-1} nifedipine (■). Means (± s.e.mean, vertical bars) of 3 separate experiments.

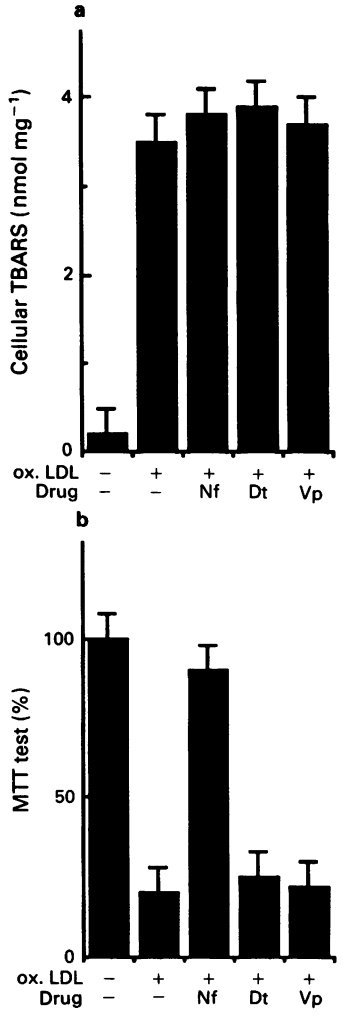

Figure 5 Tests of the effect of Ca^{2+} channel blockers on cellular TBARS formation (a) and cytotoxicity (b) (evaluated 24 h and 48 h after the beginning of the pulse, respectively). Lymphoid cells were grown in the presence (+) or absence (−) of oxidized LDL (under the conditions of Figure 3) or Ca^{2+} channel blockers (100 μmol l^{-1} verapamil, Vp; 100 μmol l^{-1} diltiazem, Dt; 10 μmol l^{-1} nifedipine, Nf). Means (s.e.mean, vertical bars) of 3 separate experiments.

tiazem) and dihydropyridine (nifedipine) series. Nifedipine was the most potent compound whereas diltiazem was only poorly active and verapamil exhibited no protective activity. The protective effect of nifedipine occurs in two different ways and two different sites: (1) by inhibiting the LDL oxidation (this antioxidant effect occuring outside the cell); (2) by blocking the cytotoxicity of oxidized LDL (inside the cell).

In our experimental model system consisting of LDL irradiated by u.v.-C radiations under the standard conditions described previously (Nègre-Salvayre *et al.*, 1990; 1991a), LDL lipid peroxidation can be efficiently prevented by nifedipine (IC_{50} around 5 ± 2 μmol l^{-1}), but not by diltiazem and verapamil. This difference between the antioxidant activity of the tested Ca^{2+} channel blockers cannot be explained by a difference of absorbance in the u.v.-C range (in our u.v.-promoted oxidation system the major band of u.v. emission is at 254 nm), since there is no correlation between the inhibition of LDL oxidation and the absorbance at 254 nm (Figures 1 and 7) and since similar results were observed in the copper-induced oxidation system. It should be noted that our results with nifedipine agree with those of Breugnot *et al.* (1991), but not those with verapamil (various batches from different sources gave similar results): we do not know if some methodological differences in oxidation conditions could explain this discrepancy. The antioxidant effect of nifedipine (inhibition of TBARS formation) is also correlated with the protection of endogenous tocopherols, in agreement with the results observed with other antioxidants (Esterbauer *et al.*, 1990; Jessup *et al.*, 1990), but higher concentrations of nifedipine were necessary for the protection of tocopherols (IC_{50} around 50 μmol l^{-1}) than for the inhibition of TBARS formation (IC_{50} 10 μmol l^{-1}). The efficacy of nifedipine as an antioxidant protecting LDL against oxidative modifications

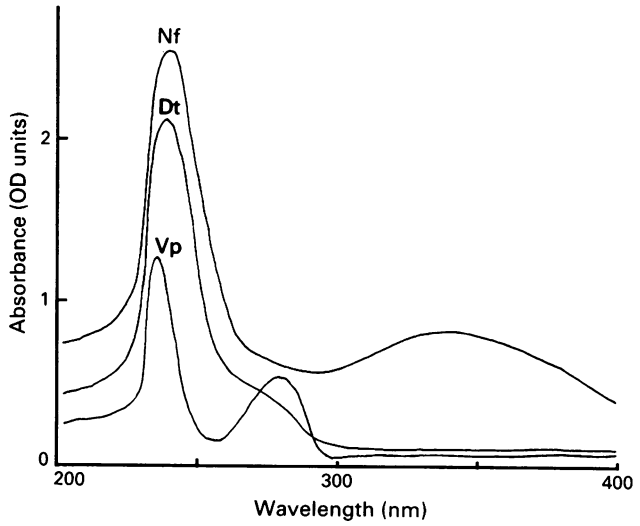

Figure 7 Comparison of the u.v.-spectra of the tested Ca^{2+} channel blockers (verapamil, Vp; diltiazem, Dt; nifedipine, Nf) using a Kontron spectrophotometer (Uvikon 930).

is in the same range as probucol (Nègre-Salvayre *et al.*, 1991a) which is known to reduce effectively LDL oxidation, macrophage uptake (Parthasarathy *et al.*, 1986) and to slow down the progression of atheroma in the Watanabe rabbit (Kita *et al.*, 1987). The antioxidant effect of nifedipine is consistent with the results of Ondrias *et al.* (1989) on liposomal systems, but not with the data reported by Janero & Burghardt (1989) who claimed that dihydropyridine Ca^{2+} channel blockers (nisoldipine, felodipine and nicardipine) exhibited antiperoxidative properties, except for nifedipine. These discrepancies could result from differences in the experimental systems employed as Janero & Burghardt (1989) used liposomes subjected to oxidation by iron/xanthine/xanthine oxidase. In our experimental model system, the antioxidant and cytoprotective effects of nifedipine occur at much higher concentrations (in the range of the effects observed in other systems by Breugnot *et al.*, 1991) than those required to block Ca^{2+} channels. The presence of voltage-dependent Ca^{2+} channels has been reported in myeloma and hybridoma cell lines, but is still disputed in B-lymphocytes (Gardner, 1990). Moreover it is possible that the molecular target of dihydropyridine (at micromolar concentrations) could be different from the classical voltage-dependent Ca^{2+} channels (Godfraind *et al.*, 1986; Hano *et al.*, 1991).

We did not study the inhibition of apoB modification because our standard conditions of u.v.-treatment promote only low levels of peroxidation products without any major alteration of apoB structure (no decrease in the TNBS reactivity and no fragmentation) (Dousset *et al.*, 1990) and no decrease of LDL uptake through the apoB/E receptor (Nègre-Salvayre *et al.*, 1990).

The study of the cytotoxicity of LDL oxidized in the presence of Ca^{2+} channel blockers showed that when LDL lipid peroxidation was inhibited, the subsequent cytotoxicity was also inhibited. These data suggest that (above a threshold level) TBARS content can be useful for predicting the cytotoxicity of oxidized LDL as previously discussed (Nègre-Salvayre *et al.*, 1990; 1991a) and that the cytotoxic effect probably results from lipid peroxidation derivatives contained in oxidized LDL (Jürgens *et al.*, 1987; Esterbauer *et al.*, 1988; Nègre-Salvayre *et al.*, 1990). Moreover, the protection of endogenous LDL-tocopherols (by nifedipine) could also participate in the prevention of oxidized LDL cytotoxicity since we have recently demonstrated that cells preincubated with vitamin E were more resistant to oxidized LDL (Nègro-Salvayre *et al.*, 1991a,b).

One of the most striking results of the present study, is that, besides its antioxidant effect, nifedipine also exhibits a direct cytoprotective effect against the cytotoxicity of oxidized LDL. This effect seems to be independent of the inhibition of LDL oxidation (described and discussed above) since LDL were oxidized in the absence of the drug before the incubation with cells. Moreover, under our experimental conditions, this direct protective effect occurred at lower concentrations (IC_{50} of $1 \pm 0.2\,\mu mol\,l^{-1}$) than the antioxidant effect (IC_{50} of TBARS inhibition is around $10 \pm 2\,\mu mol\,l^{-1}$). The dual protective effect of nifedipine occurs by inhibition of LDL oxidation, and by inhibition of the cytotoxicity of oxidized LDL, by mechanisms which are unknown at present. As recently discussed, the transduction of the cytotoxic signal of oxidized LDL is probably a multistep process associated with the formation of cellular TBARS and to a delayed and sustained $[Ca^{2+}]_i$ rise which leads finally to membrane lesions detected by the cytotoxicity tests used here (Nègre-Salvayre *et al.*, 1991a; 1992). In contrast to vitamin E which blocks the formation of cellular TBARS and the subsequent cytotoxicity (Nègre-Salvayre *et al.*, 1991a), nifedipine did not inhibit the formation of cellular TBARS induced by oxidized LDL, but blocks the $[Ca^{2+}]_i$ rise and subsequently the cytotoxicity. These results are consistent with our previous results suggesting that the $[Ca^{2+}]_i$ rise is directly involved in the genesis of the cellular lesions induced by oxidized LDL (Nègre-Salvayre *et al.*, 1992) and are in agreement with the general role of the $[Ca^{2+}]_i$ rise in various cellular injuries, reviewed by Nicotera *et al.* (1990) and Farber (1990).

We can speculate that this cytoprotective effect could be of importance *in vivo*, since the oxidized LDL are thought to play a major role in atherogenesis and cytotoxic events may be involved in the genesis of late irreversible lesions (necrotic lesion of the plaque and defects of the endothelial cell lining). This could constitute a novel approach to the prevention of atherosclerosis which should be promising since both lines of defence (prevention of LDL oxidation and direct protection of the cells) are complementary and could lead to a better therapeutic efficiency.

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S-nitroso-glutathione inhibits platelet activation *in vitro* and *in vivo*

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1 The effect of S-nitroso-glutathione (GSNO), a stable nitrosothiol, on platelet activation was examined *in vitro* and *in vivo*.

2 The adhesion of human platelets to fibrillar collagen and human endothelial cell monolayers was inhibited by GSNO.

3 GSNO caused a concentration-dependent inhibition of collagen-induced platelet aggregation *in vitro* and decreased ADP-induced aggregation in the conscious rat.

4 Inhibition of platelet aggregation *in vitro* correlated with the increase in intraplatelet cyclic GMP levels.

5 The release of NO from GSNO was enhanced by platelet lysate, native glutathione and ascorbate.

6 The results show that GSNO is a carrier of NO and therefore has pharmacological activity as an inhibitor of platelet activation.

Keywords: S-nitroso-glutathione; platelet function *in vitro* and *in vivo*

Introduction

There is now increasing evidence that the physiological and pharmacological release of nitric oxide (NO) regulates haemostatis and inhibits platelet activation (Radomski & Moncada, 1991; Stamler & Loscalzo, 1991). S-nitrosothiols such as S-nitroso-cysteine are formed by S-nitrosylation of thiols in the presence of NO or NO_2^- (Saville, 1958; Ignarro & Gruetter, 1980). With few exceptions, these compounds are usually unstable, even in the crystalline state (Mellion *et al.*, 1983; Park, 1988); however, they are potent inhibitors of platelet aggregation *in vitro* (Mellion *et al.*, 1983; Lieberman *et al.*, 1991). The biological significance of S-nitrosylation has not yet been defined. It has been suggested that highly reactive and unstable NO is stabilized by a reaction with a carrier molecule i.e. R-SH that prolongs its half-life *in vivo* and preserves its biological activity (Stamler *et al.*, 1992). S-nitroso-glutathione (GSNO, Figure 1), a stable S-nitroso-thiol, can be chemically synthesized from one of the most abundant intracellular thiols, glutathione (Meister & Anderson, 1983). This study investigates the pharmacological effects of GSNO on platelet activation both *in vitro* and *in vivo*.

Methods

In vitro

Platelet adhesion Human blood was collected and platelet-rich plasma (PRP) and prostacyclin-washed platelet suspensions (WP) were prepared (Radomski & Moncada, 1983). The adhesion of unlabelled platelets to fibrillar collagen and ^{111}In-labelled platelets to monolayers of human umbilical vein endothelial cells (SGHEC-7) was measured as described previously (Radomski *et al.*, 1987a).

Platelet aggregation and the release of ATP These were measured in a platelet-ionized calcium lumi-aggregometer (Chronolog). The $[Ca^{2+}]_i$ levels were measured in aequorin-loaded platelets prepared by the method of Yamaguchi *et al.* (1986) with a modification of substituting prostaglandin E_1 with prostacyclin (1 μM). All inhibitors of aggregation were incubated with platelets for 1 min prior to the addition of collagen ($1-2\ \mu g\ ml^{-1}$). In some experiments GSNO was incubated for 30 min at 37°C before its effect on collagen-induced platelet aggregation was measured.

Cyclic nucleotides Washed platelets were incubated in the aggregometer for 10 min at 37°C in the presence or absence of GSNO. Following incubation, EGTA (5 mM) was added and platelets lysed by 2 cycles of freezing in liquid N_2 and thawing at 37°C. The lysate was centrifuged (10,000 *g* for 5 min) and the supernatant assayed for cyclic nucleotides by the dual range acetylation enzyme immunoassay system (Amersham).

The release of NO from GSNO This was measured following incubation of GSNO with intact platelets, platelet lysate and platelet cytosol (100,000 *g* for 30 min prepared from 3×10^8 platelets) and assayed in a dual wave spectrophotometer (Shimadzu) by the haemoglobin shift method (Feelisch & Noack, 1987).

In vivo

Platelet aggregation *in vivo* was measured by use of the disappearance of single platelets as an index of aggregation (Radomski *et al.*, 1990). Male Wistar rats (250–300 g) were briefly anaesthetized with isoflurane (2%). A double-lumen catheter was implanted in the femoral vein for drug administration and a single-lumen catheter implanted in the femoral artery for blood pressure and heart rate measurements. The catheters were fed subcutaneously to exit at the lower back and were connected to the cage housing via a flexible spring. Experiments were started at least 2 h later, when the rats were conscious and unrestrained in their cages. Adenosine 5′-diphosphate (ADP, $0.1-3\ \mu mol\ kg^{-1}$, i.v.) was administered in the presence or absence of a continuous infusion of GSNO ($0.03-1\ \mu mol\ kg^{-1}\ min^{-1}$, i.v.). Blood samples (50 μl) were collected over a period of 60 min, diluted with an equal volume of Tyrode solution containing EGTA (5 mM) and immediately (1 min following each collection) counted in a

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whole blood platelet counter, Coulter T-540 (Coulter Electronics Ltd.).

Blood gases and pH levels remained within the normal range for the duration of the experiments.

Reagents

S-nitroso-glutathione (GSNO) was synthesized and human haemoglobin prepared by the methods of Hart (1985) and Paterson *et al.* (1976) respectively. Solutions of NO gas were prepared as described by Palmer *et al.* (1987). Glutathione, ascorbic acid (BDH), adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), luciferin-luciferase reagent, human thrombin, Arg-Gly-Asp-Ser, Arg-Gly-Glu-Ser (Sigma), aequorin (Dr Blinks, Mayo Foundation), prostacyclin sodium salt (Wellcome), S-nitroso-acetylpenicillamine (SNAP, Wellcome), SIN-1 (N-ethoxycarbonyl-3-morpholino-sydnonimine; Therabel) and collagen (Hormon-Chemie) were obtained from the sources indicated.

Statistics

Results are mean ± s.e.mean of at least 3 separate experiments. They were compared by analysis of variance and $P<0.05$ was considered as statistically significant.

Results

In vitro

S-nitroso-glutathione (0.1–3 μM) inhibited, in a concentration-dependent manner, platelet adhesion to fibrillar collagen (Figure 1a) and thrombin-stimulated adhesion to SGHEC-7 cells (Figure 1b). The inhibition by the maximally effective concentration of GSNO (3 μM) was completely reversed by haemoglobin (3 μM, Figure 1a,b).

Incubation of GSNO (0.01–10 μM) with PRP or WP for 1 min resulted in a concentration-dependent inhibition of the collagen-induced increase in platelet aggregation and ATP release from platelets (Figure 2a and Table 1). Incubation of GSNO in WP for 30 min did not change significantly the antiaggregating activity of this compound ($IC_{50} = 0.11 \pm 0.04$ μM). S-nitroso-glutathione was approx. 2–3 times more potent in WP than in PRP. This inhibitory activity of GSNO was reversed by haemoglobin (Figure 2b). The aggregation of aequorin-loaded platelets by collagen was also inhibited by GSNO (0.03–0.3 μM, Figure 3). A subthreshold concentration of prostacyclin (0.1 nM) acted synergistically with a threshold concentration of GSNO (0.03 μM) to inhibit aggregation (Figure 4). However, no synergistic interactions were observed between GSNO and two tetrapeptide antagonists of the fibrinogen receptor (Gartner *et al.*, 1985; Pierschbacher & Ruoslahti, 1984) Arg-Gly-Asp-Ser or Arg-Gly-Glu-Ser (3–300 μM) as inhibitors of platelet aggregation ($n = 3$, data not shown). Nitric oxide, GSNO, SNAP and SIN-1 (0.01–30 μM) all inhibited collagen-induced platelet aggregation in a concentration-dependent manner. Comparison of the antiaggregatory activity of these NO donors is shown in Table 1. The order of inhibitory potency was: GSNO > NO > SNAP > SIN-1.

The basal levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) and adenosine 3':5'-cyclic monophosphate (cyclic AMP) were 34 ± 6 and 16 ± 5 fmol/10^8 platelets respectively. Incubation of platelets with GSNO (0.01–100 μM) caused a concentration-dependent increase in intraplatelet cyclic GMP to a maximum of 2078 ± 140 fmol/10^8 platelets (Figure 5). There was a significant correlation ($r = 0.9561$, $P = 0.0439$) between inhibition of platelet aggregation and increases in cyclic GMP induced by GSNO. The levels of cyclic AMP were slightly but significantly increased ($P = 0.0412$) at concentrations of GSNO > 0.3 μM, however, there was no significant correlation ($r = 0.9102$, $P = 0.0898$) between inhibition of platelet aggregation and increase in cyclic AMP caused by GSNO.

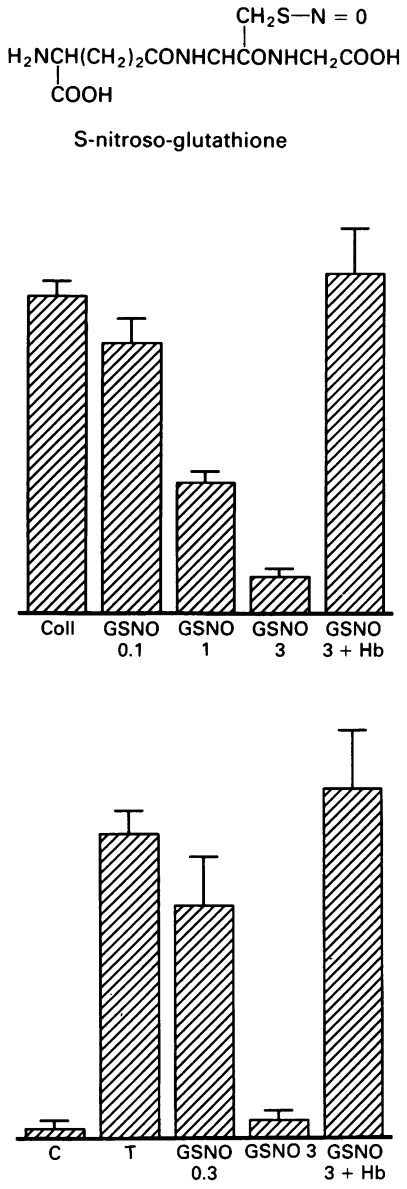

Figure 1 Inhibition of human platelet adhesion to fibrillar collagen (a) and to human umbilical endothelial cell monolayer (b) by S-nitroso-glutathione (GSNO). (a) Collagen (Coll)-induced platelet adhesion was inhibited in a concentration-dependent manner by GSNO (0.1–3 μM). The inhibitory activity of a maximally effective concentration of GSNO (3 μM) was reversed by haemoglobin (3 μM, GSNO 3 + Hb). (b) Platelet adhesion to endothelium (C) was enhanced by thrombin (0.005 u ml^{-1}, T). GSNO (0.3 and 3 μM) caused a concentration-dependent inhibition of adhesion. This action of GSNO was reversed by haemoglobin (GSNO 3 + Hb). The results are mean (± s.e.mean, vertical bars) of 4 experiments.

The release of NO from GSNO (10 μM) dissolved in Tyrode solution was low (0.01 ± 0.01 nmol min^{-1}). The addition of platelet lysate, but not intact platelets or cytosol, caused a concentration-dependent increase in the rate of NO release from GSNO (Figure 6a). Nitric oxide was also released by glutathione and ascorbic acid (Figure 6b).

In vivo

Preliminary experiments demonstrated that ADP (0.3–10 μmol kg^{-1}, i.v.) caused a dose-dependent decrease in the free platelet number and GSNO (0.03–3 μmol kg^{-1} min^{-1}, i.v.)

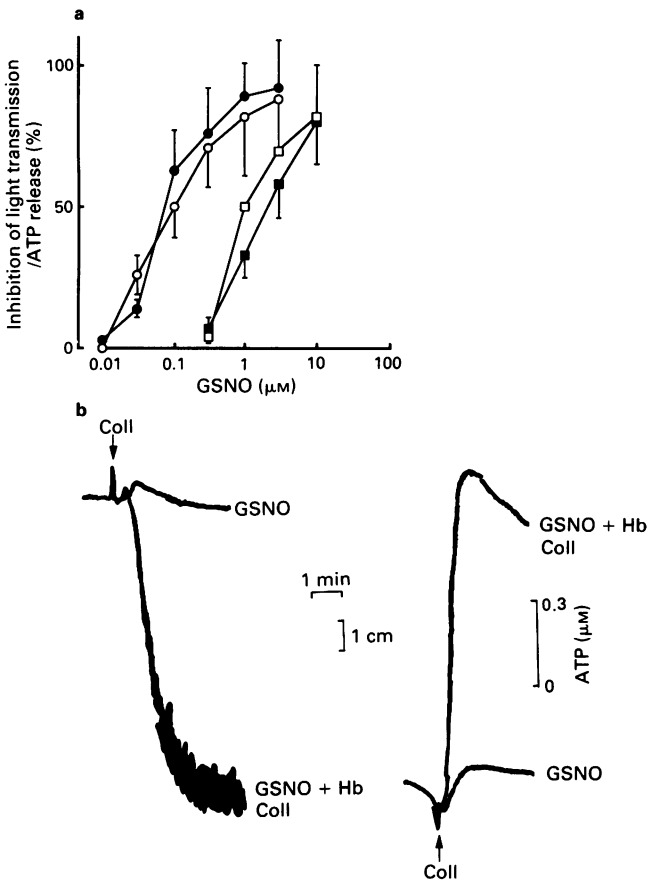

Figure 2 Inhibition of platelet aggregation and the release reaction in washed platelets (WP) and platelet-rich plasma (PRP) by S-nitroso-glutathione (GSNO) (a) and its reversal by haemoglobin (b). (a) Collagen (1 μg ml^{-1})-induced increase in light transmission in WP (○) and PRP (□) and ATP release in WP (●) and PRP (■) was inhibited by GSNO. Results are mean (± s.e.mean, vertical bars) of 3–5 experiments. (b) Haemoglobin (3 μM, GSNO + Hb) reversed the inhibitory effect of GSNO (1 μM) on collagen (1 μg ml^{-1}, Coll)-induced increases in light transmission (left hand tracings) and ATP release (right hand tracings). Tracings representative of 3 similar experiments.

Table 1 Inhibition of platelet activation by GSNO and other NO donors

Compounds	*Aggregation* IC_{50} (μM)	*ATP release*
Nitric oxide	0.36 ± 0.10	0.30 ± 0.09
S-nitroso-glutathione	0.12 ± 0.04	0.09 ± 0.04
S-nitroso-acetylpenicillamine	1.05 ± 0.27	0.79 ± 0.25
SIN-1	2.16 ± 0.41	3.41 ± 0.49

caused a dose-dependent fall in mean arterial blood pressure (data not shown).

ADP (1 μmol kg^{-1}, i.v.) caused a sub-maximal decrease in the free platelet number (27–34%) which recovered to control levels within 60 min. S-nitroso-glutathione (0.3 μmol kg^{-1} min^{-1}, i.v.) produced a small decrease in blood pressure (8 ± 3 mmHg) and inhibited significantly ($P = 0.0094$, $n = 3$) the ADP-induced fall in platelet number and its duration of action (Figure 7). These inhibitory effects were transient, since 1 h after the termination of the GSNO infusion, ADP induced a fall in platelet number similar to the control level (Figure 7).

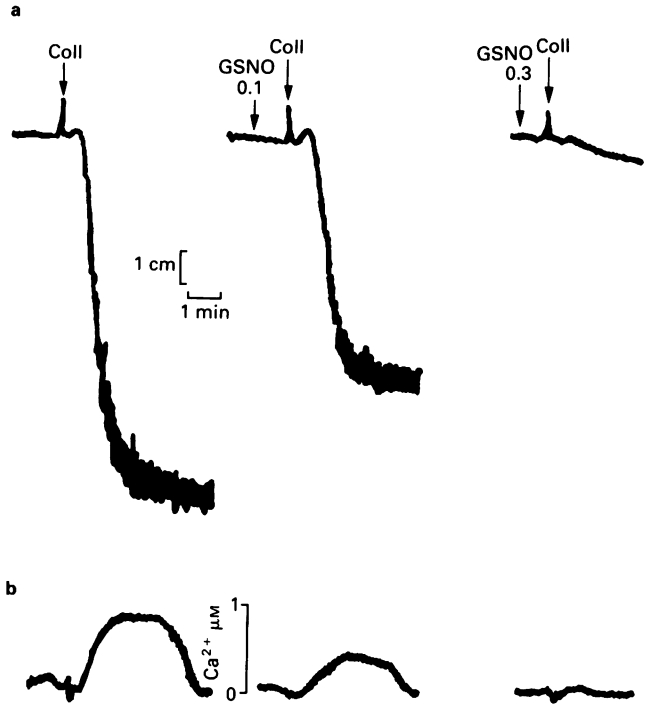

Figure 3 Inhibition of S-nitroso-glutathione (GSNO) of collagen-induced increase in $[Ca^{2+}]_i$ in aequorin-loaded platelets. Collagen (4 μg ml^{-1}, Coll)-induced increases in the light transmission (a) and $[Ca^{2+}]_i$ (b) were inhibited by GSNO (0.1 and 0.3 μM). Tracings representative of 3 similar experiments.

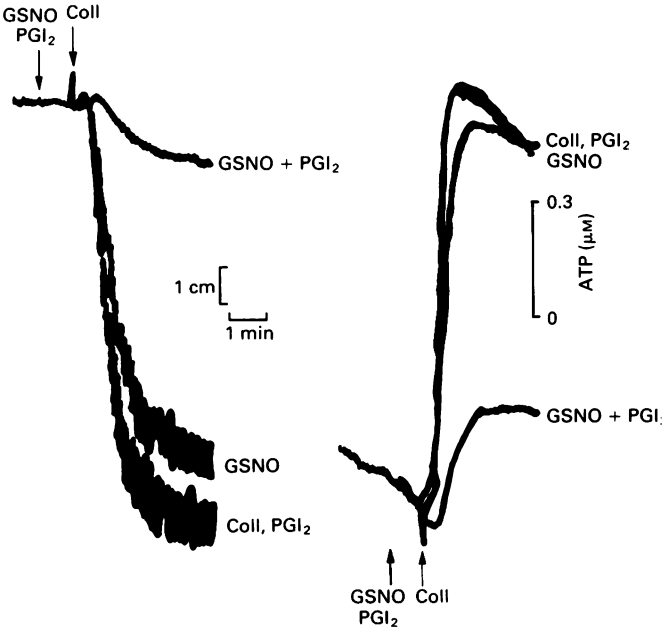

Figure 4 Interactions between S-nitroso-glutathione (GSNO) and prostacyclin as inhibitors of platelet aggregation. A subthreshold concentration of prostacyclin (PGI_2, 0.1 nM) and threshold concentration of GSNO (0.03 μM) acted synergistically (GSNO + PGI_2) to inhibit collagen (Coll, 1 μg ml^{-1})-induced increase in light transmission (left hand tracings) and ATP release (right hand tracings). Tracings representative of 3 similar experiments.

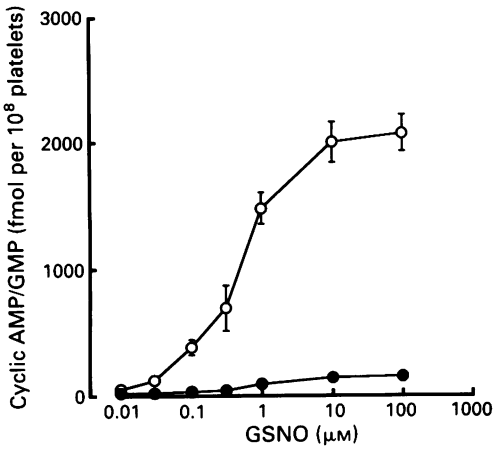

Figure 5 The increase in intraplatelet cyclic nucleotide levels by S-nitroso-glutathione (GSNO). Incubation of platelets with GSNO caused a concentration-dependent increase in cyclic GMP levels (○). A small but significant elevation of cyclic AMP (●) was observed at concentrations of GSNO > 0.3 μM. Each point is mean (± s.e.mean, vertical bars) of 3–4 experiments.

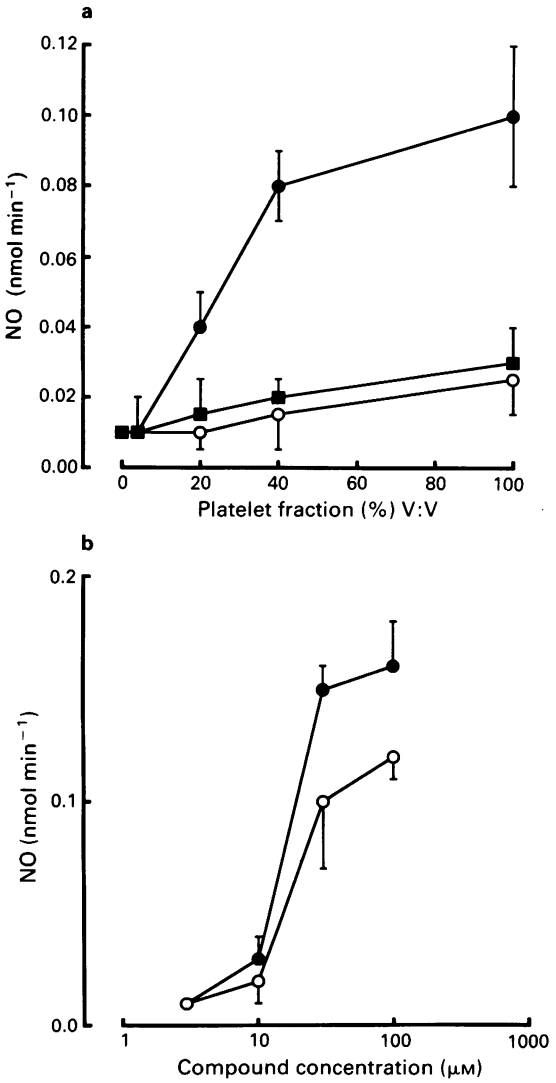

Figure 6 Release of NO from S-nitroso-glutathione (GSNO) by platelet fractions (a) or by reduced glutathione and ascorbic acid (b). (a) Spontaneous release of NO from GSNO (10 μM) was low (0.01 ± 0.01 nmol min^{-1}). The addition of platelet lysate (●) but not cytosol (○) or intact platelets (■) caused a significant increase in NO release from GSNO. (b) Reduced glutathione (●) or ascorbic acid (○) caused a concentration-dependent release of NO from GSNO (10 μM). Each point is mean (± s.e.mean, vertical bars) of 3 experiments.

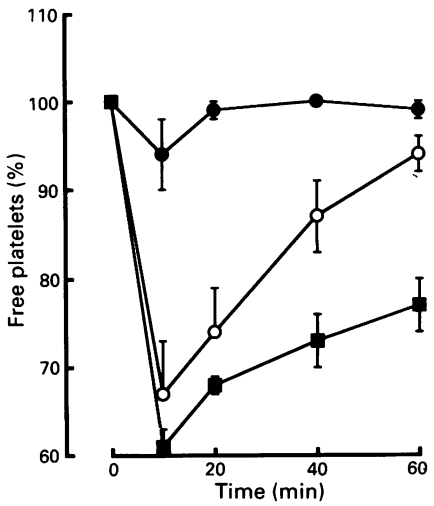

Figure 7 Inhibition of ADP-induced platelet aggregation in the conscious rat by S-nitroso-glutathione (GSNO). ADP (1 μmol kg^{-1}, i.a) caused a time-dependent decrease in free platelet numbers which recovered over the period of 60 min (○). Infusion of GSNO (0.3 μmol kg^{-1} min^{-1}, i.a) attenuated both the extent and duration of this effect (●). Following GSNO washout (1 h) the aggregating activity of ADP was restored (■). Each point is mean (± s.e.mean, vertical bars) of 3 experiments.

Discussion

We have shown that GSNO is a potent inhibitor of platelet adhesion to fibrillar collagen and to human endothelial cells (SGHEC-7). This action is similar to that reported for exogenous NO and endogenous, endothelium-derived NO (Radomski *et al.*, 1987a), confirming that this mediator is an important regulator of platelet adhesion to the vascular wall. In addition, GSNO was a potent inhibitor of collagen-induced aggregation *in vitro*. The inhibition of platelet aggregation *in vitro* has been demonstrated for S-nitrosothiols such as S-nitroso-cysteine, S-nitroso-acetylcysteine, SNAP and S-nitroso-β-D-thioglucose (Mellion *et al.*, 1983; Loscalzo, 1985; Mendelsohn *et al.*, 1990; Lieberman *et al.*, 1991). With the notable exception of SNAP, the remaining S-nitrosothiols are very unstable, which probably accounts for the reported variation in their antiaggregating potency, with IC_{50}s ranging from 6 nM (Loscalzo, 1985) to 20 μM (Lieberman *et al.*, 1991). In accordance with Park (1988), we found that the rate of NO release from GSNO dissolved in an aqueous solution was low but could be enhanced in the presence of thiols such as glutathione or ascorbate (Ignarro & Gruetter, 1980). In addition, the release of NO was significantly increased in the presence of platelet lysate but not by intact platelets or platelet cytosol. This suggests that the release of NO from GSNO may also be catalyzed by an enzyme present in platelet membranes which becomes activated following platelet stimulation. Interestingly, Kowaluk & Fung (1990) have recently suggested that an enzymic rather than spontaneous liberation of NO accounts for the relaxation of rat aortic rings by GSNO.

The biological half-life of GSNO on platelets was several times longer than that of NO (Radomski *et al.*, 1987b), although GSNO was only 2–3 times more potent as an inhibitor of aggregation. Moreover, SNAP and SIN-1, two known NO donors, were several times less potent than GSNO as inhibitors of aggregation. Thus, GSNO was a potent NO donor with a long-lasting platelet-inhibitory effect *in vitro*. Prostacyclin effectively potentiated the antiaggregating activity of GSNO, confirming well-recognized synergistic interactions between the agonists of adenylate and guanylate cyclase systems in platelets (Radomski & Moncada, 1991). Interestingly, we did not observe additive or

synergistic interactions between two tetrapeptide antagonists of the fibrinogen receptor (Pierschbacher & Ruoslahti, 1984; Gartner *et al.*, 1985) and GSNO. Whether the phenomenon is confined to the interactions between these agents *in vitro* or is a part of a general lack of synergy between agonists of guanylate cyclase, which are known to inhibit fibrinogen binding to platelets (Mendelsohn *et al.*, 1990) and inhibitors of the IIb/IIIa receptor remains to be investigated. Incubation of GSNO with WP resulted in a concentration-dependent increase in cyclic GMP and cyclic AMP levels. There was a good correlation between inhibition of platelet aggregation and increases in cyclic GMP caused by GSNO, suggesting that the biological effect of this nitrosothiol on platelets, like that of S-nitroso-cysteine (Lieberman *et al.*, 1991), depended on the stimulation of the soluble guanylate cyclase. Maurice & Haslam (1990) have recently suggested that the increases in cyclic AMP levels caused by cyclic GMP-induced inhibition of low K_m cyclic AMP phosphodiesterase contribute to the platelet-inhibitory activity of nitrovasodilators. Indeed, we have observed that GSNO increases cyclic AMP levels. However, this increase was significant only at concentrations $>0.3\,\mu M$ and failed to correlate with GSNO-induced inhibition of platelet aggregation.

S-nitroso-glutathione was also an effective inhibitor of ADP-induced platelet aggregation *in vivo*. This action was short-lasting and disappeared within 1 h following termination of the GSNO infusion which may be due to the distribution, metabolism and inactivation of GSNO by haemoglobin. It is important to note that a platelet-inhibitory dose of GSNO ($0.3\,\mu mol\,kg^{-1}\,min^{-1}$) had only a small effect on blood pressure. Thus, it may be possible to design platelet-selective NO donors based on the structure of stable S-nitrosothiols such as GSNO.

A low-molecular weight thiol, S-nitroso-cysteine has been suggested as the chemical identity of endothelium-derived relaxing factor (Myers *et al.*, 1990); however, biological generation of this compound has not been demonstrated. S-nitroso-glutathione is yet another candidate for an endogenous NO-carrying molecule. Indeed, glutathione, present in high amounts in the cell (Meister & Anderson, 1983), could be available to form GSNO and prolong the biological activity of NO. Endogenous NO has been shown to S-nitrosylate high-molecular weight thiol-containing proteins (Stamler *et al.*, 1992). Although the S-nitrosylation of low-molecular weight thiols by endogenous NO has not been yet demonstrated, it is also likely to occur. The conditions under which this might happen and the biological relevance of compounds that might act either as biological 'sinks' or carriers for NO remains to be investigated.

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Use of selective antagonists to dissociate the central cardiovascular and behavioural effects of tachykinins on NK_1 and NK_2 receptors in the rat

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1 The effects of intracerebroventricular (i.c.v.) pretreatment with selective NK_1 ((±)-CP 96,345), NK_{2a} (MEN 10,207; MEN 10,376) and NK_{2b} (R 396) tachykinin receptor antagonists on the cardiovascular and behavioural responses to i.c.v. substance P (SP) and neurokinin A (NKA) were studied in conscious rats.

2 SP and NKA (25 pmol) induced mean arterial blood pressure and heart rate increases of the same magnitude and duration. The cardiovascular responses to both peptides were accompanied by excessive face washing, sniffing, grooming and wet dog shakes.

3 The cardiovascular responses to SP but not to NKA were attenuated by pretreatment with a NK_1 receptor antagonist, (±)-CP 96,345. Of the behavioural responses, only face washing was significantly inhibited.

4 The cardiovascular and behavioural effects of NKA but not of SP were significantly attenuated by pretreatment with the selective NK_{2b} receptor antagonist, R 396.

5 The selective NK_{2a} receptor antagonists, MEN 10,207 and MEN 10,376, did not affect the cardiovascular and behavioural responses to either SP or NKA.

6 These results suggest, firstly, that the cardiovascular and behavioural effects of i.c.v. SP are mediated by NK_1 receptors; secondly, that NKA injected i.c.v. does not interact with NK_1 receptors but with another type of tachykinin receptor which may belong to the NK_{2b} subclass. These findings provide pharmacological evidence for the existence of functionally active NK_2 receptors in the rat brain.

Keywords: Substance P; neurokinin A; NK_1 and NK_2 receptors; tachykinin antagonists; blood pressure; heart rate; behaviour; central actions

Introduction

Tachykinins belong to a family of peptides characterized by the common carboxyl-terminal amino acid sequence Phe-X-Gly-Leu-Met-NH_2. Three distinct tachykinin receptors termed neurokinin$_1$ (NK_1), NK_2 and NK_3 have been characterized by pharmacological and ligand binding studies. These receptors are widely distributed in mammalian tissues and interact differently with the natural tachykinins; substance P (SP) being the preferential agonist for the NK_1 receptor, neurokinin A (NKA) for the NK_2 receptor and neurokinin B (NKB) for the NK_3 receptor (for review see Quirion & Dam, 1988; Helke *et al.*, 1990; Guard & Watson, 1991). Recently, the structures of the tachykinin receptors have been identified by molecular cloning in combination with electrophysiology (Nakanishi, 1991).

Tachykinins have been implicated in central cardiovascular regulation and in motor behaviour. In conscious rats, SP injected intracerebroventricularly (i.c.v.) or intrathecally (i.t.) increases mean arterial blood pressure and heart rate in a dose-dependent manner, mainly by activating the sympathoadrenal system (Unger *et al.*, 1985; 1988; Hassésian & Couture, 1989; Hassésian *et al.*, 1990). Increased sympathetic activity is also responsible for the cardiovascular effects of i.c.v. administered NKA while the pressor effects of i.c.v. NKB is due to the release of vasopressin from the posterior pituitary (Takano *et al.*, 1990).

The role of distinct central tachykinin receptors in mediating the cardiovascular and behavioural effects of tachykinins remains obscure. Until now, it is not clear which type of tachykinin receptor is responsible for the central effects of NKA. NKA and its precursor mRNA have been identified in several brain regions (Nawa *et al.*, 1983; 1984; Minamino *et al.*, 1984; Arai & Emson, 1986). However, the presence of NK_2 receptor binding sites in the rat brain remains controversial (Buck *et al.*, 1986; Bergstrom *et al.*, 1987; Mantyh *et al.*, 1989; Dam *et al.*, 1988; 1990; Quirion *et al.*, 1991).

Lack of potent and selective antagonists for each of the three tachykinin receptors has been one of the main reasons why the central mechanisms of these neuropeptides are so poorly understood. However, recently, considerable progress has been made in the characterization of peripheral NK_2 receptors. Two types of NK_2 receptors referred to as NK_{2a} and NK_{2b} in the present study have been characterized in peripheral organs thanks to the use of antagonists; indeed, the compound MEN 10,207 has been shown to be an antagonist of NKA on the endothelium-deprived rabbit isolated pulmonary artery while R 396 blocks the effects of NKA on the hamster isolated trachea (Maggi *et al.*, 1990). MEN 10,376 shares the same profile of affinity and selectivity as MEN 10,207 for the NK_{2a} receptor subtype but unlike MEN 10,207, MEN 10,376 is devoid of significant agonist activity (Maggi *et al.*, 1991). These studies provide pharmacological evidence for the existence of NK_2 receptor subtypes (Maggi *et al.*, 1990; 1991).

(±)-CP 96,345 has been shown to act as a competitive NK_1 receptor antagonist in various bioassays. This nonpeptide compound was able to block effectively SP induced salivation in rats, a phenomenon that is mediated by NK_1

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receptors, but did not affect the NKA-induced contraction of the rabbit isolated aorta, which represents a NK_2 receptor system. Binding studies have revealed that (±)-CP 96,345 binds to a single population of binding sites (NK_1) in guinea-pig brain but has no affinity for NK_2 or NK_3 sites (Snider *et al.*, 1991a,b; McLean *et al.*, 1991).

In the present study, the selective antagonists of the NK_{2a} (MEN 10,207, MEN 10,376) or NK_{2b} (R 396) receptors and the NK_1 receptor antagonist, (±)-CP 96,345, were used to characterize the tachykinin receptors that mediate the central cardiovascular and behavioural effects of SP and NKA in conscious rats.

Methods

Implantation of catheters and measurement of cardiovascular and behavioural parameters

The experiments were performed partly in Montreal, Canada (NK_2 receptor antagonists studies) and partly in Heidelberg, Germany (NK_1 antagonist studies). Male Wistar rats (Charles River, St. Constant, Quebec, Canada and Karl Thomae, Biberach, Riss, Germany) weighing 300 g were used. The animals were allowed free access to food and water and maintained on a 12 h light/dark cycle (lights on 06 h 00 min–18 h 00 min).

Rats were anaesthetized with an intraperitoneal (i.p.) injection of 65 mg kg^{-1} sodium pentobarbitone (Somnotol; MTC Pharmaceutical, Mississauga, Ont. Canada) and an i.c.v. cannula was implanted by use of a Kopf stereotaxic apparatus and fixed to the skull with dental cement. The coordinates were: 0.6 mm caudal to bregma, 1.3 mm lateral to the midline and 5.0 mm vertical from the skull surface. Five days later, the animals were anaesthetized again and a polyethylene catheter (PP-50) was inserted through the femoral artery into the abdominal aorta for measurement of blood pressure and heart rate. The catheter was filled with physiological saline containing heparin (50 i.u. ml^{-1}), passed through a subcutaneous tunnel and emerged at the back of the neck. Following surgery, rats were housed individually in a plastic cage with free access to food and water.

Experiments were conducted in a quiet room 48 h after the intravascular surgery on freely moving rats kept in their resident cage (40 × 23 × 20 cm). The arterial pressure was monitored through the intraarterial catheter with a Statham pressure transducer (P231D) while the heart rate was measured with a cardiac tachometer (model 7P4) and both parameters were displayed on a Grass Polygraph (model 79D) (Montreal) or with a Statham P23Db pressure transducer connected to a Gould Brush 2400 recorder (Heidelberg).

The behavioural activity was recorded over a 30 min period starting immediately with the i.c.v. injections. The frequency of the individual behavioural responses: face washing, hind limb grooming or biting, sniffing, rearing, digging and wet dog shakes, was determined according to the 15 s sampling procedure of Gipsen *et al.* (1975). A score 1 or 0 was given depending on whether the animals during 15 s period showed the specific type of behaviour or not. Summation of scores for 30 min gave the behavioural scores in each experiment. The maximal theoretical score was 120. However, only the data for face washing, hind limb grooming/biting and wet dog shakes are presented in the results. The complete behavioural data consist of the sum of all types of behavioural manifestations studied, namely face washing, hind limb grooming and biting, wet dog shakes, sniffing, rearing and digging.

Experimental protocols

Experiments started when the animal was in a resting state and basal mean arterial blood pressure (MAP) and heart rate (HR) were stable. On the first day of testing, the animals received randomly a single i.c.v. injection of either 25 pmol SP or NKA in a volume of 1 µl of artificial cerebrospinal fluid (CSF; composition in mM: NaCl 128.6, KCl 2.6, $MgCl_2$ 2.0 and $CaCl_2$ 1.4; pH adjusted to 7.2). The catheter was then flushed with 4 µl of CSF over a period of 20–30 s and the cardiovascular and behavioural responses were measured. The day after, one of the antagonists was given i.c.v. (1 µl of peptide solution flushed with 4 µl of CSF) 5 min before SP or NKA. Control animals were injected with 5 µl of CSF instead of SP or NKA. The intrinsic activity of the tachykinin receptor antagonists was tested in separate experiments. Baseline MAP and HR values were calculated 1 min before the injection of 25 pmol SP or NKA. At the end of each experiment, the position of the i.c.v. cannula was verified histologically after post mortem dissection.

Peptides and nonpeptides

(±)-CP 96,345 (the racemic mixture of (±)-*cis*-3-(2-methoxybenzylamino)-2-benzhydryl-quinuclidine) was obtained as a gift from Dr Jaw-Kang Chang, Peninsula Lab., Heidelberg, Germany and used at a dose of 5 nmol; MEN 10,207 (H-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Arg-NH_2) and MEN 10,376 (H-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Lys-NH_2) were kind gifts from Drs C.A. Maggi and P. Rovero at A. Menarini Pharmaceuticals, Florence, Italy and used at a dose of 650 pmol; R 396 (Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2) was synthesized in the laboratory of Dr D. Regoli at Sherbrooke University, Sherbrooke, Canada by conventional solid-phase methods and used at a dose of 650 pmol. The dose of (±)-CP 96,345 was chosen on the basis of its potency in displacing the labelled SP in the rat forebrain (Snider *et al.*, 1991a). The optimal doses of the selective NK_2 receptor antagonists were identified in preliminary experiments and those of SP and NKA in an earlier study (Itoi, Tschöpe, Jost, Culman, Lebrun, Stauss, Rettig and Unger, unpublished data). SP and NKA were purchased from Bachem, Bubendorf, Switzerland, and from Hükabel Scientific Ltd, Montréal, Canada. Stock solutions of MEN 10,207 (mol.wt = 1109.3), MEN 10,376 (mol.wt = 1067.3) and R 396 (mol.wt = 809.9) were made in dimethyl sulphoxide (DMSO; Fisher) and CSF was added to obtain the desired solution (the final solution contained a maximum of 1% of DMSO). (±)-CP 96,345 (conjugated with mandelate; mol.wt. = 412.6), SP and NKA were dissolved directly in CSF. The stock solutions (10 mg ml^{-1}) of peptides were divided into 100 µl aliquots and stored at − 20°C until used.

Statistical analysis of data

The results are expressed as means ± s.e.mean. Statistical differences were evaluated with Student's *t* test for paired samples. Time course effects were analysed with a two-way analysis of variance (ANOVA) in conjunction with Bonferroni confidence intervals. Only probability values (P) smaller than 0.05 were considered to be statistically significant.

Results

Both peptides, SP and NKA (25 pmol) injected i.c.v. evoked similar increases in MAP and HR which peaked within 5 min and returned gradually to pre-injection levels within 30 min (Tables 1 and 2). When compared to CSF values, both the pressor and HR responses to 25 pmol SP or NKA were significantly increased ($P < 0.001$) at 3 and 5 min post-injection. The cardiovascular responses to these peptides were accompanied by increased behavioural activity such as excessive face washing, hind limb grooming/biting and wet dog shakes (Table 3). These behavioural effects occurred at the same time as the cardiovascular responses and presented a similar time course. A similar i.c.v. injection of CSF (5 µl which contained 1% DMSO) was without appreciable effect

Table 1 Effects of selective tachykinin receptor antagonists on changes in mean arterial blood pressure (MAP) elicited by the i.c.v. injection of 25 pmol substance P (SP) or neurokinin A (NKA) in the conscious rat

				ΔMAP (mmHg) *Time (min) after SP or NKA injection*				
Antagonist	*Agonist*	n	*Baseline MAP*	3	5	10	20	30
–	CSF	8	99.8 ± 5.0	0.9 ± 0.8	0.5 ± 0.7	1.0 ± 0.4	−0.2 ± 0.8	−0.2 ± 0.5
–	SP	8	97.3 ± 7.0	16.4 ± 1.6	20.4 ± 2.7	2.0 ± 1.5	3.0 ± 2.5	−1.5 ± 0.8
(±)-CP 96,345 (5 nmol)	SP	8	99.7 ± 6.9	7.6 ± 2.3*	6.1 ± 3.3*	4.0 ± 4.5	1.8 ± 1.1	−4.0 ± 5.2
–	NKA	8	88.8 ± 4.2	12.4 ± 2.4	9.6 ± 1.8	5.1 ± 1.8	4.0 ± 1.0	3.3 ± 1.7
(±)-CP 96,345 (5 nmol)	NKA	8	90.6 ± 5.8	11.2 ± 2.2	10.0 ± 3.0	5.3 ± 3.0	4.5 ± 2.5	3.8 ± 3.2
–	SP	8	106.2 ± 5.7	15.4 ± 1.0	9.8 ± 1.0	4.2 ± 1.0	1.0 ± 0.5	−0.4 ± 0.7
MEN 10,376 (650 pmol)	SP	8	103.1 ± 5.6	13.5 ± 0.8	12.3 ± 1.1	2.9 ± 1.4	1.5 ± 1.1	−1.0 ± 0.6
–	NKA	8	98.1 ± 4.5	17.5 ± 1.1	12.7 ± 1.7	3.1 ± 0.8	−0.6 ± 0.5	−1.9 ± 0.7
MEN 10,376 (650 pmol)	NKA	8	103.1 ± 3.4	16.2 ± 2.3	12.4 ± 1.8	1.7 ± 1.0	−1.2 ± 1.1	−1.4 ± 0.6
–	SP	8	97.5 ± 6.8	13.6 ± 2.3	12.5 ± 1.2	6.7 ± 1.1	4.2 ± 1.1	−0.8 ± 0.8
R 396 (650 pmol)	SP	8	100.0 ± 7.8	10.9 ± 1.1	12.6 ± 1.4	6.7 ± 2.1	4.3 ± 1.3	2.4 ± 0.8
–	NKA	8	93.7 ± 5.9	15.4 ± 1.8	17.5 ± 2.3	9.4 ± 2.2	4.2 ± 2.0	2.7 ± 2.5
R 396 (650 pmol)	NKA	8	97.2 ± 6.3	3.9 ± 1.5**	−1.1 ± 2.1**	0.6 ± 1.1*	−0.8 ± 1.7	−1.9 ± 2.2

Values represent the means ± s.e.mean of (*n*) rats. Statistical significance of the differences between SP or NKA values in the presence and absence of the antagonist was calculated with a two-way ANOVA and is indicated by $*P < 0.05$; $**P < 0.001$.

Table 2 Effects of selective tachykinin receptor antagonists on changes in heart rate (HR) elicited by the i.c.v. injection of 25 pmol substance P (SP) or neurokinin A (NKA) in the conscious rat

				ΔHR (beats min^{-1}) *Time (min) after SP or NKA injection*				
Antagonist	*Agonist*	n	*Baseline HR*	3	5	10	20	30
–	CSF	8	300.0 ± 10.0	3.1 ± 6.7	3.2 ± 5.0	−1.4 ± 5.3	−1.8 ± 4.6	−1.3 ± 5.0
–	SP	8	307.1 ± 18.5	97.6 ± 10.7	94.6 ± 14.6	11.4 ± 10.4	−0.3 ± 2.6	−2.3 ± 9.6
(±)-CP 96,345 (5 nmol)	SP	8	316.9 ± 14.9	51.1 ± 16.3*	25.1 ± 12.1*	−8.8 ± 11.1	−20.3 ± 18.9	−23.3 ± 35.9
–	NKA	8	286.2 ± 19.4	93.3 ± 19.2	75.4 ± 25.7	14.8 ± 10.1	9.2 ± 11.6	10.4 ± 13.7
(±)-CP 96,345 (5 nmol)	NKA	8	290.0 ± 7.5	79.2 ± 11.9	52.5 ± 14.6	45.1 ± 17.5	35.0 ± 19.8	15.0 ± 19.3
–	SP	8	301.2 ± 23.9	191.2 ± 11.0	173.8 ± 16.4	50.0 ± 10.3	13.8 ± 5.8	−2.5 ± 5.5
MEN 10,376 (650 pmol)	SP	8	287.5 ± 22.1	172.5 ± 16.4	166.2 ± 14.6	61.2 ± 16.8	18.8 ± 9.3	6.2 ± 9.1
–	NKA	8	308.8 ± 26.6	175.0 ± 19.4	151.2 ± 21.8	40.0 ± 18.9	−2.5 ± 5.2	−5.0 ± 4.4
MEN 10,376 (650 pmol)	NKA	8	316.2 ± 33.5	172.9 ± 17.8	185.7 ± 12.3	62.9 ± 18.4	11.4 ± 4.9	0.0 ± 4.7
–	SP	8	306.2 ± 17.7	139.8 ± 11.4	150.5 ± 16.0	116.5 ± 21.3	74.0 ± 13.5	43.5 ± 10.1
R 396 (650 pmol)	SP	8	312.5 ± 17.3	118.6 ± 12.4	117.1 ± 10.2	100.0 ± 8.5	65.7 ± 11.3	47.1 ± 10.8
–	NKA	8	285.3 ± 17.9	83.1 ± 14.7	120.0 ± 11.5	66.2 ± 14.4	48.5 ± 14.6	30.0 ± 12.2
R 396 (650 pmol)	NKA	8	294.2 ± 20.5	30.0 ± 9.7*	15.0 ± 11.8**	6.7 ± 4.9*	0.1 ± 10.0*	1.7 ± 18.7

See footnote to Table 1.

on cardiovascular parameters (Tables 1 and 2) or on the behaviour (Table 3).

I.c.v. pretreatment with the selective NK_1 receptor antagonist, (±)-CP 96,345 (5 nmol), significantly ($P < 0.05$) attenuated the pressor and HR responses induced by 25 nmol SP at 3 and 5 min post-injection (Tables 1 and 2). (±)-CP 96,345 blocked the face washing but not the other behavioural responses to SP (Table 3). The same treatment with (±)-CP 96,345 failed, however, to affect the cardiovascular and behavioural responses to 25 pmol NKA (Tables 1–3). The NK_{2a} receptor antagonist, MEN 10,376 (650 pmol), had no significant effect on either the cardiovascular or

Table 3 Effects of selective tachykinin receptor antagonists on behavioural responses elicited by the i.c.v. injection of 25 pmol substance P (SP) or neurokinin A (NKA) in the conscious rat

Antagonist	*Agonist*	n	*Face washing*	*Grooming*	*Wet dog shake*	*Complete behaviour*
–	CSF	8	0.2 ± 0.1	0.0 ± 0.0	1.0 ± 0.9	6.3 ± 2.4
–	SP	8	12.2 ± 1.1	6.8 ± 1.4	2.6 ± 1.7	29.3 ± 3.3
(±)-CP 96,345	SP	8	4.8 ± 1.6*	6.2 ± 1.2	4.4 ± 1.3	22.5 ± 2.5
–	NKA	8	7.3 ± 1.9	5.2 ± 2.7	4.8 ± 1.3	32.4 ± 2.8
(±)-CP 96,345	NKA	8	12.1 ± 3.3	6.2 ± 0.9	4.0 ± 2.6	39.4 ± 10.2
–	SP	8	12.3 ± 1.2	5.5 ± 1.0	6.1 ± 1.4	66.9 ± 9.7
MEN 10,207	SP	8	10.0 ± 2.5	6.1 ± 1.7	5.2 ± 1.6	64.2 ± 10.5
–	NKA	8	13.1 ± 1.5	6.9 ± 0.9	9.1 ± 1.8	84.2 ± 5.0
MEN 10,207	NKA	8	12.2 ± 1.4	8.5 ± 1.9	7.7 ± 2.8	78.0 ± 6.4
–	SP	8	7.2 ± 1.4	4.8 ± 1.3	5.6 ± 0.8	64.7 ± 3.6
MEN 10,376	SP	8	6.4 ± 1.9	5.4 ± 1.7	5.0 ± 0.9	57.6 ± 11.2
–	NKA	8	11.0 ± 1.5	9.3 ± 1.6	11.5 ± 1.9	77.5 ± 5.4
MEN 10,376	NKA	8	10.8 ± 3.7	8.2 ± 1.8	9.8 ± 1.6	70.8 ± 8.1
–	SP	8	13.1 ± 1.7	6.5 ± 1.1	11.0 ± 2.6	88.2 ± 10.6
R 396	SP	8	7.3 ± 2.1	6.3 ± 2.5	13.1 ± 1.5	73.8 ± 10.2
–	NKA	8	12.9 ± 1.8	8.6 ± 1.1	8.6 ± 1.1	64.2 ± 6.0
R 396	NKA	8	5.0 ± 0.9*	2.6 ± 1.1*	6.0 ± 2.7	26.6 ± 6.0*

Values represent the frequency of the individual behavioural responses for 30 min and are indicated by the mean ± s.e.mean of (n) rats. *$P < 0.05$, antagonist-treated group as compared with its proper control using Student's t test for paired samples. See text for doses of antagonists.

behavioural responses to i.c.v. injection of either SP or NKA (Tables 1–3). The second NK_{2a} receptor antagonist, MEN 10,207 (650 pmol), was similarly ineffective against the cardiovascular (data not shown) and behavioural responses (Table 3) induced by SP or NKA.

The selective NK_{2b} receptor antagonist R 396 (650 pmol) failed to modify the cardiovascular and behavioural responses to i.c.v. SP (Tables 1–3). However, i.c.v. pretreatment with the selective NK_{2b} receptor antagonist R 396 (650 pmol) reduced the pressor response to NKA at 3, 5 and 10 min post-injection as well as the HR response at 3, 5, 10 and 20 min after NKA injection (Tables 1 and 2). The face washing, grooming and complete behaviour measured over a 30 min period after NKA injection were significantly antagonized by R 396. However, the latter antagonist had no significant effect on the NKA-induced wet dog shakes (Table 3).

The blocking effects of (±)-CP 96,345 and R 396 respectively on SP and NKA-induced cardiovascular changes and behavioural responses were completely reversed when the rats were tested again with the agonist alone 24 h later (data not shown). None of the tested antagonists had any apparent toxic actions and they all failed to cause significant changes on baseline blood pressure and heart rate (Tables 1 and 2). When given alone for a period of 30 min, these compounds were also devoid of intrinsic cardiovascular and behavioural activity (data not shown).

During the course of this study, we have also tested the compound MDL 28,564 (H-Asp-Ser-Phe-Val-Gly-Leu Ψ (CH_2 NH)Leu-NH_2), a pseudopeptide selective antagonist for the NK_{2b} receptor site (Buck *et al.*, 1990; Patacchini *et al.*, 1991). This antagonist was found to be highly toxic after i.c.v. injection in the conscious rat since at a dose of 650 pmol; 4/8 rats died within 15 min following a drastic fall of both blood pressure and heart rate. Hence MDL 28,564 is not useful for studying the NK_2 receptor in the CNS.

Discussion

Administration of SP into the lateral ventricle of conscious rats elicites an increase in mean arterial blood pressure and heart rate concurrently with increased locomotor activity, grooming behaviour and rearing. These behavioural responses are compatible with the reaction of rodents to nociceptive stimuli (Unger *et al.*, 1988). Similar cardiovascular responses can be induced by microinjections of SP into distinct hypothalamic areas suggesting that hypothalamic tachykinin receptors may mediate the central cardiovascular and behavioural effects observed after i.c.v. administration of tachykinins (Itoi *et al.*, 1991).

It is however very difficult to reach any conclusions regarding the exact localization of the tachykinin receptors that mediate the cardiovascular and behavioural responses to i.c.v. SP and NKA. Because of the fast onset of the response after i.c.v. administration of the peptides, one can speculate that these receptors may be localized in the circumventricular organs or in adjacent periventricular structures. A peripheral site of action is unlikely since i.v. injections of SP or NKA cause decreases in blood pressure secondary to systemic vasodilatation (Couture *et al.*, 1989). Additionally, selective activation of NK_2 receptors in the spinal cord failed to produce any cardiovascular changes in the conscious rat. The cardiovascular responses induced by the i.t. injection of tachykinins were ascribed to the activation of a NK_1 receptor in the spinal cord (Hassésian *et al.*, 1988). Thus, it is doubtful that the i.c.v. action of SP and NKA on the cardiovascular system is due to a diffusion of the peptide in the CSF down to the spinal cord. Furthermore, higher doses of intrathecal SP (at least 6.5 nmol) were required to induce similar cardiovascular changes in the conscious rat (Hassésian *et al.*, 1988).

As expected, the cardiovascular effects of i.c.v. SP were reduced after pretreatment of the animals with the selective NK_1 receptor antagonist, (±)-CP 96,345, but were not affected by pretreatment with selective NK_{2a} or NK_{2b} receptor antagonists. The NK_1 antagonist, (±)-CP 96,345, attenuated the cardiovascular response and only the face washing. This may suggest that the cardiovascular and behavioural effects of i.c.v. SP can be mediated by different brain structures or NK_1 receptor subclasses which may not be equally influenced by this NK_1 receptor antagonist. Since (±)-CP 96,345 is a nonpeptide compound that crosses the blood brain barrier (Picard and Couture, unpublished observations), the diffusion and distribution of this antagonist in the periventricular areas after i.c.v. administration may differ

significantly from the diffusion of SP and this may account for the absence of effect of the antagonist.

The dose of (±)-CP 96,345 used to antagonize SP was relatively high. This is in agreement with the low binding potency of this compound to NK_1 receptors in the rat brain compared to the guinea-pig, human, rabbit or bovine brains (Snider *et al.*, 1991a; Gitter *et al.*, 1991). Even these high doses of (±)-CP 96,345 were devoid of any agonistic activity with respect to changes in blood pressure, heart rate or behaviour. It has been reported that (±)-CP 96,345, albeit at high concentrations, displays non-specific effects in peripheral tissues *in vitro* (Boyle *et al.*, 1991) and also binds with high affinity to the diltiazem site on L-type calcium channels in both rat and guinea-pig CNS (Guard *et al.*, unpublished observations). In the present study, the cardiovascular and behavioural effects of NKA were not affected by (±)-CP 96,345 which suggests a specific action on NK_1 receptors although non-tachykinin receptor-mediated actions of the compound cannot be completely excluded.

The magnitude and duration of the cardiovascular response to i.c.v. NKA were similar to those of SP. Our results are in agreement with the finding of Takano *et al.* (1990), who however used anaesthetized animals and higher doses of tachykinin peptides. Of all the selective tachykinin receptor antagonists tested, only the NK_{2b} receptor antagonist R 396 attenuated the cardiovascular effect of i.c.v. NKA. The two NK_{2a} receptor antagonists MEN 10,207 and MEN 10,376 did not affect the cardiovascular response to i.c.v. NKA. The selective NK_{2b} receptor antagonist R 396 (Maggi *et al.*, 1990; Patacchini *et al.*, 1991) affected in a similar way both the cardiovascular and the behavioural responses to NKA, except that of wet dog shakes. Wet dog shakes was claimed to be a typical behavioural manifestation induced by the activation of central NK_3 receptors (Elliott & Iversen, 1986; Stoessl *et al.*, 1988) which can be stimulated by NKA (Regoli *et al.*, 1988).

The pressor response to i.c.v. SP or NKA is brought about by sympathoadrenal activation whereas increased release of vasopressin is responsible for an increase in blood pressure after i.c.v. NKB (Takano *et al.*, 1990). The NK_1 antagonist, (±)-CP 96,345, failed to modify the cardiovascular response to NKA which was, however, blocked by R 396, a fairly selective antagonist of NK_2 receptors that is weakly active on NK_3 receptors (Maggi *et al.*, 1990). Thus, the central effects of NKA are unlikely to be mediated by either NK_1 or NK_3 receptors. Our present data strongly suggest that the presence of a discrete population of functionally active NK_2 receptors in the brain which may belong to the NK_{2b} receptor subclass (Maggi *et al.*, 1991; van Giersbergen, 1990). Hence, the present functional study sheds light on the conflicting results regarding the presence of NK_2 receptor binding sites in the CNS (Buck *et al.*, 1986; Saffroy *et al.*, 1987; Mantyh *et al.*, 1989; Dam *et al.*, 1988; 1990; Quirion *et al.*, 1991; Dietl & Palacios, 1991).

In conclusion, selective and potent antagonists of NK_1 and NK_2 receptors have been used to characterize the receptor subtypes which are responsible for the central cardiovascular and behavioural effects of SP and NKA. Although the integrated cardiovascular and behavioural response patterns to both peptides injected i.c.v. were identical, our findings show that the response to SP was mediated by NK_1 receptors and that to NKA by NK_2 receptors which belong to NK_{2b} subclass (similar to that found in the hamster isolated trachea). Hence, our data provide evidence for the existence of distinct population of functionally active NK_1 and NK_2 receptors in the adult rat brain.

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Nitric oxide is involved in 5-HT-induced relaxations of the guinea-pig colon ascendens *in vitro*

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1 In the guinea-pig colon ascendens, 5-hydroxytryptamine (5-HT) induces contractions, mediated by 5-HT_2, 5-HT_3 and 5-HT_4 receptors, and relaxations, through a 5-HT_1 receptor subtype, that triggers the release of an inhibitory neurotransmitter. Nitric oxide (NO) is one of the main candidates of NANC inhibitory neurotransmission in the gut. The aim of this study was to establish whether NO is involved in 5-HT-induced relaxations of the guinea-pig colon ascendens.

2 Antagonists to block the contractile responses to 5-HT *via* 5-HT_2, 5-HT_3 and 5-HT_4 receptors were present throughout the experiments and methacholine was administered to precontract the strips. Under these conditions, 5-HT concentration-dependently induced relaxations from 10 nM onwards (EC_{50} = 258 (172–387) nM). The relaxations were inhibited by metergoline (10 nM) and methiothepine (100 nM) and abolished by tetrodotoxin (TTX, 320 nM). Guanethidine (3.2 μM) did not affect them.

3 N^G-nitro-L-arginine (L-NNA) inhibited the responses to 5-HT (IC_{50} = 18.7 (13.3–26.3) μM); at the highest 5-HT concentration a maximum inhibition of about 75% was observed with 320 μM L-NNA. This inhibition was reversed with L-arginine. Relaxations to glyceryl trinitrate (GTN) were not inhibited by L-NNA.

4 Haemoglobin (32 μM) inhibited the relaxations to 5-HT and GTN, but not those to isoprenaline (Iso). Methylene blue (10 μM) inhibited the relaxations to 5-HT but did not affect those caused by GTN or Iso.

5 It is concluded that 5-HT induces relaxations that involve NO. We also confirmed that 5-HT induces these relaxations via (a) 5-HT_1 receptor subtype(s), located on neurones.

Keywords: Guinea-pig colon ascendens; longitudinal muscle; nitric oxide; methylene blue; haemoglobin; N^G-nitro-L-arginine; 5-hydroxytryptamine; 5-HT_1-receptors

Introduction

Inhibitory enteric neurones play an important role in gut motility. They are involved in reflex relaxation of the lower oesophageal and the internal anal sphincter, the receptive and adaptive relaxation of the stomach and the descending inhibition during intestinal peristalsis. The exact mechanisms underlying these phenomena remain to be elucidated.

In isolated colon ascendens preparations of the guinea-pig, 5-hydroxytryptamine (5-HT), a neurotransmitter of the enteric nerves, induces both contractions and relaxations (Costa & Furness, 1979). It has previously been shown that the contractions are mediated *via* 5-HT_2, 5-HT_3 and 5-HT_4 receptors (Briejer *et al.*, 1991; Sato *et al.*, 1991; Elswood *et al.*, 1991), and the relaxations through a 5-HT-receptor different from 5-HT_2, 5-HT_3 or 5-HT_4 receptors (Briejer *et al.*, 1991; Sato *et al.*, 1991) but sensitive to 5-HT_1 receptor antagonists (Kojima, 1991; Elswood & Bunce, 1992). Since TTX abolished these relaxations, they were presumed to be mediated by nerves (Gershon, 1967). In the guinea-pig proximal (Costa & Furness, 1979) and distal colon (Onori *et al.*, 1984), 5-HT-evoked relaxations were not affected by incubation with guanethidine, which suggests that the relaxations are mediated by the intrinsic NANC (non-adrenergic, non-cholinergic) inhibitory neurones. Several compounds have been proposed as a neurotransmitter of these NANC inhibitory neurones: ATP (Burnstock, 1972), vasoactive intestinal peptide (VIP) (Makhlouf, 1985) and nitric oxide (NO) (Moncada *et al.*, 1991).

Evidence for NO as a transmitter of inhibitory motor neurones in the gastrointestinal tract have been obtained by several authors in various tissues: canine ileocolonic junction (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1990), canine terminal ileum (Bogers *et al.*, 1991), guinea-pig ileum (Osthaus & Galligan, 1992), guinea-pig stomach (Desai *et al.*, 1991; Meulemans *et al.*, 1992), rat gastric fundus (Li & Rand, 1990), rat stomach (Lefebvre *et al.*, 1992), opossum internal anal sphincter (Rattan & Chadker, 1992; Tøttrup *et al.*, 1992), human ileum (Maggi *et al.*, 1991), human sigmoid colon and internal anal sphincter (Burleigh, 1992).

Much of this evidence is based on the use of compounds that selectively modify the 'nitrergic' neurotransmission. NO is formed from L-arginine by NO-synthase (Palmer *et al.*, 1988), which has been immunohistochemically demonstrated in the myenteric plexus of the rat (Bredt *et al.*, 1990). NO readily diffuses through the presynaptic membrane into the target cell, where it is thought to activate the soluble guanylate cyclase, thus giving rise to increased guanosine 3':5'-cyclic monophosphate (cyclic GMP)-levels (Arnold *et al.*, 1977; Feelish & Noack, 1987a; Moncada *et al.*, 1991; McCall & Vallance, 1992). This L-arginine-NO-pathway can be inhibited at several levels. Analogues of L-arginine inhibit the synthesis of NO (Moncada *et al.*, 1991; McCall & Vallance, 1992). N^G-nitro-L-arginine has been shown to be a potent inhibitor of the NO-synthesis (Moore *et al.*, 1990; Mülsch & Busse, 1990). Haemoglobin captures NO after it has diffused out of the neurone, which will prevent it from diffusing into the smooth muscle cells (Martin *et al.*, 1985;

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Kelm & Schrader, 1990). The dye and oxidant methylene blue selectivity inhibits the target of NO, the soluble guanylate cyclase (Gruetter *et al.*, 1981; Martin *et al.*, 1985) which is present in the smooth muscle cells.

The aim of this study was to establish whether NO is involved in relaxations of the guinea-pig colon ascendens evoked by 5-HT.

Methods

Tissue preparation

Dunkin-Hartley guinea-pigs of either sex, weighing 400–600 g, were killed by cervical dislocation. The ascending colon was removed, and the luminal contents were washed out with De Jalon solution (mM: KCl 5.6, $CaCl_2$ 0.5, $NaHCO_3$ 6.0, NaCl 155, glucose 2.78). The mesentery was carefully removed. Starting at the proximal end, about 1 cm distal from the caecum, the colon was divided into four segments of circa 2.5 cm. These intact segments were individually mounted vertically for isotonic measurement into a tissue bath containing 20 ml De Jalon solution. This solution was kept at 37°C and gassed with 95% O_2:5% CO_2. The strips were subjected to a preload of 2 g and allowed to stabilize for 30 min. Ketanserin (320 nM) and tropisetron (ICS 205–930, 3.2 μM) were continuously present in the De Jalon solution during all experiments to block contractile responses mediated by 5-HT_2, 5-HT_3 and 5-HT_4 receptors. After stabilization, the response of the longitudinal muscle to 320 nM methacholine was measured. After washing and 10 min stabilization, the procedure was repeated in order to stabilize the response of the tissue.

Protocols

For the construction of concentration-response curves, agonists and antagonists were added directly to the tissue bath (added volume $\leqslant 3\%$ of tissue bath volume). Methacholine (320 nM) was added to the bath in order to precontract the muscle. The mean decrease in length was 5.78 ± 0.24 mm.

5-Hydroxytryptamine Since pilot experiments had revealed that relaxations to 5-HT are subject to tachyphylaxis, non-cumulative concentration-response curves were established, beginning 10 min after the administration of methacholine. The size of the 5-HT-induced relaxation was measured as the maximum relaxation induced during an incubation period of approximately 2 min. Then, the agonists were washed out by replacing the bathing fluid twice. Stabilization of 10 min was allowed. This 20 min dosing cycle was repeated, applying ascending concentrations of 5-HT. The response to repetitive administration of methacholine was perfectly stable throughout the experiment. On each segment, first a concentration-response curve to 5-HT was constructed in the absence of an antagonist (ketanserin and tropisetron present) (= first series). Consecutively, the measurements were repeated in the presence of an antagonist (= second series). Antagonists were added 10 min prior to the methacholine-induced precontraction, and were re-added directly after each washout. The antagonists had no effect on the methacholine-induced contractions, except for methiothepin. When L-arginine was tested against N^G-nitro-L-arginine, they were added together. Four segments were taken per animal; each fourth strip was used as a control, i.e. vehicle control was added during the second series. In these control strips, the concentration-response curve to 5-HT was perfectly reproducible as compared to the curves of the first series. All responses were expressed as a percentage of the relaxation induced by 5-HT 10 μM of the first series for each individual segment. 5-HT (10 μM) induced relaxations of 3.8 ± 0.2 mm ($n = 32$; mean ± s.e.mean).

Glyceryl trinitrate and isoprenaline For the construction of concentration-response curves to glyceryl trinitrate and isoprenaline, the compounds were added in a cumulative fashion, 10 min after precontraction to methacholine (320 nM). After washing and stabilization for 30 min (washing every 10 min), a second concentration-response curve was established in the presence of an antagonist. The responses induced by glyceryl trinitrate or isoprenaline were expressed relative to the response to either glyceryl trinitrate 10 μM or isoprenaline 3.2 μM of the first series for each segment. The relaxations induced by these concentrations were respectively 7.6 ± 0.5 mm ($n = 18$) and 10.5 ± 1.0 mm ($n = 12$).

Chemicals

Methiothepin maleate (Hoffman-La Roche, Switzerland), methacholine HCl (E. Merck, Germany), 5-hydroxytryptamine HCl, tetrodotoxin (Serva, Germany), bovine haemoglobin (Sigma, Belgium), glyceryl trinitrate (Federa, Belgium), metergoline (Gruppo Montedison Farmitalia, Italy), isoprenaline tartrate, methylene blue, N^G-nitro-L-arginine and L-arginine (Janssen Chimica, Belgium) were added to the tissue bath solution in volumes less than 3% of the bath volumes. Ketanserin tartrate (Janssen Pharmaceutica, Belgium) and tropisetron HCl (ICS 205–930; Sandoz, Switzerland) were continuously present in the De Jalon solution. All compounds were dissolved in distilled water, except for 5-hydroxytryptamine (stock containing ascorbic acid 250 nM). This vehicle had no effect on the tone of the strips. Glyceryl trinitrate was further diluted with distilled water from a stock solution of 1% in ethanol. The solution of haemoglobin was always freshly prepared, kept on ice during the experiment and protected from light. The stock solution of TTX was prepared in advance and kept frozen (−25°C) in aliquots of 100 μM. It was kept on ice during the experiment.

Statistical analysis

For graphical representation means ± standard error of the means were calculated. EC_{50} and IC_{50} values were determined by linear regression analysis. Differences between mean values were tested by the two-tailed Student's t test for unpaired observations. Values of $P < 0.05$ were considered to be statistically different.

Results

5-HT induced concentration-dependent relaxations from 10 nM onwards (Figure 1). The relaxations were fast in onset. In about 1 out of 3 preparations, the relaxations induced by 5-HT in the concentration-range of 320 nM to 3.2 μM were multiphasic, i.e. an initial relaxation was followed by a quick

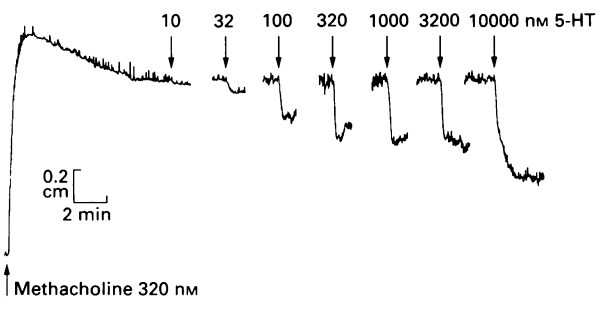

Figure 1 Tracing of relaxations of the guinea-pig colon ascendens evoked by non-cumulative concentrations of 5-hydroxytryptamine (5-HT). The preparation was precontracted by incubation with methacholine, and antagonists were continuously present to block contractile responses elicited *via* 5-HT_2, 5-HT_3 and 5-HT_4 receptors.

small contraction and consecutively a larger, more sustained relaxation. The concentration-response curve to control 5-HT appeared non-monophasic, starting a second phase at about 1.0 μM (Figure 2). The EC_{50} value calculated over the entire concentration-response curve to 5-HT was 258 (172–387) nM. TTX (320 nM) contracted the strips (18.4 ± 3.8% ($n = 7$) of the methacholine-induced contractions), and it abolished the relaxations to 5-HT, 10 μM ($n = 6$, not shown). The 5-HT_1 receptor antagonist, metergoline (10 nM), inhibited the relaxations to 5-HT (Figure 2). Methiothepin (100 nM) also inhibited the relaxations to 5-HT (by about 45–70% depending on the applied 5-HT concentration) but it inhibited the methacholine-induced contractions as well (by about 25%). Guanethidine (3.2 μM) did not affect the concentration-response curve to 5-HT ($n = 4$, results not shown).

NO-antagonists

N^G-nitro-L-arginine concentration-dependently depressed the concentration response curve to 5-HT, the inhibitory effect being statistically significant from 10 μM N^G-nitro-L-arginine onwards (Figure 3). The IC_{50} of the inhibitory effects of N^G-nitro-L-arginine against 5-HT-induced relaxations was 18.7 (13.3–26.3) μM (calculated at 5-HT 320 nM). N^G-nitro-L-arginine 320 μM abolished relaxations to 5-HT up to 320 nM. However, the relaxations caused by 5-HT > 320 nM were not completely blocked: about 25% of the control response remained (Figure 3). The high concentrations of N^G-nitro-L-arginine contracted the preparations slightly (27.2 ± 4.5% ($n = 6$) of the methacholine-induced contractions). L-Arginine (100 μM) did not affect the concentration-response curve to 5-HT ($n = 3$, results not shown). L-Arginine (320 μM) completely reversed the inhibition by N^G-nitro-L-arginine (10 μM) of 5-HT (100 nM)-induced relaxations ($n = 6$, results not shown).

Glyceryl trinitrate concentration-dependently induced relaxations from 3.2 nM onwards (Figure 4) that were fast in onset. TTX (320 nM), N^G-nitro-L-arginine (100 μM) and L-arginine (100 μM) did not inhibit the responses to glyceryl trinitrate (Figure 4).

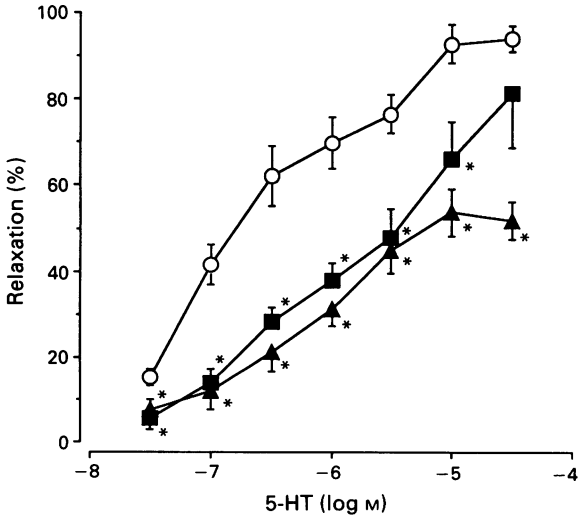

Figure 2 Concentration-response curves to 5-hydroxytryptamine (5-HT): control (○), or in the presence of metergoline 10 nM (■) or methiothepin 100 nM (▲). The preparation was precontracted by incubation with methacholine, and antagonists were continuously present to block contractile responses elicited *via* 5-HT_2, 5-HT_3 and 5-HT_4 receptors. Values ($n = 6$) that are significantly different from control values ($P < 0.05$) are marked with an asterisk. The relaxations are expressed as a percentage of the relaxations of 5-HT (10 μM).

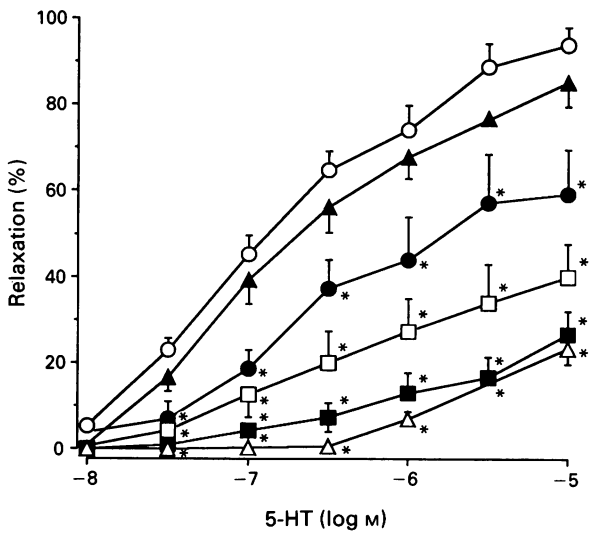

Figure 3 Concentration-response curves to 5-hydroxytryptamine (5-HT): control (○), or in the presence of N^G-nitro-L-arginine 3.2 μM (▲), 10 μM (●), 32 μM (□), 100 μM (■) and 320 μM (△). The preparation was precontracted by incubation with methacholine, and antagonists were continuously present to block contractile responses elicited *via* 5-HT_2, 5-HT_3 and 5-HT_4 receptors. Values (all $n = 6$; control $n = 12$) that are significantly different from control values ($P < 0.05$) are marked with an asterisk. The relaxations are expressed as a percentage of the relaxations of 5-HT (10 μM).

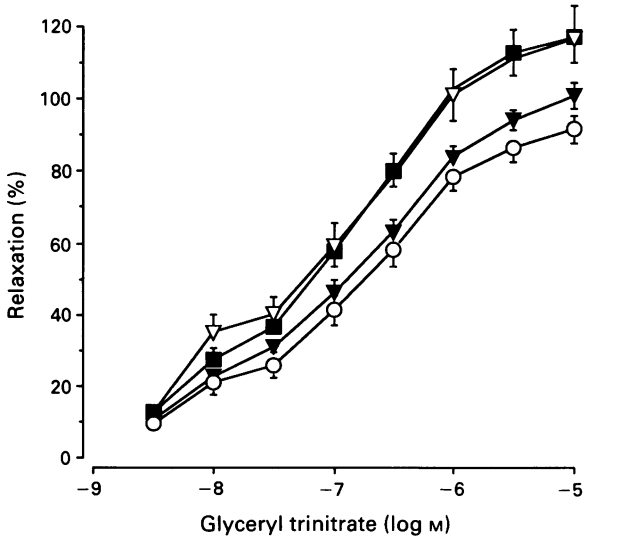

Figure 4 Concentration-response curves to glyceryl trinitrate in the absence of an antagonist (○), or in the presence of L-arginine 100 μM (▼), N^G-nitro-L-arginine 100 μM (■) or tetrodotoxin 320 nM (▽). The preparation was precontracted by incubation with methacholine, and antagonists were continuously present to block contractile responses elicited *via* 5-HT_2, 5-HT_3 and 5-HT_4 receptors. All values $n = 6$. The relaxations are expressed as a percentage of the relaxations to glyceryl trinitrate (10 μM).

Haemoglobin 10 μM tended to inhibit the 5-HT-induced relaxations, whereas haemoglobin 32 μM significantly inhibited them (Figure 5). Isoprenaline relaxed the preparations from 1 nM onwards. The relaxations were not fast in onset. Haemoglobin 32 μM did not affect the concentration-response curves to isoprenaline (Figure 5). In contrast, it depressed the concentration-response curve to glyceryl trinitrate (Figure 5). Haemoglobin slightly contracted the muscle (4.6 ± 1.6% of

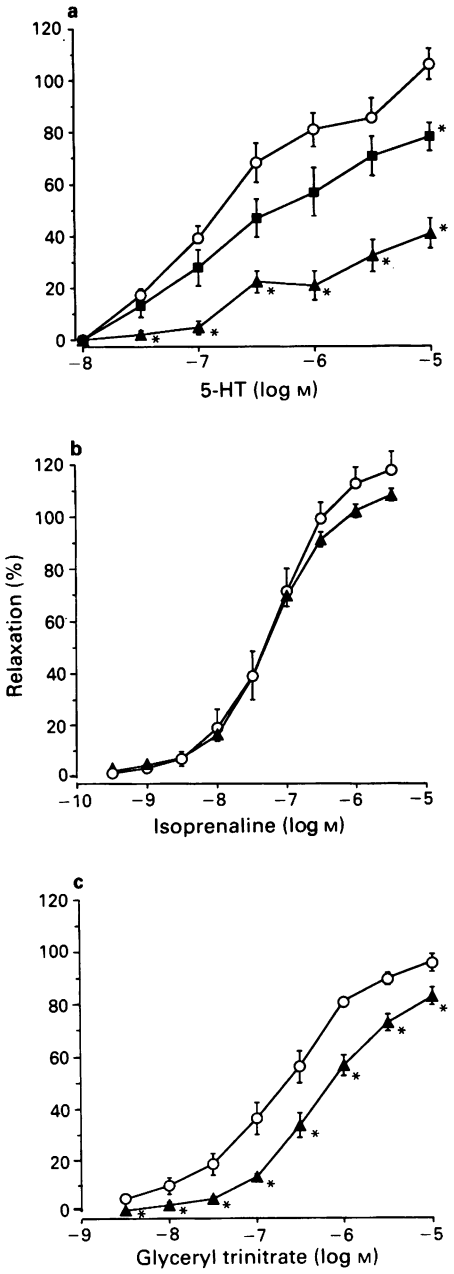

Figure 5 Concentration-response curves to 5-hydroxytryptamine (5-HT) (a), isoprenaline (b) and glyceryl trinitrate (c): controls (○), or in the presence of haemoglobin 10 μM (■) and 32 μM (▲). The preparation was precontracted by incubation with methacholine, and antagonists were continuously present to block contractile responses elicited *via* 5-HT_2, 5-HT_3 and 5-HT_4 receptors. Values ($n = 6$) that are significantly different from control values ($P < 0.05$) are marked with an asterisk. The relaxations to 5-HT, isoprenaline or glyceryl trinitrate are expressed as a percentage of the relaxations of 5-HT (10 μM), isoprenaline (3.2 μM) or glyceryl trinitrate (10 μM) respectively.

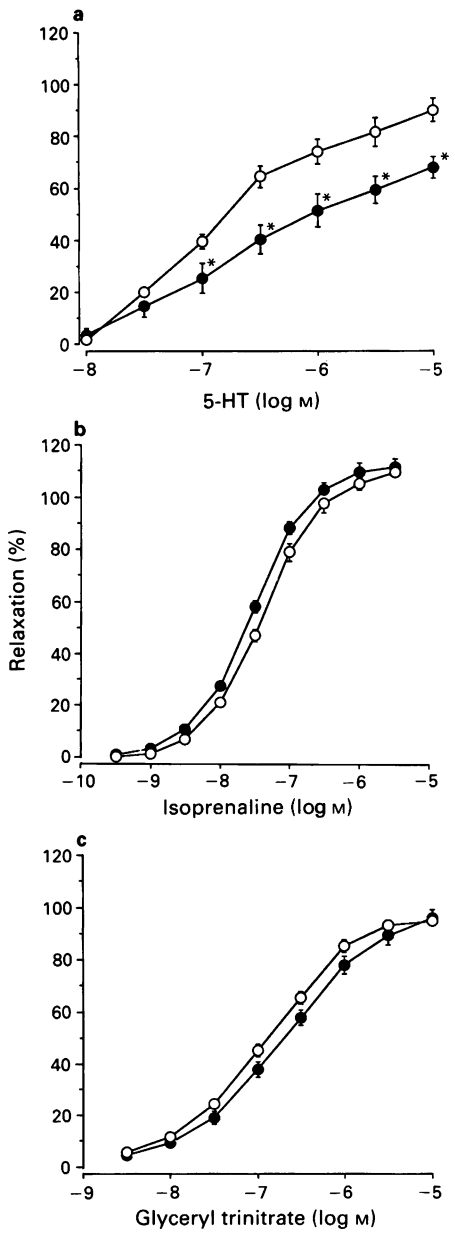

Figure 6 Concentration-response curves to 5-hydroxytryptamine (5-HT) (a), isoprenaline (b) and glyceryl trinitrate (c): controls (○), or in the presence of methylene blue 10 μM (●). The preparation was precontracted by incubation with methacholine, and antagonists were continuously present to block contractile responses elicited *via* 5-HT_2, 5-HT_3 and 5-HT_4 receptors. Values ($n = 6$) that are significantly different from control values ($P < 0.05$) are marked with an asterisk. The relaxations to 5-HT, isoprenaline or glyceryl trinitrate are expressed as a percentage of the relaxations of 5-HT (10 μM), isoprenaline (3.2 μM) or glyceryl trinitrate (10 μM) respectively.

methacholine-induced contractions, $n = 10$). Methylene blue (10 μM) inhibited the responses to 5-HT from 100 nM onwards (Figure 6). The concentration-response curves to isoprenaline or glyceryl trinitrate however were not affected (Figure 6). Methylene blue (10 μM) contracted the muscle slightly (6.2 ± 2.1% of the methacholine-induced contractions, $n = 6$). Higher concentrations of methylene blue could not be tested because they markedly inhibited the methacholine-induced contractions.

Discussion

The selective 5-HT_1 receptor antagonists, methiothepin and metergoline, both inhibited the relaxations caused by 5-HT. The selective 5-HT_2 receptor antagonist ketanserin and the 5-HT_3 and 5-HT_4 receptor antagonist, tropisetron, were present throughout our experiments. It therefore seems likely that, according to the criteria for 5-HT receptor classification as proposed by Bradley *et al.* (1986), the relaxations to 5-HT are mediated *via* 5-HT_1 receptors. Kojima (1991) and Elswood & Bunce (1992) also found a 5-HT_1 receptor to be

involved in 5-HT-induced relaxations in the guinea-pig colon. It was however not established which 5-HT_1 receptor subtype is concerned. In intestinal tissues of the guinea-pig, rat and rabbit, receptors with 5-HT_{1A} (Fozard & Kilbinger, 1985; Matsuyama *et al.*, 1990; Wade *et al.*,; 1991; Galligan, 1992), putative 5-HT_{1P} (Mawe *et al.*, 1986; Branchek *et al.*, 1988; Wade *et al.*, 1991), 5-HT_{1C} and 5-HT_{1D} (Baez *et al.*, 1991; Kalkman & Fozard, 1991) characteristics have been demonstrated. Further studies are required to elucidate which 5-HT_1 receptor subtype is involved in the 5-HT-induced relaxations of the guinea-pig colon.

TTX abolished relaxant responses to 5-HT, which confirms that nerves are involved. Since guanethidine did not affect the 5-HT-induced relaxations (Costa & Furness, 1979; present results), it is not likely that noradrenaline is the inhibitory neurotransmitter involved. In the canine terminal ileum and ileocolonic junction, it has been demonstrated that 5-HT-induced relaxations are mediated by NO (Bogers *et al.*, 1991). In our study, evidence was obtained which strongly suggests that NO is involved in 5-HT-induced relaxations in the guinea-pig colon ascendens as well. This evidence consists of three sets of observations.

Firstly, the L-arginine analogue, N^G-nitro-L-arginine, which is known to be a potent inhibitor of NO-synthase (Moore *et al.*, 1990; Mülsch & Busse, 1990), inhibited the relaxations to 5-HT but not those to glyceryl trinitrate. Furthermore, the inhibition could be reversed by L-arginine. These data suggest that NO is involved in the 5-HT-induced relaxations.

Secondly, both the relaxations to glyceryl trinitrate and 5-HT but not those to isoprenaline were inhibited by haemoglobin. Although the exact mechanism of action is still under debate, glyceryl trinitrate is thought to react with thiol groups of co-factors (cysteine) of the soluble guanylate cyclase, which yields the generation of NO (Ignarro *et al.*, 1981; Feelish *et al.*, 1988). NO in turn activates the soluble guanylate cyclase to increase the intracellular cyclic GMP content (Feelish & Noack, 1987a,b). Haemoglobin is a scavenger of NO (Martin *et al.*, 1985; Kelm & Schrader, 1990). It has been shown that haemoglobin does not bind or inactivate glyceryl trinitrate and that it does not affect the reaction between glyceryl trinitrate and the thiols (Martin *et al.*, 1986; Feelish & Noack, 1987b). In our experiments, neither TTX nor N^G-nitro-L-arginine or L-arginine inhibited the relaxations to glyceryl trinitrate, which is in good agreement with the above mechanism of action. Due to its large size, haemoglobin is likely to be confined to the extracellular space. Since NO readily crosses cell membranes, haemoglobin might avidly bind the extracellular NO which will lower its extracellular concentration, thus increasing the net rate of diffusion of NO out of the cell (Martin *et al.*, 1985; 1986). This might explain the observed inhibition by haemoglobin against the relaxations to glyceryl trinitrate. Other authors have also found haemoglobin to inhibit relaxations to glyceryl trinitrate: Martin *et al.* (1985, 1986; rabbit aorta), Boeckxstaens *et al.* (1990; canine ileocolonic junction), Bogers *et al.* (1991; canine terminal ileum and ileocolonic junction). Isoprenaline, on the other hand, induces relaxations through an increase in intracellular cyclic AMP-content (Martin *et al.*, 1985). Hence, the inhibition by haemoglobin of the relaxations to 5-HT and glyceryl trinitrate, but not isoprenaline, strongly suggests that NO is involved in the action of 5-HT.

Thirdly, the relaxations to 5-HT were inhibited by the oxidant methylene blue, whereas the responses to glyceryl trinitrate and isoprenaline were not. Methylene blue is a selective inhibitor of the target of NO, the soluble guanylate cyclase (Gruetter *et al.*, 1981; Martin *et al.*, 1985). Hence, it would be expected that the responses to glyceryl trinitrate would also be inhibited, as it does so in other tissues (Gruetter *et al.*, 1981; Martin *et al.*, 1985; Desai *et al.*, 1991). We do not have an explanation for this observation. The inhibition of the responses to 5-HT but not to isoprenaline however provides additional proof for the involvement of NO.

Methylene blue, N^G-nitro-L-arginine, haemoglobin and also the neurotoxin TTX all contracted the strips. This might indicate that NO constantly leaks from enteric inhibitory nerves in small quantities.

The concentration-response curve to 5-HT does not seem to be monophasic. Furthermore, N^G-nitro-L-arginine was not able to abolish the responses to 5-HT. Both these findings suggest that, at higher concentrations of 5-HT, inhibitory endogenous substances might be co-released with NO. Whether other candidates for inhibitory NANC-neurotransmission, which implicates VIP and ATP, are involved, needs further investigation.

It is concluded that in the guinea-pig colon ascendens, NO is involved in the relaxation that is induced by 5-HT. Our studies confirm that the relaxations are mediated by (a) 5-HT_1 receptor subtype(s). The involvement of VIP and/or ATP cannot be excluded.

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(±)-CP-96,345, a selective tachykinin NK_1 receptor antagonist, has non-specific actions on neurotransmission

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1 The non-specific effects of the non-peptide tachykinin receptor antagonist (±)-CP-96,345, were assessed in several smooth muscle-nerve preparations. The preparations were the iris sphincter muscle of the rabbit and the taenia coli, vas deferens and seminal vesicle of the guinea-pig.

2 (±)-CP-96,345 concentration-dependently inhibited the elctrically evoked, tachykinin-mediated contractile responses of the iris sphincter and the taenia coli. The pIC_{50} values were 5.4 ± 0.2 (mean ± s.e.mean) and 5.7 ± 0.08 respectively.

3 (±)-CP-96,345 also inhibited non-tachykinin-mediated contractile responses to electrical stimulation of the iris sphincter, taenia coli, vas deferens and seminal vesicle. The pIC_{50} values were 4.3 ± 0.02, 4.8 ± 0.03, 4.7 ± 0.02 and 4.4 ± 0.05 respectively. These values differ significantly from the pIC_{50} values of the inhibition of the tachykinin-mediated response in the iris sphincter and taenia coli.

4 (±)-CP-96,345 was without effect on carbachol- and noradrenaline-evoked contractions of the iris sphincter but inhibited carbachol- and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-evoked contractions of the taenia coli.

5 We suggest that (±)-CP-96,345, apart from its NK_1 receptor blocking activity, induces non-specific suppression of neurotransmission, exerted at both pre- and post-junctional sites.

Keywords: NK_1 receptor antagonist; non-specific action on neurotransmission; rabbit iris; guinea-pig taenia coli; guinea-pig vas deferens; guinea-pig seminal vesicle

Introduction

The mammalian tachykinins, substance P, neurokinin A and neurokinin B, exert numerous biological activities in the central nervous system and in peripheral organs. The available evidence to date indicates that three tachykinin receptors, named NK_1, NK_2 and NK_3 receptors, mediate the biological effects of these peptides in mammalian tissues (for reviews see Maggio, 1988; Guard & Watson, 1991).

The characterization of different tachykinin receptor subtypes has been based mainly on work with selective tachykinin receptor agonists (Regoli *et al.*, 1988; Brunelleschi *et al.*, 1990; Hagan *et al.*, 1991; Hall *et al.*, 1991). Selective and potent tachykinin receptor antagonists are much needed to corroborate current concepts concerning tachykinin receptors. Very recently, a non-peptide antagonist of NK_1-receptors, CP-96,345 has attracted considerable interest (Snider *et al.*, 1991). Using various isolated organs and smooth muscle preparations, (±)-CP-96,345 was found to be a competitive NK_1-receptor antagonist while being devoid of activity at NK_2 or NK_3 receptors (Snider *et al.*, 1991; Rouissi *et al.*, 1991; Håkanson *et al.*, 1991; Lecci *et al.*, 1991; Gitter *et al.*, 1991). Also, results of *in vivo* experiments have confirmed that (±)-CP-96,345 acts as a selective NK_1 receptor antagonist (Snider *et al.*, 1991; Lecci *et al.*, 1991; Radhakrishnan & Henry, 1991).

However, some effects of (±)-CP-96,345 which are not related to the antagonism to tachykinins have been observed (Boyle *et al.*, 1991; Lecci *et al.*, 1991). In the present study, a series of *in vitro* experiments was performed to investigate whether (±)-CP-96,345 exerted non-specific effects on neurotransmission.

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Methods

General

Adult pigmented rabbits of either sex (1.5–3.0 kg) and male guinea-pigs (200–250 g) were used. The animals were killed by a blow on the neck and exsanguinated. The iris sphincter muscle of the rabbit and the vas deferens and seminal vesicle of the guinea-pig were prepared and mounted vertically on a Perspex holder in a 8 ml organ bath as described elsewhere (Stjernquist *et al.*, 1983; Wahlestedt *et al.*, 1985). One end of the preparation was attached to a rigid support and the other to a Grass FT03 force displacement transducer. The preparation was stretched with a force of 1.5 mN (iris sphincter) or 5 mN (vas deferens and seminal vesicle). The modified Krebs solution (Wahlestedt *et al.*, 1985) was bubbled with a gas mixture of 7% CO_2 in O_2 giving a pH of 7.2–7.3 at 37°C.

The guinea-pig taenia coli preparation, consisting of longitudinal smooth muscle with the attached myenteric plexus (Leander *et al.*, 1981), was placed in modified Krebs solution and kept at 4°C for about 60 min. The preparation was then mounted vertically on a Perspex holder in a 8 ml organ bath maintained at 33°C. One end of the preparation was attached to a rigid support and the other to a lever connected via a spring to a Grass FT03 force-displacement transducer. The load on the muscle was set at 0.2 g.

Two preparations from the same animal were mounted in separate baths, one being exposed to the antagonist and the other being exposed to the vehicle (as control preparation).

Studies of electrically evoked smooth muscle contractions

The mechanical activity of each preparation was recorded continuously on a Grass model 7 polygraph. Before the start of each experiment the preparation was allowed to equilibrate for 60–90 min. Electrical stimulation with square wave

pulses (25 V, voltage drop 14–17 V over the electrodes, 0.3–1 ms duration) was applied by means of platinum electrodes connected to a Grass S4C stimulator. The preparations were stimulated either with single pulses or with trains of pulses lasting 3–20 s, the pulse frequency varying from 1–20 Hz. All electrically evoked responses were abolished by 10^{-6} M tetrodotoxin (TTX). (±)-CP-96,345 was given in a cumulative manner. The preparations were exposed to each concentration of the antagonist for 10 min before the electrical stimulation.

Studies of drug-evoked smooth muscle contractions

We investigated the effects of (±)-CP-96,345 on the contractions produced by carbachol or noradrenaline in the iris sphincter muscle and by carbachol or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the taenia coli. Repeated applications (30 min intervals) of each of carbachol, noradrenaline and $PGF_{2\alpha}$ caused reproducible contractions in the preparation tested. (±)-CP-96,345 was applied 10 min before the application of the agonist.

Drugs

(±)-CP-96,345 was a gift from AB Astra Pain Control, Södertälje, Sweden. It is a racemic mixture containing both [(2**R**, 3**R**)-*cis*- and (2**S**,3**S**)-*cis*-2-(diphenylmethyl)-*N*-(2-methoxyphenyl)-methyl]-l-azabicyclo[2.2.2]octan-3-amine. Only the (2**S**,3**S**)-*cis*- form is thought to possess antagonistic potency (Snider *et al.*, 1991). Atropine was from Alcon, TX, U.S.A. and guanethidine from CIBA-Geigy, Basel, Switzerland. TTX, which is a blocker of nerve conduction (Kao, 1966), was from Sankyo, Japan. Carbachol and noradrenaline were purchased from Sigma, MO, U.S.A. (±)-CP-96,345 was dissolved in 0.1 N acetic acid (10^{-2} M solution).

Analysis of results

Concentration-response curves were constructed and the pIC_{50} values (the negative logarithm of the molar concentration of the antagonist producing 50% inhibition of the electrically evoked contraction) were estimated by linear regression analysis of the results in the 10–90% response interval. Statistical analysis was made by Student's *t* test. A probability of $P < 0.05$ was considered statistically significant.

Results

Studies of tachykinin antagonism

In the presence of 10^{-6} M atropine and 5×10^{-6} M guanethidine, the contractile response of the rabbit iris sphincter to electrical stimulation (20 Hz, 25 V, 10 s) is mediated by tachykinins (Wahlestedt *et al.*, 1985). (±)-CP-96,345 inhibited the tachykinin-mediated contraction concentration-dependently (Figure 1a). The pIC_{50} value is given in Table 1.

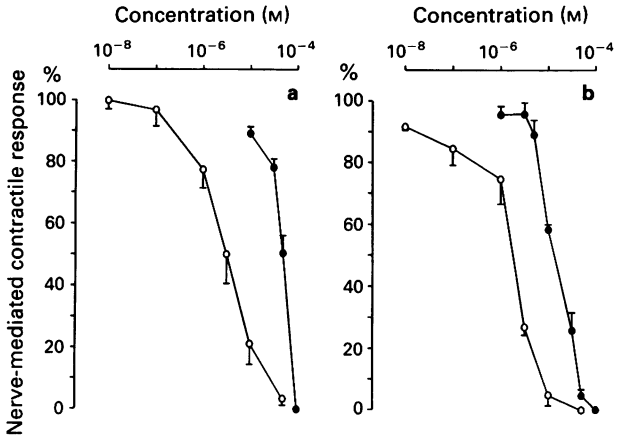

Figure 1 (±)-CP-96,345 concentration-dependently inhibited both tachykinin-mediated (○) and cholinergic (●) contractile responses of the rabbit iris sphincter (a) and guinea-pig taenia coli (b) to electrical stimulation. The contractile responses to electrical stimulation before the application of (±)-CP-96,345 were considered as 100%. Means of 5–6 experiments. Vertical bars give s.e.mean.

Also, electrical stimulation (3 Hz, 25 V, 3 s) of the atropinized guinea-pig taenia coli induces a contractile response that is mediated by tachykinins (Leander *et al.*, 1981). (±)-CP-96,345 inhibited the tachykinin-mediated contraction concentration-dependently (Figure 1b). The pIC_{50} value is given in Table 1.

Studies of non-specific actions of neurotransmission

The iris sphincter muscle responds to single pulse stimulation (1 pulse/60 s, 25 V) with a twitch-like contraction. This contraction can be blocked by 10^{-6} M atropine (Leander & Håkanson, 1985). (±)-CP-96,345 inhibited the cholinergic contractile response concentration-dependently (Figures 1a, 2a). At 10^{-4} M, (±)-CP-96,345 abolished the contraction. The pIC_{50} is given in Table 1; it was about 1 log unit lower than the pIC_{50} of the inhibition of the tachykinin-mediated response ($P < 0.01$). After extensive washing, the contractile response to electrical stimulation showed complete recovery (not shown).

The taenia coli responds to electrical field stimulation (1 Hz, 25 V, 3 s) with a contraction that reflects the release of acetylcholine from cholinergic nerve fibres (Leander *et al.*, 1981). This contraction was abolished by 10^{-6} M atropine (not shown). (±)-CP-96,345 inhibited the cholinergic contractile response concentration-dependently (Figure 1b, 2b). At 10^{-4} M, (±)-CP-96,345 abolished the contraction. The pIC_{50} is given in Table 1; it was about 1 log unit lower than the pIC_{50} of the inhibition of the tachykinin-mediated response ($P < 0.01$). After extensive washing, the contractile response to electrical stimulation showed complete recovery (not shown).

Table 1 Effect of (±)-CP-96,345 on contractile responses of various smooth muscle preparations to electrical stimulation

	pIC_{50}	
	Tachykinin response	*Non-tachykinin response*
Rabbit iris sphincter muscle	5.4 ± 0.20 (5)**	4.3 ± 0.02 (6)
Guinea-pig taenia coli	5.7 ± 0.08 (6)**	4.8 ± 0.03 (6)
Guinea-pig vas deferens	–	4.7 ± 0.02 (4)
Guinea-pig seminal vesicle	–	4.4 ± 0.05 (4)

Means ± s.e.mean. Numbers in parentheses indicate the number of experiments (animals).
**Indicates $P < 0.01$ (difference between pIC_{50} for tachykinin and non-tachykinin-mediated responses).

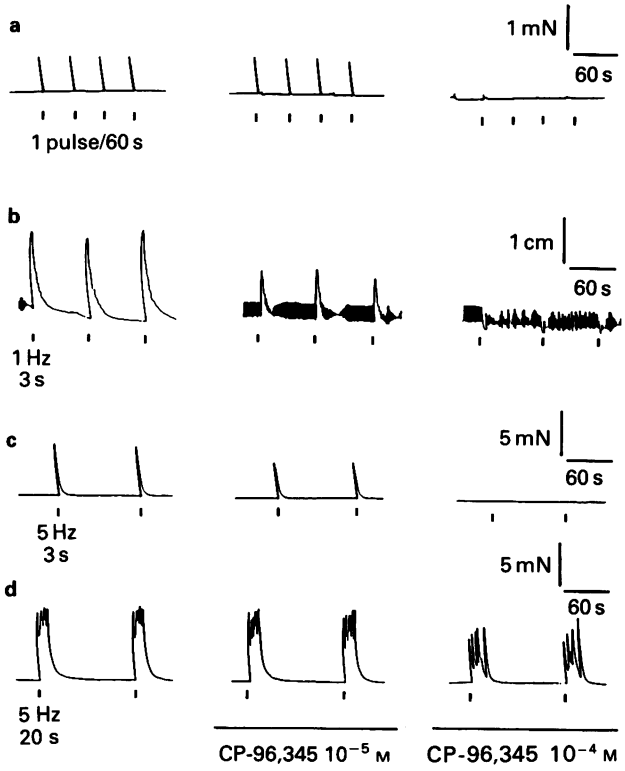

Figure 2 Original tracings showing the inhibitory effect of (±)-CP-96,345 on the contractile non-tachykininergic responses to electrical stimulation of different smooth muscle preparations: (a) rabbit iris sphincter; (b) guinea-pig taenia coli; (c) guinea-pig vas deferens; (d) guinea-pig seminal vesicle. The application of (±)-CP-96,345 increased the spontaneous activity of the guinea-pig taenia coli; the vehicle used to dissolve (±)-CP-96,345 had same effect (not shown). In the other preparations, the vehicle was without effect on the contractile responses (not shown).

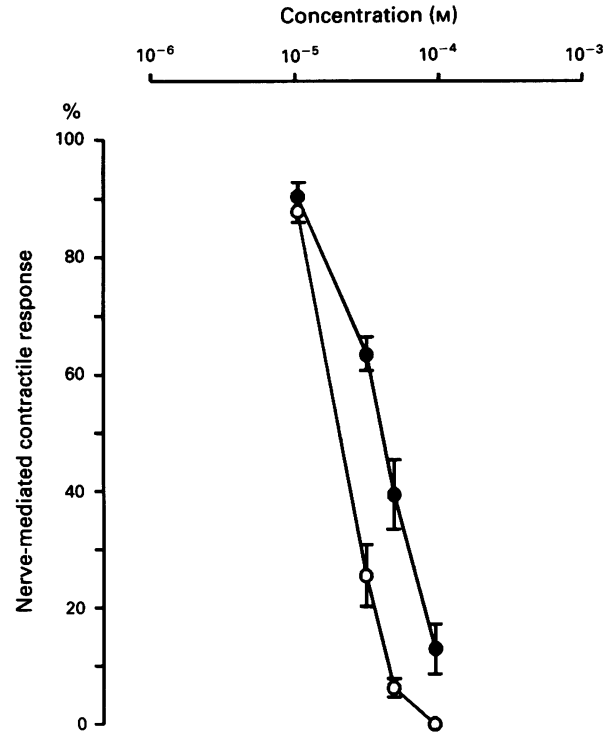

Figure 3 (±)-CP-96,345 concentration-dependently inhibited the non-tachykininergic contractile responses of the guinea-pig vas deferens (○) and seminal vesicle (●) to electrical stimulation. The contractile responses to electrical stimulation before the application of (±)-CP-96,345 were considered as 100%. Means of 4 experiments. Vertical bars give s.e.mean.

The vas deferens responds to low frequency stimulation (5 Hz, 25 V, 3 s) with a twitch-like contraction (Stjernquist *et al.*, 1983). This contraction was unaffected by 10^{-6} M atropine but abolished by 5×10^{-6} M guanethidine (not shown). (±)-CP-96,345 inhibited the contraction concentration-dependently (Figure 2c, 3); the contraction was abolished at 10^{-4} M. The pIC_{50} is given in Table 1. After extensive washing, the contractile response to electrical stimulation showed complete recovery (not shown).

The seminal vesicle preparation responds to electrical stimulation (5 Hz, 25 V, 20 s) with a contractile response which is unaffected by atropine and guanethidine. The neurotransmitter involved has not been identified (Stjernquist *et al.*, 1983). (±)-CP-96,345 concentration-dependently inhibited the 'tonic' component of the contractile response (Figures 2d, 3). The twitch contractions that were superimposed on the 'tonic' contraction were not suppressed

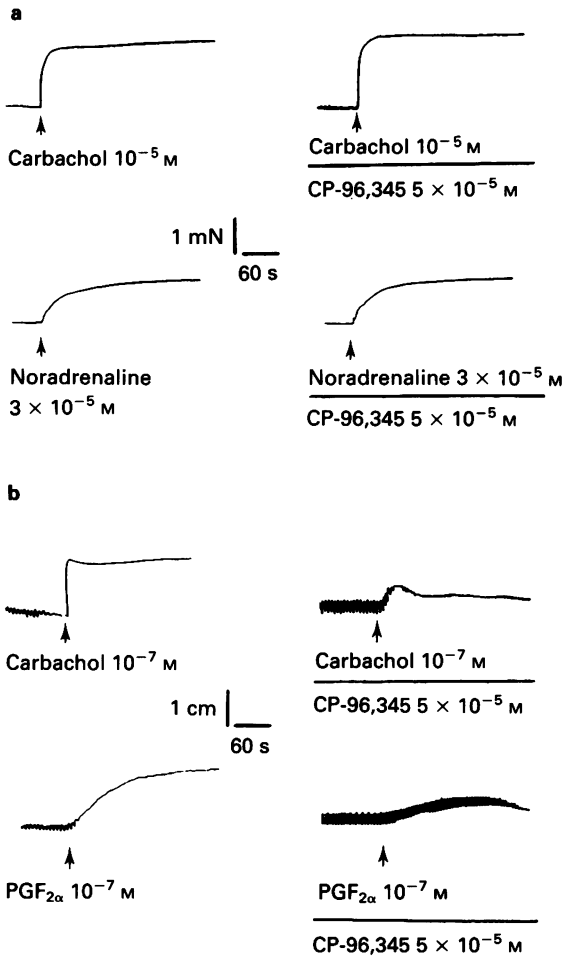

Figure 4 The tracings illustrate the effects of (±)-CP-96,345 on the contractions evoked by carbachol (10^{-5} M) and noradrenaline (3×10^{-5} M) in the rabbit iris sphincter (a) and on the contractions evoked by carbachol and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (10^{-7} M of each) in the guinea-pig taenia coli (b). The concentrations of carbachol, noradrenaline and $PGF_{2\alpha}$ are submaximal.

(Figure 2d). The pIC_{50} is given in Table 1. After extensive washing, the contractile response showed partial recovery (not shown).

Studies of the effect of (±)-CP-96,345 on drug-evoked contractions

Both carbachol (10^{-5} M) and noradrenaline (3×10^{-5} M) contracted the rabbit iris sphincter muslce. (±)-CP-96,345 5×10^{-5} M, was without effect on the contraction produced by either carbachol or noradrenaline (Figure 4a).

Both carbachol and $PGF_{2\alpha}$ (10^{-7} M each) contracted the guinea-pig taenia coli; 5×10^{-5} M (±)-CP-96,345 inhibited both contractile responses greatly (Figure 4b).

Discussion

Snider *et al.* (1991) claimed that (±)-CP-96,345 is the most potent antagonist of the NK_1 receptor identified to date. In addition, the non-peptide nature of CP-96,345 makes it an attractive tool with which to study the pharmacotherapeutic potential of selective NK_1 receptor antagonists. In this context, it would be of interest to identify additional, non-specific actions of the drug. Some properties of (±)-CP-96,345 other than the antagonism of tachykinins have been described previously, e.g. its ability to produce unspecific depression of contractility in the guinea-pig ileum and an unspecific smooth muscle relaxing activity in the rabbit pulmonary artery, hamster trachea and rat portal vein (Boyle *et al.*, 1991; Lecci *et al.*, 1991).

In the present study, (±)-CP-96,345 was found to inhibit tachykinin-evoked contractile responses of the rabbit iris sphincter muscle and guinea-pig taenia coli. It was also found to inhibit non-tachykinin-mediated contractile responses to electrical stimulation of the iris sphincter muscle of the rabbit and of the taenia coli, vas deferens and seminal vesicle of the guinea-pig. In all instances, the non-specific action of (±)-CP-96,345 was concentration-dependent and reversible. However, the concentration of (±)-CP-96,345 needed to inhibit non-tachykinin responses was ten times higher than that needed to inhibit tachykinin-mediated responses. (±)-CP-96,345 did not affect the contractions produced by carbachol and noradrenaline in the iris sphincter but inhibited the contractions produced by carbachol and $PGF_{2\alpha}$ in the taenia coli. Hence, the possibility cannot be excluded that it acts at both pre- and post-junctional sites. Our results suggest that racemic (±)-CP-96,345 exerts non-specific effects on neurotransmission, perhaps related to a local anaesthetic action. It remains to be shown if these non-specific effects are associated with the active or with the inactive enantiomer or both.

Note added in proof

Recently, the active enantiomer of (±)-CP-96,345 was placed at our disposal through the courtesy of Dr R.M. Snider, Pfizer Inc. The ability of this agent to inhibit tachykinin-mediated and non-tachykinin-mediated responses was very similar to that of the racemate. An example: the pIC_{50} value for the tachykinin-mediated response of the rabbit iris sphincter muscle was 5.6 ± 0.01 and for the non-tachykinin-mediated response 4.3 ± 0.02.

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The effect of *in vivo* oestrogen pretreatment on the contractile response of rat isolated detrusor muscle

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1 The effect of oestradiol pretreatment was investigated on the response of rat isolated detrusor muscle to cholinergic, electrical and 5-hydroxytryptamine (5-HT) stimulation with and without diethystilboestrol (DES) (2 μM) in the organ bath.

2 Virgin female Wistar rats were injected subcutaneously for 8 days with oestradiol benzoate 150 μg kg^{-1}. Control rats received no injections or injection only with the vehicle, ethyl oleate.

3 Detrusor muscle from treated rats showed a decreased sensitivity to acetylcholine (ACh) and carbachol-induced contractile responses. The dose-response curves to these agonists showed a 44% reduction in maximum contractile response for ACh ($P<0.001$), and a 38% reduction in maximum contractile response for carbachol ($P<0.05$). The addition of 2 μM DES to the bathing medium further significantly reduced the maximum contractile response by 56 and 57% of control respectively.

4 Electrically stimulated detrusor muscle from treated rats showed a significant 49% reduction in the maximum contractile response ($P<0.001$). The addition of 2 μM DES to the bathing medium further significantly reduced the maximum contractile response by 66% of control. The tetrodotoxin resistant responses were smaller in pretreated rats, suggesting a reduced sensitivity of the smooth muscle to direct electrical stimulation.

5 The response to 5-HT stimulation by detrusor muscle samples from oestradiol-treated rats showed a non-significant reduction in maximum contractile response, but the addition of 2 μM DES to the bath chamber resulted in a 67% reduction in the response ($P<0.001$).

6 Oestradiol pretreatment did not affect the potassium dose-response curve.

7 Oestradiol pretreatment reduced the rat detrusor muscle sensitivity to the blocking effect of atropine on the response to electrical field stimulation. Pretreatment also reduced the potentiating effect of physostigmine on the same response.

8 These results suggest that oestradiol pretreatment had a modulating effect on cholinergic responses. The addition of oestrogen to the tissue environment enhances this inhibitory effect.

Keywords: Oestrogen; bladder; carbachol; 5-hydroxytryptamine

Introduction

Oestrogens have been used for a number of years to treat urinary symptoms especially those associated with the lower urinary tract such as atrophic urethritis. Their place in the management of motor urge incontinence has never been established (Miodrag *et al.*, 1988) despite the fact that high affinity oestradiol receptors have been isolated in human (Iosif *et al.*, 1981; Batra & Iosif, 1983), rabbit (Urner *et al.*, 1983; Shapiro, 1986; Batra & Andersson, 1989) and baboon detrusor muscle (Weaker *et al.*, 1983). Treatment with oestradiol decreased the muscarinic receptor density in rabbits (Shapiro, 1986) but the muscarinic response to carbachol and the cholinergic neurogenic response following electrical field stimulation was not greatly decreased despite a markedly decreased density in muscarinic receptors (Batra & Andersson, 1989).

Earlier experiments in our laboratories have shown that diethylstilboestrol (DES) added directly to the organ bath had a profound effect on the contractile response following cholinergic, 5-hydroxytryptamine (5-HT), calcium ion, potassium and electrical field stimulation of rat and human detrusor muscle. This was probably due to a reduction in calcium ion uptake by the detrusor muscle cells rather than an effect on intracellular calcium release (Elliott *et al.*, 1992). If *in vivo* treatment with oestradiol had similar effects on muscarinic receptors in rats as in rabbits (Shapiro, 1986) then pretreatment and direct oestrogen would have different mechanisms of action and thus a summation of effects might be seen on cholinergic stimulation. Because there appear to be no reports illustrating the effects of pretreatment and direct oestrogen treatment on the same detrusor muscle, the present experiments were performed.

Methods

Virgin female wistar rats were injected subcutaneously with oestradiol benzoate 150 μg kg^{-1}, twice a day for 8 days. Treatment was initiated when the rats were in the dioestrus phase, as judged by vaginal smears. The 8 day treatment regimen covered two cycles, after which the rats were killed by a blow to the head. The bladders were removed and dissected free of fat and serosa. Strips of bladder muscle 7 mm by 4 mm were suspended in a 50 ml organ bath chamber containing Krebs solution at 37°C and aerated with 95% oxygen and 5% carbon dioxide. The bladder base was attached to a fixed hook in the chamber and the apex by a thread attached to an isometric transducer connected to a two channel Washington oscillograph. The tissues were allowed to equilibrate for 1 h under a tension of 10 mN.

After equilibration, acetylcholine (ACh) (10^{-8} M–2×10^{-4} M), carbachol (10^{-8} M × 10^{-4} M) or potassium chloride (KCl) (10 mM–60 mM) was injected cumulatively into the bath chamber to obtain dose-response curves. 5-Hydroxytryptamine (5-HT) (10^{-8} M–10^{-5} M) was injected at 5 min intervals and samples were washed between doses to avoid tachyphylaxis. For electrical field stimulation, muscle strips were

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passed through two parallel circular electrodes connected to a Digitimer stimulator. The stimulator delivered 1–80 pulses s^{-1} at 4–6 V and a 1 ms pulse width in 10 s trains at 2 min intervals. Frequency-response curves were obtained by stimulating the tissue with 1, 5, 10, 20, 40, 60, 80 pulses per second. The effect of oestradiol pretreatment plus the presence of 2 μM DES in the external medium was investigated by repeating the dose-response curves after the addition of DES to the bath chamber. Effects of bath-applied DES were not easily reversible. Even after several washes the response did not return to pre-applied levels.

Tetrodotoxin (TTX, 1.6×10^{-6} M) was used to distinguish between nerve-mediated contractile responses, and those due to direct muscle stimulation in controls and pretreated samples.

Different bladder muscle samples were used for each agonist. Control dose-response curves were obtained from rat bladder muscle taken from untreated rats in the dioestrus phase. Samples were also taken from rats injected only with the vehicle ethyl oleate. Dose-response curves for the comparison of bladders from rats pretreated with oestradiol and controls are presented as concentration of agonist against the increase in tension, and not percentage of maximum response. This is to demonstrate the absolute decrease in response obtained after oestradiol treatment which would not be apparent when calculating percentage of maximum response if the same bladder samples were used as its own control.

Solutions and chemicals

Krebs solution contained (mM): NaCl 119, KCl 4.4, $NaHCO_3$ 20, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5 and glucose 11.

ACh chloride (Sigma), carbamylcholine chloride (Sigma), atropine sulphate (Sigma), physostigmine (Sigma) and 5-HT (Sigma) were all dissolved in distilled water and made up on the day of the experiment. Diethylstilboestrol (DES) (Sigma) was dissolved in ethanol and the concentration of ethanol in the organ bath chamber did not exceed 3 mM. Oestradiol benzoate (Paines & Byrne) was supplied in vials containing 5 mg ml^{-1}. TTX (Sigma) was made up in distilled water and stored at −20°C in 1 ml aliquots.

For each experiment the results were the mean of 5 different bladder muscle samples, unless otherwise stated. Statistical analysis was carried out with Student's *t* test.

Results

Following 8 days oestradiol treatment the rat uterus showed marked hypertrophy compared to non-treated animals. This was taken as an indication of oestrogenisation. The bladders removed from treated rats were also hypertrophic (mean weight 94.32 ± 8.60 mg) compared to the non-treated animals (mean weight 66.65 ± 2.54 mg) ($P<0.05$). The response of the detrusor muscle in control rats and those treated with vehicle only did not differ.

Effect of oestrogen on electrical field stimulation

The spontaneous contractions normally exhibited by rat detrusor muscle were markedly reduced in frequency and amplitude in samples taken from oestradiol treated rats. The frequency dose-response curve of the detrusor muscle of such rats to electrical field stimulation showed a 49% reduction in maximum response compared to control ($P<0.001$). When 2 μM DES was added to the surrounding medium, the result was a further significant reduction of maximum response (66%, Figure 1). The maximum response obtained by electrical field stimulation was 61.4% of the maximum response obtained with 10^{-4} M carbachol in controls and 61.5% in pretreated rats.

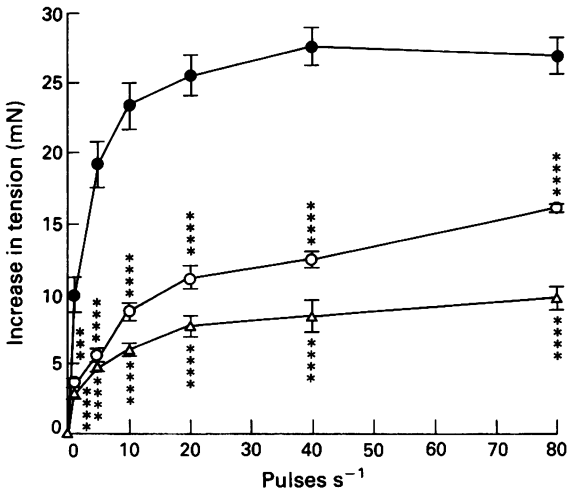

Figure 1 Dose-response curves to electrical field stimulation in rat isolated detrusor muscle: (●) control; (○) after 8 days oestradiol treatment; (△) after 8 days oestradiol treatment plus 2 μM diethylstilboestrol in the organ bath ($n = 5$). Vertical bars represent s.e. mean. ***$P<0.01$; ****$P<0.001$.

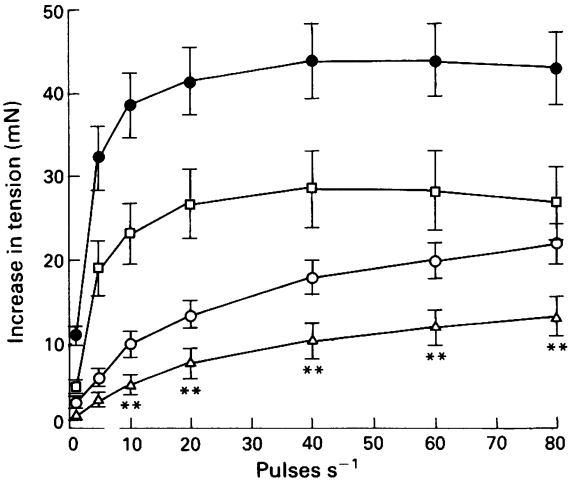

Figure 2 Frequency-response curves showing the effect of oestradiol pretreatment on the tetrodotoxin-resistant response to electrical field stimulation in rat isolated detrusor muscle: (●) control; (○) control after TTX; (□) after 8 days treatment; (△) after 8 days treatment and TTX, ($n = 5$). Vertical bars represent s.e.mean. **$P<0.05$.

Effect of oestrogen on tetrodotoxin sensitivity

Tetrodotoxin blocked about 50% of the maximum contractile response to electrical field stimulation at our parameters. At lower frequencies (10 pulses s^{-1}) about 75% of the contractile response was blocked. The TTX-resistant responses were significantly smaller in rats pretreated with oestradiol than in control rats ($P<0.05$, Figure 2).

Effect of oestrogen on acetylcholine and carbachol response

The contractile response of detrusor muscle to ACh stimulation was reduced in amplitude in oestradiol-treated rats compared to controls. The dose-response curve showed a 44% reduction in maximum response ($P<0.001$). The addition of 2 μM DES to the water bath resulted in a further significant

reduction in maximum response (56%, Figure 3a). Oestradiol pretreatment resulted in similar effects on carbachol-induced contractions with a reduction of 38% in maximum response being obtained ($P<0.05$), which was reduced further with the addition of 2 μM DES (57%, Figure 3b).

Effect of oestrogen on 5-hydroxytryptamine response

Rat detrusor muscle response to 5-HT stimulation was phasic and much reduced in amplitude compared to cholinergic and electrical field stimulation responses. Detrusor muscle from oestradiol-treated rats showed a non significant reduction in maximum response of 22%, but with the addition of 2 μM DES to the bath chamber this response was significantly reduced by 67% ($P<0.001$), Figure 4.

Effect of oestrogen on atropine sensitivity

Detrusor muscle from control rats, stimulated electrically, showed a 19% reduction in maximum contractile response in the presence of 10 μM atropine (Figure 5a). Atropine exerted its inhibitory effect mainly on high frequency induced contractile responses.

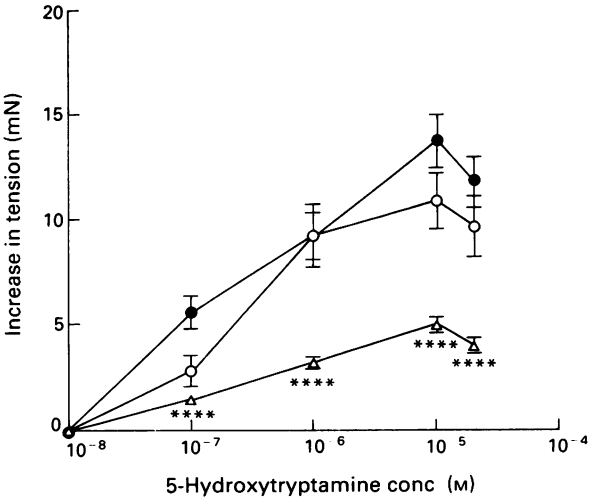

Figure 4 Dose-response curves to 5-hydroxytryptamine (5-HT) stimulation of rat isolated detrusor muscle: (●) control; (○) after 8 days oestradiol treatment; (△) after 8 days oestradiol treatment plus 2 μM diethylstilboestrol in the organ bath ($n = 5$). Vertical bars represent s.e.mean. ****$P<0.001$.

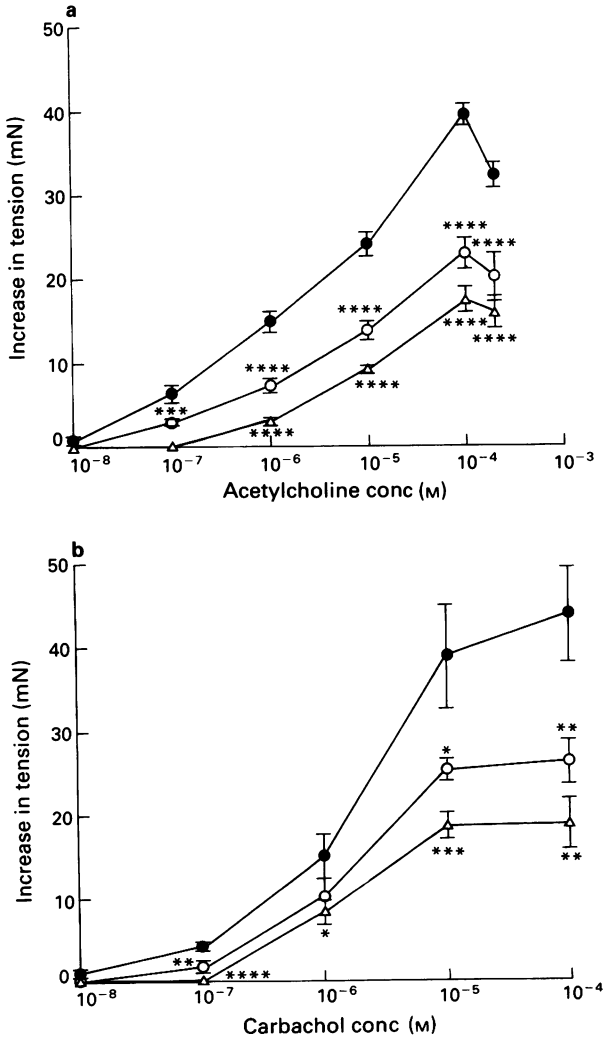

Figure 3 (a) Dose-response curves to acetylcholine stimulation in rat isolated detrusor muscle: (●) control ($n = 8$); (○) after 8 days oestradiol treatment; (△) after 8 days oestradiol treatment plus 2 μM diethylstilboestrol (DES) in the organ bath ($n = 9$). (b) Dose-response curves to carbachol stimulation in rat isolated detrusor muscle: (●) control; (○) after 8 days oestradiol treatment; (△) after 8 days oestradiol treatment plus 2 μM DES in the organ bath ($n = 5$). Vertical bars represent s.e.mean. *$P<0.10$; **$P<0.05$; ***$P<0.01$; ****$P<0.001$.

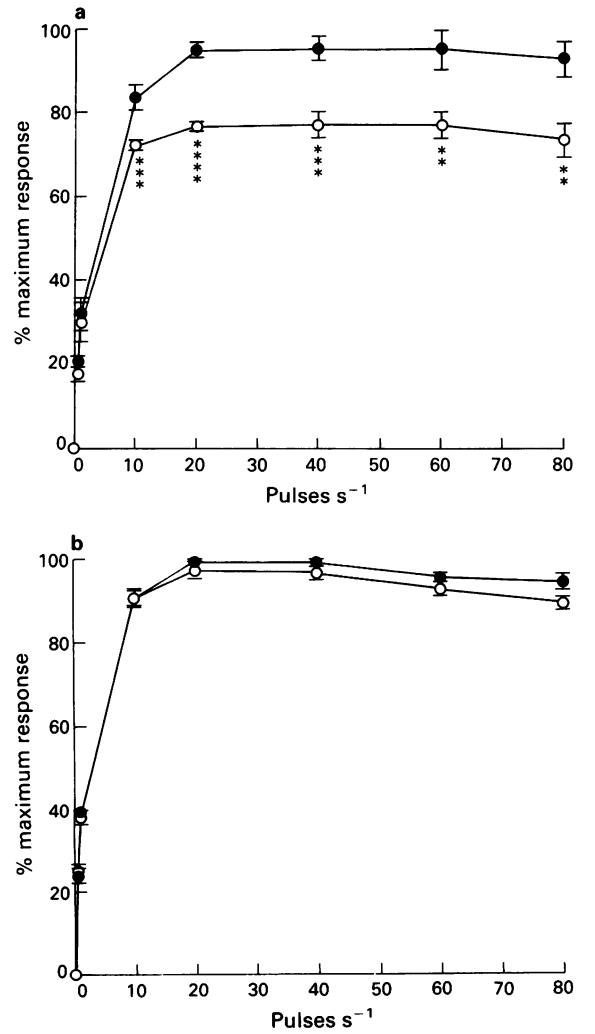

Figure 5 (a) Dose-response curves to electrical field stimulation in rat isolated detrusor muscle from untreated rats ($n = 4$). (b) Dose-response curves to electrical field stimulation in rat isolated detrusor muscle from rats pretreated with oestradiol for 8 days: (●) control; (○) after 10 μM atropine ($n = 5$). Vertical bars represent s.e.mean. **$P<0.50$; ***$P<0.01$; ****$P<0.001$.

The sensitivity of the detrusor muscle to blockade of the cholinergic component of electrical stimulation was almost totally abolished in bladder muscle samples taken from oestradiol-treated rats. The dose-response curve showed no significant difference before and after the addition of atropine 10 μM (Figure 5b).

Effect of oestrogen on physostigmine potentiation

Control detrusor muscle samples showed potentiation of electrically induced contractile responses in the presence of 0.01 μM physostigmine. The maximum contractile response was increased by 30% ($P<0.001$) (Figure 6a).

Bladder muscle from oestradiol pretreated rats had lost its sensitivity to physostigmine. The electrically induced contractile responses were not significantly different in the presence or absence of the drug (Figure 6b).

Effect of pretreatment on KCl response

Oestradiol pretreatment did not affect the KCl dose-response curve.

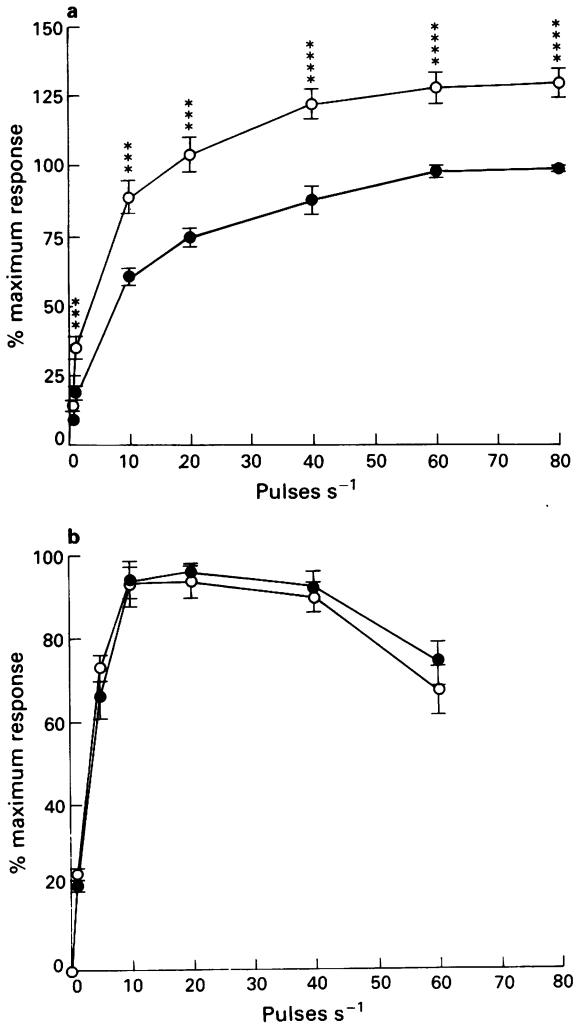

Figure 6 (a) Dose-response curves to electrical field stimulation in rat isolated detrusor muscle from untreated rats ($n = 5$). (b) Dose-response curves to electrical field stimulation in rat isolated detrusor muscle from rats pretreated with oestradiol for 8 days ($n = 5$): (●) control; (○) after the addition of 0.01 μM physostigmine. Vertical bars represent s.e.mean. ***$P<0.01$; ****$P<0.001$.

Discussion

Previous results from our laboratory have shown that the addition of DES to the organ bath had a profound effect on the contractile response of rat and human detrusor muscle. The results suggested that DES affected the movement of extracellular calcium ions into the muscle cells (Elliott *et al.*, 1992). If previous studies were plotted on the present dose- and frequency-response curves, the resultant curve would be situated between the control and pretreatment curves.

The present results showed that there was a significant decrease in the contractile response to muscarinic receptor stimulation after pretreatment with oestradiol and that this reduction in contractility could be further enhanced by the direct addition of DES to the tissue environment. Since there was no difference between the effect of oestradiol on the responses to ACh and carbachol, pretreatment is unlikely to have affected cholinesterase activity. The decrease in contraction following electrical stimulation was due predominantly to an effect on the cholinergic component since pretreatment with oestradiol almost abolished the atropine sensitive component of electrical field stimulation. The enhancing effect of physostigmine on this response was also greatly reduced after oestradiol pretreatment. In man it is likely that the effect of oestradiol pretreatment will be even greater as the contraction following electrical field stimulation is mainly cholinergic (Sjögren *et al.*, 1982; Sibley, 1984; Kinder & Mundy, 1985). Pretreatment did not affect the KCl response and it is unlikely therefore that pretreatment is affecting calcium ion permeability. This is in contrast to the direct effect of DES (Elliott *et al.*, 1992). The results with TTX show that, with our parameters of stimulation, the nerve-mediated response was 50% of the contractile response to electrical field stimulation at high frequencies and that 50% was direct muscle action. This is true for both control and pretreated rats. The TTX-sensitive response was reduced after pretreatment with oestradiol, suggesting that oestradiol decreases the sensitivity of detrusor muscle to direct electrical stimulation. Brading & Williams (1990) have clearly shown that the predominant mechanical response to intrinsic nerve stimulation of rat and guinea-pig detrusor was through non-muscarinic receptors and that contractile responses resistant to atropine are most clearly seen in the early response to electrical field stimulation. Conversely neostigmine (Brading & Mostwin, 1989) and physostigmine potentiated electrical field stimulation at low although less than at high frequencies. There is now good evidence that the non-cholinergic transmitter is adenosine 5'-triphosphate (ATP) (Brading & Mostwin, 1989; Brading & Williams, 1990; Parija *et al.*, 1991), but whether pretreatment affects this mechanism is unknown at present.

Pretreatment with oestradiol in the rat also had no effect on the contractile response to 5-HT. Chen (1990) has shown that there is a cholinergic component to 5-HT stimulation in the rabbit in addition to non-adrenergic, non-cholinergic excitatory neurotransmission. The present results would suggest that this cholinergic component was relatively unimportant following 5-HT stimulation in the rat. The addition of DES to the tissue environment caused a significant reduction in contractile response to 5-HT possibly due to changes in cell membrane permeability to calcium ions (Elliott *et al.*, 1992).

The most likely explanation for the inhibitory effect of pretreatment with oestradiol on the cholinergic response was that there was down-regulation of muscarinic receptors, although there may have been a minor effect on the sensitivty to direct electrical stimulation. There was no overall decrease in contractility of the pretreated detrusor muscles in the present study, despite considerable hypertrophy of the bladder. As early as 1977, Roberts *et al.* showed that pretreatment with oestradiol could increase α-adrenoceptor densities in the rabbit uterus. Larsson *et al.* (1984) reported that this increase in α-adrenoceptors could also be induced in the female rabbit urethra. They clearly showed that the increase

in receptors was not proportional to the increase in the weight of the tissue as it was in the uterus. Shapiro (1986) found that pretreatment with oestradiol decreased the muscarinic cholinoceptor density in the rabbit bladder; this was despite a marked increase in the weight of the bladder body. Batra & Andersson (1989) also using the rabbit confirmed previous work that muscarinic receptor density was reduced following oestradiol treatment but were unable to show that contractile responses to electrical field stimulation and carbachol were significantly decreased. The difference between their results and the present ones could represent species differences, but is unlikely to be due to inadequate oestrogen-treatment since Batra & Andersson (1989) produced a reduction in muscarinic receptor density of 90% after 4 weeks. Anderson & Marks (1982) showed that only a small fraction of the cholinoceptors needed to be occupied to produce contractile responses, and thus there was a large receptor reserve. They further argued that the rate limiting step for regulation of the contractile response to carbachol was neither muscarinic receptor occupation nor membrane calcium channel opening but the intracellular mechanisms which regulate the responsiveness of the myofibrils to calcium ions.

In conclusion, pretreatment with oestradiol in rats significantly reduced muscarinic receptor-stimulated contractions of detrusor muscle although the exact mechanism by which this was brought about is uncertain. The addition of DES directly to the organ bath in pretreated animals caused a further decrease in contractile response. This combined effect of oestradiol has not been shown before but if confirmed in man would clearly mimic the situation in women given oestradiol therapy long-term for over-activity of the detrusor muscle. In such women detrusor contractions are associated with urge incontinence and thus pretreatment with oestradiol may have a very significant clinical role in the control of urinary incontinence.

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Effects of neuropeptide Y and agonists selective for neuropeptide Y receptor sub-types on arterioles of the guinea-pig small intestine and the rat brain

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1 The actions of neuropeptide Y (NPY) and agonists selective for NPY receptor subtypes were examined on arterioles from the guinea-pig small intestine and the rat pia in order to characterize the receptors mediating the vasoconstrictor and potentiating effects of NPY.

2 A method was developed for measuring the potentiating effects of NPY in situations where it was not possible to obtain a full concentration-response relationship for the vasoconstrictor. NPY, 50 nM, had a greater potentiating effect on the guinea-pig intestinal arterioles than those from the rat pia.

3 NPY and the Y_1-selective agonist, NPY[Leu^{31},Pro^{34}], potentiated the constrictor responses to U46619 in both arterioles and responses to noradrenaline in the guinea-pig arterioles. There was marked desensitization of the potentiating effect, and cross-desensitization between NPY and NPY[Leu^{31},Pro^{34}]. Both NPY and NPY[Leu^{31},Pro^{34}] caused constriction of the rat pial arterioles but not of those from the guinea-pig intestine.

4 The Y_2-selective agonist PYY(13–36) caused no potentiation or vasoconstriction and did not affect the potentiation by NPY or NPY[Leu^{31},Pro^{34}].

5 The potentiating and vasoconstrictor effects of NPY on these arterioles were mediated by Y_1 receptors.

Keywords: Vascular smooth muscle; arteriole; neuropeptide Y receptors; NPY[Leu^{31},Pro^{34}]; PYY(13–36)

Introduction

Neuropeptide Y (NPY) is a 36-amino-acid peptide that is found in a variety of central and peripheral neurones. It occurs in sympathetic nerves supplying the heart and blood vessels, and this has led several investigators to consider its possible role in cardiovascular regulation. NPY injected into the systemic circulation produces a moderate but prolonged rise in arterial blood pressure but the normal plasma level of NPY is low (Morris *et al.*, 1986; 1987; Corder *et al.*, 1988) and it seems unlikely that it functions as a circulating hormone (Pernow *et al.*, 1987). When it is applied to isolated blood vessels the most prominent action of NPY is the potentiation of the effects of a variety of vasoconstrictor substances or vasoconstrictor nerve stimulation. NPY itself will also cause vasoconstriction in some vessels but the effect if often small and requires higher concentrations of NPY than are needed to produce potentiation (Morris & Murphy, 1988; Abel & Han, 1989). In at least one arteriole, NPY causes potentiation at nanomolar concentrations, but negligible constriction in concentrations up to 1μM (Neild & Kotecha, 1990). The other major peripheral action of NPY is on nerve terminals, where it reduces neurotransmitter release (Lundberg & Stjarne, 1984; Potter, 1984). The physiological importance of this action was most clearly demonstrated in the dog heart, where NPY from sympathetic nerves reduced acetylcholine release from the vagus (Potter *et al.*, 1989).

There is now clear evidence that there are at least two types of neuropeptide Y receptor (Wahlestedt & Hakanson, 1986; Wahlestedt *et al.*, 1990) termed Y_1 and Y_2. Y_1 receptors appear to be present on many types of vascular smooth muscle, where they mediate muscle contraction and the potentiation of the vasoconstrictor effects of other substances. Y_2 receptors are found mainly on neuronal tissue, including sympathetic nerves supplying blood vessels, where they modulate neurotransmitter release. The different NPY receptors can be activated selectively by certain NPY analogues. Modifications of the amino acid sequence near the C-terminal end of the NPY molecule has produced Y_1-selective agonists (Fuhlendorff *et al.*, 1990); removal of amino acids from the N-terminal end of the molecule produces a varying degree of selectivity for Y_2 receptors (Potter *et al.*, 1989; Michel *et al.*, 1990).

When injected systemically Y_1 agonists cause a rise in blood pressure similar to that caused by NPY and this would be expected from their vasoconstrictor and potentiating effects on vascular smooth muscle. Y_2 agonists also produce a small rise in blood pressure, but this is the opposite of what would be expected if their action were to reduce neurotransmitter release from nerves. By reducing sympathetic neurotransmitter output they should cause a fall in peripheral resistance. Y_2-selective agonists do have some action on Y_1 receptors which could result in vasoconstriction, but at least 100 fold higher concentrations are required to obtain effects comparable to those of NPY (Wahlestedt & Hakanson, 1986; Rioux *et al.*, 1986; Modin *et al.*, 1991) or Y_1-selective analogues (Schwartz *et al.*, 1989), and it seems unlikely that this could account for their pressor action. Another possibility is that there are significant numbers of Y_2 receptors on vascular smooth muscle in some tissues which mediate constriction or potentiation. Although on larger arteries the effects of NPY are mediated by Y_1 receptors, Wahlestedt *et al.* (1990) have drawn attention to a possible parallel with the α-adrenoceptor system, where the predominantly presynaptic α_2 receptors are found to mediate smooth muscle constriction in some arteries and particularly in the smaller arterioles (Faber, 1988). As the small arteries and arterioles are the region where the nervous system exerts its major influence on circulatory control mechanisms, this is where nerve-released NPY will have its main physiological effect. In the pig,

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Modin et al. (1991) have shown vasoconstrictor effects in the spleen which seem to be mediated by Y_2 receptors.

The experiments in this paper were performed to see if Y_2 receptors mediating vasoconstriction or potentiation could be found on arteriolar smooth muscle. We chose two types of arteriole that showed different responses to NPY and which might differ in the types of NPY receptors that they possessed. Arterioles from the submucosa of the guinea-pig small intestine show only the potentiating effect of NPY, with no effect on smooth muscle membrane potential and no direct constrictor effect. Arterioles in the pia of the rat brain show potentiation, constriction, and smooth muscle depolarization, and might have had multiple NPY receptor types. Our results, however, show that all these effects in both arterioles are mediated by Y_1 receptors.

Methods

Sheets of connective tissue containing arterioles were dissected from the guinea-pig small intestine or the rat brain. Guinea-pigs (Monash outbred strain) of either sex weighing 200–300 kg were used. They were killed by a heavy blow to the head followed by exsanguination by section of the jugular veins, and a piece of ileum was removed. It was cut open and the submucous connective tissue layer removed by first peeling off the mucosa and then peeling the submucous layer from the underlying muscle. Rats (Wistar, 250–300 g) were given an intraperitoneal injection of sodium pentobarbitone (40 mg kg^{-1}) sufficient to induce deep surgical anaesthesia and exsanguinated by section of the abdominal aorta and vena cava. The brain was removed and the pial connective tissue containing the middle cerebral artery and its branches was gently dissected free.

The connective tissue sheet was pinned to transparent silicone rubber on the base of a small chamber (volume 1.0 ml) mounted on an inverted compound microscope, and continuously superfused with warmed oxygenated physiological saline at 6.0 ml min^{-1}. The saline contained (mmol l^{-1}): Na^+ 146, K^+ 5, Ca^{2+} 2.5, Mg^{2+} 2, Cl^- 134, HCO_3^- 25, $H_2PO_4^-$ 1 and glucose 11, and was equilibrated with 95% O_2/5% CO_2. The temperature in the chamber was 30°C. High potassium solution for determining maximal arteriolar constriction (Neild & Kotecha, 1989) was made by replacing 95 mM of Na^+ with K^+ to give a K^+ concentration of 100 mM.

Vasoconstrictor drugs were applied to the tissue from a micropipette placed within 50 μM of the arteriole. Noradrenaline was ejected by ionophoresis; U46619 was ejected from lower resistance pipettes by pressure. The duration of the ejection pulse and the position of the pipette were changed to grade the size of the constrictor response. NPY and its analogues were applied by adding them to the superfusion solution to produce a known concentration.

The diameter of the arterioles was monitored by computer analysis of a television image from a camera attached to the microscope (Neild, 1989).

Catecholamine-containing nerves were demonstrated histochemically by the FAGLU method of Furness et al. (1977).

Drugs used were: noradrenaline bitartrate (Sigma), 9,11-dideoxy-9α,11α-methanoepoxy-prostaglandin $F_{2\alpha}$ (U46619, Cayman Chemical Co), neuropeptide Y (porcine sequence, synthesized by Monash University Department of Biochemistry), NPY[Leu^{31},Pro^{34}], PYY(13–36) (Auspep, Melbourne).

A quantitative indication of the potentiating effect of NPY or its analogues was required for this study. We could not use a method based on concentration-response curves for NPY or vasoconstrictors, as these arterioles showed marked tachyphylaxis and desensitization to noradrenaline, U46619, NPY, and combinations of these. Concentration-response relationships could only be obtained by use of single concentrations at intervals of at least 20 min. A method for measuring potentiation was devised, and is explained in the Appendix. A parameter P was calculated as an index of the potentiating effect; values greater than 1 indicated potentiation. The mean of P and its standard error were calculated for particular combinations of vasoconstrictor and potentiator, and significance of differences between Ps was determined using Student's t test, with $P<0.05$ taken to indicate significance. P values in the text are given ± the standard error of the mean.

Results

Arterioles from the guinea-pig small intestine

Noradrenaline (NA) was applied to the arteriole by ionophoresis from a micropipette every 5 min, and it produced a brief constriction of consistent amplitude as shown in Figure 1. When 50 nM NPY was added to the superfusing solution starting 2 min before an application of NA, the response to NA was increased. From 23 experiments the mean value of P for this potentiating effect of NPY was 4.74 ± 0.40. The NPY was left in contact with the arteriole for a total of 7 min, and it can be seen that the potentiating effect declined, so that the second response to NA in the presence of NPY was smaller than the first. In addition to this tachyphylaxis there was a profound desensitization to NPY, such that a second application of NPY 18 min after the end of the first application produced significantly less potentiation (P = 2.22 ± 0.40, $n = 8$) than the first. The results from these and similar experiments using receptor-specific agonists are summarized in Table 1.

An agonist selective for NPY Y_1 receptors (Fuhlendorff et al., 1990), NPY[Leu^{31},Pro^{34}], had very similar effects to NPY. Exposure of the arteriole to 50 nM NPY[Leu^{31},Pro^{34}] potentiated the response to NA (P = 6.65 ± 1.27, $n = 8$) and the

Table 1 Potentiation of responses of guinea-pig intestinal arterioles to noradrenaline (NA) by neuropeptide Y (NPY), the Y_1-selective agonist NPY[Leu^{31},Pro^{34}] and the Y_2-selective agonist PYY(13–36)

Substance applied first	P	*Substance applied second 18 min after first*	P	*Significant reduction of second response?*
NPY	4.74 ± 0.40 $n = 23$	NPY	2.22 ± 0.40 $n = 8$	Yes
		Y_1ag	3.93 ± 0.46 $n = 5$	No
Y_1ag	6.65 ± 1.27 $n = 8$	Y_1ag	2.51 ± 0.51 $n = 6$	Yes
		NPY	1.86 ± 0.14 $n = 5$	Yes
Y_2ag	1.06 ± 0.06 $n = 7$			
		NPY	6.65 ± 0.84 $n = 5$	No

Data from experiments using the same protocol as that illustrated in Figure 1.
Y_1ag = NPY[Leu^{31},Pro^{34}]; Y_2ag = PYY(13–36).

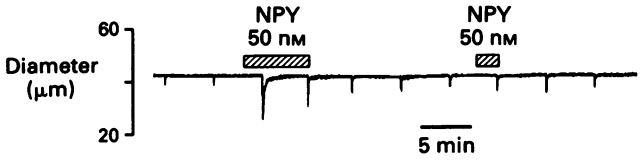

Figure 1 Effect of 50 nM neuropeptide Y (NPY) on the contractile responses caused by brief applications of noradrenaline (NA) to an arteriole from the guinea-pig small intestine. NA was applied to the arteriole every 5 min by ionophoresis from a micropipette. The duration of the ionophoretic current was 1 s, and the resulting constriction shows as a brief downward deflection on the diameter record. The initial exposure to NPY for 2 min before a pulse of NA caused an increase in the amplitude of the constriction, but a subsequent application of NPY 18 min after the first had a much smaller effect.

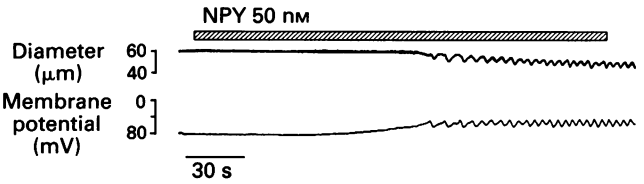

Figure 2 Effects of 50 nM neuropeptide Y (NPY) on the diameter and smooth muscle membrane potential of an arteriole from the rat pia. NPY caused a constriction and depolarization that developed after a delay of about 60 s. There were oscillations of both membrane potential and diameter, with the peaks of depolarization preceding the peaks of contraction.

response to a second exposure to NPY[Leu^{31},Pro^{34}] 18 min later was significantly reduced ($P = 2.51 \pm 0.51$, $n = 6$). The initial potentiating effect ($P = 6.65$) was not significantly different from the effect of the same concentration of NPY ($P = 4.74$). There was interaction between the effects of NPY and NPY[Leu^{31},Pro^{34}]. Prior exposure of the artery to 50 nM NPY[Leu^{31},Pro^{34}] significantly reduced the P value for the potentiation caused by a subsequent application of NPY; 50 nM NPY reduced the potentiating effect of NPY[Leu^{31}, Pro^{34}] but the reduction of P was not significant.

The similarity of the effects of NPY and NPY[Leu^{31},Pro^{34}] and their interaction strongly suggest that they were acting via the same receptors and internal chemical pathways to cause their potentiating effect.

An agonist selective for Y_2 receptors (Wahlestedt & Hakanson, 1986), 50 nM PYY(13–36), caused no potentiation ($P = 1.06 \pm 0.06$, $n = 7$, not significantly different from 1), and did not reduce the potentiating effect of a subsequent application of 50 nM NPY 18 min later ($P = 6.56 \pm 0.84$, $n = 5$). Higher concentrations of PYY(13–36) were tried in a few experiments, but were also without effect (100 nM, $n = 2$; 200 nM $n = 1$).

In all the experiments described above there was no evidence of constriction caused by NPY, NPY[Leu^{31},Pro^{34}], or PYY(13–36) alone.

Arterioles from the rat brain

NPY 50 nM applied to the arterioles in the isolated pial connective tissue of the rat brain produced depolarization of the arteriolar smooth muscle and constriction of the arteriole. These effects developed slowly after a delay of approximately 2 min, as shown in Figure 2. The mean constriction was $20.2 \pm 6.47\%$ of the maximum, and the membrane potential changed from a mean of -53 ± 0.94 mV to -39.5 ± 0.75 mV ($n = 8$). Similar effects were obtained with NPY-[Leu^{31},Pro^{34}], but PYY(13–36) caused no constriction or depolarization in concentrations up to 1 μM. The constriction and depolarization caused by 100 nM NPY (constriction 24.2 ± 3.60, membrane potential from -52.0 ± 0.81 to -38.4 ± 2.14; $n = 7$) were not significantly greater than those caused by 50 nM.

The constriction caused by NPY complicated the analysis of the potentiating effects, so subsequent experiments were performed using 12.5 nM NPY, which did not cause arteriolar constriction in this preparation. The stable thromboxane analogue U46619 was used as a vasoconstrictor, as these arterioles do not constrict in response to NA. The constrictor responses were complex, consisting of an initial rapid constriction followed by a slower component with superimposed oscillations (Figure 3). Measurements were made on the initial component, as this part of the response closely resembled the responses obtained from the guinea-pig small intestine arterioles.

As shown in Figure 3, both NPY ($P = 2.44 \pm 0.26$, $n = 10$) and NPY[Leu^{31},Pro^{34}] ($P = 2.67 \pm 0.31$, $n = 7$) caused

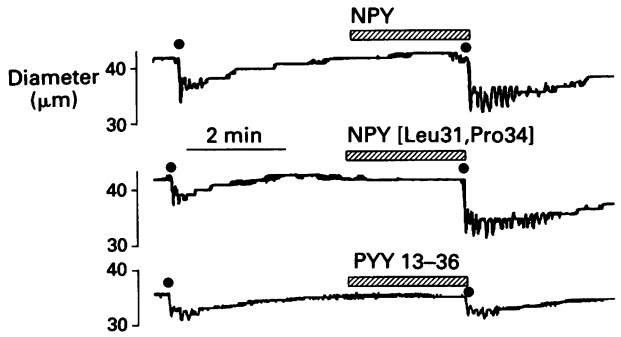

Figure 3 Effects of neuropeptide Y (NPY), NPY[Leu^{31},Pro^{34}], PYY(13–36) on the contractile responses of the rat pial arterioles to U46619. U46619 was applied as a 1 s pulse from a micropipette at the times marked by (●). NPY and NPY[Leu^{31}, Pro^{34}] caused significant potentiation, but PYY(13–36) did not. Traces from 3 different arterioles.

significant potentiation of the vasoconstrictor responses, whereas PYY(13–36) had no effect ($P = 1.02 \pm 0.02$, $n = 5$). Three experiments with 25 nM PYY(13–36) still produced no evidence of a potentiating effect ($P = 1.09 \pm 0.04$, $n = 3$), nor did single experiments with concentrations of 50 nM, 500 nM, and 1 μM.

Comparison of the effects of neuropeptide Y on the two types of arteriole

In order to compare the potentiating effect of NPY on the two types of arteriole a series of experiments were conducted on the guinea-pig intestinal arterioles using the same protocol that was used for the rat pial arterioles i.e. U46619 as the vasoconstrictor, and 12.5 nM NPY. Under these conditions P for the potentiating effect of NPY in the guinea-pig intestinal arterioles was 4.92 ± 1.10, $n = 7$, which was significantly greater than the value of 2.44 found for the rat arterioles. We conclude therefore that the guinea-pig intestinal arterioles are more sensitive to NPY than the rat pial arterioles.

Previous studies on the innervation of the rat pial arteriole have shown that the sympathetic innervation of these vessels does not extend to all the arterioles in our preparation (Hill *et al.*, 1986). In contrast, all the arterioles in our intestinal preparation receive a sympathetic innervation. Furthermore, the sympathetic innervation density is at least three times higher in the intestinal arterioles (Neild, 1984) than in the most densely innervated pial arterioles (Hill *et al.*, 1986). As the sympathetic nerves contain NPY, we thought it possible that their presence may influence the sensitivity of the arterioles. We therefore examined the effects of NPY on 17 rat pial arterioles that were checked after the experiment for the presence of catecholamine-containing nerves. The potentiating effect of 12.5 nM NPY on the constriction to U46619 did not depend on the presence of sympathetic nerves; P for innervated arterioles was 2.1 ± 0.19, $n = 8$, and P for non-innervated arterioles was 2.85 ± 0.87, $n = 9$.

Discussion

Our results show that in both the guinea-pig intestinal arterioles and the rat pial arterioles the potentiating effect of NPY was mediated entirely by Y_1 receptors. NPY[Leu^{31}, Pro^{34}], a well characterized Y_1 agonist with very little activity at Y_2 receptors (Fuhlendorf *et al.*, 1990), was as effective as NPY in producing potentiation of responses to NA or U46619. The lack of effect of PYY(13–36) showed not only that there were no Y_2 receptors involved but also that the concentrations used did not activate Y_1 receptors in this tissue. Although it is generally agreed that NPY analogues based on shortened C-terminal sequences show some selectivity for Y_2 over Y_1 receptors (Wahlestedt *et al.*, 1990), there have been suggestions that they sometimes showed significant activity at Y_1 receptors also. In particular, they can raise blood pressure in anaesthetized rats (Potter *et al.*, 1989), whereas a compound acting only on Y_2 receptors would be expected to lower blood pressure by reducing neurotransmitter release from perivascular sympathetic nerves. The hypertensive effect of short C-terminal is perhaps therefore due to Y_2-mediated vasoconstriction by an action on the arteriolar muscle. The coronary vessels show some vasoconstriction in response to high concentrations of NPY(16–36) and NPY(19–36) (Rioux *et al.*, 1986), but this alone would be insufficient to produce the observed hypertensive effects. We feel our studies make it unlikely that the intestinal or cerebral vascular beds are involved, unless there is marked heterogeneity of responses in different regions of these tissues.

The magnitude of the potentiating effect of 12.5 nM NPY when U46619 was the vasoconstrictor in the guinea-pig intestinal (P = 4.92) and rat pial (P = 2.44) arterioles was different. We have not been able to discern the reason for this difference, other than to show that it is not due simply to differences in the sympathetic innervation, as the sensitivity of the pial arterioles to NPY was the same in sympathetically innervated and non-innervated vessels. However, regions that did not receive a sympathetic innervation were probably still innervated by parasympathetic NPY-containing nerves from the pterygopalatine ganglion (Cavanagh *et al.*, 1990). These nerves also contain vasoactive intestinal polypeptide (VIP) and are probably vasodilator. The function of the NPY in them is not known; in the uterine artery NPY reduces the vasodilator effect of VIP (Morris, 1990).

The variation in sensitivity to NPY in different vascular beds may be related to different sources of endogenous ligand. It is usually assumed that in arteries, NPY released from the sympathetic nerves acts as a co-transmitter, and this is probably the case for the intestinal arterioles that we have studied. However, the finding of high levels of NPY in the cerebrospinal fluid of rabbits has led to the suggestion that NPY in cerebrospinal fluid may be a modulator of cerebral vascular tone (McDonald *et al.*, 1988). Our finding that the sensitivity of smooth muscle in the pial arterioles to NPY is independent of their sympathetic innervation is compatible with that view.

Appendix

Comparison of the magnitude of potentiating effects–T.O. Neild

It is generally agreed that potentiation (also called synergy) is best detected and quantitated by experiments which determine iso-effective combinations of the interacting substances (Berenbaum, 1989). In our case this was not possible, because NPY alone caused no constriction of the intestinal arterioles. It was therefore obvious that the effect of NPY was synergistic rather than additive, but the ideal analysis by determining iso-effective combinations and plotting isoboles could not be carried out. We have, therefore, developed a new method of analysis that enabled us to quantitate the potentiating effect of NPY on transient constrictions caused by brief applications of vasoconstrictors. Unlike the isobole method, it cannot distinguish a potentiating effect from an additive effect when the interacting substances both cause constriction of the arteriole. It uses an arbitrary equation to derive an index which increases with increasing potentiation and can be used to detect quantitative differences in potentiation.

Individual arterioles were stimulated with a constant constrictor stimulus at regular intervals. The amplitude of the constriction was measured in control conditions (c) and in the presence of one concentration of NPY (n). The maximum constriction (max) that the arteriole could produce was also measured, and used to normalize data from different experiments. The amount of vasoconstrictor applied was varied between experiments to give a range of control response amplitudes; the smaller control responses were increased more than larger responses that were closer to the maximum. If the data were plotted with normalized control response amplitude on the abscissa scale and the ratio of potentiated to control amplitude on the ordinate scale, the points fell around a curve as shown in Figure 4. Data from all types of experiment conformed to this pattern, but where the potentiating effect was greater the curve intersected the ordinate at a higher value.

An equation was found (equation 2) which produced a curve that matched the distribution of the data points and contained only 1 free parameter. It was derived from the expression:

$$P = \left(\frac{n}{max-n}\right) / \left(\frac{c}{max-c}\right) \qquad \text{equation 1}$$

We have used this expression to calculate the parameter P for use as a quantitative index of the potentiating effect. A value of P applies to one concentration of the potentiating substance and the whole range of concentrations of the vasoconstrictor, e.g. P for 50 nM NPY and noradrenaline was 4.74. Differences in P between experimental situations indicate differences in the magnitude of the potentiating effect;

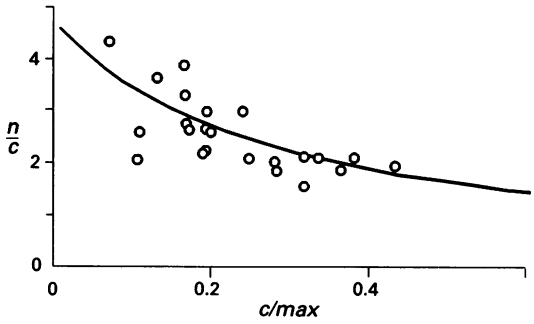

Figure 4 Plot of the ratio of potentiated (n) to control (c) responses against normalized control response amplitude. These data were from experiments using noradrenaline as the vasoconstrictor, with potentiation caused by 50 nM neuropeptide Y (NPY). A trace from one of these experiments is shown in Figure 1. The curve was plotted using equation 1 with a value of 4.74 for P. This value of P was found by taking the mean of individual values calculated from each experiment; it was not derived by fitting the curve to the points shown in this graph.

a value of 1 indicates no potentiation, with higher values for greater potentiating effect.

Re-arranging equation 1 gives:

$$\frac{n}{c} = \frac{max}{c + \frac{(max-c)}{P}} \qquad \text{equation 2}$$

which was used to produce the curve in Figure 4. The limit of this expression as c approaches max is 1, as would be expected intuitively. A control response that is already maximum cannot be increased in amplitude, no matter how great the potentiating effect. As c approaches 0 the expression approaches P, showing that P indicates the greatest factor by which a particular potentiating influence can increase a response. Control responses of intermediate size will be increased by some factor less than P, depending on their size.

In practice we prefer to find the mean value of P for a particular set of data by calculating the mean of individual values calculated for each experiment rather than by finding the best fit of the curve

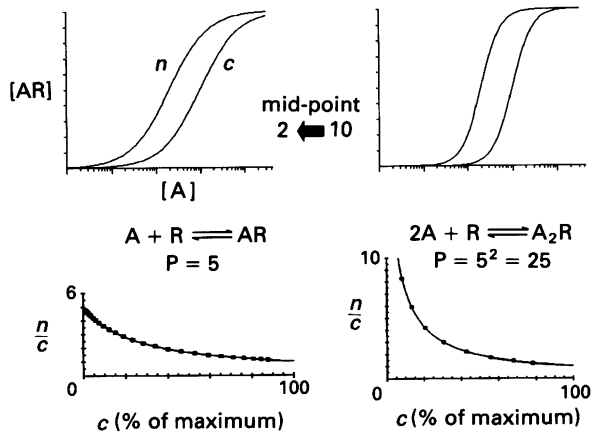

Figure 5 Simulation to show the P values obtained from the analysis of a situation in which the potentiating mechanism shifts the stimulus-response curve to the left (upper plots). A simple logistic function was used to calculate sigmoid curves corresponding to first order (left) and second order (right) binding reactions. Concentration of the agonist [A] is in arbitrary units; potentiation was represented by shifting the mid-point of the curves from 10 units to 2. The lower plots of n/c against c are the type that would be used to analyse experimental data, as shown in Figure 4. The simulated data points taken from the sigmoid curves fit exactly to a curve drawn using equation 2 with P equal to the ratio of the midpoints of the sigmoid curves raised to the power of the order of reaction.

given by equation 2 to the whole data set. Both sides of equation 2 contain terms that are subject to experimental error, and a true best fit with an estimate of the variance of P cannot be easily obtained. When P is calculated from the mean of values from individual experiments standard statistical methods can be used to test for differences between Ps and between P and 1.

The application of this analysis to simulated situations in which a graph of log (stimulus) and response was sigmoidal, and potentiation shifted this graph to the left, as shown in Figure 5. The stimulus-response curves were calculated from logistic equations for the fraction of binding sites occupied by various concentrations of a ligand. 'Control values' were taken from one curve and 'potentiated values' taken from the shifted curve at the same 'stimulus' value. P was calculated for each pair of values using equation 1, as would be done for experimental data, and the mean value of P was used to plot a curve through the measured points. The results using first and second order binding reactions are shown in Figure 5.

These simulations show that a value of P obtained experimentally is related to a shift in the stimulus-response relationship, even though the full relationship had not been obtained. In the case of a first order system, P would be the ratio of mid-points of the two curves; in the case of higher order systems P would be equal to this ratio raised to the power of the order.

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A pharmacological study of NK_1 and NK_2 tachykinin receptor characteristics in the rat isolated urinary bladder

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1 We have estimated potencies of tachykinin receptor agonist and antagonist analogues in order to determine the recognition characteristics of tachykinin receptors mediating phasic contractile responses of the rat isolated urinary bladder *in vitro*.

2 The NK_1-selective synthetic agonists, substance P methyl ester and GR73632, the synthetic NK_2-selective agonists [β-Ala^8]-NKA(4-10) and GR64349, and the mammalian tachykinins, neurokinin A and neurokinin B, were assayed relative to substance P and were found to be approximately equipotent. The NK_3-selective agonist, senktide, was inactive (10 μM).

3 Potencies of all these agonists were not significantly different ($P > 0.05$) when experiments were carried out in the presence of the neutral endopeptidase inhibitor, phosphoramidon, and the kininase II inhibitor, enalaprilat (both 1 μM).

4 The NK_1-selective antagonist, GR82334, inhibited responses to substance P methyl ester in a competitive manner in the rat urinary bladder and the rat ileum, and also in the guinea-pig ileum. Markedly different pK_B estimates were obtained in the rat bladder (6.38) and rat ileum (6.56) compared to the guinea-pig ileum (7.42). GR82334 (3 μM) was inactive against responses of the rat bladder to [β-Ala^8]-NKA(4-10).

5 The NK_1-selective antagonist (±)-CP-96,345 also inhibited responses of the rat bladder and guinea-pig ileum to substance P methyl ester; however, in the rat bladder at 1 μM, this antagonist reversibly inhibited responses both to the NK_2-selective agonist [β-Ala^8]-NKA(4-10) and to the muscarinic agonist carbachol ($P \leq 0.01$), thus showing evidence of some non-selective depressant actions.

6 The NK_2-selective antagonists, MEN10207 and L-659,874, competitively inhibited responses of the rat bladder to the NK_2-selective agonist [β-Ala^8]-NKA(4-10) giving pK_B estimates of 5.75 and 6.68, respectively. Both antagonists (10 μM) were inactive against responses to the NK_1-selective agonist substance P methyl ester.

7 These results support the proposal of a mixed population of NK_1 and NK_2 receptors mediating contraction of the rat isolated urinary bladder. The NK_2 receptor is characterized by a relatively low affinity for the NK_2-selective antagonist MEN10207 but a high affinity for L-659,874. The NK_1-mediated responses are inhibited by (±)-CP-96,345: this compound however, has non-specific depressant effects in the rat bladder at high concentration (1 μM). In contrast, the NK_1-receptor peptide antagonist GR82334, did not have non-specific depressant effects and competitively inhibited NK_1 responses in the rat bladder and rat ileum with an affinity significantly lower than at the NK_1-receptors in the guinea-pig ileum.

Keywords: Neurokinin; substance P; urinary bladder (rat); tachykinin receptors; neurokinin antagonists; tachykinin; NK_1 receptors; NK_2 receptors; (±)-CP-96,345, GR82334

Introduction

The currently accepted classification scheme for tachykinin receptors (see, Henry, 1987; Guard & Watson, 1991) identifies three distinct types, NK_1, NK_2 and NK_3, the structural sequences of which have been elucidated (Masu *et al.*, 1987; Yokota *et al.*, 1989; Shigemoto *et al.*, 1990; Nakanishi, 1991). It is now becoming clear that this scheme may need to be refined to take into account species homologues of the same receptor type. In particular, Maggi and co-workers (Maggi *et al.*, 1990) have reported that the NK_2-selective antagonist [Tyr^5,D-Trp^{6,8,9},Arg^{10}]-NKA(4-10) (MEN10207) (Rovero *et al.*, 1990) has high affinity for NK_2 tachykinin receptors in the rabbit pulmonary artery ($pA_2 = 7.65$), but has relatively low affinity for the NK_2 receptors of the hamster trachea ($pA_2 = 5.80$). In contrast, the NK_2-selective hexapeptide antagonist Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2 (Maggi *et al.*, 1990) shows a reversed selectivity (pA_2 in hamster trachea 7.67, as compared to 5.42 in the rabbit pulmonary artery). These and other observations, (Buck *et al.*, 1990; van Giersbergen *et al.*, 1991), have led to speculation as to the existence of subtypes or species homologues of NK_2 receptors (Maggi *et al.*, 1990; Patacchini *et al.*, 1991). Very recently, the novel non-peptide NK_1-selective antagonist (±)-CP-96,345 (Snider *et al.*, 1991) has been reported to have variable affinities at NK_1 receptors when compared between a number of different preparations (Beresford *et al.*, 1991; Gitter *et al.*, 1991; McLean *et al.*, 1991; Rouissi *et al.*, 1991; Snider *et al.*, 1991). It has been suggested that such differences in affinities of antagonists at NK_1 and NK_2 receptors may be related to between-species differences in recognition properties of these tachykinin receptors (Beresford *et al.*, 1991; Gitter *et al.*, 1991; Hall *et al.*, 1992; Watling *et al.*, 1991).

The isolated urinary bladder from a number of species contracts in response to substance P and related tachykinins (Falconieri-Erspamer *et al.*, 1973), and there is evidence suggesting a physiological or pathophysiological role for endogenous tachykinins, released from afferent endings of sensory nerves, in the micturition reflex (Maggi & Meli, 1986; Maggi *et al.*, 1987a). The post-junctional receptors on rat bladder smooth muscle that are involved in the phasic contractile response to tachykinins have been characterised in terms of potencies of agonist analogues in functional studies

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(Watson et al., 1983; Maggi et al., 1987a,b; 1988) and further defined in radioligand binding studies (Burcher & Buck, 1986; Nimmo et al., 1992). Such studies have indicated the presence of both NK_1 and NK_2 receptors, predominantly on the smooth muscle cells. Although NK_2-selective antagonist ligands have been tested in the rat isolated urinary bladder preparation against responses to substance P and neurokinin A (Maggi et al., 1991), at the present time there have been no reports of quantitative pharmacological analysis of the post-junctional tachykinin receptors using recently available selective ligands.

The aim of this study, therefore, was to investigate further the recognition characteristics of the NK_1 and NK_2 tachykinin receptors mediating phasic contractile responses of the rat isolated urinary bladder, using a range of both naturally occurring agonist and snythetic agonist and antagonist ligands. In particular, the affinities of the NK_1-selective antagonists GR82334 (Hagan et al., 1991a) and (±)-CP-96,345 (Snider et al., 1991), and the NK_2-selective antagonists L-659,874 (McKnight et al., 1988) and MEN10207 (Rovero et al., 1990) were determined. Because the rat urinary bladder is not a monoreceptor system, to obtain reliable antagonist affinity estimates at tachykinin receptors, it was necessary to test these analogues against agonists established as being highly selective for the tachykinin receptor types. The two agonists chosen were, for NK_1 receptors substance P methyl ester (Cascieri et al., 1981; Watson et al., 1983), and for NK_2 receptors, [β-Ala^8]-NKA(4-10) (Rovero et al., 1989). The antagonist affinities estimated in this study are compared with those obtained from preparations taken from the same and another species, in order to gain evidence regarding possible species-dependent differences in receptor recognition properties.

Preliminary accounts of this work have been communicated to the British Pharmacological Society (Flowers et al., 1991) and the European Neuropeptide Club (Hall et al., 1992).

Methods

General

Male Wistar rats (200–400 g) were killed by stunning and exsanguination. The urinary bladder was excised and superficial fat and connective tissue were removed. The lower third of the bladder was discarded and four preparations of the detruser muscle (with adhering mucosa) were cut longitudinally from the bladder.

Preparations were mounted under 0.75 g initial tension in 2.5 ml silanised glass organ baths and responses were measured isometrically with Grass FT03B transducers coupled to Grass Model 7E polygraphs or to JJ Instruments Cr452 potentiometric flat-bed recorders (after d.c. amplification).

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

In all experiments, preparations were left to equilibrate for 60 min, followed by estimation of the maximal response to carbachol.

Agonist studies

Four preparations were set up in parallel, with two bathed in Krebs solution containing the neutral endopeptidase inhibitor phosphoramidon and the kininase II inhibitor, enalaprilat (both 1 μM), and two in Krebs solution without peptidase inhibitors. A randomised dosing protocol (agonist and concentration) was adopted to compare the potencies of tachykinins (1 nM–3 μM) in two series of experiments. In the first series, the NK_1-selective agonists substance P methyl ester and GR73632 were assayed relative to substance P as standard; and in the second series the mammalian tachykinins neurokinin A and neurokinin B were assayed along with the NK_2-selective agonists [β-Ala^8]-NKA(4-10) and GR64349 also relative to substance P. A 5 min dose-cycle was used for drug concentrations up to 0.1 μM, extended, as necessary, to 10–20 min for drug concentrations greater than 0.1 μM. Preparations were washed as soon as peak phasic responses had been achieved. The NK_3-selective agonist succ-[Asp^6,Me-Phe^8]-SP(6-11) (senktide; Wormser et al., 1986) was found to be inactive (10 μM), so was not included in any formal bioassay procedure.

Antagonist studies

A three-point log concentration-response curve was obtained for the agonist under study using serial dosing, which was repeated either in the presence of increasing concentrations of tachykinin receptor antagonist (test) or no antagonist (concurrent time control). A 20 min dose cycle with a 15 min antagonist incubation time was used, and up to four antagonist concentrations were tested in each individual preparation. A 15 min antagonist incubation time was chosen since preliminary studies indicated no change in pK_B estimates when a longer incubation period was used (data not shown). Experiments with MEN10207 and L-659,874 were carried out in the presence of the phosphoramidon and enalaprilat (both 1 μM).

NK_1 antagonist studies

In the rat bladder, the NK_1-selective antagonists GR82334 (0.3–10 μM) and (±)-CP-96,345 (0.1–3 μM) were tested against responses to both substance P methyl ester and neurokinin A. To assess specificity, GR82334 (3 μM) and (±)-CP-96,345 (1 μM) were also tested against responses to submaximal concentrations of [β-Ala^8]-NKA(4-10) and carbachol. Three control submaximal responses to [β-Ala^8]-NKA(4-10) and carbachol were obtained, and these were then repeated after a 15 min incubation with antagonist. If inhibition was seen, responses to control doses of agonist were redetermined following a 60 min washout period. For comparison, the antagonists (±)-CP-96,345 (10 nM –0.3 μM) and GR82334 (30 nM –3 μM) were also assayed using Schild plot analysis of antagonism of substance P methyl ester on the longitudinal smooth muscle of the guinea-pig ileum and GR82334 (1–10 μM) in the intact rat ileum (for methods, see Hall & Morton, 1991).

NK_2 antagonist studies

In the rat bladder, the NK_2-selective antagonist MEN10207 (1-10 μM) was tested against responses to [β-Ala^8]-NKA(4-10). The NK_2-selective antagonist L-659,874 (0.1-10 μM) was tested against responses to [β-Ala^8]-NKA(4-10) and neurokinin A. Both antagonists (10 μM) were tested for selectivity against responses to submaximal concentrations of substance P methyl ester. Three control submaximal responses to substance P methyl ester were obtained and these were repeated following a 15 min incubation with antagonist.

Source of drugs

Agents were obtained as follows: carbachol, atropine sulphate, ibuprofen and hexamethonium bromide (Sigma, U.K.), mepyramine maleate (May & Baker, U.K.), enalaprilat (Merck, Sharp & Dohme, U.S.A), cimetidine (Smith, Kline & French, U.K.), guanethidine sulphate (Ciba, U.K.). δ-aminovaleryl-[L-Pro^9,N-MeLeu^{10}]-SP(7-11) (GR73632) and [Lys^3,Gly^8-R-γ-Lactam, Leu^9]-NKA(3-10) (GR64349) (Glaxo

Group Research, U.K), [β-Ala^8]-NKA(4-10) (A. Menarini Pharmaceuticals, Italy; or Bachem, U.K), [Tyr^5,D-Trp^{6,8,9}, Arg^{10}]-NKA(4-10) (MEN10207) (A. Menarini Pharmaceuticals, Italy or Peninsula Laboratories, Europe), (±)-CP-96,345 ([(2S,3S)-*cis*-2-(diphenylmethyl)-*N*-[2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine], A. Menarini Pharmaceuticals, Italy), Ac-Leu-Met-Gln-Trp-Phe-Gly-NH_2 (L-659,874) (Cambridge Research Biochemicals, U.K.), [D-Pro^9 [Spiro-γ-Lactam]Leu^{10},Trp^{11}]-physalaemin(1-11) (GR82334) (Peninsula Laboratories Europe or Glaxo Group Research), substance P methyl ester and phosphoramidon (Peninsula Laboratories Europe, U.K.). All other peptides were obtained from Bachem, U.K. All salts used were of analytical grade and were obtained from B.D.H., U.K.

Ibuprofen was dissolved in 5% Na_2CO_3. (±)-CP-96,345, senktide, L-659,874 and neurokinin B were dissolved in DMSO. All other peptides and agents were dissolved in distilled water. Peptides were stored under N_2 at −20°C.

Expression of results and statistical analysis

Contractile responses are expressed as % of maximal response to carbachol, and the estimates are shown as means ± s.e.mean. Relative activities were calculated from differences in mean pD_2 (-log EC_{50}, 50% carbachol maximum) estimates within each series. Tests for significance were made by Student's *t* test for paired- or two-independent samples as appropriate. The sensitivity of the rat bladder both to substance P methyl ester and to [β-Ala^8]-NKA(4-10) tended to decrease after construction of the first control curve, but then stabilised for subsequent curves (data not shown). Consequently, individual dose-ratio estimates (x) between test and concurrent control preparations where measured, and from these values, individual pK_B estimates (± s.e.mean) were obtained for competitive interactions from the Gaddum-Schild equation, $pK_B = \log_{10}(x\text{-}1) - \log_{10}[A]$, where [A] is the antagonist concentration. Conventional linear regression analysis of Schild plots was used to test for significant departure of the estimated slope (b) from a population value (β) of unity slope. The coefficient of linear correlation was used as a measure of goodness-of-fit. Throughout, *n* refers to the number of estimates (pK_B), or number of preparations (all other estimates), and in every case represents data obtained from 3–8 animals.

Results

General

Contractile responses of the rat bladder to tachykinins reached a peak phasic response at a rate that depended somewhat on concentration, but was generally within 2 min, after which time preparations were washed until they regained base-line tension.

Agonist studies

Figure 1 shows log concentration-response curves obtained in the two series of experiments in the rat bladder where a range of tachykinins were assayed relative to substance P; in the first series (Figure 1a) for the NK_1-selective agonists; and in the second for the mammalian tachykinins and NK_2-selective agonists (Figure 1b). Mean pD_2 values were estimated at 50% the maximal response to carbachol since maximal responses to individual peptides were not obtained, and these estimates along with activities relative to substance P calculated from these data are shown in Table 1. The peptidase inhibitors phosphoramidon and enalaprilat were without significant effect on agonist pD_2 estimates ($P > 0.05$; see Table 1; Hall *et al.*, 1990).

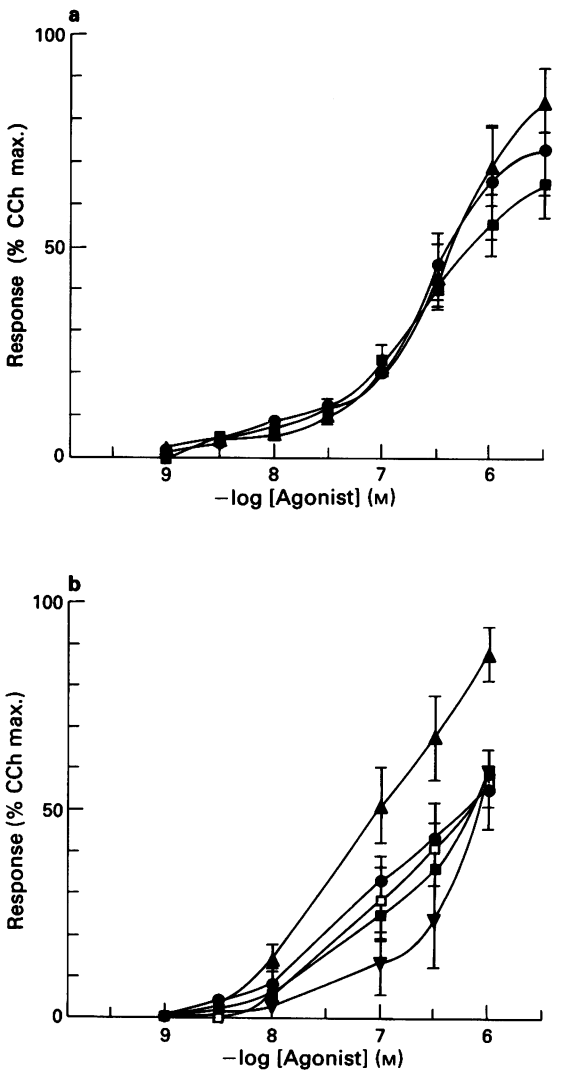

Figure 1 Mean log concentration-response curves in the rat isolated urinary bladder for agonists active at NK_1 or NK_2 receptors. (a) Shows the NK_1 selective agonists, GR73632 (▲) and substance P methyl ester (■); (b) shows the NK_2 selective agonists GR64349 (□) and [β-Ala^8]-NKA(4-10) (■), along with neurokinin A (▲) and neurokinin B (▼). Substance P (●) was included as an internal standard in both series. Each point expressed as % maximal response to carbachol (CCh) is the mean ± s.e.mean (vertical bars) for 6 preparations.

NK_1 antagonist studies

In the rat bladder and rat ileum, the NK_1-selective antagonist, GR82334, competitively antagonized responses to substance P methyl ester, and pK_B estimates of 6.38 ± 0.13 ($n = 22$) and 6.56 ± 0.09 ($n = 14$) respectively were obtained (see Figure 2a and Table 2). It should be noted that the rat ileum preparation did not appear to offer many advantages as an example of rat NK_1 preparation, in that like the bladder it is not a monoreceptor system, and furthermore then NK_1-receptor-induced contractions were much weaker and rather more variable than in the bladder. GR82334 also competitively antagonised responses to substance P methyl ester in the guinea-pig ileum preparation, but with an affinity that was significantly ($P < 0.001$) higher ($pK_B = 7.42$, Figure 2a) than that obtained in the rat bladder and rat ileum. Over a range of concentrations (0.3-10 μM) in the bladder, GR82334 showed variable antagonism of responses to neurokinin A (see Table 2). In tests for selectivity in the bladder, GR82334 (3 μM) was inactive against responses to

Table 1 Activity estimates for neurokinin receptor agonists in the rat urinary bladder

Agonist	Without inhibitors: pD_2 (± s.e.mean)	Without inhibitors: Relative activity	With inhibitors: pD_2 (± s.e.mean)
Substance P	6.03 (0.24)	1.00	6.26 (0.12)
GR73632	6.23 (0.18)	1.59	6.28 (0.22)
Substance P methyl ester	5.70 (0.29)	0.47	6.07 (0.13)
Substance P	6.65 (0.30)	1.00	6.39 (0.11)
Neurokinin A	7.05 (0.17)	2.5	6.79 (0.10)
Neurokinin B	6.29 (0.15)	0.47	6.08 (0.20)
GR64349	6.50 (0.20)	0.71	6.59 (0.30)
[β-Ala^8]-NKA(4-10)	6.51 (0.25)	0.72	6.09 (0.23)

The pD_2 values for each series of experiments, were calculated from $-\log EC_{50}$ estimates in 6 individual preparations: these estimates were unchanged by the presence of the peptidase inhibitors phosphoramidon and enalaprilat (both 1 μM; $P>0.05$). Activities of agonists relative to substance P (1.00) were calculated from differences in mean pD_2 estimates. The difference in absolute activity of substance P in the two series is due to biological variation, and was not significant ($P>0.05$).

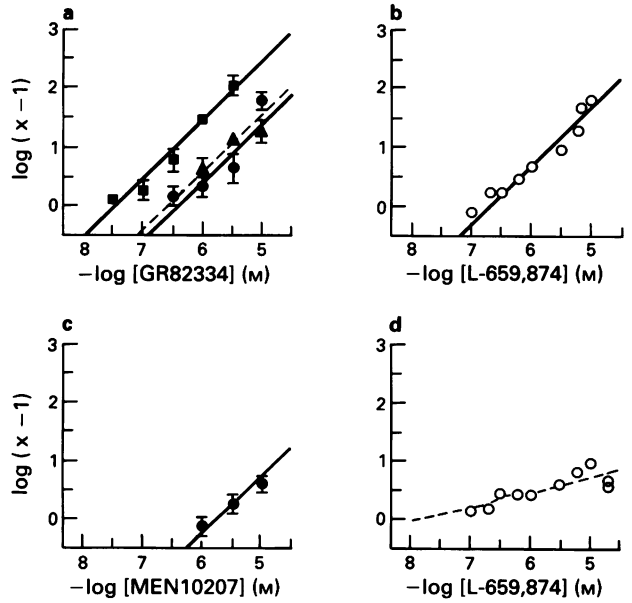

Figure 2 Schild plot analyses, in the rat urinary bladder, rat ileum, or the guinea-pig ileum, for NK_1 or NK_2 selective antagonists. Throughout, symbols are solid for means (with vertical bars denoting s.e.mean) or open for individual observations: circles are for experiments with rat urinary bladder, triangles for rat ileum, and squares for guinea-pig ileum. b = estimated coefficient of linear regression ± s.e.mean, P the significance of b (estimated slope) versus β (population slope value = 1.00) as estimated by conventional linear regression analysis of Schild plots.
(**a**) The NK_1-selective antagonist, GR82334, against the NK_1-selective agonist, substance P methyl ester, in the rat bladder (●) (b = 1.04 ± 0.27; $P>0.05$), rat ileum (▲) (b = 0.65 ± 0.22; $P>0.05$) or guinea-pig ileum (■) (b = 1.09 ± 0.11; $P>0.05$). (**b**) The NK_2-selective antagonist, L-659,874 against the NK_2-selective agonist, [β-Ala^8]-NKA(4-10) in the rat bladder (○) (b = 0.98 ± 0.06; $P>0.05$). (**c**) The NK_2-selective antagonist, MEN10207, against [β-Ala^8]-NKA(4-10) in the rat bladder (●) (b = 0.743 ± 0.11; $P>0.05$). (**d**) L-659,874 against the non-selective agonist neurokinin A in the rat bladder (○) (b = 0.24 ± 0.07; $P<0.001$).

carbachol and [β-Ala^8]-NKA(4-10) (see Figure 3b and Table 2).

In the rat bladder, (±)-CP-96,345 was found also to attenuate responses to substance P methyl ester and to neurokinin A (data not shown), and Schild regressions for both agonists were compatible with competition at a single site, yielding pK_B estimates of 6.79 and 6.61, respectively. However, in tests for selectivity in the bladder, (±)-CP-96,345 (1 μM) caused a small but significant inhibition ($P\leq 0.01$) of submaximal responses to carbachol and [β-Ala^8]-NKA(4-10) (see Figure 3a). For these reasons, interpolation from these data is potentially misleading. In the guinea-pig ileum preparation, (±)-CP-96345 competitively antagonized responses to substance P methyl ester with an affinity ($pK_B = 9.00$) that was significantly higher that its apparent affinity in the bladder ($P<0.001$)(data not shown).

NK_2 antagonist studies

The NK_2-selective antagonist, L-659,874, competitively antagonized contractile responses to the NK_2-selective agonist [β-Ala^8]-NKA(4-10). These data are displayed as a Schild plot in Figure 2b. A pK_B of 6.68 was calculated (Table 2). L-659,874 (10 μM) was inactive against responses to the NK_1-selective agonist substance P methyl ester ($P>0.05$, Table 2). When tested against the subtype non-selective agonist neurokinin A, L-659,874 (0.1–20 μM) attenuated responses, but Schild analysis showed a shallow regression line (b = 0.24) that differed significantly from unity ($P<0.01$) (see Figure 2d), compatible with an action of neurokinin A at a site additional to the NK_2-receptor.

In the bladder, the NK_2-selective antagonist MEN10207 competitively antagonized responses to the NK_2-selective agonist [β-Ala^8]-NKA(4-10), though with a relatively low affinity ($pK_B = 5.75$; see Figure 2c and Table 2), but was inactive (10 μM) against contractile responses to substance P methyl ester (Table 2). At 10 μM, MEN10207 showed partial agonist activity (*ca.*5% CCh maximum; data not shown; see also Maggi *et al.*, 1991).

Table 2 Affinity estimates in the rat isolated urinary bladder for three receptor-selective antagonists when tested against the NK_1-selective agonist substance P methyl ester (SPOMe), the NK_2-selective agonist [β-Ala^8]-NKA(4-10), and the non-selective agonist neurokinin A (NKA).

Antagonist	*Agonist*		
	SPOMe pK_B	*[β-Ala^8]-NKA(4-10)* pK_B	*NKA* pK_B
GR82334	6.38 (0.13;22)	NA (3 μM;7)	NC† (0.3-10 μM;11)
MEN10207	NA (10 μM;5)	5.75 (0.06;9)	NT
L-659,874	NA (10 μM;5)	6.68 (0.04;9)	NC# (0.1-20 μM;10)

Estimates of pK_B (with s.e.mean and n, in parentheses) are shown only where Schild plot regressions showed no significant departure ($P > 0.05$) from unity slope. NC denotes interaction not competitive with respect to a single site over the range shown in parentheses, as judged from shallow Schild regression, b = 0.24 (#), or variable antagonism (†). NA denotes not active (at the concentration shown in parentheses), NT denotes not tested.

Discussion

The present analysis, using selective agonists provides evidence of both NK_1 and the NK_2 receptor types, in the rat isolated urinary bladder. The lack of appreciable activity of the highly NK_3-selective agonist senktide (Wormser *et al.*, 1986) discounts any major contribution from the NK_3 receptor type under the conditions of this study (see also, Maggi *et al.*, 1988; Pietra *et al.*, 1990). Both the neutral endopeptidase inhibitor phosphoramidon and the kininase II inhibitor enalaprilat were without significant effect on agonist potencies, suggesting that peptide breakdown had not distorted agonist activity estimates. We have previously shown aminopeptidase inhibition with bestatin (100 μM) to be without effect on agonist potencies in this preparation (Hall *et al.*, 1990).

The data obtained with tachykinin-receptor antagonists, in general conform to these conclusions, since both NK_1-and NK_2-selective antagonists competitively blocked responses to their respective receptor-selective agonists. Affinities of antagonists for both the NK_1 and the NK_2 sites differed appreciably from those found in some other preparations (see below).

NK_1 receptor characteristics

The NK_1-selective agonists, substance P methyl ester and GR73632, were both active in the rat urinary bladder, confirming earlier reports (Maggi *et al.*, 1988; Pietra *et al.*, 1990). Both these agonists are established as selective for the NK_1 receptor type, over the NK_2 and NK_3 types, as determined in monoreceptor systems (Hagan *et al.*, 1989; 1991b; Hall & Morton, 1991). These results therefore confirm the presence of NK_1 receptors in the rat urinary bladder.

The characteristics of the NK_1 receptors were further established by use of selective antagonists. Thus, the NK_1-selective antagonist, GR82334, competitively antagonized responses to substance P methyl ester in the rat urinary bladder. The pK_B obtained in the rat bladder ($pK_B = 6.38$) was similar to that estimated in rat ileum ($pK_B = 6.56$), and is about one log unit lower than the pK_B estimate against NK_1-mediated contraction in guinea-pig ileum ($pK_B = 7.42$). This pK_B value obtained in the guinea-pig ileum with GR82334 is in agreement with earlier reports ($pK_B = 7.64$; Hagan *et al.*, 1991a). These results suggest that GR82334 distinguishes between the NK_1 receptors in these two species. The variable antagonism of responses to neurokinin A by GR82334 is as expected since this agonist interacts with both NK_1 and NK_2 receptors.

The presence of NK_1 receptors in the bladder appeared to be supported by results with the NK_1-selective non-peptide antagonist (±)-CP-96,345, which was also much more potent as an antagonist of NK_1 receptors in the guinea-pig ileum as compared to the rat urinary bladder (pK_B estimates of 9.00 and 6.79, respectively). The pK_B estimate we obtained for (±)-CP-96,345 in the guinea-pig ileum ($pK_B = 9.00$) is comparable to that reported by other workers ($pK_B = 8.89$, Beresford *et al.*, 1991; $pK_B = 8.11$, Lecci *et al.*, 1991). Furthermore, the affinity for (±)-CP-96,345 in the guinea-pig ileum is similar to its affinity found in some other NK_1 preparations, the rabbit aorta ($pK_B = 8.81$; Beresford *et al.*, 1991) and rabbit iris ($pK_B = 8.85$; Hall *et al.*, 1992), the dog carotid artery ($pK_B = 8.7$, Snider *et al.*, 1991). The relatively low pK_B estimate we obtained in the rat urinary bladder is similar to that determined in another rat preparation, the neonatal rat spinal cord ($pK_B = 7.13$, Beresford *et al.*, 1991).

Our finding that the affinities for (±)-CP-96,345 and GR82334 differ significantly in the guinea-pig as compared to the rat preparations, suggests that they both can discriminate between NK_1 receptors in preparations taken from the two species. Indeed, the ability of (±)-CP-96,345 to distinguish between the NK_1 receptors in a number of different tissues from several species has been demonstrated (Beresford *et al.*, 1991; 1992; Gitter *et al.*, 1991; Snider *et al.*, 1991). However, it should be noted that (±)-CP-96,345 (unlike GR82334) also inhibited contractile responses to the NK_2-selective agonist, [β-Ala^8]-NKA(4-10), as well as those to carbachol in the rat bladder, so suggesting a non-specific component to its action as an antagonist; a characteristic also reported in some other preparations (Lecci *et al.*, 1991; Boyle *et al.*, 1991), including the guinea-pig ileum at concentrations (0.5 μM) in the range of those used in the present study. Furthermore, non-specific effects with both the active (CP-96,345) and inactive (CP-96,344) enantiomers of this compound have been reported (Donnerer *et al.*, 1992; Schmidt *et al.*, 1992), which are apparently related to L-type calcium-channel blockade (Guard & Watling, 1992; Schmidt *et al.*, 1992). The usefulness of this antagonist as an analytical tool in pharmacological studies is therefore limited, though it is clear that it produces little antagonism of responses to substance P methyl ester in the rat bladder at concentrations causing marked antagonism in a number of preparations from other species; e.g. the guinea-pig ileum and trachea, rabbit aorta and iris (Beresford *et al.*, 1991; 1992; Hall *et al.*, 1992).

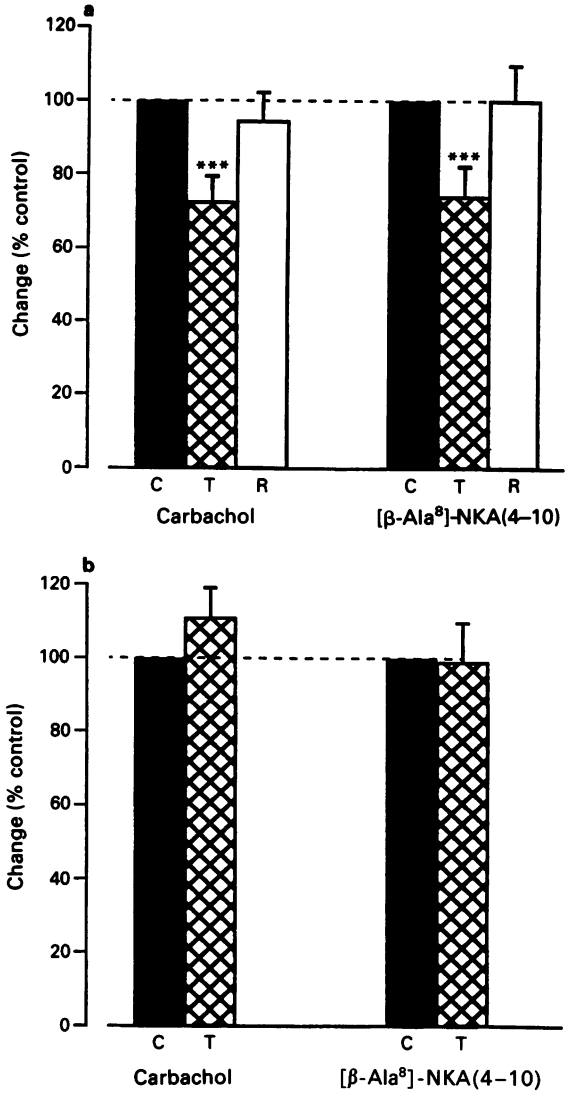

Figure 3 Tests for selectivity of antagonism by NK_1 antagonists of contractile responses in the rat urinary bladder to submaximal concentrations of carbachol and [β-Ala^8]-NKA(4-10). In (a) results with (±)-CP-96,3435 (1 μM): control (C) pre-antagonist responses (solid column) are normalized to 100% (shown by a dashed-line), and mean test (T) responses (± s.e.mean) after 5 min are shown (cross-hatched columns) followed by post-antagonist re-test (R) after washing for 60 min (open bar). The depression of responses to each of the two agonists was highly significant ($P \leq 0.01$; paired t test). In (b) a similar test series with GR82334 (3 μM) is shown, where there was no significant change in the magnitude of contractile response ($P > 0.05$; paired t tests).

NK_2 receptor characteristics

The NK_2 selective agonists, GR64349 and [β-Ala^8]-NKA(4-10), were both potent spasmogens in the rat urinary bladder; the results with GR64349 confirm a previous report (Pietra *et al.*, 1990). Both agonists are established as selective for the NK_2 receptor type, being weakly inactive at NK_1 and NK_3 receptors (Hagan *et al.*, 1989; 1991b; Hall & Morton, 1991). Further evidence for the presence of NK_2 receptors in the rat urinary bladder was provided by the observation that the NK_2-selective antagonist, MEN10207, competitively antagonized responses to [β-Ala^8]-NKA(4-10). The relatively low affinity estimate ($pK_B = 5.75$) we obtained for this antagonist, suggests that the receptors in the rat urinary bladder resemble those in the hamster trachea preparation where this analogue has a reported pA_2 of 5.80 (Maggi *et al.*, 1990), rather than the receptors in the rabbit pulmonary artery where it has a higher affinity ($pA_2 = 7.65$, Maggi *et al.*, 1990). The NK_2 selective antagonist, L-659,874 also competitively antagonized responses to the NK_2-selective agonist, [β-Ala^8]-NKA(4-10), yielding a pK_B estimate of 6.68; a value similar to that reported for this antagonist by McKnight *et al.* (1988) in the rat vas deferens ($pA_2 = 6.8$).

In experiments designed to show dual receptor interaction by neurokinin A in the rat bladder, L-659,874 antagonized responses to neurokinin A with a shallow Schild regression, as might be expected were this agonist also to interact with NK_1 receptors. This observation may therefore be taken to provide further evidence for the presence of both NK_1 and NK_2 receptors in the rat bladder.

In conclusion, the results from the present study support the proposal that both NK_1 and NK_2 receptors are involved in the contractile response of the rat isolated urinary bladder preparation to tachykinins. These data are in agreement with information gained using molecular biology techniques, where expression of mRNA for both NK_1 and NK_2 receptors has been shown in rat preparations including the urinary bladder (Hershey *et al.*, 1991; Ohkubo & Nakanishi, 1991; Takeda & Krause, 1991). Pharmacologically, the NK_2-receptor type is relatively resistant to MEN10207 whilst being relatively sensitive to L-659,874. NK_1-receptor mediated responses in the rat bladder and ileum are antagonized by GR82334, albeit with a significantly lower affinity compared with the guinea-pig ileum. Thus, such data provide evidence of heterogeneity within both the NK_1- and NK_2-receptor classes in different preparations. This heterogeneity may, at least in part, prove to be attributable to inter-species differences, as might be anticipated from the between-species variations demonstrated in known primary structural sequences of tachykinin receptors. As an analytical tool, GR82334 appears valuable for the further study of NK_1 receptor heterogeneity, in as much as it has reasonably high affinity without the undesirable effects of some antagonists, in particular the non-specific depressant effects observed with CP-96,345.

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Pharmacological profile of a high affinity dipeptide NK_1 receptor antagonist, FK888

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1 In our search for compounds that inhibit the binding of [^{3}H]-substance P (SP) to guinea-pig lung membranes, the dipeptide SP antagonist, FK888, was developed by chemical modification of the parent compound, (D-Pro^4, D-Trp^{7,9,10}, Phe^{11})SP_{4-11}.

2 In a [^{3}H]-SP binding assay using guinea-pig lung membranes and rat brain cortical synaptic membranes, FK888 displaced [^{3}H]-SP binding with a K_i value of 0.69 ± 0.13 nM and 0.45 ± 0.17 μM, respectively, in a competitive manner.

3 FK888 inhibited the contraction of guinea-pig isolated ileum induced by SP in the presence of atropine and indomethacin (a NK_1 receptor bioassay) with a pA_2 value of 9.29 (8.60– 9.98).

4 FK888 inhibited contractions of rat vas deferens by NKA (a NK_2 receptor bioassay) and of rat portal vein by NKB (a NK_3 receptor bioassay) at concentrations at least 10,000 times greater than that required to inhibit contractions of guinea-pig ileum.

5 FK888 also inhibited SP-induced airway oedema in guinea-pig after both intravenous and oral administration.

6 These data demonstrate that FK888 is a potent and selective NK_1 antagonist which is active both *in vitro* and *in vivo*.

Keywords: Substance P; tachykinin; antagonist; NK-receptor; airway oedema

Introduction

The discovery of several highly selective tachykinin receptor antagonists has recently been reported. Snider *et al.* (1991) and Garret *et al.* (1991) described the NK_1 receptor selective antagonists, CP-96,345 and RP 67589, respectively, and Maggi *et al.* (1991) and Emonds-Alt *et al.* (1992) described NK_2 receptor selective antagonists, MEN-10376 and SR 48968, respectively. Substance P (SP) and its related peptides (neurokinin A, neurokinin B) elicit a wide range of biological actions in mammals via three different receptors (NK_1, NK_2 and NK_3) (Guard & Watson, 1991). For example, they cause hypotension as a result of vasodilatation (NK_1), increase vascular permeability (NK_1) and contract smooth muscle (NK_1, NK_2, NK_3). The involvement of tachykinins in the pathophysiology of many disease states has been suggested (Pernow, 1983). The discovery of novel, selective SP antagonists will be useful both experimentally and clinically because it will allow further studies aimed at clarifying the role of tachykinins in physiological and pathophysiological conditions. We have been undertaking research to discover new SP antagonists for several years and have previously described the cyclic peptide antagonist FK224, a NK_1 and NK_2 dual type antagonist isolated from fermentation products (Morimoto *et al.*, 1992b; Murai *et al.*, 1992), and the synthetic tripeptide NK_1 receptor antagonist, FR113680, which was produced by chemical modification of the parent compound, (D-Pro^4, D-Trp^{7,9,10}, Phe^{11})SP_{4-11} (Hagiwara *et al.*, 1991; Morimoto *et al.*, 1992a). After further modification of FR113680, we have successfully produced a very potent dipeptide NK_1 receptor antagonist FK888, N^2-[(4*R*)-4-hydroxy-1-(1-methyl-1H-indol-3-yl)carbonyl-L-prolyl]-*N*-methyl-*N*-phenylmethyl-3-(2-naphthyl)-L-alaninamide, (Figure 1). In this paper, we describe the pharmacological properties of FK888 in both *in vitro* and *in vivo* experiments.

Methods

Receptor binding

Guinea-pig lung membranes were prepared according to the method described by Norman *et al.* (1987) with the following modification. Male Hartley guinea-pigs weighing 300–400 g were killed, and whole lungs together with the airway tracts were removed. The isolated whole lung tissues were homogenized in 10 volumes of ice cold Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose and 0.1 mM EDTA using a Polytron PT-10. The homogenate was centrifuged at 1,000 *g* for 10 min and the supernatant was collected. The supernatant obtained was further centrifuged at 35,000 *g* for 20 min. The pellet was washed twice with 9 vol of 5 mM Tris-HCl buffer (pH 7.5) and the final pellet was re-suspended in 50 mM Tris-HCl buffer (pH 7.5) and stored at – 70°C until use.

Rat cerebral cortical synaptic membranes were prepared according to the methods described by Zukin *et al.* (1974). Male Sprague-Dawley rats weighing 250–300 g were killed, and the cerebral cortex was rapidly removed and homogenized in 9 vol of ice cold 1 mM phosphate buffer (pH 7.5)

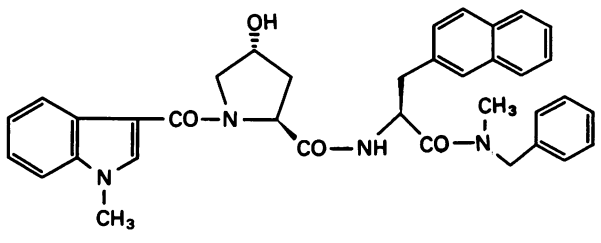

Figure 1 Chemical structure of FK888.

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containing 0.32 M sucrose and 0.1 mM EDTA in a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 1,000 g for 10 min and the supernatant was collected. The supernatant was then centrifuged at 20,000 g for 20 min. The crude synaptosomal pellet was resuspended in distilled water to lyse the synaptosomes. The suspension was then centrifuged at 8,000 g for 20 min. The supernatant was removed and the soft buffy uppercoat of the pellet was collected by careful rinsing with the supernatant. The combined supernatant and buffy coat layer was then centrifuged at 35,000 g for 20 min and the pellet was washed twice with 9 vol of 5 mM Tris-HCl buffer (pH 7.5). The final pellet was stored at − 70°C until use.

The radioligand binding experiments were performed according to the methods described by Lee *et al.* (1983) with a slight modification. Binding was initiated by adding 100 µl of the membrane preparations (final concentration, circa 0.6 mg protein/tube) in a final volume of 500 µl of 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM $MnCl_2$, 200 µg ml^{-1} bovine serum albumin (BSA), 5 µg ml^{-1} chymostatin, 4 µg ml^{-1} leupeptin, 40 µg ml^{-1} bacitracin, 10 µM phosphoramidon, 1 nM [^{3}H]-SP and various concentrations of FK888. Assays were performed in duplicate. The reaction mixtures were incubated at 25°C for 30 min. At the end of the incubation period, 5 ml of ice cold 50 mM Tris-HCl buffer was added to each tube and its content was filtered immediately under reduced pressure through Whatman GF/B glass filters pretreated with 0.1% polyethyleneimine solution for 3 h before use. Each of the filters was then washed three times with 5 ml of ice cold 50 mM Tris-HCl buffer and radioactivity measured by liquid scintillation spectrometry. Non-specific binding was defined as binding in the presence of 5 µM SP. Specific binding was calculated by subtracting non-specific binding from total binding. Specific binding corresponded to about 85% of total binding in all cases. The protein concentration was determined by the method of Lowry *et al.* (1951) with BSA used as a standard. IC_{50} values were determined by the data from three independent experiments. Scatchard analysis of saturation data was performed by the regression analysis using the data from three independent experiments.

Functional assays

In order to evaluate receptor selectivity, the following three experiments were performed: contraction of guinea-pig ileum by SP in the presence of atropine and indomethacin (a NK_1 receptor bioassay), contraction of rat vas deferens by NKA (a NK_2 receptor bioassay) and contraction of rat portal vein by NKB (a NK_3 receptor bioassay), (Lee *et al.*, 1982; Mastrangelo *et al.*, 1986; Regoli *et al.*, 1988). Tissues were obtained from male guinea-pigs (Hartley, 300–400 g) and male rats (Sprague-Dawley, 250–350 g). Strips of guinea-pig ileum were suspended in 10 ml organ baths filled with warm (37°C), oxygenated (95% O_2/5% CO_2) Tyrode solution containing 5.2 µM atropine sulphate and 4.1 µM indomethacin under a tension of 0.5 g. Strips of rat vas deferens and rat portal vein were suspended in Tyrode solution (not supplemented with atropine and indomethacin) under a tension of 0.3 g and 0.5 g, respectively. Tension change was monitored isometrically with a force-displacement transducer connected to a polygraph system. The contraction of each tissue was induced by addition of a submaximal concentration of each agonist (SP 1 nM, NKA 100 nM and NKB 100 nM). Test drugs were applied 10 min before agonists. To obtain pA_2 values in the guinea-pig ileum, dose-response curves to SP (0.1 nM–100 µM) in the absence and the presence of FK888 were obtained. Results were expressed as mean ± s.e.mean from 5–6 preparations and the IC_{50} values were determined by the probit method. The pA_2 values and slopes were determined by regression analysis of Schild plots as described by Arunlakshana & Schild (1959), using the data from 5–6 experiments in each dose.

Airway oedema in guinea-pig

Male albino guinea-pigs weighing 260–440 g were given a solution of SP (1 nmol kg^{-1}) or capsaicin (320 nmol kg^{-1}) containing Evans Blue dye (20 mg kg^{-1}) and heparin (200 iu kg^{-1}) by i.v. administration. Ten min later animals were stunned, bled and perfused through the pulmonary artery with 50 ml saline. The trachea and main bronchi were removed, blotted dry and weighed. The trachea and main bronchi were then incubated at 37°C in 0.5 ml of 1 N KOH overnight and Evans Blue dye was then extracted by addition of 4.5 ml of 0.6 N H_3PO_4: acetone (5:13) solution. After centrifugation at 3,000 r.p.m. for 15 min, the concentration of extracted Evans Blue dye in the supernatant was quantified from light absorbance at 620 nm by interpolation on standard curve of dye concentrations in the range 0–4 µg ml^{-1}. Test drugs or control vehicles were administered i.v. 2 min before an agonist challenge. Increased amount of leaked Evans Blue dye was calculated by subtracting the Evans Blue content obtained from animals injected with Evans Blue dye and heparin solution without agonist. Results were expressed as mean ± s.e.mean and statistical analysis was performed by a Student's t test for unpaired data.

Materials

Tris; 2-amino-2-(hydroxymethyl)-1,3-propanediol, acid, BSA, chymostatin, leupeptin, bacitracin and capsaicin were purchased from Sigma Chemical Company (St. Louis, U.S.A.); polyethyleneimine, histamine and atropine sulphate were obtained from Nakarai Tesque Chemical Company (Kyoto, Japan). Phosphoramidon and substance P were from Peptide Institute Inc. (Osaka, Japan). Evans Blue dye was from E. Merck (Darmstadt, Germany). [^{3}H]-SP (1.65 TBq mmol^{-1}) was purchased from Amersham International plc (Amersham, U.K.). (±)-CP-96,345 was synthesized in our laboratories as a racemic mixture according to the method described by Lowe *et al.* (1991).

Results

Effect of FK888 on tachykinin receptors

FK888 inhibited [^{3}H]-SP (1 nM) binding to guinea-pig lung membranes in a dose-dependent manner with an IC_{50} value of 6.9 ± 1.3 nM (K_i = 0.69 ± 0.13 nM). FK888 also dose-dependently inhibited [^{3}H]-SP (1 nM) binding to rat brain cortical synaptic membranes but with a lower affinity (IC_{50} = 1.8 ± 0.7 µM, K_i = 0.45 ± 0.17 µM). The specificity of FK888 in the binding assay was tested in the following experiments: [^{3}H]-quinuclidinyl benzilate (1 nM), [^{3}H]-leukotriene D_4 ([^{3}H]-LTD_4, 0.3 nM) and [^{3}H]-bradykinin (0.1 nM) binding to guinea-pig lung membranes; [^{125}I]-cholecystokinin ([^{125}I]-CCK, 50 pM) binding to rat pancreas membranes (CCK_A receptor) and guinea-pig brain cortical membranes (CCK_B receptor); [^{3}H]-angiotensin II (1 nM) binding to rat lung membranes; [^{3}H]-Arg-vasopressin (0.5 nM) binding to rat liver membranes (V_1 receptor) and rat kidney medullary membranes (V_2 receptor); [^{125}I]-endothelin-1 (50 pM) binding to a membrane preparation from CHO cells transfected with bovine ET_A receptor cDNA and rat ET_B receptor cDNA. FK888 (10 µM) did not inhibit ligand binding in any of these assays (data not shown).

Unlabelled tachykinins displaced [^{3}H]-SP binding to guinea-pig lung membranes with the following order of potency: SP > physalaemin > > eledoisin > NKA > NKB, suggesting that binding was predominantly to the NK_1 receptor. (±)-CP-96,345, which is reported to be an NK_1 antagonist (Snider *et al.*, 1991), also inhibited [^{3}H]-SP binding to guinea-pig lung membranes with an IC_{50} value of 4.7 ± 0.2 nM

($K_i = 0.47 \pm 0.02$ nM). The mechanism of FK888 inhibition was studied by Scatchard analysis (Figures 2 and 3). In guinea-pig lung membrane, FK888 (0, 1, 3.2, 10 nM) increased K_D values (0.11, 0.16, 0.34, 0.99 nM) without changing the B_{max} values (Figure 2). Similarly in rat brain cortical synaptic membranes, FK888 (0, 1, 3.2 μM) also increased K_D values (0.34, 0.51, 0.90 nM) without changing the B_{max} values (Figure 3). These results suggest that the inhibition of [^{3}H]-SP binding in guinea-pig lung membranes or rat brain cortical synaptic membranes by FK888 was competitive.

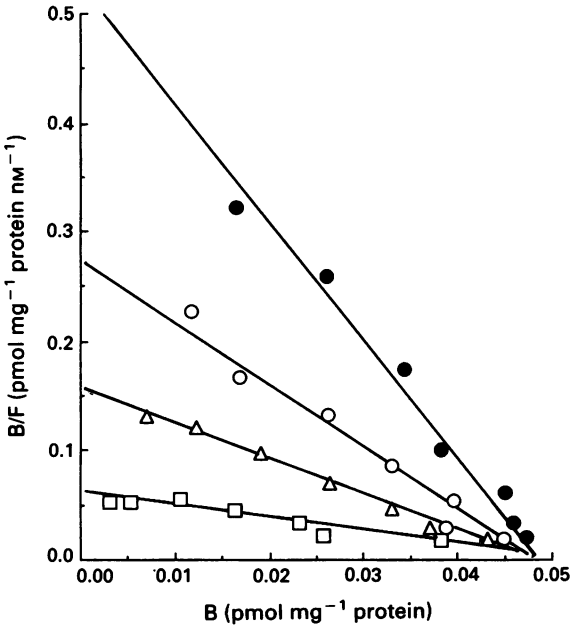

Figure 2 Scatchard analysis of specific [^{3}H]-substance P ([^{3}H]-SP) binding to guinea-pig lung membranes in the absence (●) and in the presence of FK888 at 10^{-9} M (○), 3.2×10^{-9} M (△) and 10^{-8} M (□). Data represent a typical experiment that was performed in duplicate and repeated three times with similar results.

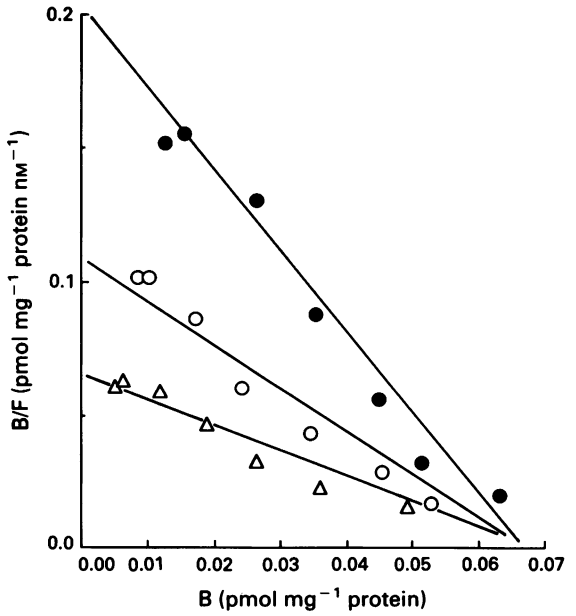

Figure 3 Scatchard analysis of specific [^{3}H]-substance P ([^{3}H]-SP) binding to rat brain cortical synaptic membranes in the absence (●) and the presence of FK888 at 10^{-6} M (○) and 3.2×10^{-6} M (△). Data represent a typical experiment that was performed in duplicate and repeated three times with similar results.

The selectivity of FK888 for SP receptor subtypes (NK_1, NK_2 and NK_3) was tested in functional experiments using contraction of guinea-pig ileum by SP (1 nM) in the presence of atropine and indomethacin (NK_1 receptor), contraction of rat vas deferens by NKA (100 nM) (NK_2 receptor) and contraction of rat portal vein by NKB (100 nM) (NK_3 receptor) (Lee *et al.*, 1982; Mastrangelo *et al.*, 1986; Regoli *et al.*, 1988). FK888 dose-dependently inhibited the NK_1 receptor-mediated response with an IC_{50} value of 0.64 nM. Figure 4 shows the dose-response curves for SP-induced contraction of guinea-pig isolated ileum in the absence and in the presence of FK888. FK888 dose-dependently shifted the dose-response curve of SP to the right without suppression of the maximum response. The Schild plot gave a slope of -0.78 (-1.02 to -0.54), which was not significantly different from -1 and a pA_2 value of 9.29 (8.60–9.98). In this experiment, the pD_2 value for SP was calculated as 8.34. On the other hand, NK_2 and NK_3 receptor-mediated responses were inhibited only by very high concentrations of FK888, with IC_{50} values of 11 μM and >32 μM, respectively (data not shown).

(±)-CP-96,345 also showed selectivity for NK_1 receptors in these experimental systems with an IC_{50} value in guinea-pig isolated ileum of 1.5 nM. Higher concentrations of (±)-CP-96,345 (1 μM–32 μM) also exhibited inhibitory actions in the NK_2 and NK_3 receptor assays. However, this was considered to be non-specific because at the same concentrations (±)-CP-96,345 inhibited contraction of rat vas-deferens and portal vein induced by noradrenaline. FK888, by itself, did not induce any change in the resting tension of the tissues used in the study at any of the concentrations tested, 0.1 nM–10 μM.

Effect of FK888 on airway oedema in guinea-pig

The effect of FK888 on plasma extravasation in guinea-pig airway (airway oedema) was studied *in vivo*. As shown in Figure 5, FK888 given intravenously dose-dependently (i.v.) inhibited airway oedema induced by an i.v. injection of SP (1 nmol kg^{-1}) with an ED_{50} value of 0.011 mg kg^{-1}. The inhibitory activity of FK888 on airway oedema induced by SP was also observed after oral administration (p.o.) and the ED_{50} value was 4.2 mg kg^{-1}. Airway oedema induced by histamine (320 nmol kg^{-1}) was not affected by FK888 at the dose of 1 mg kg^{-1} (i.v.) and 100 mg kg^{-1} (p.o.), suggesting that the effect of FK888 was selective for SP induced-airway oedema.

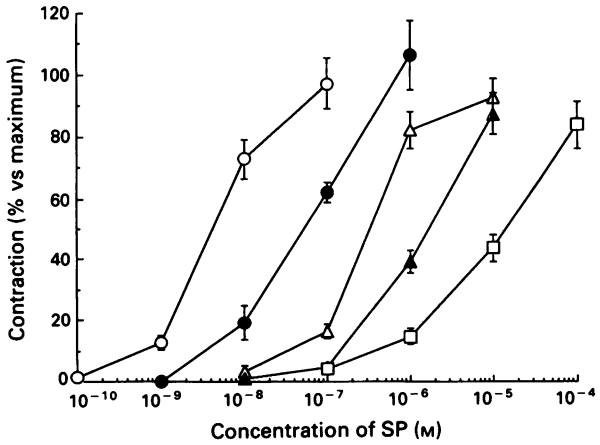

Figure 4 Dose-response curves to substance P (SP) in guinea-pig isolated ileum in the absence (○) and the presence of FK888 at 10^{-8} M (●), 10^{-7} M (△), 10^{-6} M (▲) and 10^{-5} M (□). The maximum contraction was induced by histamine (3.2×10^{-6} M). Each point shows the mean of 5–6 experiments.

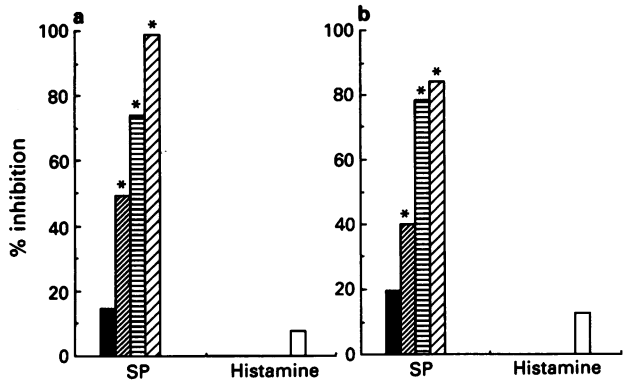

Figure 5 Effect of FK888 on substance P (SP)- and histamine-induced airway oedema in guinea-pigs after intravenous (a) and oral (b) administration. In (a), ■ 0.003 mg kg^{-1}; ▨ 0.01 mg kg^{-1}; ▤ 0.03 mg kg^{-1}; ▨ 0.1 mg kg^{-1}; □ 1 mg kg^{-1}. In (b), ■ 1 mg kg^{-1}; ▨ 3.2 mg kg^{-1}; ▤ 10 mg kg^{-1}; ▨ 32 mg kg^{-1}; □ 100 mg kg^{-1}. Results are expressed % inhibition [(1-mean of treated group/mean of control group) × 100], from experiments using 5–6 animals in each group. Significantly different from the control, $^*P<0.01$.

Discussion

We have attempted to discover novel antagonists at the SP receptor using [^{3}H]-SP receptor binding in guinea-pig lung membranes as the primary screening system. We have taken two different approaches in our research: one involving random screening of fermentation products and the other one involving a drug design study based on chemical modification of the parent compound, octapeptide (D-Pro^4, D-Trp^{7,9,10}, Phe^{11})SP$_{4-11}$ (Mizrahi *et al.*, 1984). The first approach identified actinomycin D as a SP antagonist (Fujii *et al.*, 1991) followed by the cyclic peptide SP antagonist, WS119326A. The catalytic hydrogenation of WS119326A produced FK224 which was about 10 times more potent than the original compound (Morimoto *et al.*, 1992b; Murai *et al.*, 1992). The second approach has produced a tripeptide antagonist, FR113680 (Hagiwara *et al.*, 1991; Morimoto *et al.*, 1992a), and a dipeptide antagonist, FK888, which is described in this paper. The IC_{50} values for (D-Pro^4, D-Trp^{7,9,10}, Phe^{11})SP$_{4-11}$, FR113680 and FK888 in the [^{3}H]-SP binding assay using guinea-pig lung membranes are 600 nM, 85 nM and 6.9 nM, respectively, indicating that the drug design study has achieved a 100 fold increase in potency following chemical modification of the original octapeptide antagonist to the dipeptide antagonist, FK888.

To evaluate the selectivity of FK888 for the three distinct SP receptors (NK_1, NK_2 and NK_3), we tested FK888 in three bioassays. In the NK_1 receptor bioassay (contraction of guinea-pig ileum by SP), FK888 exhibited high affinity with a pA_2 value of 9.29. FK888 has at least 10,000 times higher affinity at the guinea-pig ileum NK_1 receptor compared to rat NK_2 (contraction of vas-deferens by NKA) and rat NK_3 (contraction of portal vein by NKB) receptors. In contrast, in the same experimental systems, the cyclic peptide antagonist FK224 exhibited similar inhibitory effects on NK_1 and NK_2 receptor-mediated responses with pA_2 values of 6.88 and 7.52, respectively (Morimoto *et al.*, 1992b). There is therefore a critical difference in receptor selectivity between FK888 and FK224. This difference is also observed between FK888 and the original parent octapeptide which inhibited NK_1 and NK_2 receptor-mediated responses [contraction of guinea-pig ileum by SP (1 nM) and contraction of rat vas deferens by NKB (100 nM)] with IC_{50} values of 0.27 μM and 1.34 μM, respectively, suggesting that only NK_1 antagonist potency has been potentiated by a large degree during the course of drug design.

Recently, the existence of species differences in NK_1 receptors has been proposed. CP-96,345 was reported to displace [^{3}H]-SP binding to brain cerebral cortical membranes prepared from rabbit, guinea-pig, human, bovine, hamster and gerbil with an approximately 100 fold greater affinity than from rat and mouse membrane preparations (Beresford *et al.*, 1991; Gitter *et al.*, 1991). In contrast, unlabelled SP and physalaemin displace [^{3}H]-SP binding to the same preparation with similar affinity. The peptide SP antagonist (D-Pro^4, D-Trp^{7,9})SP$_{4-11}$ has also been reported to exhibit a higher affinity for [^{3}H]-SP binding sites on guinea-pig brain and ileum membranes than for binding sites on rat membranes (Fardin & Garret, 1991). FK888 showed a similar species difference in its affinity for the NK_1 receptor to that seen with CP-96,345, and inhibited [^{3}H]-SP binding to guinea-pig lung membrane with an approximately 100 fold higher affinity than to rat brain cortical membranes. On the other hand, the recently described NK_1 antagonist, RP 67580 (Garret *et al.*, 1991), is considered to have a distinct receptor selectivity when compared with FK888 and CP-96,345. RP 67580 has been reported to be a potent inhibitor of [^{3}H]-SP binding to rat brain membrane preparations ($K_i = 4.16$ nM) whereas activity in the SP-induced guinea-pig ileum contraction bioassay is relatively weak ($pA_2 = 7.16$).

The pharmacological profile of FK888 as an NK_1 selective antagonist was also studied in *in vivo* experiments. FK888 given i.v. or by oral administration significantly inhibited airway oedema induced by SP in guinea-pigs. SP-induced plasma extravasation is considered to be an NK_1 receptor-mediated response because SP is the most potent agonist of the tachykinin peptide family. FK888 also inhibited another vascular reaction mediated by the NK_1 receptor, SP-induced systemic hypotension in guinea-pigs, with a similar potency to SP-induced airway oedema (data not shown).

FK888 is therefore a selective NK_1 receptor antagonist both *in vitro* and *in vivo*, and will be a useful tool for clarifying the role of SP in physiological and pathophysiological conditions.

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Membrane potential and current responses to neurotensin in the longitudinal muscle of the rectum of the fowl

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1 The effects of neurotensin (NT) on membrane potential and membrane current of the longitudinal smooth muscle of chicken rectum were investigated by intracellular recording and whole-cell voltage clamp.

2 NT (3 nM–1.2 μM), when applied via the bathing medium, produced a concentration-dependent membrane depolarization with an EC_{50} of 18 ± 2 nM ($n = 7$) which was accompanied by an increase in the membrane conductance. The effect was biphasic: an initial, rapid depolarization reached a peak within 2–3 min and then declined to a lower but still elevated level which was sustained until washout.

3 Excitatory junction potentials (e.j.ps), which were non-adrenergic non-cholinergic (NANC) in nature, were decreased in amplitude and total duration in the presence of NT (0.6 μM). The depression of the e.j.p. was due mainly to the reduction of the membrane resistance.

4 When NT was applied locally by means of pressure ejection from a micropipette containing NT, some cells responded with a membrane depolarization and some failed to respond, whereas e.j.ps could invariably be elicited from all of them.

5 In single muscle cells enzymatically isolated from the muscle and dialyzed under voltage clamp at − 50 mV with a CsCl-rich solution, NT (5 or 10 μM) produced an inward current. NT-induced inward currents were obtained with inclusion of 10 mM EGTA in the pipette solution and their reversal potential was around 0 mV. In cells dialyzed under voltage clamp at 0 mV with a KCl-rich solution, NT (5 μM) produced a brief outward current followed by abolition of spontaneous transient outward currents.

6 The present results suggest that the membrane depolarization, which may arise from activation of non-selective cation channels, and release of calcium from internal stores produced by neurotensin are responsible for its contractile activity in the longitudinal smooth muscle of chicken rectum. Further, the depolarizing effect may provide support for the involvement of NT in the NANC transmission in this preparation.

Keywords: Neurotensin; smooth muscle; fowl; membrane potential; membrane current; voltage clamp; rectum

Introduction

In the search for the transmitter of non-adrenergic, non-cholinergic (NANC) excitatory nerves in the rectum of the chicken (Komori *et al.*, 1986a,b), a neurotensin-like peptide was isolated from chicken rectum which has different amino acid composition from bovine neurotensin but indistinguishable biological activities (Iwabuchi *et al.*, 1987). This peptide is identical to chicken neurotensin isolated by Carraway & Bhatnagar (1980a,b), from chicken small intestine. Neurotensin has been proposed as a candidate for the excitatory neurotransmitter of the NANC nerves in chicken rectum (Komori *et al.*, 1986a). This proposal rests on the following findings: neurotensin has a high potency in contracting the rectal muscle and the ED_{50} is about 2 nM; its presence in Remak ganglia and the rectum in which cell bodies and axon terminals of the NANC neurones are located, respectively, and its ability to abolish the contractile responses of the rectal muscle to stimulation of NANC neurones as well as the peptide itself. However, membrane responses to neurotensin, changes in membrane potential, membrane resistance and membrane current, have not been measured as yet, although evidence suggests that the NANC e.j.p. is mediated by an increase in membrane permeability to ions, mainly Na ions (Komori & Ohashi, 1982;1988a).

We have now investigated the effects of neurotensin using microelectrodes and the voltage clamp technique. The peptide was applied locally by pressure ejection from a micropipette or by adding to the bathing medium and its effects on the membrane properties of the longitudinal muscle cells of chicken rectum were studied. A possible involvement of neurotensin in the NANC transmission has also been examined. Throughout the present study, synthetic bovine neurotensin was used, because synthetic chicken neurotensin is not available.

Methods

Preparation of smooth muscle strips and single smooth muscle cells

White Leghorn chickens (*Gallus domesticus*, aged more than 80 days and weighing 0.8–2.0 kg) were stunned and bled to death. The rectal region of the intestine was excised, from which strips (1–2 mm wide and 15–20 mm long) of the longitudinal muscle were cut and used for recording of membrane potential changes. Single smooth muscle cells were obtained enzymatically from small pieces (0.5 × 0.5 mm) of the longitudinal muscle layer isolated from chicken rectum: tissue pieces were incubated in a physiological salt solution (PSS, composition given below) with 30 μM added Ca^{2+} plus collagenase (1.25 mg ml^{-1}), papain (4.5 mg ml^{-1}) and bovine serum albumin (5 mg ml^{-1}) for 30–50 min at 37°C. The tissue pieces were then transferred to a PSS with 120 μM added Ca^{2+} but no added enzymes, and gently agitated to disperse single smooth muscle cells. Single cells were collected by

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centrifuging at 700 r.p.m. for 2 min, suspended in a PSS with 0.8 mM added Ca^{2+}, and stored in a small aliquot on glass cover-slips in a moist atmosphere at 4°C until use. The cells were used in experiments on the day of preparation.

Intracellular recordings of membrane potential

The muscle strip preparation was mounted, mucosal side up, in a 2 ml organ bath with two chambers, one for recording and the other for stimulation, as described by Abe & Tomita (1968). The part in the recording chamber was pinned to a black rubber piece. The two chambers were filled and irrigated at a flow rate of 2–3 ml min^{-1} with Tyrode solution (composition given below) preheated to 33 ± 1°C and bubbled with air to keep the pH at 7.4. A small hole (about 1.5 mm in diameter) in the partition between the two chambers through which the muscle strip had been passed also served to allow Tyrode solution to flow from the recording chamber to the stimulating chamber. Overflowing solution from the stimulating chamber was sucked away with an aspirator.

Membrane potential changes were recorded intracellularly with microelectrodes filled with 3 M KCl solution and having a resistance of 40–80 MΩ. Electrotonic potentials were evoked by applying current pulses of varied durations to the tissue through the stimulating plates. Voltage gradients in the solution of the stimulating chamber produced by passing current pulses were monitored by one silver electrode. Excitatory junction potentials were elicited by electrical field stimulation of intramural nerves of the tissue with square-wave pulses of 0.1 ms duration at an appropriate intensity (10–50 V). The stimulus pulses were delivered by a stimulator (Nihon Kohden, SEN-3013) through a pair of Ag-AgCl electrodes (1 mm in diameter). One electrode, which was insulated with Araldite (Nagase) except for the tip, was placed in the centre of the tissue 1.5–2.0 mm away from the stimulating partition in contact with it. The other electrode, which was uninsulated, was placed in the bathing solution.

For local application of neurotensin to the tissue, glass micropipettes (3–10 μm in tip diameter) filled with neurotensin (0.3 mM) made up in Tyrode solution were used. The drug-containing pipette was connected by polyethylene tubing to a nitrogen tank via a three-way solenoid valve (CKD corp., UMGI-T1) so that the drug solution could be ejected by pressure (2.5–3.5 kg cm^{-1}). Ejected amounts of the drug solution can be controlled by varying the duration of current pulses (8–1000 ms) delivered from a stimulator (Nihon Kohden, MES-3) to turn on the solenoid valve. The relationship between the pulse duration and the drug amount was determined as described by Komori & Ohashi (1988b). The drug-containing pipette was manipulated to place the tip in a position as close as possible to the tissue surface and 50 μm away from the recording microelectrode.

This experimental apparatus permitted electrotonic potentials, excitatory junction potentials and responses to pressure ejection of neurotensin to be recorded from the same cell (Komori & Ohashi, 1988b). The potential changes were displayed on an oscilloscope and photographed.

To Tyrode solution were added atropine (0.5 μM), methysergide (2 μM), pyrilamine (1.3 μM), isoprenaline (1.3 μM) and methoxyverapamil (D 600, 10 μM) and if necessary, guanethidine (2 μM) throughout the course of the experiments in which changes in membrane potential were recorded. Atropine, methysergide and pyrilamine served to block effects of acetylcholine, 5-hydroxytryptamine and histamine, respectively, possibly released from the tissue; guanethidine to block noradrenaline release and isoprenaline and D 600 served to suppress electrical spike activity and development of tension in the smooth muscle. The concentrated solutions of these drugs were made up and stored at − 20°C. Final dilutions of the stock solution were made in Tyrode solution just before use and the solutions were frequently renewed.

Recordings of membrane current

A glass cover-slip with cells was placed in a small organ bath (0.6 ml) and then irrigated with PSS. Whole-cell membrane currents were recorded by standard patch-clamp techniques (Hamill *et al.*, 1981) and patch pipettes filled with a solution (composition given below) with a resistance of 4–7 MΩ. Membrane currents were amplified by an amplifier (List, EPC-7), stored on FM tape by a recorder (Sony, FR-3215W) and replayed onto a thermal array recorder (Nihon Kohden, RTA-1100M) for illustration and analysis.

Drugs and solutions

Drugs used were atropine sulphate (Tanabe), methysergide hydrogen maleate (Sandoz), pyrilamine maleate (Sigma), (−)-isoprenaline sulphate (MercK), bovine neurotensin (Peptide Institute Inc., Osaka), methoxyverapamil (D 600, Knoll), adenosine 5'-triphosphate (ATP, Sigma), tetrodotoxin (Sankyo) and guanethidine sulphate (Ciba-Geigy). The stock solutions of all drugs were dissolved in distilled water, made up at 1000 or more times higher concentrations than those used for the experiments, and stored at − 20°C. A certain amount of the concentrated drug solution was added to the solution in the organ bath or the reservoir to give the final desired concentration, and the drug was washed away by replacing the bathing solution with fresh solution.

PSS contained (in mM): NaCl 126, KCl 6, $CaCl_2$ 2, $MgCl_2$ 1.2, glucose 14 and HEPES 10.5 (titrated to pH 7.2 with NaOH). Tyrode solution contained (in mM): NaCl 137, KCl 2.7, NaH_2PO_4 0.4, $NaHCO_3$ 12, $MgCl_2$ 1, $CaCl_2$ 1.8 and glucose 5.6. The solution in the patch pipette contained (in mM): CsCl 134, $MgCl_2$ 1.2, MgATP 1, EGTA 0.05, glucose 14 and HEPES 10.5 (titrated to pH 7.2 with NaOH). To record outward K^+ current, the CsCl in the pipette solution was replaced with KCl.

Means are expressed with ± the standard error of the mean. The statistical significance was evaluated by Student's *t* test for paired samples and *P* values of 0.05 or less were considered significant.

Results

Membrane potential response to neurotensin

Bath application The average resting membrane potential of the smooth muscle cells was − 54 ± 0.4 mV (mean ± s.e.mean, range − 40 to − 65 mV, $n = 140$). Bath application of neurotensin (NT) resulted in a concentration-dependent membrane depolarization with EC_{50} of 18 ± 2 nM ($n = 7$). When 0.6 μM NT was applied, the concentration required to produce a nearly maximal response, it produced a biphasic membrane depolarization: the initial depolarization reached a maximum of more than 20 mV within 2–3 min and then it declined to a lower but still elevated level (about 10 mV more positive than the resting membrane potential) which was sustained throughout the period of NT application (Figure 1). The effect of NT was reversible and tetrodotoxin (0.25 μM)-insensitive, indicating a direct action of the peptide on the muscle membrane.

Electrotonic potentials were evoked by applying inward current pulses and recorded from smooth muscle cells at a distance of less than one fifth of the length constant (1.6 mm) measured by spatial decay of electrotonic potentials (Komori & Ohashi, 1982) from the stimulating site. After application of 0.6 μM NT they were decreased in amplitude to 56 ± 10% ($n = 10$) at the peak depolarization and to 63 ± 5% ($n = 10$) during the sustained membrane potential (10 min after NT application) of the amplitude of electrotonic potentials recorded immediately before application of the peptide (Figure 2a). As the tissue has cable-like properties (Komori & Ohashi, 1982), a decrease in membrane resistance to about

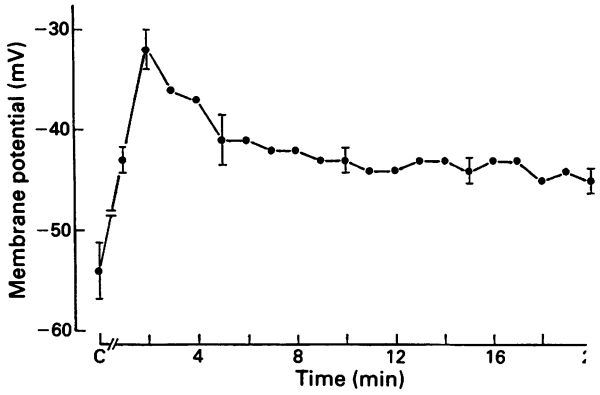

Figure 1 Membrane potential change recorded intracellularly from smooth muscle cells of the longitudinal muscle layer of chicken rectum induced by 0.6 μM neurotensin (NT). Each point represents the mean of measurements from 4–17 cells in different preparations and is plotted against time after NT application. C, the membrane potential before exposure to NT. Vertical bars indicate s.e.mean. Bathing solution containing atropine (0.5 μM), methysergide (2 μM), pyrilamine (3 μM), isoprenaline (1.3 μM) and D 600 (10 μM) was used throughout the experiment.

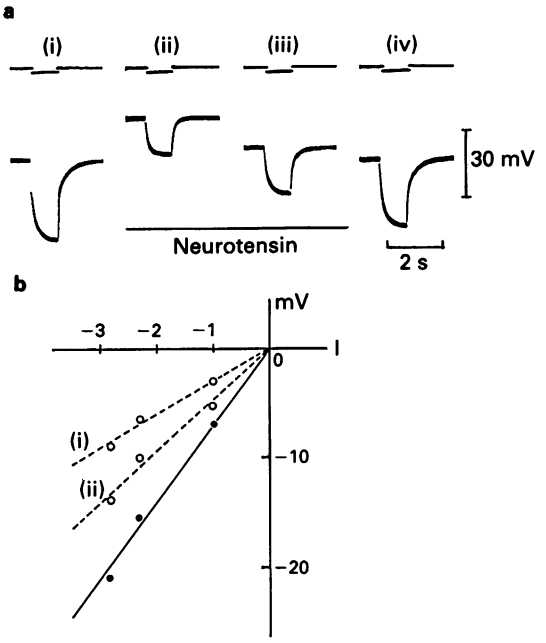

Figure 2 The effect of neurotensin (NT) on electrotonic potentials. (a) Electrotonic potentials evoked by applying inward current pulses of 1 s duration using partition stimulation and recorded intracellularly. All responses were from the same cell: (i) control; (ii) and (iii), 4 and 15 min after application of 0.6 μM NT; and (iv) 6 min after its washout. Upper trace, applied current strength in terms of $V\,cm^{-1}$; lower trace, potential records. (b) Plots of electrotonic potential amplitude (mV) against applied current strength (I, arbitrary unit) before (●, solid line) and after (○, dashed line) application of 0.6 μM NT. (i) and (ii), 5 and 15 min after application of NT. Lines fitted to the data points by eye. The bathing solution contained the same drugs as described in Figure 1.

31% and 40%, respectively can be calculated from the reduction of electrotonic potential amplitude. Figure 2b shows the result of an experiment in which current-voltage relationships were studied before and 5 and 15 min after application of 0.6 μM NT. The current-voltage relationship was linear at the three different times and the slopes were clearly less steep in the presence of NT, also indicating a decrease in the membrane resistance.

Excitatory junction potentials (e.j.ps) were elicited by electrical stimulation of the intramural nerves, which were non-adrenergic, non-cholinergic (NANC) in nature, as previously reported (Takewaki & Ohashi, 1977; Komori & Ohashi, 1982). When e.j.ps were recorded from muscle cells 2 mm away from the electrode for nerve stimulation, their amplitude was 5.4 ± 0.2 mV ($n = 34$). Application of 0.6 μM NT resulted in a decrease of the amplitude of e.j.ps to 48 ± 8% ($n = 9$) and 66 ± 7% ($n = 10$) at the two respective levels of the peak and sustained depolarization produced by NT. The total duration of the e.j.ps was also significantly reduced from the control value of 840 ± 30 ms ($n = 10$) to 687 ± 56 ms ($n = 10$) at the peak depolarization and to 720 ± 54 ms ($n = 10$) during the sustained depolarization (10–15 min later). When the sustained membrane depolarization was eliminated artifically by applying inward current, the e.j.p. remained unchanged. In the absence of NT, displacement of the membrane potential from the resting level to a similar level to the sustained membrane potential produced by NT resulted in a decrease in the amplitude of the e.j.p. only by less than 10%, and no or a very slight decrease in total duration. To investigate the precise relationship between the effects of NT on the e.j.p. and on the membrane resistance, e.j.ps and electrotonic potentials were recorded from the same cell at varied intervals after application of 0.6 μM NT. As shown in Figure 3, e.j.ps and electrotonic potentials were decreased in amplitude by NT. The effects of NT on both potentials gradually faded in a parallel manner during early the period of NT application, 20 min from the start of NT application, the electrotonic potential amplitude had returned to the control level but the e.j.p. amplitude was still reduced. After that, electrotronic potentials continued to increase in amplitude and reached a level greater than the control whereas the e.j.p. amplitude did not change any more.

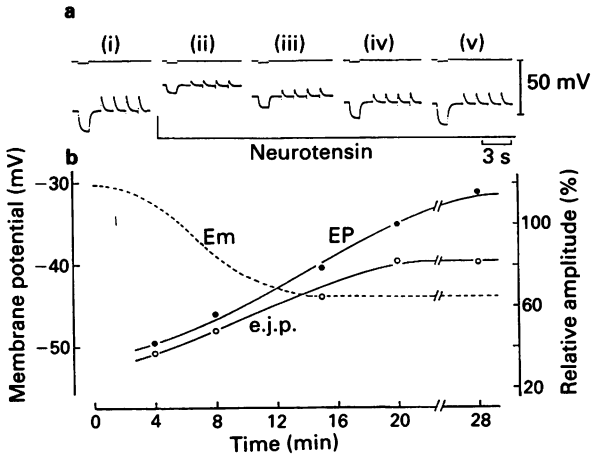

Figure 3 The effects of neurotensin (NT) on e.j.ps and electrotonic potentials. (a) E.j.ps and electrotonic potentials recorded intracellularly from the same cell before and after application of 0.6 μM NT. E.j.ps were evoked by electrical field stimulation of intramural nerves with a train of 4 square-wave pulses of 0.1 ms duration at 1 Hz and electrotonic potentials were evoked by applying inward current pulses of 1 s duration. (i) Control; (ii)–(v) 4, 8, 15 and 20 min after application of NT. Upper trace, applied current strength in terms of $V\,cm^{-1}$; lower trace, potential records. (b) Changes in e.j.p. amplitude (○, solid line, e.j.p.), electrotonic potential amplitude (●, solid line, EP) and membrane potential (dashed line, Em) in the continued presence of 0.6 μM NT. E.j.p., EP and Em are plotted against time after NT-induced peak depolarization; e.j.p. and EP are expressed as percentages of those measured immediately before NT application. The bathing solution contained guanethidine in addition to the drugs listed for Figure 1.

Local application Local application of NT by means of pressure ejection from a micropipette filled with solution containing NT (0.3 mM) caused either no detectable membrane responses (less than 2 mV) or membrane responses with different features from smooth muscle cells in the area exposed to NT. Further, in some records, small and irregular fluctuations of membrane potential preceded the response, which was occasionally followed by dislodgement of the microelectrode. Cells responding to NT include those showing a membrane depolarization with an amplitude of 2–34 mV, a latency of 0.07–0.3 s and a time taken to reach a peak (time to peak) of 0.1–1.5 s (NT-sensitive cells) (Figures 4, 5 and 6), and those showing a membrane depolarization with a smaller amplitude of 2–5 mV, a longer latency of 1–3 s and a longer time to peak of 1.0–2.5 s (less NT-sensitive cells) (Figure 4b). The former cells were much smaller in number than the latter cells. In addition, the responses in NT-sensitive cells were evoked with a pulse duration of 8–200 ms, but those in less NT-sensitive cells needed a longer pulse duration of 200–1000 ms. As the pulse duration was prolonged to increase the amount of ejected NT, the response increased in amplitude and duration, but not in latency and time to peak. Figure 5a shows membrane responses to NT ejected with varied pulse durations in a NT-sensitive cell. The relationship between the pulse duration and the response amplitude is shown in Figure 5b, suggesting that the upper limit of the response amplitude may be approx. 35 mV. A time to peak of 0.1–0.2 s was measured in some NT-sensitive cells, which was as short as in the e.j.p. elicited by stimulation of intramural nerves, as shown in Figure 4a. However, their latency of 0.07–0.18 s was much longer than the latency of about 0.01 s for the e.j.p. Figure 6 shows successive NT responses when NT was repeatedly applied by pressure pulses of 10 ms duration. It can be seen that the amplitude and time course of the response is little affected by repetitive application at up to 0.8 s interval. However, on careful comparison of two successive responses, the later response was very slightly smaller in amplitude than the preceding one. At application intervals shorter than 0.8 s, successive responses summed up to a sustained membrane depolarization.

In all of NT-insensitive and less NT-sensitive cells, e.j.ps could be elicited by electrical stimulation of the intramural nerves and their amplitude and time course were similar to those recorded from NT-sensitive cells. Thus, clearly the low sensitivity to the local application of NT is not attributable to any damage to the cells.

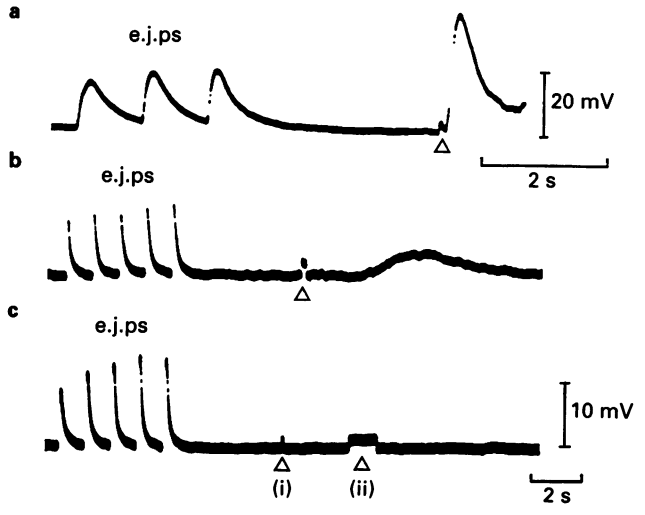

Figure 4 Comparison of membrane responses to local application of neurotensin (NT) and e.j.ps recorded from the same cell. (a), (b) and (c) Records from different preparations. E.j.ps were evoked by electrical field stimulation of intramural nerves with square-wave pulses of 0.1 ms duration and NT-induced responses were evoked by pressure ejection at triangles with varied durations from a 0.3 mM NT-filled micropipette. (a) A large depolarization was evoked with a short latency developing as rapidly as does an e.j.p.; (b) a slow depolarization was evoked with a long latency; (c) there was no response to NT. Pressure pulses of a 200 ms duration were used in (a) and (b) and those of 50 and 1000 ms for (i) and (ii), respectively, in (c). The bathing solution contained the same drugs as listed for Figure 3. Note that cells which were less or insensitive to NT could sustain e.j.ps.

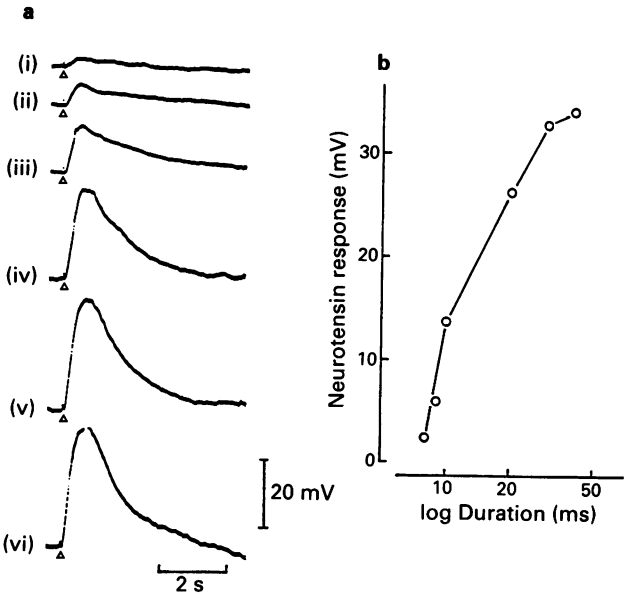

Figure 5 Dose-related membrane depolarizations in response to local application of neurotensin (NT). (a) Potential records from a cell in response to NT ejected from a 0.3 mM NT-filled micropipette by pressure pulses of varied durations ((i)–(vi), 8, 9, 10, 20, 30 and 40 ms, respectively). (b) A plot of the amplitude of NT responses against pressure pulse duration (logarithmic scale). Atropine (0.5 μM), isoprenaline (1.3 μM) and D 600 (10 μM) were present in the bathing solution.

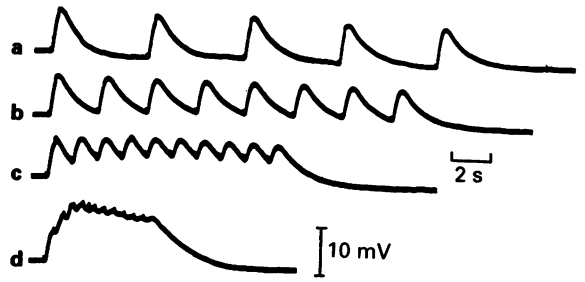

Figure 6 Membrane responses to repeated local applications of neurotensin (NT). NT was ejected at varied intervals by pressure pulses of 10 ms duration from a 0.3 mM NT-filled micropipette. (a) Five pulses at 0.2 Hz; (b) 8 pulses at 0.4 Hz; (c) 10 pulses at 0.8 Hz; (d) 12 pulses at 2 Hz. Potential records from the same cell.

Membrane current response to neurotensin

Single smooth muscle cells were held under voltage clamp at − 50 mV which was close to the mean resting potential of − 54 ± 0.4 mV (see above), and patched with pipettes filled with a CsCl-rich solution to block outward K^+ current. Bath application of NT (5 or 10 μM) produced an inward current (11 cells) (Figure 7a) or no detectable current (58 cells). NT-induced inward currents declined with time in the continued presence of the peptide. The peak amplitude of the

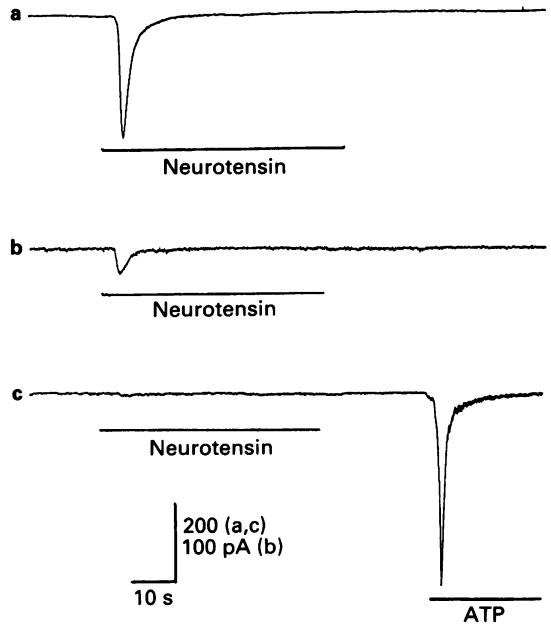

Figure 7 Inward current responses to neurotensin (NT) and ATP in single muscle cells enzymatically isolated from the longitudinal muscle layer of chicken rectum. The cells were bathed in PSS and dialyzed under voltage clamp at − 50 mV with a CsCl-rich solution. (a) A large inward current was evoked in response to 5 μM NT; (b) a small inward current was evoked in response to 5 μM NT; (c) no current in response to NT occurred but a large inward current response was evoked by 10 μM ATP. See text for details.

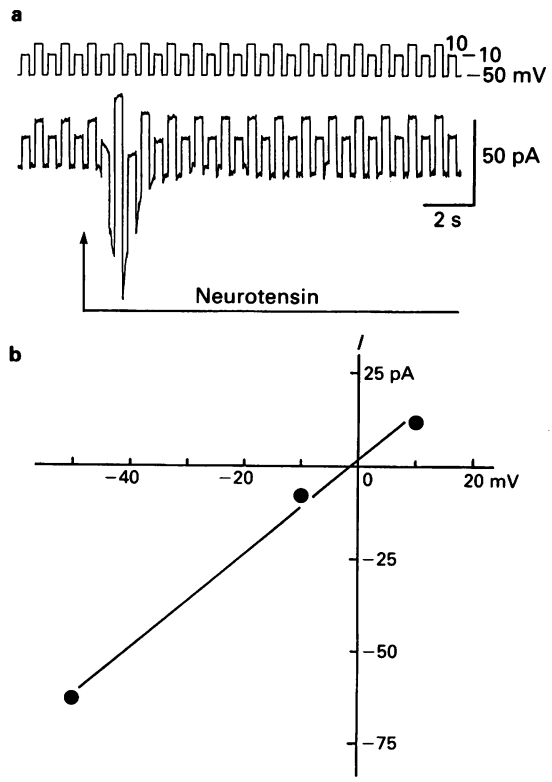

Figure 8 Estimation of the reversal potential of the inward current response to neurotensin (NT) in a single muscle cell. The cell was bathed in PSS and dialyzed under voltage clamp at − 50 mV with a CsCl-rich solution. (a) Current response to 5 μM NT recorded during stepping from − 50 mV to test potentials of − 10 and 10 mV. Upper trace, voltage step protocol; lower trace, current record. (b) A plot of NT-induced current response amplitude against clamp potential. The calculated regression line is gven as $y = 1.3x + 1.5$ ($r = 0.997$, $P < 0.01$), where y is current response amplitude (pA) and x is clamp potential (mV).

inward currents varied from 20 pA up to 400 pA in the 11 cells. Similar current responses to NT were obtained with inclusion of 10 mM EGTA in the pipette solution (2 cells), suggesting that the current response may result from activation of calcium-independent ionic channels. In all of the cells, whether they responded to NT or not, a brief inward current with an amplitude of 100–800 pA was recorded when ATP (10 μM) was applied (Figure 7c). It is therefore unlikely that variations in sensitivity to NT of different cells is due solely to some damage arising during the enzymatic dispersion.

In order to estimate the reversal potential of the NT-induced inward current, the membrane potential was stepped from the holding potential of − 50 mV to test potentials of − 10 mV and 10 mV during application of the peptide (Figure 8a). Figure 8b shows a plot of the amplitude of the NT-induced current against clamp potential. It can be seen that the NT-induced current is inward at − 50 mV and − 10 mV but outward at 10 mV, giving a reversal potential of about − 1.5 mV. In three other cells, reversal potentials of NT-induced currents were estimated to be between − 4 and 3 mV. The mean reversal potential was -1.0 ± 1.3 mV ($n = 4$).

As calcium-activated K^+ current can be employed as a probe for monitoring a rise in calcium concentration in the cytosol (Komori & Bolton, 1990), cells were patched with pipettes containing a KCl-rich solution, held at 0 mV and exposed to NT. Under these conditions, 3 cells discharged spontaneous transient outward currents (STOCs, Benham & Bolton, 1986) and responded to NT (5–10 μM) with a brief outward current followed by abolition or reduction of STOC discharges (Figure 9a). The effects of NT resembled those of caffeine and carbachol in other types of intestinal cell in which these drugs cause a massive release of calcium from internal stores (Komori & Bolton, 1990; Komori *et al.*, 1992). However, in five other cells, NT was without effect or, if anything, it caused only an increase of STOCs in amplitude and discharge rate (Figure 9b).

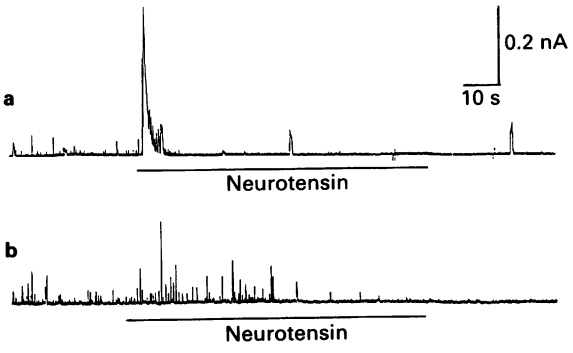

Figure 9 Outward current responses to 5 μM neurotensin (NT) in single muscle cells dialyzed under voltage clamp at 0 mV with a KCl-rich solution. (a) A brief outward current was evoked followed by abolition of spontaneous transient outward currents (STOCs); (b) an increase in amplitude and discharge rate of STOCs was evoked and then abolished. (a) and (b) are records from different cells.

Discussion

The present results demonstrated that NT, applied by addition to the bathing solution or locally by pressure ejection from a micropipette, can cause the membrane of the longitudinal muscle of chicken rectum to depolarize. A similar

effect of NT on the membrane potential has been observed on the smooth muscle of the guinea-pig taenia coli (Kitabgi *et al.*, 1979). The membrane response to NT in chicken rectum smooth muscle was found to be biphasic on prolonged exposure to NT. The first phase of depolarization was shorter in duration and larger in amplitude than the second steady-state phase of depolarization. The depolarization was accompanied by an increase in membrane conductance, indicating that NT opens ion channels present in the cell membrane. This is strongly supported by the fact that in single smooth muscle cells held under voltage clamp at − 10 or − 50 mV, NT induced an inward current which consisted of an initial, peak phase followed by a gradually declining phase and was accompanied by a decrease in input resistance and an increase in current noise. The reversal potential of the NT-induced current of about 0 mV suggests that NT may activate ion channels through which either Cl^- alone (distributed equally on both sides of the cell membrane) or more than one kind of ion can flow. It is very likely that the inward current is carried by a mixture of cations such as Na^+, K^+ and Ca^{2+} rather than a calcium-activated Cl^- current, as it was generated when EGTA (10 mM) was included in the patch pipette.

The whole-cell patch clamp experiments also revealed that NT elicited a brief outward K^+current followed by inhibition of STOCs when the cell was held under voltage clamp at 0 mV and the patch pipette contained a KCl-rich solution. The outward current response was substantially similar to current responses to caffeine and carbachol (Komori & Ohashi, unpublished data). In many types of smooth muscle cell, such current responses to caffeine, muscarinic receptor agonists and adrenoceptor agonists are believed to result from a massive release of calcium from internal stores (Benham & Bolton, 1986; Komori *et al.*, 1992). Therefore, it is highly probable that NT, like caffeine and carbachol, can also release calcium from internal stores.

The present results provide evidence in favour of NT as a possible neurotransmitter of non-adrenergic, non-cholinergic (NANC) nerves in chicken rectum (Komori *et al.*, 1986a). NT can cause the membrane of the longitudinal muscle of the chicken rectum to depolarize. Membrane depolarizations evoked by locally applied NT in some cells were very similar in property to e.j.ps evoked by NANC nerve stimulation. The rising phase of NT responses was as rapid as that of e.j.ps recorded from the same cell. Trains of membrane depolarizations evoked by repetitive jets of locally ejected NT were comparable with trains of e.j.ps evoked by repetitive NANC nerve stimulation. The finding that cells, which failed to respond to locally applied NT, exhibited e.j.ps following NANC nerve stimulation does not preclude the possibility that NT is a transmitter for the NANC nerves. Cells in the smooth muscle of the chicken rectum are electrically coupled to each other (Komori & Ohashi, 1982), and e.j.ps evoked in cells in which the transmitter acts may spread electrotonically to other cells. Depression of e.j.p. amplitude produced by NT in a relatively high concentration is mainly attributed to reduction of membrane resistance but not to desensitization of NT receptors, since effects of NT on both e.j.ps and electrotonic potentials faded almost in parallel. NT-induced membrane depolarization results in a decrease in driving force for the e.j.p. which has a reversal potential of − 15 mV (Komori & Ohashi, 1988a) and this also contributes to the depression of e.j.p. amplitude.

Prolonged exposure to NT revealed that NT may have a dual action on the membrane conductance, as an activator and as an inhibitor of ion channels in the longitudinal muscle of chicken rectum. The membrane conductance was first increased and then gradually declined to normal approx. 20 min after the start of NT exposure. After that, the membrane conductance continued to reduce to beyond control. The change in membrane conductance occurred even after the membrane depolarization reached a steady level. As previously reported (Kitabgi & Freychet, 1978; 1979; Kitabgi, 1982), if NT acts indirectly through release of active substances other than acetylcholine, 5-hydroxytryptamine and histamine from neural and/or non-neural elements, the dual effect on the membrane conductance may be not merely the result of activation of NT receptors located on the smooth muscle. Another possibility is that multiple subtypes of NT receptor mediating different membrane responses (Quirion *et al.*, 1980) are present in the smooth muscle, since the peptide has been reported to act directly on the smooth muscle to relax or contract in a variety of gastrointestinal preparations (Carraway & Leeman, 1973; Rökaeus *et al.*, 1977; Kitabgi & Freychet, 1978; Quirion *et al.*, 1980; Kitabgi & Vincent, 1981). More strategic tools such as specific antagonists are required for confirmation of the dual action of NT on the membrane conductance. Thus, there is scope for further study on the hypothesis that NT acts as a transmitter of NANC neurones in the chicken rectum (Komori *et al.*, 1986a).

Following bath application of NT, a decreased membrane potential could be recorded from all the cells tested. When NT was locally applied, only a fraction of the cell population of the muscle was sensitive to NT. Similar results were obtained in enzymatically dispersed single muscle cells. However, in the case of ATP, all the cells in the muscle respond to its local application with a membrane depolarization (Komori & Ohashi, 1988b) and in the present experiments, most of the enzymatically dispersed single cells showed a good sensitivity to ATP. If one makes allowance for some limitations imposed by pressure ejection and enzyme treatment, the present results suggest that NT-sensitive cells, cells possessing NT receptors, may be a fraction of the cell population and distributed in a tessellated manner in the longitudinal muscle layer of chicken rectum. The muscle appears to have highly developed intercellular coupling because of its length constant of 1.6 mm measured by spatial decay of electrotonic potentials (Komori & Ohashi, 1982). The presence of electrical coupling between cells possessing NT receptors and cells lacking NT receptors serves to elicit a modest membrane depolarization, and may account for a large part of the consistency of membrane responses, when NT was applied by addition to the bathing solution.

The longitudinal muscle of chicken rectum belongs to unitary muscles according to Bozler's classification (Bozler, 1948) and is spontaneously active. In this type of smooth muscle, membrane depolarization can increase the discharge rate of propagated action potentials leading to Ca entry through voltage-dependent Ca channels without causing depolarization block and thus it is effective in producing tension. The contractile activity of neurotensin in the rat fundus is sensitive to nifedipine and related calcium channel blockers, and it has been suggested that it may be coupled to voltage-dependent calcium channels (Donoso *et al.*, 1986). Thus, it can be well understood that NT, which causes a modest membrane depolarization, is a very potent agent in contracting the longitudinal muscle of chicken rectum (Komori *et al.*, 1986a).

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Antagonism of kinin effects on epithelia by Hoe 140: apparently competitive and non-competitive interactions

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1 Hoe-140, a potent kinin receptor antagonist, was investigated for its ability to inhibit the effects of lysylbradykinin (kallidin) on a cultured colonic epithelium, HCA-7 Colony 29, derived from a human adenocarcinoma.

2 Measurements of electrogenic chloride secretion (as short circuit current), and of intracellular Ca^{2+} (from Fura-2 fluorescence) were used to assess the action of lysylbradykinin in the absence and presence of Hoe 140.

3 From short circuit current data, Hoe 140 appeared to be a competitive antagonist with a K_i value of 5 nM. However, with measurements of intracellular Ca^{2+} Hoe 140 was apparently a non-competitive antagonist with a K_i of between 4–6 nM.

4 Because of the unexpected finding of non-competitive antagonism, measurements were made with a second antagonist pair, histamine and mepyramine. Mepyramine behaved as a competitive antagonist against responses to histamine with a K_i value of $\approx$5 nM when short circuit current measurements were evaluated. However, when intracellular Ca^{2+} concentration was used as a measure mepyramine, 30 nM, produced a near parallel shift in the response curve, but at 100 nM the maximal response was depressed.

5 The reasons why the apparent type of antagonism depends upon the method of measurement is discussed, bearing in mind that the increase in intracellular Ca^{2+} is a signal which precedes the increase in short circuit current.

Keywords: Hoe 140; lysylbradykinin; kinin receptor antagonist; epithelial chloride transport; intracellular Ca^{2+}

Introduction

The second generation, high affinity, kinin receptor antagonist, Hoe 140 (Wirth *et al.*, 1991; Hock *et al.*, 1991) is the concern of this paper. Pioneering studies by Stewart & Vavrek showed that antagonist activity could be introduced into the kinin peptides by replacement of l-proline at position 7 with a D-amino acid, such as D-Phe. In this way antagonists with IC_{50} values in the range 0.1-1.0 μM were obtained, for example D-Arg-[Hyp^2, Thi^{5,8},D-Phe^7] bradykinin (Vavrek & Stewart, 1985). Further modifications have resulted in a compound with an IC_{50} in the nanomolar range, namely D-Arg-L-Arg-L-Pro-L-[(4**R**)-4-hydroxyprolyl]-Gly-L-[3-(2-thienylalanyl]-L-Ser-D-(1, 2, 3, 4-tetrahydroisoquinolin-3'yl-carbonyl] -L-[(3a**S**,7a**S**)-octahydroindol-2-yl-carbonyl] -L-Arg, (D-Arg-[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK), also known as Hoe 140.

Hoe 140 inhibited bradykinin responses with IC_{50} values of between 1 and 10 nM in a variety of smooth muscle preparations, in endothelium-derived relaxing factor (EDRF) release and intracellular calcium release (Ca_i) in endothelial cells, and in ligand binding assays in membrane preparations (Wirth *et al.*, 1991; Hock *et al.*, 1991). No information is available about the antagonist action of Hoe 140 against kinins upon epithelial ion transport. Since mammalian epithelia are important loci of kinin actions, resulting in stimulation of electrogenic anion secretion (Cuthbert & MacVinish, 1989; Gaginella & Kachur, 1989) it is of interest to know if these effects can be inhibited by Hoe 140. A colonic epithelial cell line, HCA-7 Colony 29, has been used for this work, requiring that the kinin receptors must be located upon the epithelial cells themselves, and not on sub-epithelial elements in the lamina propria considered to be another site of action for kinins in some instances (Warhurst *et al.*, 1987). We have measured two types of kinin response: the increase in electrogenic chloride secretion measured as short circuit current (SCC) and the increase in intracellular calcium (Ca_i) measured by Fura-2 fluorescence.

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Methods

General

All experiments have been done with HCA-7 Colony 29 cells derived from a human colonic adenocarcinoma. They were maintained in culture as described previously (Cuthbert *et al.*, 1987; Pickles & Cuthbert, 1991). Epithelial monolayers were cultured on collagen coated Millipore filters as described originally by Cuthbert *et al.*, (1985a). Each monolayer had an area of 0.2 cm^2. These monolayers were used to measure the electrogenic transepithelial transport of chloride (electrogenic chloride secretion) by measuring short circuit current (SCC) by standard methods (Cuthbert *et al.*, 1985a,b; 1987; Pickles & Cuthbert, 1991). The cells were also cultured on plastic slips in Leighton tubes and were used to measure intracellular Ca^{2+} concentration (Ca_i) by using Fura-2 fluorescence as described by Pickles & Cuthbert (1991, 1992). The plastic slips were cut in half to yield two identical monolayers to serve as test and control when paired preparations were required.

Experimental design

The experiments in this paper were designed to measure the kinetic parameters for two antagonists on epithelial cell function. A conventional experimental design is precluded because of the rapid and intense desensitization caused by one of the agonists, lysylbradykinin (LBK) (Cuthbert *et al.*, 1987). It was not practicable to add LBK more than once to each side of the epithelium. LBK stimulates chloride secretion when receptors on the apical or the basolateral side are activated and these two receptor pools can be activated separately (Cuthbert *et al.*, 1985b). Pairs of epithelial monolayers from the same batch of cultures were mounted in Ussing chambers for SCC measurements. One tissue was equilibrated with the antagonist for 30 min before both were exposed to LBK, usually first on one side and then the other followed by a standard concentration of histamine, 10 μM.

Thus preparations were as comparable as possible, except for the presence of antagonist in one of the pair. To examine the effect of Hoe 140 on Ca_i slips from Leighton tubes were cut into two halves, cells were loaded with Fura-2 and one half was equilibrated with the antagonist for 30 min. Each half was then mounted in the spectrofluorimeter and exposed to LBK and then to a standard concentration of histamine, 100 μM. In this way the two halves of a single culture were matched as closely as possible, the only difference being the presence of antagonist in one preparation. The Ca_i measurements following LBK in this configuration represent the signal from activating both apical and basolateral kinin receptors. Full details of the justification for converting fluorescence emission ratios into values of Ca_i, with allowance for autofluorescence and methods of calibration are given in Pickles & Cuthbert (1991; 1992). Hoe 140, 10 μM, and mepyramine, 1 μM, concentrations far greater than used in the quantitative experiments, had no effect on Fura-2 fluorescence.

Analysis of data

Concentration-response relationships were analysed by fitting the data to a hyperbola by use of a weighted, non-linear regression method (Wilkinson, 1961). Basically, the data were fitted to S/R versus S plots, where S is drug concentration and R the response, to obtain estimates of K_m and R_{max} where K_m is the apparent dissociation constant for the drug receptor complex, i.e. the EC_{50}. These estimates are then used to fit the data to a hyperbola and an iterative procedure used to minimize the weighted, least squares deviation. Comparing the concentration-response relations in the presence and absence of an antagonist then gave the type of antagonism and the K_i for the antagonist. A computer programme was used to process the data. The relevant equation for a competitive antagonist is

$$K_i = \frac{i}{\frac{K_p}{K_m} - 1}$$

where i is the antagonist concentration and K_m and K_p are the apparent dissociation constants in the absence and presence of antagonist respectively. For non-competitive antagonism the equation is

$$K_i = \frac{i}{\frac{R_{max}}{R_p} - 1}$$

where R_{max} and R_p are the maximal responses in the absence and presence of the antagonist respectively. Both equations are adapted from considerations of enzyme-substrate interactions (Dixon & Webb, 1979) and values of K_i especially in the non-competitive case, must be interpreted cautiously. In the pharmacological sense, non-competitive means inhibition is not surmountable by increasing agonist concentrations.

The date for the antagonism of SCC responses to LBK by Hoe 140 were more appropriately handled by an analysis of variance (Goldstein, 1964) as there were too few points for curvilinear fitting.

Solution

The solution used to bathe the tissues for both Ca_i and SCC measurements was as follows (mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.0, KH_2PO_4 0.4, $MgSO_4$ 0.3, HEPES buffer (pH 7.4) 10 and glucose 1.2. The solution was equilibrated with 100% O_2.

Drugs

Hoe 140 was supplied by Hoechst AG. LBK, histamine, mepyramine and Des-Arg-Leu-BK were from Sigma. Forskolin was supplied by Calbiochem, piroxicam by Pfizer and cimetidine by Smith Kline Beecham.

Results

The nature of the response

Colony 29 monolayers grown on plastic slips have been used to measure intracellular $Ca^{2+}(Ca_i)$, whereas monolayers grown on permeable supports were used to measure chloride secretion as SCC. Typical changes in Ca_i in response to LBK and histamine at different concentrations are shown in Figure 1a and b. Two types of measurements were made from changes in Ca_i following agonist. They were peak increase in Ca_i above the baseline and the area under curve (AUC), for the 3 min following addition of agonist. Clearly the Ca_i responses are concentration-related and transient, even though the agonist was not removed.

In a number of situations described later it was important to have paired preparations so that agonists effects in the presence and absence of antagonist could be measured. To ensure test and control preparations were as similar as possible, single plastic slips from Leighton tubes with cultured

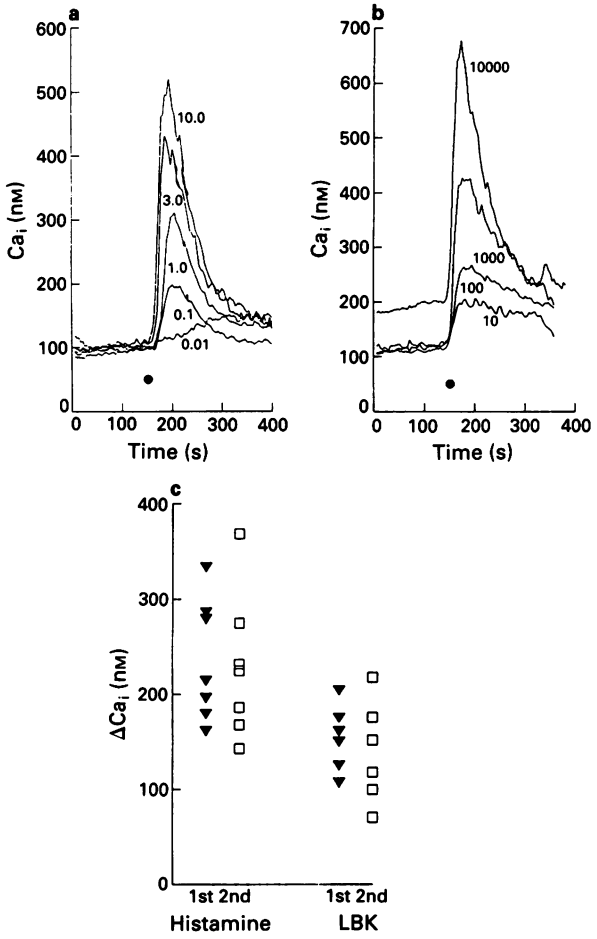

Figure 1 Responses to lysylbradykinin (LBK) and histamine in Colony 29 monolayers. In (a) LBK at the concentrations and time indicated was added to the solution perfusing the monolayer and remained present during the rest of the recording. All monolayers were from the same batch. In (b) the same protocol is illustrated this time with histamine. In (a) and (b) drug concentrations are in μM. In (c) monolayers were loaded with Fura-2 and the plastic slide cut into two pieces. One half was exposed to histamine (100 μM) or LBK (1 μM) and some 20 min later the other half was exposed to the same agonist, i.e. first and second responses. Note the scatter of Ca_i increases was the same in the two halves.

monolayers were cut in half, to give two preparations grown under identical conditions. Figure 1c shows peak Ca_i responses to histamine and LBK in the two halves of identical cultures. The responses of the second halves, made approximately 30 min after the first responses, show a similar distribution of values so justifying the use of this experimental paradigm.

Figure 2 shows SCC records from two epithelial monolayers of the same batch. In this situation agonists can be applied separately to the apical and basal surfaces. Hoe 140, 10 nM, was added to the solutions bathing both surfaces in one preparation. Both monolayers were then exposed sequentially to LBK basolaterally then apically and finally histamine on the basolateral side. Again peak height above baseline and AUC for the first 3 min of the response were the measures made from SCC records of this type.

Antagonism of responses to lysylbradykinin by Hoe 140

Paired monolayer halves were used in 14 separate experiments in which a concentration of Hoe 140 of 10 nM was equilibrated with one-half of each monolayer. Thirty min were allowed for equilibration. The magnitude of the calcium responses versus LBK concentration in the presence and absence of Hoe 140 are shown in Figure 3. Antagonism does not appear to be surmountable and linearisation procedures suggest it is non-competitive. The values for K_i from analysis

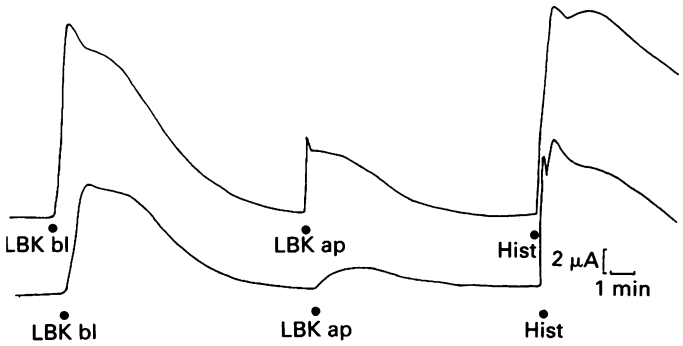

Figure 2 Short circuit records from two Colony 29 monolayers (0.2 cm^2). Each was exposed to lysylbradykinin (LBK, 0.3 μM) applied on the basolateral (bl) side and then on the apical (ap) side and finally to histamine (Hist, 100 μM) on the basolateral side. In the experiment illustrated by the lower trace Hoe 140, 10 nM, was present in the bathing solution for 30 min before the agonists were added.

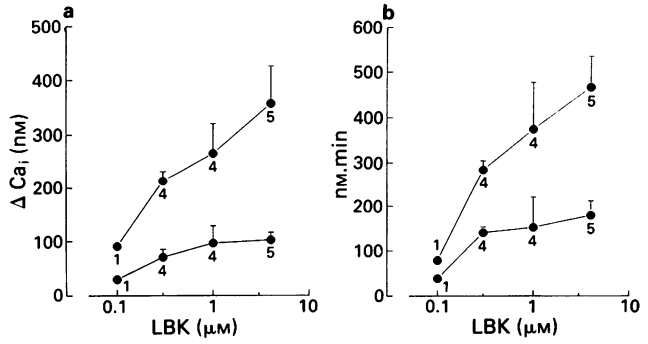

Figure 3 Antagonism of lysylbradykinin (LBK) by Hoe 140 on Δ Ca_i changes in Colony 29 monolayers: (a) shows the relation between Δ Ca_i and LBK concentration, whereas (b) shows AUC for 3 min after LBK was applied. In both (a) and (b) the lower curve was from tissues previously incubated with Hoe 140, 10 nM, for 30 min at the time LBK was added. The number of observations obtained for each point is indicated. Each culture yielded two preparations, one used for control and one treated with antagonist.

was 4.2 nM based on peak Ca_i and 6.0 nM based on AUC (Table 1).

In the experiments of Figure 3 a second agonist, histamine, 100 μM, was added once the response to LBK had disappeared. The reason for this was to normalize the LBK responses to those for histamine, allowing variation in responsiveness to be eliminated. It was discovered that while responses to histamine did not vary in respect of the LBK concentration they were smaller in the presence of Hoe 140, 10 nM. Histamine responses in controls were 313 ± 36 nM ($n = 14$) and in the presence of Hoe 140 were 219 ± 20 nM ($n = 14$) using peak values for Ca_i. By use of AUC the values were 652 ± 63 nM min ($n = 13$) in controls and 460 ± 34 ($n = 13$) in the presence of Hoe 140. The differences in these pairs of values were significant ($P < 0.05$).

Although histamine was less useful for normalization than expected since its effects were depressed by Hoe 140, we recalculated each LBK response as a percentage of the histamine response. However linearisation of these data as S/R versus S plots indicated that the apparent K_m values in the presence and absence of Hoe 140 were different (i.e. as with competitive antagonism) and maximal response values were also different (i.e. as with non-competitive antagonism). The same was true if AUC values for Ca_i were used instead of peak values. These findings indicate antagonism by Hoe 140 may be mixed, assuming diminution of the histamine responses is due to a non-specific effect of Hoe 140. Alternatively if the mean value of the reduction of the histamine response by Hoe 140 is used to correct the LBK responses in the presence of Hoe 140, then non-competitive antagonism is found with a K_i of 9.3 nM.

Since monolayers were equilibrated with Hoe 140 during the period of Fura-2-AM uptake and throughout the rest of the experiment there was no opportunity to see if Hoe 140 had partial agonist activity. This was investigated in separate experiments where it did cause minor increases in Ca_i in some but not all experiments. The details are as follows: (Hoe 140 concentration, fraction of monolayers showing responses and mean Ca_i increases in the responders) – 10 nM, 1/6, 45 nM; 30 nM, 2/4, 29 nM; 100 nM, 2/9, 29 nM; 1 μM, 3/6 43 nM.

To examine if inhibition of LBK by Hoe 140 was altered when prostaglandin formation by LBK was inhibited, measurements of Ca_i were made in monolayers preincubated with piroxicam. In the presence of piroxicam there were no major qualitative or quantitative differences in the responses, a finding confirmed by the data given in Table 2. Thus blockade of prostaglandin formation does not alter the type of antagonism shown by Hoe 140 against LBK.

Turning attention to SCC measurements as a further indication of kinin action the relationships were markedly different. Only one response to LBK could be obtained in each tissue because of the rapid and prolonged desensitization which occurs (Cuthbert *et al.*, 1987). A design analogous to a four-point assay was chosen, with two concentrations of LBK on the linear part of the concentration-response curve and data obtained in the presence or absence of a fixed concentration of Hoe 140. With basolaterally applied LBK there was a parallel shift of the concentration-response curve to the right when Hoe 140 was present. An analysis of variance (Goldstein, 1964) for the peak heights of responses for the data of Figure 4 was carried out. Of the total variance around 60% was accounted for by regression, while only 2% was due to deviation from parallelism. F values were for regression 49.2 (d.f. 1,28) $P < 0.01$, for the difference between the regression lines in the presence and absence of Hoe 140, 10.6 (d.f.1,28) $P < 0.01$ and for deviations from parallelism, 1.93 (not significant). Thus a competitive type interaction is indicated. The 'dose-ratio' from the variance analysis gave a value of K_i of 5.4 nM (or 5.3 nM using AUC) (Table 1). No agonist activity of Hoe 140 on chloride secretion was detected in three experiments with concentrations ranging from 1 to 7.5 μM. No diminution in the SCC responses to histamine in the presence of Hoe 140 was recorded. Res-

Table 1 Summary of kinetic values from experiments with Hoe 140 and lysylbradykinin (LBK)

Type of measurement	*Conditions*	*Maximal response*	*Apparent* K_I	*Type of antagonism*
Ca_i peaks	Control	369 nM	–	–
	Hoe 140 10 nM	110 nM	4.2 nM	Non-competitive
Ca_i AUC, 3 min	Control	491 nM min	–	–
	Hoe 140, 10 nM	184 nM min	6.0 nM	Non-competitive
SCC peaks	Control	–	–	–
	Hoe 140, 10 nM	–	5.4 nM	Competitive
SCC AUC, 3 min	Control	–	–	–
	Hoe 140, 10 nM	–	5.3 nM	Competitive

Abbreviations: AUC,3 min, area under curve for 3 min after addition of agonist. An analysis of variance was used to give the 'dose ratio' for the antagonism of LBK by Hoe 140 on SCC. K_i was calculated from 'dose ratio' = $A/K_i + 1$, where A was the antagonist concentration.

ponses to histamine, 10 μM, in the absence of antagonist were (mean ± s.e.mean (*n*)) 28.1 ± 3.4 μA cm^{-2} (8) and after Hoe 140, 27.2 ± 3.4 μA cm^{-2} (8). No significant differences were detected when AUC measures were used, the values being 45.5 ± 5.4 nEq (8) for controls and 40.0 ± 4.5 nEq (8) in the presence of the antagonist.

Apical receptors are more sensitive to kinin than those on the basolateral side (Pickles & Cuthbert, 1991; Simmons, 1992) but the responses are smaller and at the concentrations used in this study are already maximal. Although quantitative data cannot therefore be obtained from the results, marked inhibition of these responses by 10 nM Hoe 140 was observed which was not overcome by relatively high concentrations of bradykinin (Figure 4).

To examine for the presence of B_1 receptors in Colony 29 epithelia, monolayers were incubated with the B_1 receptor antagonist Des-Arg-Leu-BK, 1 μM (Regoli & Barabé, 1980) for 15 min. SCC responses to LBK (0.3 μM) in controls were 24.1 ± 6.2 μA cm^{-2} (6) while in the presence of the antagonist the responses were 21.2 ± 3.9 μA cm^{-2} (9). Clearly these responses do not suggest that B_1 receptors are present.

As before, the effect of inhibition of prostaglandin synthesis on the responses to LBK were investigated. Epithelial monolayers were preincubated with or without piroxicam and single SCC responses to basolateral application of LBK were recorded. Table 2 shows the values of both peak responses and AUC responses. Piroxicam causes a significant reduction in both measurements. There was little change in the overall form of the responses except for size. However, piroxicam reduced the sizes of basolateral responses to a size too small for quantitative analysis under these conditions.

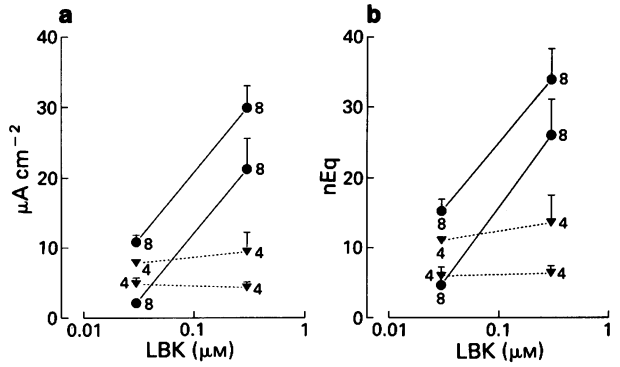

Figure 4 Antagonism of lysylbradykinin (LBK) by Hoe 140 on short circuit current increases in Colony 29 monolayers. Peak increases in SCC are shown on the left while AUC for first 3 min after LBK added is shown on the right: (●) are responses to LBK applied basolaterally, while (▼) are for apically applied kinin. The number of observations for each point are given. Paired preparations, were used; that is, one culture served as a control while the other was exposed to Hoe 140. In both (a) and (b) the lower one of each pair of curves represent tissues pre-equilibrated with Hoe 140 (10 nM) before LBK was added.

Table 2 Effects of piroxicam (5 μM for 30 min (a) or 15 min (b)) on: (a) Ca_i responses to lysylbradykinin (1.0 μM); (b) SCC responses to lysylbradykinin (0.1 μM)

a	Δ *Ca$_i$* (nM)	*AUC* (nM min)
Control	177 ± 24 (5)	235 ± 26 (5)
Plus piroxicam	179 ± 31 (5)	200 ± 40 (5)
b	Δ *SCC* (μA)	*AUC* (nEq)
Control	10.9 ± 1.5 (11)	10.5 ± 1.7 (8)
Plus piroxicam	4.2 ± 0.6 (8)	3.8 ± 0.8 (8)

Data are given as changes in the parameters Ca_i or SCC. Either peak responses or AUC for 3 min are given. Means ± s.e.mean are given for the number of observations, shown in parentheses. For the SCC experiments lysylbradykinin was added basolaterally.

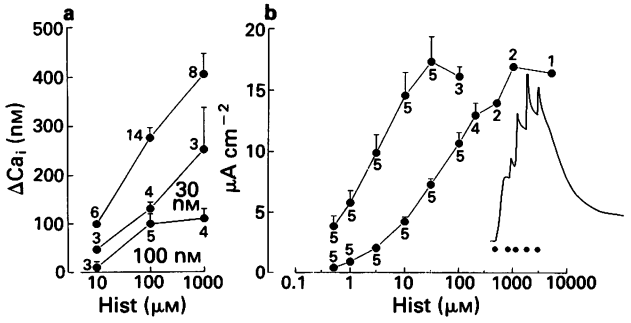

Figure 5 (a) Peak Ca_i responses to histamine in the absence (upper curve) and presence of mepyramine (30 nM, middle, or 100 nM, bottom, curves). Mepyramine was allowed to equilibrate for 30 min with the monolayers before effects of histamine were measured. (b) Concentration-response curves to histamine in Colony 29 monolayers using peak SCC values. Histamine was added cumulatively on the basolateral side. The peak responses measured from the preceding trough were added together to give the concentration-related responses (see inset). The lower curve was obtained after tissues were equilibrated with mepyramine, 100 nM, for 30 min. In (a) and (b) the number of observations is given by each point.

Antagonism of responses to histamine by mepyramine

Because of the contradictory results with Hoe 140 it was decided to examine the antagonism of histamine by mepyramine using the same methods as before. An identical procedure as with Hoe 140 gave the data shown in Figure 5. By use of the same analytical methods it appeared that mepyramine, 30 nM, was a non-competitive antagonist with a K_i of 9.4 nM (for Ca_i peaks) and 3.4 nM (for AUC). Yet, with mepyramine, 30 nM, the curves are close to parallel with a dose-ratio of approximately 10, both for Ca_i peaks and for AUC (not shown) suggesting at this concentration antagonism might be competitive with a K_i of around 3 nM. With 100 nM mepyramine analysis indicated competition was of the mixed type. A second agonist to normalize the responses to histamine was not used in this series, as large histamine concentrations deplete the stores to reduce subsequent responses to LBK (Pickles & Cuthbert, 1991). Potential non-specific actions of mepyramine were checked with LBK. Peak Ca_i responses to LBK (1.0 μM) were 197 ± 22 nM (mean ± s.e.mean) ($n = 6$), while following histamine 100 μM in the presence of mepyramine 100 nM, peak Ca_i responses to LBK (1.0 μM) were 212 ± 28 nM (mean ± s.e.mean) ($n = 12$).

For SCC measurements with histamine, cumulative addition could be used as the responses did not desensitize so rapidly. Traces, as illustrated in Figure 5b, could be analysed in two ways; either the values of the peaks above baseline were measured, or each incremental increase could be added cumulatively to the preceding ones. Either way gave virtually identical values for K_i. Analysis of the data of Figure 5b indicated competitive antagonism with a value of K_i of 6.3 nM or 4.5 nM. In the presence of cimetidine, 1 μM, responses to histamine, 10 μM, were (mean ± s.e.mean; n) 15.0 ± 1.5 μA cm^{-2} (7) while in controls a value of 15.9 ± 2.3 μA cm^{-2} (8) was obtained, clearly indicating histamine responses in Colony 29 monolayers are mediated via H_1-receptors. With mepyramine the maximal response values, obtained from the linearisation procedure, indicate competitive-type antagonism when SCC responses are considered, whereas this was not so for measurements of Ca_i.

Discussion

No previous quantitative study has been made of the antagonism of kinin actions on epithelia by Hoe 140. As it is now known that kinins cause electrogenic anion secretion in many epithelia, including those of the gastrointestinal tract, the airways, the genito-urinary tract among others (see Cuthbert & MacVinish, 1989), the availability of a potent antagonist is of significance, both for therapeutic potential and as a research tool. If the cultured colonic epithelium used is typical of human epithelia, then it appears that Hoe 140 is effective in the nanomolar range. This represents a 100 to 1,000 fold increase in potency compared to the Stewart compounds (Vavrek & Stewart, 1985). In the comprehensive study of *in vitro* and *in vivo* actions of Hoe 140 as a kinin receptor antagonist by the German group (Wirth *et al.*, 1991; Hock *et al.*, 1991) pA_2 values of around 9 were given. In this study, electrogenic chloride secretion, measured as SCC and increases in Ca_i were used as two separate measures of LBK action. There is a large body of evidence which relates these measures in a variety of epithelia (Yada *et al.*, 1989; Wong *et al.*, 1989; Morris *et al.*, 1990), and this is true too of HCA-7 Colony 29 cells (Pickles & Cuthbert, 1991).

An increase in intracellular Ca^{2+} may affect transepithelial chloride secretion by several mechanisms. For example, apical chloride channels can be Ca^{2+}-sensitive (Cliff & Frizzell, 1990). Alternatively an increase in Ca^{2+} opens basolateral Ca^{2+}-sensitive K^+channels, with consequent hyperpolarization and an increase in the electrical gradient for chloride efflux from the cell. Inhibition of eicosanoid synthesis does not affect the calcium signal but severely attenuates the SCC response. Prostaglandins activate epithelial adenylate cyclase (Cuthbert *et al.*, 1984) which in turn causes apical chloride channels to open (Henderson *et al.*, 1992). Furthermore in Colony 29 epithelial cells, calcium release from intracellular stores provokes a further calcium influx from outside the cell (Pickles & Cuthbert, 1991). These mechanisms militate against there being a linear relationship between Ca_i and the SCC response (Pickles & Cuthbert, 1991). In rat colon epithelium the relationship between SCC and Ca_i is best fitted by a cubic function, over a limited range of Ca_i, suggesting that there is a calmodulin involvement in the SCC response (Cuthbert, 1985; Worrell & Frizzell, 1991). It is known that Colony 29 epithelia can show oscillatory Ca_i responses with agonists (Pickles *et al.*, 1991), but usually this is not synchronized across the whole epithelium. If the frequency or amplitude of these oscillations are an important signalling mechanism, then the average Ca_i recorded here is unlikely to be simply related to agonist concentration.

Clearly the generation of intracellular signals, such as elevation of Ca_i, is a necessary event which must occur before the transepithelial transport of chloride, measured as SCC, can be activated. That is, Ca_i increase is an event in a sequence closer to the kinin-receptor interaction than the final effector process.

Kinins have been shown to increase phosphatidylinositol hydrolysis to bring about an increase Ca_i and there may be a regenerative aspect to the Ca_i increase (Finch *et al.*, 1991). However, kinin receptors can be linked, through G proteins, to the activation of both phospholipase C and phospholipase A_2, a mechanism to increase both Ca_i and eicosanoid formation (Burch & Axelrod, 1987). We can only discover B_2 receptors in Colony 29 cells, implying a single type of receptor is linked to the activation of the Ca_i increase and prostaglandin formation.

The complexity of the Ca_i response to kinins applies in much the same way to the actions of histamine on this cell line (Pickles & Cuthbert, 1991), a situation in which only a single type of receptor, H_1 is present. Classically, mepyramine is a competitive antagonist, while here it may be so at low concentrations but have mixed actions at higher concentrations. Given the complexities of the Ca_i response in epithelia it may be that the apparent non competitive actions of mepyramine, and perhaps too of Hoe 140, do not accurately report the interaction at the receptors. Receptor binding studies in epithelial cells would be needed to resolve the nature of the receptor interaction. Particular care was taken in these studies to ensure equilibrium was established between the tissue and antagonists. However both agonists used, particularly LBK, cause rapid desensitization, so it is unlikely equilibrium is achieved between receptors, agonists and antagonists when responses are measured, a situation which is aggravated by high agonist concentrations. However, these same arguments apply equally to experiments in which SCC was recorded.

In recent reports (Rhaleb *et al.*, 1992; Griesbacher & Lembeck, 1992) it was concluded that Hoe 140 acted non-competitively in a number of smooth muscles. In other instances (Field *et al.*, 1992) concentration-response curves with depressed maxima were obtained in guinea-pig taenia caeca and tracheal muscles in the presence of Hoe 140.

Since SCC responses indicated that Hoe 140 was a competitive antagonist by the usual criteria and since a SCC increase is a downstream event from Ca_i increase, the inescapable conclusion is that this result is fortuitous if the action of the antagonist is truly non-competitive. The maximal transporting capacity for chloride secretion is determined by the basolateral sodium pump, while signal generation continues to increase at agonist concentrations which are supramaximal for the SCC response (Pickles & Cuthbert, 1991).

Two comments about this situation from an exhaustive list of possibilities, can be made here. First, it may be an inhe-

rent epithelial property to respond in a hyperbolic fashion to different concentrations of secretagogues, showing a threshold, a maximal response and a roughly log-linear relation in between. Although this behaviour can be represented by a simple mass action formulation this is only one of many possibilities, a point emphasized long ago by Clark (1933). An antagonist, by changing threshold, may simply move the curve to the right with the maximal response being determined by a factor unconnected with events at the receptor, in this instance the sodium pump.

Secondly, if the increase in Ca_i is unrelated to the increase in anion transport, although this is highly unlikely, then Hoe 140 may be a true competitive antagonist when SCC is measured. The drawback of this explanation is that the non-competitive nature of the Ca_i measurement must then be mediated by a different receptor. No evidence for more than one type of receptor exists for this epithelium, although B_3 receptors have been postulated to exist in the major airways (Farmer *et al.*, 1989).

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A possible role of decreased relaxation mediated by β-adrenoceptors in bladder outlet obstruction by benign prostatic hyperplasia

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1 To explore mechanisms of urinary obstruction in benign prostatic hyperplasia (BPH), the features of contraction and relaxation in human hyperplastic and non-hyperplastic (control) prostatic tissues were investigated for β- and α_1-adrenoceptors by radioligand binding and *in vitro* isometric tension experiments.

2 Hyperplastic and control prostatic tissues had a similar number (per mg protein) of prazosin binding sites with similar affinities. Noradrenaline (NA) induced dose-dependent contraction in both tissues. Contraction induced by either exogenous NA or transmural stimulation was inhibited by prazosin in both tissues, indicating that the same contractile mechanisms mediated by α_1-adrenoceptors exist in hyperplastic and control tissues.

3 The number of dihydroalprenolol (DHA) binding sites (per mg protein) was less in hyperplastic tissues than in controls, whereas the affinity to the ligand was identical in both tissues. Isoprenaline caused a marked relaxation of the tonic contraction induced by KCl in control tissues, but not in hyperplastic tissues. Propranolol enhanced exogenous NA- or transmural stimulation-induced contraction more in control tissues than in hyperplastic tissues. Both tissues, however, similarly responded to forskolin by relaxation.

4 These results indicate that decreased β-adrenoceptor-mediated relaxation in hyperplastic prostatic tissues, which is attributable at least in part to the decreased number of β-adrenoceptors, may play a role in the urinary obstruction of BPH in addition to mechanical compression of the urethra by the enlarged prostate.

Keywords: Benign prostatic hyperplasia; α_1-adrenoceptor; β-adrenoceptor; adenylate cyclase

Introduction

Benign prostatic hyperplasia (BPH) occurs in the majority of the aging male population and the bladder outlet obstruction caused by BPH is one of the most commonly encountered disorders at urology clinics. However, the specific features of the prostatic adenoma predisposing to the development of bladder outlet obstruction have not been well explored.

Human hyperplastic prostatic tissues have been found to reveal a marked contractile response to noradrenaline (NA), which is antagonized by pretreatment with phentolamine, an α-adrenoceptor antagonist (Caine *et al.*, 1975). These findings have led to a proposal that bladder outlet obstruction by BPH is composed of static and dynamic components. The former component is the physical obstruction produced by the presence of the hyperplastic tissue itself, whereas the latter factor is the tone of the prostatic smooth muscle regulated by the autonomic nervous system (Caine, 1986). A very dense network of adrenergic nerve fibres has been found histologically distributed within the smooth muscle layer of the prostatic glandular stroma (Vaalasti & Hervonen, 1980) and adrenoceptors of human prostatic tissue have been characterized and quantitated by radioligand receptor binding methods and *in vitro* isometric tension experiments (Lepor & Shapiro, 1984; Hedlund *et al.*, 1985; Yokoyama *et al.*, 1985; Yamada *et al.*, 1987). These studies demonstrated that human prostatic tissue contained a substantial number of high affinity α_1-adrenoceptors and that contraction of prostatic smooth muscle was mediated by α_1-adrenoceptors.

There have been very few studies, however, on the properties of β-adrenoceptors in human prostates. In the present study, we have investigated the binding and functional properties of β- as well as α_1-adrenoceptors in hyperplastic and non-hyperplastic prostatic tissues with radioligand binding studies and *in vitro* isometric tension experiments and the results indicate a possible role of the reduced relaxation of prostatic smooth muscle mediated by β-adrenoceptors contributing to the bladder outlet obstruction by BPH.

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Methods

Prostatic tissues

Human prostatic tissues were obtained at operation from 45 males aged from 42 to 82 years of age (median 70.0 years); 32 by open subcapsular prostatectomy as surgical treatment of BPH and 13 by radical cystoprostatectomy for bladder cancer. The latter patient group included those who had neither symptoms of bladder outlet obstruction nor an enlarged prostate by rectal examination and served as controls. All of the patients with BPH had urinary obstructive symptoms such as urinary retention, residual urine greater than 50 ml, urinary frequencies and low urinary flow rate by uroflowmetry. Each sample of prostatic tissue was divided into two or three portions of approximately equal volume and used for radioligand binding assay and *in vitro* isometric tension experiments.

Radioligand receptor binding assay

Preparation of crude membrane fractions Immediately after removal, a portion of the tissue was immersed in 50 mM ice-cold phosphate buffer (pH 7.5), minced with scissors and twice homogenized with a Polytron Homogenizer at maximum speed for 30 s in 15 volumes of ice-cold phosphate

buffer (pH 7.5). The homogenates were then centrifuged at 1,000 g for 10 min at 4°C. The supernatants were recentrifuged at 100,000 g for 60 min at 4°C. The final pellets were resuspended in fresh phosphate buffer as a crude membrane fraction and were stored at −80°C until use.

Protein concentration of each membrane fraction was measured with bovine serum albumin as a standard by the method of Lowry et al. (1951). Then, each fraction was adjusted with phosphate buffer (pH 7.5) to contain 100 μg protein 100 μl^{-1} for binding assay of [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) or 250 μg protein 100μl^{-1} for [^{3}H]-prazosin assay.

Saturation analysis Radiolabelled ligands used for saturation analyses were [^{3}H]-DHA (95.9 Ci $mmol^{-1}$) for β-adrenoceptors and [^{3}H]-prazosin (76.6 Ci $mmol^{-1}$) for α_1-adrenoceptors. The medium for the assay was adjusted to 250 μl containing 100 μl crude membrane fractions, 150 μl phosphate buffer (pH 7.5) and radiolabelled ligands at concentrations specified in Figures 2 and 5. Each assay medium was shaken for 30 min at 37°C and filtered through Whatman GF/B glass fibre filters. The filter discs were then rinsed three times with 2 ml of ice-cold phosphate buffer and placed in 16 ml of scintillation fluid (Econofluor; New England Nuclear Research Products). Radioactivities were counted with a Packard 460-CD scintillation spectro-fluorometer.

In vitro *isometric tension experiments*

Immediately after removal, another portion of the prostatic tissue was placed in ice-cold modified Krebs solution (pH 7.4) which contained (mM); NaCl 115.0, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_4PO_4 1.2, $NaHCO_3$ 25.0 and glucose 10.0. After removal of fat and connective tissue, tissue strips (about 5x15 mm, 0.2 g wet weight) were prepared and mounted vertically in organ baths containing 20 ml of modified Krebs solution which was continuously bubbled with 95% O_2 and 5% CO_2 at 37°C. One end of each strip was anchored to a metal hook at the bottom of the organ bath and the other end was connected to a force-displacement transducer (TB611T, Nihon Kohden Kogyo CO.). Isometric changes in tension were recorded on a pen-writing oscillograph (R-64, Rika Denki Co.). The length of the strips was adjusted several times until there was a stable basal tissue tension of 1.0 g. Before starting the experiment, the strips were allowed to equilibrate for at least 40 min in the organ baths and the bath solution was replaced every 20 min. Each agonist or antagonist was applied cumulatively in the organ baths as described in each figure. When subjected to electrical field stimulation, the tissue strips were mounted between two parallel platinum ring electrodes in the organ baths. Intramural nerve stimulation was performed by means of an electronic stimulator delivering square pulses of 0.2 ms duration at supramaximum voltage (80 V over the electrodes) and 20 Hz for 5 s. The interval between trains is specified in each figure legend. As shown in Figure 1, the almost complete inhibition of the response by tetrodotoxin (3×10^{-7} M) confirmed that the contractions induced by transmural stimulation were nerve-mediated.

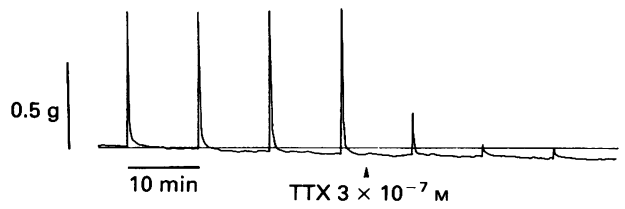

Figure 1 Inhibition of transmural stimulation-induced contraction by tetrodotoxin (TTX) in a control human prostatic tissue strip. Transmural electric stimulation was given every 10 min. TTX (3×10^{-7} M) was applied after the response had stabilized. Similar records were obtained on hyperplastic tissue preparations.

Chemicals

[^{3}H]-dihydroalprenolol ([^{3}H]-DHA) and [^{3}H]-prazosin were purchased from New England Nuclear Research Products, noradrenaline bitartrate, prazosin hydrochloride, isoprenaline hydrochloride, propranolol hydrochloride and tetrodotoxin from Sigma Chemical Co. and forskolin from Calbiochem Co. Ltd. All other reagents used were of analytical grade and water was redistilled.

Statistical analysis

All data are expressed as the mean ± s.e.mean. Statistical analysis was by Student's t test. Regression lines were calculated by the least squares method.

Results

Contraction mediated by α_1-adrenoceptors

α_1-adrenoceptors were studied using binding of [^{3}H]-prazosin in hyperplastic and control prostatic tissue homogenates. The binding of [^{3}H]-prazosin was saturable with high affinity. Scatchard plot analysis revealed that the α_1-adrenoceptor binding sites in the prostatic tissues constituted a single population (Figure 2). The dissociation equilibrium constant (K_d) and the receptor density (B_{max}) for [^{3}H]-prazosin binding were identical in both hyperplastic and control prostatic tissues (Table 1).

Isometric tension recordings were made from several tissue strips prepared from different patients with either BPH or bladder cancer. Exogenous NA induced a dose-dependent contraction of both hyperplastic and control prostatic tissues

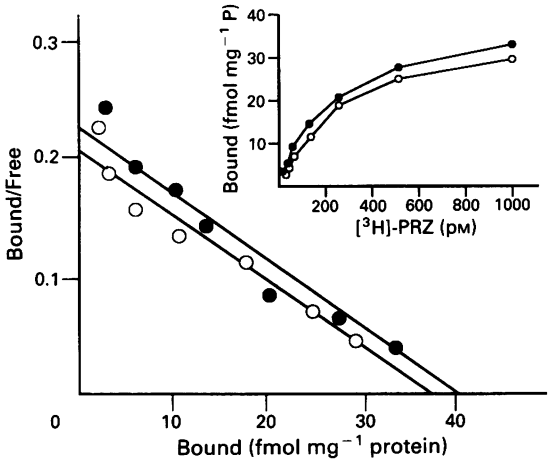

Figure 2 Representative Scatchard plots for [^{3}H]-prazosin binding to crude membrane fractions of prostatic tissues. Tissue preparations from hyperplastic (●) and control (○) prostates were incubated with seven different concentrations (16.25-1040 pM) of [^{3}H]-prazosin in duplicate under the conditions described in the text. Non-specific binding was determined in the presence of 10^{-4} M cold prazosin. Inset, saturation curves for [^{3}H]-prazosin binding in prostatic tissue homogenates.

Table 1 [^{3}H]-prazosin binding in prostatic tissue homogenates

Tissue	n	K_d	B_{max}
BPH	15	177.5 ± 7.7	39.7 ± 6.2
Control	6	197.1 ± 18.9	42.4 ± 7.7

K_d and B_{max} are expressed as pM and fmol mg^{-1} protein, respectively.

and the dose-response curve was shifted in parallel to the right by prazosin without any reduction in the maximum tension (Figure 3). Prazosin dose-dependently inhibited the transient contraction induced by transmural nerve stimulation of both hyperplastic and control prostatic tissues (Figure 4). The results indicate that either transmural nerve stimulation or exogenous NA induced α_1-adrenoceptor-mediated contraction of both hyperplastic and control prostatic tissues.

Relaxation mediated by β-adrenoceptors

Receptor binding studies with [^{3}H]-DHA as a radiolabelled ligand were performed to examine the properties of β-adrenoceptors in hyperplastic and control prostatic tissues. The binding of [^{3}H]-DHA was saturable with high affinity. Scatchard plot analysis indicated that β-adrenoceptors in the prostatic tissues have a single population of binding sites (Figure 5). The B_{max} for [^{3}H]-DHA on the basis of protein was significantly less in hyperplastic prostatic tissues than in controls, while K_ds of both tissue preparations for [^{3}H]-DHA were identical (Table 2).

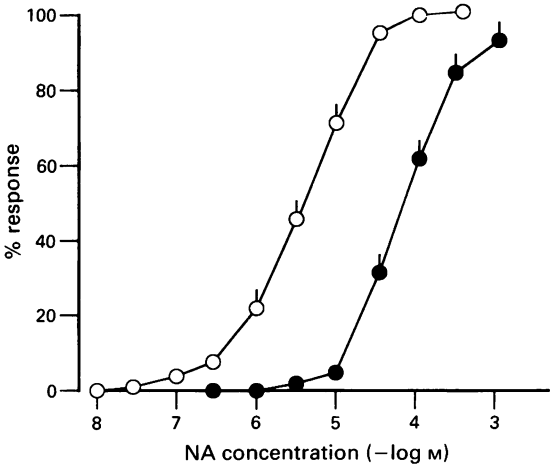

Figure 3 Mean dose-response curves of contraction of prostatic tissue strips to noradrenaline (NA) in the presence (●) or absence (○) of an α_1-adrenoceptor antagonist, prazosin (PRZ). Ten prostatic tissue strips were prepared from eight patients with BPH. The dose-response curves to NA were constructed by increasing the concentration (10^{-9} to 10^{-3} M) of NA in a cumulative fashion. Contractions were expressed as a percentage of the maximum contraction by NA. Inhibition of NA-induced contraction was obvious after prior exposure of prostatic tissue strips to 10^{-7} M PRZ for 20 min. A similar result was obtained in control prostatic tissues.

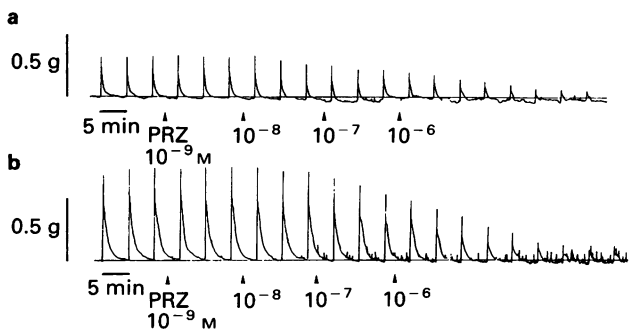

Figure 4 Representative traces of the inhibitory effect of prazosin (PRZ) on the contraction induced by transmural nerve stimulation in hyperplastic (a) and control (b) prostatic tissue strips. Electrical stimulation was given as described in the text at intervals of 5 min. After the response had stabilized, PRZ was applied in a cumulative fashion every 20 min. Addition of PRZ inhibited, dose-dependently, transmural neurogenic contractions of both hyperplastic and control prostatic tissues.

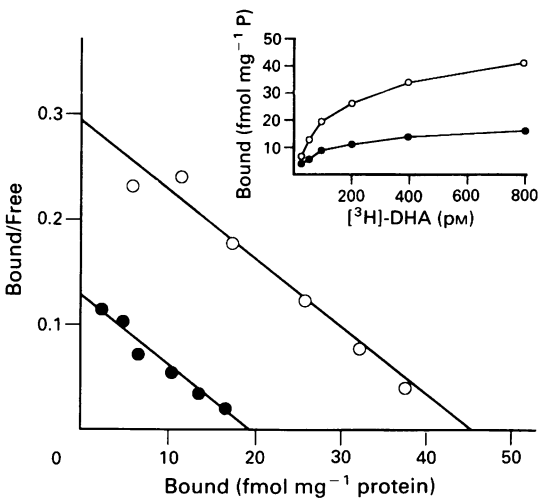

Figure 5. Representative Scatchard plots for [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) binding to crude membrane fractions of prostatic tissues. Tissue preparations from hyperplastic (●) and control (○) prostates were incubated with six different concentrations (25–800 pM) of [^{3}H]-DHA in duplicate under the conditions described in the text. Non-specific binding was determined in the presence of 10^{-4} M propranolol. Inset, saturation curves for [^{3}H]-DHA binding in prostatic tissue homogenates.

Table 2 [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) binding in prostatic tissue homogenates

Tissue	n	K_d	B_{max}
BPH	15	140.2 ± 9.4	21.5 ± 1.0*
Control	6	142.8 ± 11.0	36.9 ± 5.2

K_d and B_{max} are expressed as pM and fmol mg^{-1} protein, respectively. *P<0.05.

Isometric tension was used to study β-adrenoceptor-mediated relaxation in hyperplastic and control prostatic tissue strips. The relaxation response of prostatic tissues to the β-adrenoceptor agonist, isoprenaline, was not obvious under basal tonus even at high concentration (10^{-4} M) and was therefore examined under tonic contraction produced by 60 mM KCl. The agonist induced a dose-dependent relaxation of the tonic contraction in control prostatic tissues but not in hyperplastic tissues (Figure 6). The β-adrenoceptor antagonist, propranolol, almost abolished the isoprenaline-induced relaxation of control prostatic tissue at 10^{-6} M. The antagonist also enhanced contractions induced by either exogenous NA or transmural nerve stimulation in control prostatic tissues but the effect was not obvious in hyperplastic prostatic tissues as shown in Figure 7.

To examine the responsiveness of the intracellular signalling coupled with β-adrenoceptors, adenylate cyclase was stimulated by forskolin. As shown in Figure 8, the agent induced marked relaxation of KCl-induced tonic contraction of both hyperplastic and control prostatic tissues, while the former tissues were not responsive to isoprenaline.

The above results are summarized in Figure 9. In contrast to smaller responses of hyperplastic tissues to agents acting on β-adrenoceptors, the response to direct stimulation of adenylate cyclase by forskolin was greater in hyperplastic prostatic tissues than in controls.

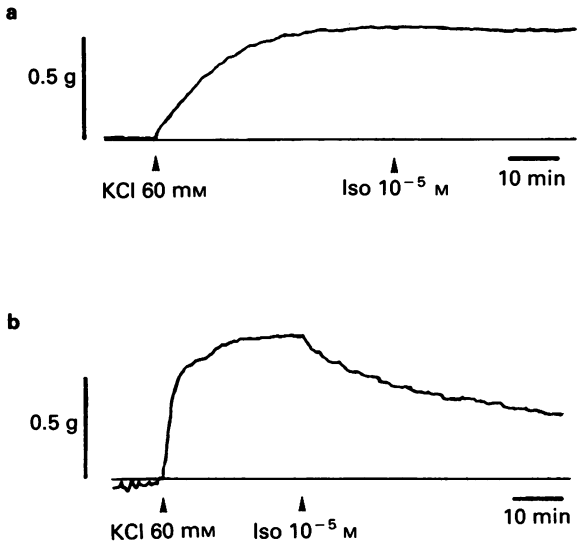

Figure 6 Representative relaxation response of KCl-induced tonic contraction by isoprenaline (Iso) of tissue strips from prostatic hyperplasia (a) and control prostate (b). Iso 10^{-5} M was applied to the organ bath after a stable tonic contraction to 60 mM KCl had been attained.

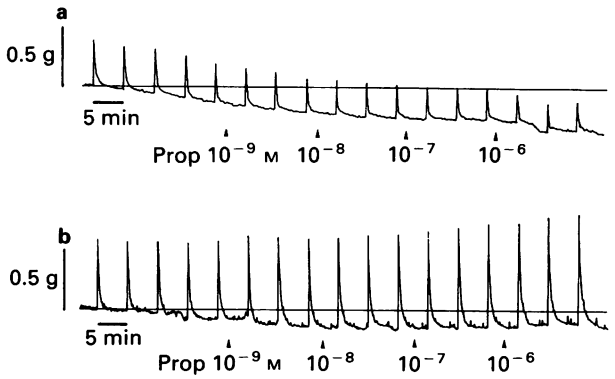

Figure 7 Representative examples of the effect of propranolol (Prop) on the contraction induced by transmural nerve stimulation in tissue strips from hyperplastic (a) and control prostate (b). The β-adrenoceptor antagonist propranolol, was applied every 15 min in a cumulative fashion.

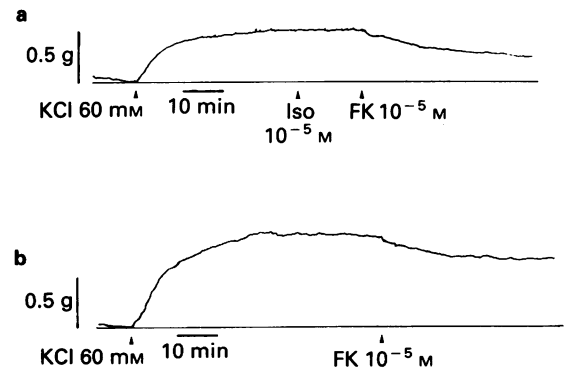

Figure 8 Representative example of the relaxation induced by forskolin (FK) of the KCl-induced contraction of tissue strips from hyperplastic (a) and control prostate (b). The adenylate cyclase stimulant, FK, was applied to the organ bath at 10^{-5} M after a stable tonic contraction to 60 mM KCl had been attained.

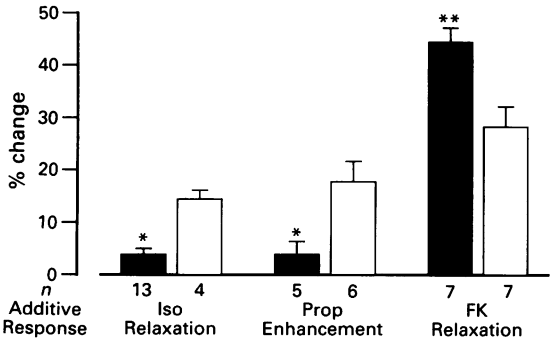

Figure 9 The effects of isoprenaline (Iso), forskolin (FK) and propranolol (Prop) on prostatic tissue strips in isometric tension experiments. The effects of Iso and FK are expressed as percentage relaxation of contraction induced by 60 mM KCl, while the effect of Prop is expressed as percentage enhancement of noradrenaline (NA)-induced contraction by measuring the amplitudes of contraction induced by 10^{-5} M NA, before and after addition of Prop. Open columns; control and closed columns; hyperplastic tissues. The number of experiments is indicated below the columns. Vertical bars indicate s.e.mean.
$^{*}P<0.05$; $^{**}P<0.01$.

Discussion

The present study with NA, prazosin and transmural nerve stimulation confirms that α_1-adrenoceptors mediate contraction of prostatic smooth muscles. Furthermore, the results indicate that the α_1-adrenoceptor-mediated responsiveness of hyperplastic and control prostatic tissues are equivalent, confirmed by the identical B_{max} and K_d for prazosin binding in both tissues. Gup *et al.* (1989, 1990) reported that the B_{max} for [^{125}I]-HEAT was similar in homogenates prepared from prostatic tissues of asymptomatic and symptomatic BPH and that the magnitude of the contractile response and the potency of phenylephrine-induced contractions of both prostatic tissues were similar. On the other hand, Yokoyama *et al.* (1985) and Yamada *et al.* (1987) found that there was a significant increase in the B_{max} for [^{3}H]-prazosin in hyperplastic prostates compared to control tissues. The present results, however, indicate that the increased urethral resistance by prostatic hyperplasia is not due to changed properties of the α_1-adrenoceptor-mediated contraction of prostatic smooth muscles.

The present receptor binding experiments with [^{3}H]-DHA indicate that the number of β-adrenoceptors in normal prostatic tissues is comparable to α_1-adrenoceptors and that it is significantly less in hyperplastic prostatic tissues than in controls, while K_ds are identical in both tissues. Consistent with the present findings, Yokoyama *et al.* (1985) have demonstrated high affinity [^{3}H]-DHA binding sites in human prostate with lower B_{max} in hyperplastic tissues than controls.

The present results demonstrate β-adrenoceptor-mediated relaxation of prostatic smooth muscles under tonic contraction in control tissues, while Caine *et al.* (1975) and Kitada (1983) have failed to observe any relaxation response to isoprenaline of human prostatic tissue in the resting state. Further, the relaxation response is apparently lost in hyperplastic tissues. It could be assumed that the almost non-existent responsiveness to β-adrenoceptor stimulation in hyperplastic tissues is attributable to impaired signal transduction in addition to the decreased number of β-adrenoceptors in hyperplastic tissues. Adensoline 3',5'-cyclic-monophosphate (cyclic AMP) has been demonstrated to be a second messenger in the β-adrenoceptor-mediated relaxation in most types of smooth muscles, including urinary bladder (Rohner & Hannigan, 1980; Levin *et al.*, 1986). As demonstrated in the present results, an adenylate cyclase stimulant, forskolin, induced marked and significantly greater relaxation

in hyperplastic tissues than controls. Therefore, it appears likely that the decreased number of β-adrenoceptors in hyperplastic tissues observed in the present study contributes at least in part to the reduced responsiveness of hyperplastic tissues. The role of changes in G-protein, another intermediate indispensible for signal transduction between β-adrenoceptors and adenylate cyclase, remains to be investigated.

In conclusion, decreased β-adrenoceptor-mediated relaxation in hyperplastic prostatic tissues appears to be involved in urinary obstruction by causing a continuous contractile state of the hyperplastic tissues in BPH in addition to the mechanical compression of the urethra by BPH.

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Neurokinin A-induced contraction of guinea-pig isolated trachea: potentiation by hepoxilins

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1 Hepoxilin A_3 (8**R** and 8**S** isomers) (HxA_3), hepoxilin A_3-C (8**R** and 8**S** isomers) (HxA_3-C) and trioxilin A_3 (8**S** isomer) ($TrXA_3$, the stable derivative of HxA_3) were tested for their effects on helicoidal strips of guinea-pig isolated tracheae.

2 None of the compounds (10^{-9}–10^{-6} M) tested had a direct effect on resting tension of trachea.

3 HxA_3 (8**S**) and HxA_3-C (8**R**) (10^{-8} M) produced a significant leftward shift of the log concentration-response curves to neurokinin A (NKA) (EC_{50} (nM), control = 29.0 ± 2.8, HxA_3 (8**S**) = 21.7 ± 3.7, HxA_3-C (8**R**) = 13.8 ± 3.8, $n = 6$ for each). Also the maximal response to NKA was significantly increased when the tissues were exposed to these hepoxilins (% of the maximal response to NKA, control = 100, HxA_3 (8**S**) = 114.5 ± 5.3, HxA_3-C (8**R**) = 139.0 ± 6.2, $n = 6$ for each). The threshold concentrations for both hepoxilins was 10^{-8} M and their effects were dose-related.

4 Stereochemical specificity was observed. The 8**S**-isomer of HxA_3 was active in potentiating the NKA-induced contraction of the trachea while the 8**R** isomer was inactive. In contrast, the 8**R** isomer of HxA_3-C was active while the 8**S** isomer was inactive. The trihydroxy metabolite of the active isomer of HxA_3 (8**S**), i.e. $TrXA_3$ (8**S**) (10^{-6} M), was inactive in potentiating the NKA-induced contraction of the trachea.

5 It is concluded that hepoxilins sensitize the guinea-pig isolated trachea to the potent bronchoconstrictor, NKA.

Keywords: Hepoxilin A_3; hepoxilin A_3-C; contraction; guinea-pig trachea; potentiation; neurokinin A

Introduction

Neurokinin A (NKA) belongs to a family of peptides called tachykinins. Three distinct tachykinin receptors denoted as neurokinin$_1$ (NK_1), NK_2 and NK_3 have been characterized by pharmacological and radioligand binding studies. These receptors are widely distributed in mammalian tissues and interact differently with the natural tachykinins, substance P (SP) being the preferential agonist for the NK_1 receptor, NKA for the NK_2 receptor and neurokinin B (NKB) for the NK_3 receptor (Regoli *et al.*, 1988; Helke *et al.*, 1990).

Tachykinins have been implicated in the non-adrenergic and non-cholinergic nervous system which exerts potent control of airways (Naline *et al.*, 1989). SP and NKA have been co-localized in a population of primary sensory C-fibres which have peripheral branches in various tissues including the respiratory tract (Hua *et al.*, 1985; Lundberg & Saria, 1987). Local release of SP and/or NKA from sensory fibres seems to play an important role in neurogenic inflammation (vasodilatation and plasma protein extravasation), airway oedema and bronchoconstriction (Hua *et al.*, 1984; 1985; Lundberg *et al.*, 1983a,b). NKA was more potent than SP and other tachykinins in causing bronchoconstriction *in vivo* in the guinea-pig (Hua *et al.*, 1984; Martling *et al.*, 1987a), rat (Joos *et al.*, 1986), sheep (Abraham *et al.*, 1991) and man (Evans *et al.*, 1988). Similarly, NKA appears to be particularly active on human isolated bronchi (Martling *et al.*, 1987b; Naline *et al.*, 1989) and on the guinea-pig isolated trachea which has been particularly useful in understanding the mechanisms that could control airway narrowing (Uchida *et al.*, 1987; Devillier *et al.*, 1988).

The enhancement of vascular permeability and stimulation of mucus secretion are also important physiological effects that contribute to airway narrowing. A variety of mediators such as eicosanoids are released during these physiological processes that lead to an inflammatory reaction. Among arachidonic acid metabolites studied, leukotrienes have received a lot of attention because they cause airway contraction both *in vivo* and *in vitro* (Hedqvist *et al.*, 1980). Also leukotrienes C_4 and D_4 promote plasma leakage (Dahlén *et al.*, 1981) and leukotriene B_4 is a potent chemokinetic and chemotactic agent released from polymorphonuclear leukocytes (Ford-Hutchinson *et al.*, 1980) all contributing to the process of inflammation. Our recent studies have demonstrated that a particular group of lipoxygenase metabolites, hepoxilins, also play a significant role in the modulation of the physiological events that take place during the process of inflammation (Dho *et al.*, 1990; Laneuville *et al.*, 1991a,b; 1992). Two hepoxilins have been isolated, i.e. 8-hydroxy-11,12-epoxy eicosa-5Z, 10E, 14Z-trienoic acid (hepoxilin A_3) and 10-hydroxy-11,12-epoxy eicosa-5Z, 8Z, 14Z-trienoic acid (hepoxilin B_3); these metabolites are formed from the 12-lipoxygenase product, 12-HPETE (Pace-Asciak *et al.*, 1983; Pace-Asciak & Martin, 1984). Biological activities of the hepoxilins relate to the modulation of vascular tone, the increase of vascular permeability in the skin and activation of human neutrophils. Based on these studies we investigated their possible role in the modulation of muscular tone in the guinea-pig isolated trachea.

Methods

Preparation of tracheal smooth muscle

Male guinea pigs (purchased from Charles River, St-Constant, Quebec, Canada) weighing 300–450 g were killed

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by stunning and exsanguination through sectioning of the carotid arteries. The trachea was rapidly removed and placed in Krebs solution (composition in mM: NaCl 118.1, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25.0, $MgSO_4$ 1.2, $CaCl_2$ 2.5, D-glucose 5.5) kept at 37°C and continuously gassed with 95% O_2, 5% CO_2 mixture. Helicoidal strips were cut from the trachea according to Constantine (1965), and mounted in organ baths filled with 10 ml Krebs solution maintained at 37°C and oxygenated. The lower end of the tracheal strip was attached to a glass hook at the base of the chamber by a loop of silk thread. The upper end was attached in the same manner to a force transducer (Grass FT.03C) for continuous recording of isometric tension by a Grass polygraph (model 79D). Each trachea was cut into four strips of equal dimensions; 4 mm wide by 25 mm long. Tissues were stretched at a tension of 3.0 g and equilibrated for 90 min during which time the bath solution was replaced every 15 min.

Effects of hepoxilins on neurokinin A-induced contraction of the trachea

Preliminary experiments showed that the addition of hepoxilins to the chamber at a final concentration of up to 10^{-6} M failed to cause muscle contraction or to relax NKA precontracted guinea-pig tracheal strips. To determine whether hepoxilins alter the NKA-mediated contraction, the response to NKA was first tested and its stability and reproducibility was established through three consecutive concentration-response curves. A period of 10 min was left between each dose-response curve which was constructed by cumulative addition of NKA. As the dose-response curves were not significantly different from each other, the third curve was used as the preadministration (control) value. Hepoxilins were then added and 45 min later, a concentration-response curve to NKA was obtained. Cumulative dose-responses to each hepoxilin (10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M) were carried out on the same preparations. No measurements were made of reversibility of effect on wash-out of hepoxilins. Only one hepoxilin was tested on each preparation. In control experiments, the effect of the vehicle, dimethyl sulphoxide (DMSO, 13 mM) on NKA-induced contraction was assessed.

Expression of results and statistical analysis

Contractions are expressed as the percentage of the maximal response to NKA obtained before the addition of hepoxilins. Sensitivity to NKA in the trachea was expressed as EC_{20} and EC_{50} values, EC_{20} being the concentration of agonist (NKA) required to give 20% of maximal response of the trachea, EC_{50} representing half-maximal response of agonist. EC_{50} values were obtained by logit/log regression analysis of each individual preparation and averaged. Results are expressed as mean ± s.e.mean of six separate experiments. The differences between the values, obtained in the same strip, were tested for significance by use of Student's t test for paired observations. Probabilities (P) smaller than 0.05 were considered to be significant.

Drugs

Neurokinin A (Institut Armand Frappier, Laval, Canada) was dissolved in saline (NaCl 0.9%) to give a stock solution of 13 nM to 13 μM. NKA doses were added in a volume of 100 μl to the bath. Authentic hepoxilins prepared chemically were kindly provided by Prof. E.J. Corey (Harvard University, Cambridge, U.S.A.); HxA_3 and $TrXA_3$ were used as the methyl ester forms and dissolved in DMSO (Calbiochem, Toronto, Canada) and HxA_3-C was dissolved in a mixture of phosphate buffer and ethanol (4:1) (Corey & Su, 1984; 1990). Purity was greater than 95% as judged by thin layer chromatography and high performance liquid chromatography. Hepoxilins were diluted with Krebs solution to a final concentration of DMSO or ethanol not higher than 0.02%.

Results

Potentiation by hepoxilins of the neurokinin A-induced contraction of the trachea

The contraction of the isolated trachea to NKA consisted of slow increase in tension which persisted at a plateau level as long as the peptide was kept in contact with the tissue (Figure 1). The addition of NKA caused concentration-dependent contraction of the guinea-pig trachea (Figure 2). The minimum concentration of NKA needed to evoke a detectable increase in tension was >0.13 nM (Figure 1) and the sensitivity of the tissues as calculated by the EC_{50} varied between 15.9–35.9 nM (Table 1) which were comparable to earlier values obtained on the same preparation (Dion *et al.*, 1987). Tension of the trachea returned to the resting level when the bath fluid was replaced with fresh Krebs buffer. The level of contraction of the trachea obtained by the addition of NKA remained unaltered both in its sensitivity and in its maximal response to NKA in the presence of the vehicle of hepoxilins, DMSO (Table 1). The dose-response curves to NKA were quite reproducible throughout the experiments. A series of experiments was conducted over a period of 4 h where a dose-response curve to NKA was performed every 65 min. The EC_{50} for the 5 curves generated and the maximal contraction to NKA were not significantly different from each other reflecting the stability of the preparation in its response to NKA throughout the experiment (Table 1). Typical variations around the maximal response to NKA within a series of dose-responses on one preparation were 101.1 ± 2.0 ($n = 6$).

Figure 1 shows tracings of cumulative dose-dependent contractions of the trachea to NKA before and after the administration of vehicle (DMSO) and 10^{-8} M concentration of HxA_3 (8S). The 8S isomer of HxA_3 significantly reduced the threshold concentration at which NKA caused a contraction, while the tissue was slightly contracted by the addition of 1.43 nM NKA, it contracted much more when the same dose of NKA (1.43 nM) was added in the presence of HxA_3 (8S) 10^{-8} M (Table 1). It should also be noted that the maximal contraction of the trachea obtained with 366.5 nM NKA reached 114.5 to 121.9% of control in the presence of 10^{-8} to 10^{-6} M (8S) (Table 1). The cumulative dose-response curve to NKA was significantly shifted to the left in the presence of HxA_3 (8S) (Figure 2a) and the sensitivity of the tissues (EC_{50} values) was markedly enhanced (Table 1). EC_{20} values are also shown giving a measure of threshold concentrations of NKA.

The stable trihydroxy metabolite of HxA_3 (8S), i.e. $TrXA_3$ (8S), added at a concentration where HxA_3 (8S) significantly potentiated the contraction of the trachea to NKA (10^{-6} M) was inactive in modifying the NKA response (Table 1). This suggests that the effects of HxA_3 (8S) are not mediated by its trihydroxy metabolite, $TrXA_3$ (8S).

The effects of the glutathionyl conjugate of hepoxilin A_3, HxA_3-C, on the potentiation of NKA-induced contraction of the guinea-pig trachea were more pronounced than those of its precursor HxA_3. Figure 1 compares the effects of an equal dose of both HxA_3 (8S) and HxA_3-C (8R) at 10^{-8} M concentration on the contraction of the trachea exposed to NKA. Note that the contraction of the trachea obtained with 1.43 nM NKA was much larger in the presence of HxA_3-C (8R) ($38.9 \pm 7.5\%$) than it was in the presence of HxA_3 (8S) ($11.3 \pm 3.5\%$). The maximal response to NKA was increased to a greater extent when the trachea was exposed to HxA_3-C (8R) 10^{-8} M ($134.0 \pm 6.2\%$) as compared to HxA_3 (8S) 10^{-8} M ($114.5 \pm 5.3\%$) (Table 1). Higher concentrations (10^{-7} and 10^{-6} M) of hepoxilins A_3-C were also tested and the effects measured were proportionately greater from the preadministration values. The dose-response curve to NKA was shifted further to the left, as reflected by a lower EC_{50} value, when higher concentrations of HxA_3-C (8R) were added (Table 1).

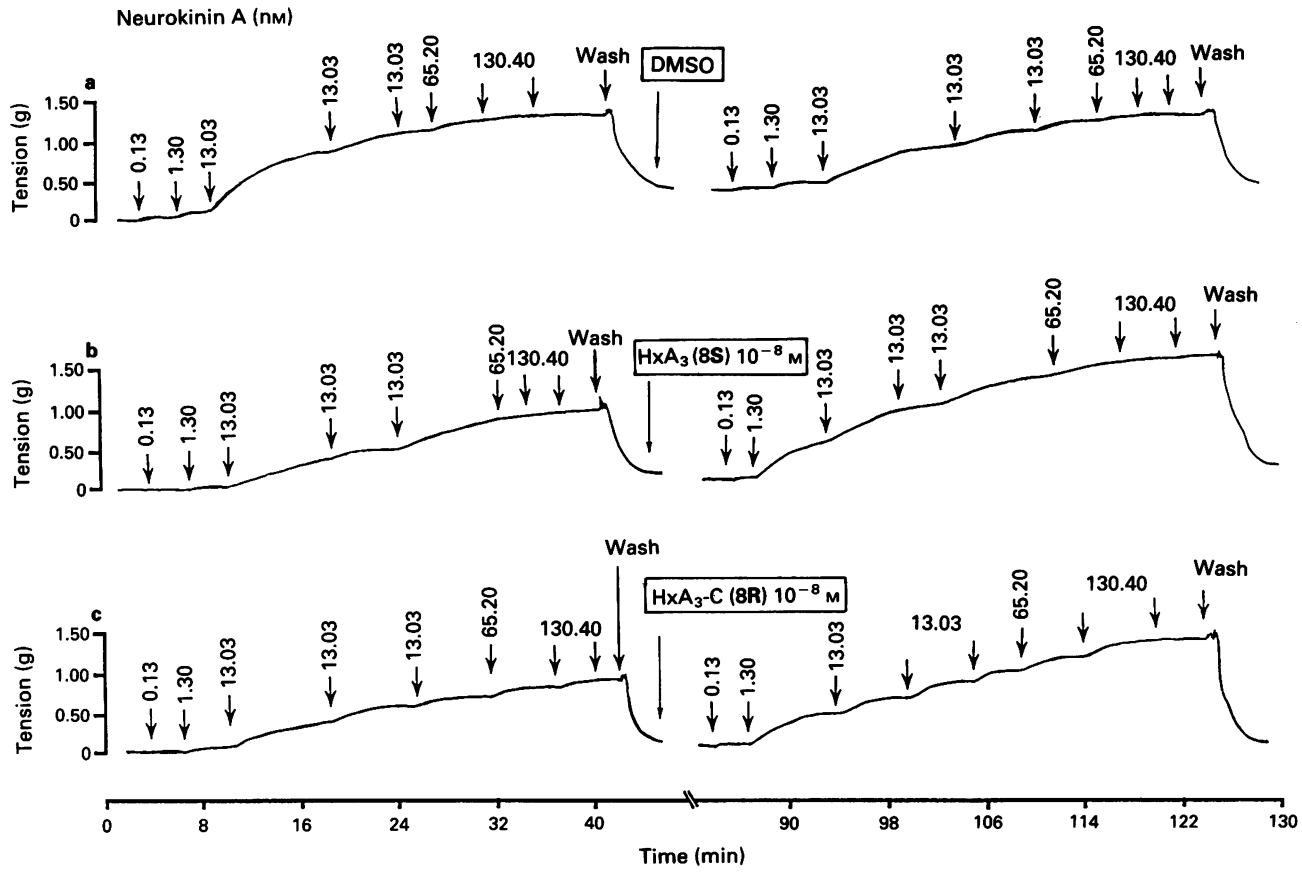

Figure 1 Effect of hepoxilins on contractions of strips of guinea-pig trachea induced by cumulative doses of neurokinin A (NKA). Three successive complete dose-response curves to NKA (0.13 to 366.54 nM) were obtained (the last one is shown here in each panel) followed by tissue washout. Subsequent application was made of dimethylsulphoxide vehicle (a), hepoxilin A_3 (8S) (HxA_3 (8S)) (b) or HxA_3-C (8R) (c) followed 45 min later by another cumulative dose-response curve to NKA. Note that in the presence of HxA_3 (8S) (b) or HxA_3-C (8S) (c), the contraction of the tissue to NKA was enhanced so that significant contraction takes place at 1.43 nM NKA.

Table 1 EC_{20} and EC_{50} values for neurokinin A (NKA) in the presence and absence of dimethylsulphoxide (DMSO, 13 mM) and of the 8-enantiomers of HxA_3, $TrXA_3$ and HxA_3-C in the guinea-pig trachea

Treatment	*EC_{20} (nM)*	*EC_{50} (nM)*	*Maximal response to NKA*
Preadministration			
DMSO after 20 min	8.6 ± 2.6	29.0 ± 2.8	100
after 85 min	10.0 ± 3.7	31.4 ± 3.7	103.5 ± 4.1
after 150 min	9.6 ± 2.8	28.7 ± 3.0	104.2 ± 3.3
after 215 min	9.1 ± 4.8	29.9 ± 2.5	101.6 ± 2.8
Preadministration	9.3 ± 5.2	31.9 ± 5.5	105.4 ± 7.2
HxA_3 (8S) Me 10^{-9} M	7.5 ± 3.0	30.4 ± 4.0	100
10^{-8} M	6.3 ± 3.5	21.8 ± 3.9*	107.7 ± 2.3
10^{-7} M	4.8 ± 2.9	21.7 ± 3.7*	114.5 ± 5.3*
10^{-6} M	3.8 ± 2.7*	14.0 ± 4.9**	120.5 ± 7.6**
Preadministration	4.0 ± 2.2*	13.5 ± 4.6***	121.9 ± 10.5**
HxA_3 (8R) Me 10^{-8} M	8.8 ± 3.4	35.9 ± 2.8	100
10^{-6} M	8.8 ± 2.9	36.1 ± 6.7	94.7 ± 4.3
Preadministration	7.0 ± 3.4	32.6 ± 3.4	93.1 ± 5.0
$TrXA_3$ (8S) Me	6.9 ± 3.5	33.2 ± 5.3	100
10^{-6} M	8.4 ± 4.0	31.1 ± 6.5	105.1 ± 6.4
Preadministration	4.0 ± 2.9	15.9 ± 5.7	100
HxA_3-C (8S) 10^{-6} M	6.5 ± 3.1	20.6 ± 4.8	106.5 ± 10.7
Preadministration	5.0 ± 3.5	26.4 ± 6.3	100
HxA_3-C (8R) 10^{-9} M	10.0 ± 3.6*	21.4 ± 5.0	99.7 ± 6.6
10^{-8} M	3.6 ± 2.0	13.8 ± 3.8**	134.0 ± 6.2***
10^{-7} M	3.0 ± 3.0*	11.4 ± 4.1***	142.7 ± 7.1***
10^{-6} M	2.2 ± 1.8**	6.3 ± 4.8***	170.6 ± 7.6***

HxA_3: hepoxilin A_3; $TrXA_3$: trioxilin A_3
Values represent the mean ± s.e.mean for six separate tissue preparations from six different animals. Each treatment was compared to the preadministration value for each tissue by use of Student's t test for paired samples.
*$P<0.05$; **$P<0.01$ and ***$P<0.005$ with respect to corresponding control values.

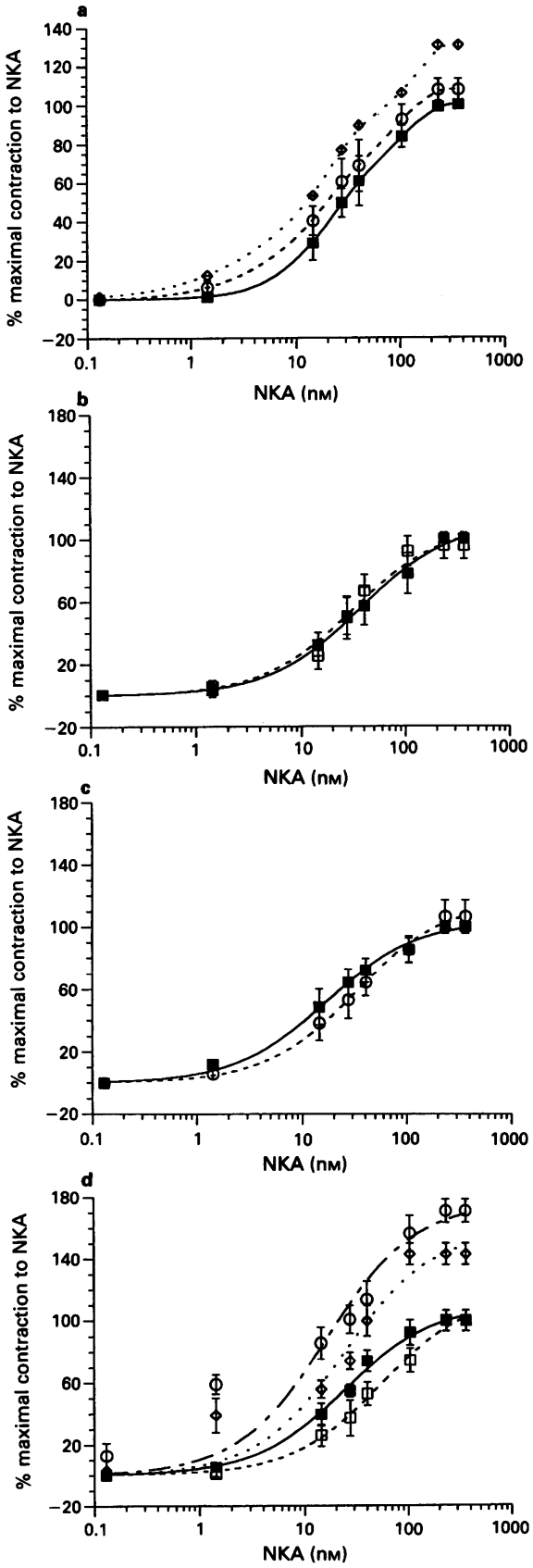

Figure 2 Dose-response curves to neurokinin A (NKA, $n = 6$) in the presence of (a) hepoxilin A_3 (8S) (HxA_3 (8S)), (b) HxA_3 (8R), (c) HxA_3-C (8S), (d) HxA_3-C (8R). Note the leftward shift to NKA in the presence of 10^{-6} M concentration of HxA_3 (8S) or HxA_3-C (8R). Also note the increase of the maximal response to NKA in the presence of HxA_3 (8S) or HxA_3-C (8R). In (a): (◇) HxA_3 (8S) Me, 10^{-7} M; (○) HxA_3 (8S) Me, 10^{-9} M; (■) control (preadministration). In (b): (□) HxA_3 (8R) Me, 10^{-6} M; (■) control (preadministration). In (c): (○) HxA_3-C (8S), 10^{-6} M; (■) control (preadministration). In (d): HxA_3-C (8R), (○) 10^{-6} M; (◇) 10^{-8} M; (□) 10^{-9} M; (■) control (preadministration).

Stereospecificity of the hepoxilin-induced potentiation in the contraction of the trachea to neurokinin A

In contrast to the 8S isomer, the 8R isomer of HxA_3 was essentially inactive in potentiating the NKA-induced contraction of the trachea at a concentration as high as 10^{-6} M (Figure 2a,b, Table 1). Conversely, the 8R isomer of the glutathione conjugate of HxA_3, i.e. HxA_3-C, was active but not the 8S isomer (Figure 2c,d, Table 1).

Discussion

As reported by Dion *et al.* (1987), the tracheal smooth muscle contracted in a concentration-dependent manner to NKA and is a classical pharmacological preparation used to study the NK_2 receptor. In contrast to the human trachea where only the NK_2 receptor is present (Naline *et al.*, 1989), in guinea-pig trachea, contractile responses to tachykinins can be induced by activation of both NK_1 and NK_2 receptors (Devillier *et al.*, 1988; Maggi *et al.*, 1991; Ireland *et al.*, 1991).

From the results described here, we conclude that (1) hepoxilins are not active on their own in changing the muscular tone of the trachea; (2) NKA-induced contraction of the guinea-pig trachea is potentiated by hepoxilins; (3) HxA_3 and HxA_3-C decrease both the EC_{20} and EC_{50} and increase the maximal contraction to NKA; (4) the glutathione conjugate of hepoxilin A_3, HxA_3-C, is more active than its precursor, HxA_3, and finally; (5) hepoxilin-induced potentiation is stereospecific.

We have recently shown that hepoxilins potentiate the contractile effects of noradrenaline on the rat isolated thoracic aorta and portal vein (Laneuville *et al.*, 1992). Although the threshold doses of hepoxilins described here are identical, 10^{-8} M, the following differences were observed. Firstly, the maximal contraction to the constrictor agent was unchanged in the blood vessels while it was significantly increased in the tracheal preparation, and secondly, the potentiation of contractions by hepoxilins in blood vessels showed a narrow dose-response (one log dose) in comparison to the wider response range seen for the trachea. Whether these differences can be explained by the fact that a different preparation of smooth muscles was used or by the fact that a different constrictor agent was used remains to be investigated.

The stereospecificity of action of hepoxilins described here is similar to that previously reported for the vascular preparation, i.e. the 8S isomer of HxA_3 is active while the 8R isomer is inactive and the 8R isomer of HxA_3-C is active while the 8S isomer is inactive (Laneuville *et al.*, 1992). It has been shown in human neutrophils that the hepoxilin-mediated actions on intracellular calcium and second messenger release are inhibited by pertussis toxin, suggesting that hepoxilin action may be under receptor control coupled to G-protein activation (Dho *et al.*, 1990; Nigam *et al.*, 1990).

Hepoxilins are recent messengers identified in cellular communication. The present data suggest that in addition to the 5-lipoxygenase products, leukotrienes, the 12-lipoxygenase pathway may also be important in modulating the control of inflammation and contraction in the trachea through formation of the hepoxilins. However, we do not know whether the hepoxilin effect is general to other bronchoconstrictors or whether the hepoxilins affect only the NKA-induced contraction.

No information is currently available on the metabolism of hepoxilins by the trachea. The present experiments showing stereospecificity of action of the hepoxilins suggest that the hepoxilins are either stable during the 45 min exposure of the tissue or that the hepoxilins induced a time-dependent cellular change which facilitates contraction by NKA. The fact that considerable time (longer than 30 min) is required to elicit a potentiation of the NKA-induced contractile response

suggests that hepoxilins may have an effect on protein synthesis. Further studies are needed to define the mechanism involved. An overproduction of hepoxilins may sensitize airways to sensory neurotransmitters (e.g. SP and NKA) and other bronchoconstrictors. Thus, elucidation of this mechanism may provide new insight in the control of airway function and into the pathophysiology of asthma and bronchial hyperreactivity.

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Electrophysiological effects of diprafenone, a dimethyl congener of propafenone on guinea-pig ventricular cells

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1 The effects of diprafenone and propafenone on transmembrane action potential were examined and compared in papillary muscles and single ventricular myocytes isolated from guinea-pig hearts.

2 In papillary muscles, both diprafenone and propafenone $\geq 10^{-6}$ M caused a significant and dose-dependent decrease in the maximum upstroke velocity ($\dot{V}_{max}$) of the action potential.

3 In the presence of either drug, trains of stimuli at rates ≥ 0.1 Hz led to an exponential decline in $\dot{V}_{max}$. A time constant (τR) for $\dot{V}_{max}$ recovery from the use-dependent block was 15.5 s for diprafenone and 8.8 s for propafenone.

4 The use-dependent block of $\dot{V}_{max}$ with diprafenone was enhanced when the resting potential was depolarized by high (8, 10 mM) $[K^+]_o$, whereas that with propafenone was virtually unchanged. τR with diprafenone was shortened by the depolarization, while that with propafenone was rather prolonged.

5 In single myocytes perfused with diprafenone or propafenone, 10 ms conditioning clamp to 0 mV caused a significant decrease in $\dot{V}_{max}$ of subsequent action potential. A prolongation of the clamp pulse duration resulted in a modest enhancement of the $\dot{V}_{max}$ inhibition with diprafenone, while a large enhancement of the $\dot{V}_{max}$ inhibition occurred with propafenone.

6 These findings suggest that diprafenone, like propafenone, may block the sodium channel during both the activated and inactivated states. The relative contribution of inactivation block is less important for diprafenone than for propafenone. The different voltage-dependence of use-dependent block with diprafenone from propafenone would contribute to its high antiarrhythmic potency.

Keywords: Diprafenone; propafenone; ventricular cells; action potential; $\dot{V}_{max}$; sodium channel

Introduction

Diprafenone is a relatively new antiarrhythmic agent having a chemical structure close to propafenone (Gülker *et al.*, 1985). *In vivo* studies in dogs have demonstrated that this compound slows conduction through all the parts of the atrio-ventricular conducting system, prolongs the atrial and ventricular refractory periods, and prevents tachyarrhythmias induced by trains of stimuli, acute ischaemia or delayed reperfusion (Gülker *et al.*, 1985; Thale *et al.*, 1987). The antiarrhythmic potency of diprafenone in these animal models was appreciably higher than propafenone. In several clinical reports a higher therapeutic potential for diprafenone than for propafenone against supraventricular and ventricular tachyarrhythmias has been observed (Manz *et al.*, 1986; Gülker *et al.*, 1987; Heuer *et al.*, 1987; Geibel *et al.*, 1988).

In vitro experiments in guinea-pig ventricular muscles have shown that diprafenone reduces the maximum upstroke velocity ($\dot{V}_{max}$) of the action potential without affecting the resting membrane potential, and that the $\dot{V}_{max}$ inhibition is enhanced in a frequency-dependent manner (Kohlhardt & Seifert, 1983; Kohlhardt & Fichtner 1988a). Diprafenone was also shown to have β-adrenoceptor blocking activity, which was several times higher than propafenone (Greenberg *et al.*, 1989; Groschner *et al.*, 1991). These findings suggest that diprafenone, like propafenone, possesses Class-I as well as Class-II antiarrhythmic actions (Vaughan Williams, 1984).

Recently, direct evidence of sodium channel block by diprafenone has been presented in experiments using single channel recording (Kohlhardt & Fichtner, 1988a,b; Kohlhardt *et al.*, 1989). However, much remains to be elucidated as to the precise mode of action of diprafenone on cardiac sodium channels in relation to its antiarrhythmic activity. In the present study, the effects of diprafenone and propafenone on the transmembrane action potential were investigated and compared in right ventricular papillary muscles, as well as in single ventricular myocytes isolated from guinea-pig hearts. The modulation of drug-induced $\dot{V}_{max}$ inhibition by stimulation frequencies and by membrane potential was studied in detail so as to compare their sodium channel blocking characteristics.

Methods

Papillary muscles

Guinea-pigs of either sex weighing 200 to 250 g were killed by a blow on the head and hearts were quickly removed. Papillary muscles 2 to 3 mm in length and 0.3 to 0.4 mm in diameter were dissected from the right ventricle. The preparation was mounted in the tissue bath and superfused continuously with Krebs-Ringer solution kept at 34°C and gassed with 95% O_2 and 5% CO_2. The composition of the solution was as follows (in mM): NaCl 120.3, KCl 4.0, $CaCl_2$ 1.2, $MgSO_4$ 1.3, $NaHCO_3$ 25.2 and glucose 5.5 (pH 7.4). The preparations were stimulated by a pair of 1.0 mm platinum wire electrodes placed 1.0 mm apart of either side of the muscles. Pulses used for stimulation were 0.5–1.0 ms in duration and 20% higher than the diastolic threshold in intensity unless otherwise specified. Equipment for recording transmembrane potential was the same as described previously by Kodama *et al.* (1985) and Toyama *et al.* (1987).

To study the use-dependent effects of diprafenone and propafenone on the maximum upstroke velocity ($\dot{V}_{max}$) of action potentials, the preparation was stimulated repetitively at varying rates ranging from 0.1 to 2.0 Hz. Rest periods of 90 s, which were sufficient to ensure full recovery from the rate-dependent decrease in $\dot{V}_{max}$, were interposed between the

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trains of stimuli. Single cell impalements of the microelectrodes were maintained throughout each experiment. This experimental protocol is able to detect the existence of two types of drug-induced $\dot{V}_{max}$ inhibition, tonic and use-dependent block. The former is defined by the decrease in $\dot{V}_{max}$ of the first action potential preceded by the rest period, and the latter in the decrease of $\dot{V}_{max}$ during the train (from the value of the first action potential to the new steady-state level).

The recovery of $\dot{V}_{max}$ from the use-dependent block was studied by applying a single test stimulus at various coupling intervals following a stimulation train for 60 s at 1.0 Hz. The intensity of the test stimulus was adjusted to obtain a constant latency from the stimulus artifact to the initiation of the action potential upstroke.

In experiments to examine the influence of resting membrane potential on the tonic and use-dependent $\dot{V}_{max}$ inhibition by the drugs, the K^+ concentration in the superfusate was elevated from 4.0 up to 8.0 and to 10.0 mM by adding KCl.

Single ventricular myocytes

Single ventricular myocytes were isolated enzymatically from guinea-pig hearts by the same procedure as described in our previous study (Kodama *et al.*, 1990). A few drops of cell suspension was placed in a recording chamber attached to an inverted microscope. The chamber was perfused at a rate of 2 ml min^{-1} with normal Krebs solution of the following composition (mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, HEPES 5.0 and glucose 5.0; pH was adjusted to 7.4 by adding NaOH, and the solution was equilibrated with 100% O_2. The temperature was maintained at 35°C.

Following the increase in calcium concentration of the medium to 1.8 mM (normal Krebs solution), 30 to 40% of myocytes deteriorated into round-shaped cells due to irreversible contracture. The remaining cells were tolerant to calcium; their intact rod-shape was maintained without spontaneous beating, and the experiments were carried out on these myocytes.

The single-pipette, whole cell clamp method was employed to control and record membrane potential using single cell/single channel amplifier (List-Medical, L/M-EPC7). The pipettes were heat polished and filled with internal solution that gave a resistance ranging from 2 to 3 Mohm. The pipette solution consisted of (mM): KCl 120.0, NaH_2PO_4 10.0, EGTA 1.0, MgATP 5.0 and HEPES 10.0; pH was adjusted to 7.2 by adding KOH. Action potentials were recorded by the current-clamp mode by passing a short stimulus current (2 to 4 ms in duration) through the electrodes. Transition from the voltage-clamp mode to the current-clamp mode was regulated by a pulse generator through an electronic relay. Details of the experimental protocols are given in the Results section.

Drugs and data analysis

Diprafenone HCl and propafenone HCl were kindly donated by Yamanouchi Pharmaceutical Co. Ltd. (Tokyo, Japan). These compounds were dissolved in deionized water and diluted with Krebs solution to achieve the final concentration required. Values are presented as means or means ± s.e.mean unless otherwise stated. Data were analysed by *t* test, analysis of variance, Dunnett's test and regression analysis. Differences were considered significant at $P<0.05$.

Results

Action potentials of papillary muscle

Effects of diprafenone (3×10^{-7} M to 10^{-5} M) and propafenone (3×10^{-7} M to 10^{-5} M) on the action potential configuration were examined in each of six papillary muscles constantly stimulated at 1.0 Hz (Figure 1, Table 1).

After exposure to diprafenone, 3×10^{-7} M for 40 min, action potential duration at 80% repolarization (APD_{80}) was slightly prolonged with no significant change in other parameters. $\dot{V}_{max}$ was decreased significantly at 10^{-6} M. At higher concentrations of diprafenone (3×10^{-6} M, 10^{-5} M) APD at 30% and 80% repolarization were shortened in association with a further reduction of $\dot{V}_{max}$. Resting potential (RP) was unaffected even at 10^{-5} M.

Qualitatively, similar results were obtained with propafenone. Thus, it caused a concentration-dependent decrease in $\dot{V}_{max}$ at above 10^{-6} M without affecting RP. APD_{30} and APD_{80} tended to be prolonged at the lower concentrations (3×10^{-7} M, 10^{-6} M), but the changes were statistically insignificant. At the higher concentrations of propafenone, both APD_{30} and APD_{80} were shortened. IC_{20} of $\dot{V}_{max}$ inhibition induced by diprafenone and propafenone, which was obtained by interpolation on a graph of log molar drug concentration versus response, was 3.1×10^{-6} M and 1.6×10^{-6} M respectively.

Use-dependent effects on $\dot{V}_{max}$

The effects of diprafenone and propafenone on $\dot{V}_{max}$ were examined with stimulation trains at different rates separated from each other by 90 s rest period. In control preparations,

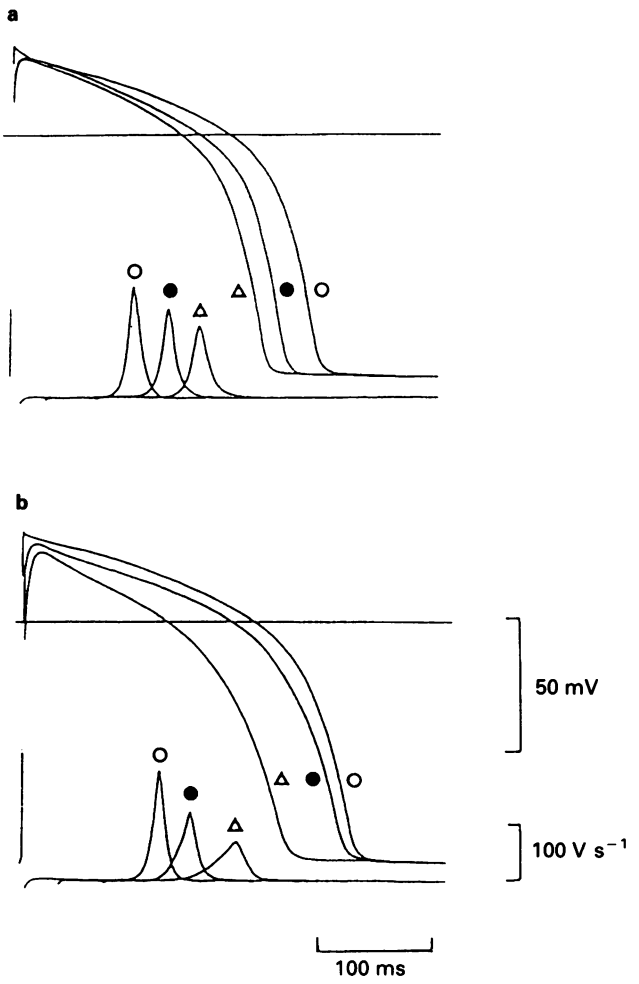

Figure 1 Effects of diprafenone (a) and propafenone (b) on transmembrane action potentials of papillary muscles. Action potentials and their differentiated upstroke spikes were recorded before (○) and 40 min after application of diprafenone or propafenone at 3×10^{-6} M (●) and 10^{-5} M (△). The preparation was constantly stimulated at 1.0 Hz.

the value of $\dot{V}_{max}$ was almost unchanged with stimulation trains at rates from 0.1 to 2.0 Hz. After treatment with diprafenone or propafenone, $\dot{V}_{max}$ of the first action potential in each train was decreased indicating a minimal or modest tonic block (Table 2). Further decline of $\dot{V}_{max}$ during the repetitive activity (use-dependent block) was dependent on the stimulation frequency; the higher the frequency, the greater the block (Figure 2).

Table 1 Effects of diprafenone and propafenone on action potential characteristics

	RP (mV)	$\dot{V}_{max}$ (Vs^{-1})	APD_{30} (ms)	APD_{80} (ms)
Control ($n = 6$)	− 92.2 ± 1.2	218 ± 15	198 ± 16	250 ± 13
Diprafenone				
3×10^{-7} M	− 93.1 ± 1.3	214 ± 20	206 ± 12	263 ± 9*
10^{-6} M	− 92.5 ± 0.9	204 ± 13*	194 ± 16	247 ± 14
3×10^{-6} M	− 92.0 ± 1.1	174 ± 15*	184 ± 10*	240 ± 8*
10^{-5} M	− 91.8 ± 1.1	109 ± 19*	141 ± 12*	225 ± 11*
Control ($n = 6$)	− 93.4 ± 1.8	211 ± 11	202 ± 15	262 ± 18
Propafenone				
3×10^{-7} M	− 92.8 ± 1.1	215 ± 14	207 ± 16	270 ± 21
10^{-6} M	− 92.5 ± 1.0	184 ± 10*	206 ± 11	268 ± 13
3×10^{-6} M	− 92.1 ± 0.8	138 ± 10*	191 ± 8*	263 ± 13
10^{-5} M	− 92.8 ± 1.5	87 ± 16*	153 ± 11*	227 ± 14*

Values are means ± s.e.mean of six experiments for each drug. The preparations were constantly stimulated at 1.0 Hz. Data were obtained before (control) and 40 min after application of diprafenone or propafenone at a given concentration. RP: resting membrane potential; $\dot{V}_{max}$: maximum upstroke velocity of action potential; APD_{30} and APD_{80}: action potential duration from the upstroke to 30% and 80% repolarization.
*Significantly different from control at $P < 0.05$.

Table 2 Tonic and use-dependent block of $\dot{V}_{max}$ by diprafenone and propafenone

	n	*Tonic block* (%)	*Onset rate of use-dependent block* 1.0 Hz (AP^{-1})	2.0 Hz (AP^{-1})
Diprafenone				
3×10^{-6} M	(5)	8.1 ± 2.5*	0.09 ± 0.03	0.06 ± 0.01#
10^{-5} M	(5)	15.5 ± 0.9*	0.13 ± 0.01#	0.10 ± 0.01#
Propafenone				
3×10^{-6} M	(5)	5.9 ± 2.0	0.14 ± 0.01	0.12 ± 0.01
10^{-5} M	(5)	10.9 ± 0.4*	0.35 ± 0.01	0.31 ± 0.01

Values are means ± s.e.mean. n = number of preparations. AP^{-1}: per action potential.
*The value was statistically significant ($P < 0.05$); #significantly different from propafenone at the same concentration ($P < 0.05$).

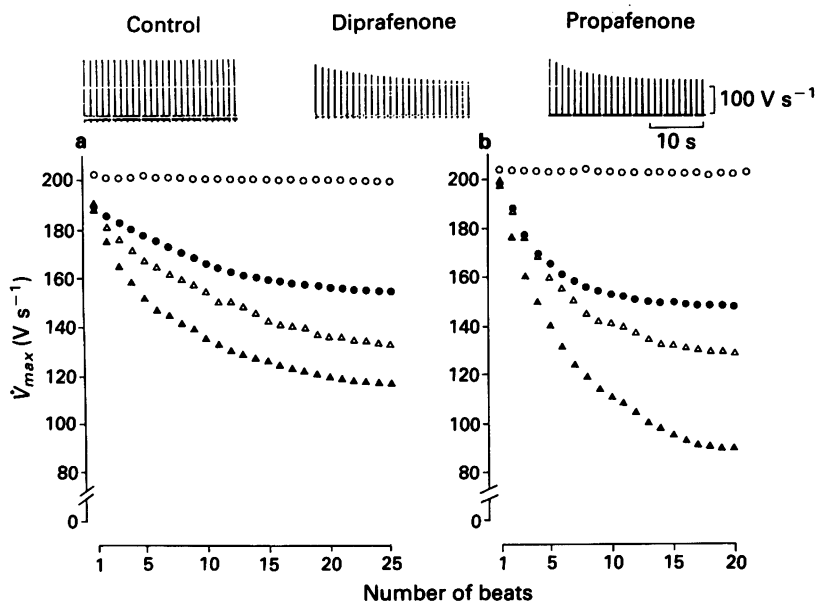

Figure 2 Rate-dependent decrease of the maximum upstroke velocity ($\dot{V}_{max}$). Upper panels: differentiated upstroke spikes of action potentials during stimulation trains at 1.0 Hz after 90 s rest. The records were obtained before, and 40 min after application of diprafenone (3×10^{-6} M) or propafenone (3×10^{-6} M). Lower graphs: beat-to-beat change in $\dot{V}_{max}$ at onset of stimulation trains. Ordinate scales: $\dot{V}_{max}$. Abscissa scales: number of beats (action potentials) from initiation of stimulation train. Frequencies of stimulation were 1.0 Hz under control conditions (○), and 0.5 Hz (●), 1.0 Hz (△) and 2.0 Hz (▲) in the presence of (a) diprafenone (3×10^{-6} M) or (b) propafenone (3×10^{-6} M).

Figure 3 summarizes the percentage decrease of $\dot{V}_{max}$ from the first action potential to the new steady-state level, which was attained at around the 25th to 30th action potential for diprafenone and 15th to 20th action potentials for propafenone. The use-dependent block was larger at higher drug concentrations.

The beat-to-beat decline of $\dot{V}_{max}$ fitted a single exponential curve well (Figure 2), so that the onset rate per action potential (AP^{-1}) at which $\dot{V}_{max}$ fell to the new steady-state level could be calculated in each experiment (Table 2). The rate of onset with diprafenone was slower than that with propafenone when compared at the same drug concentration and at the same stimulation frequency (Table 2).

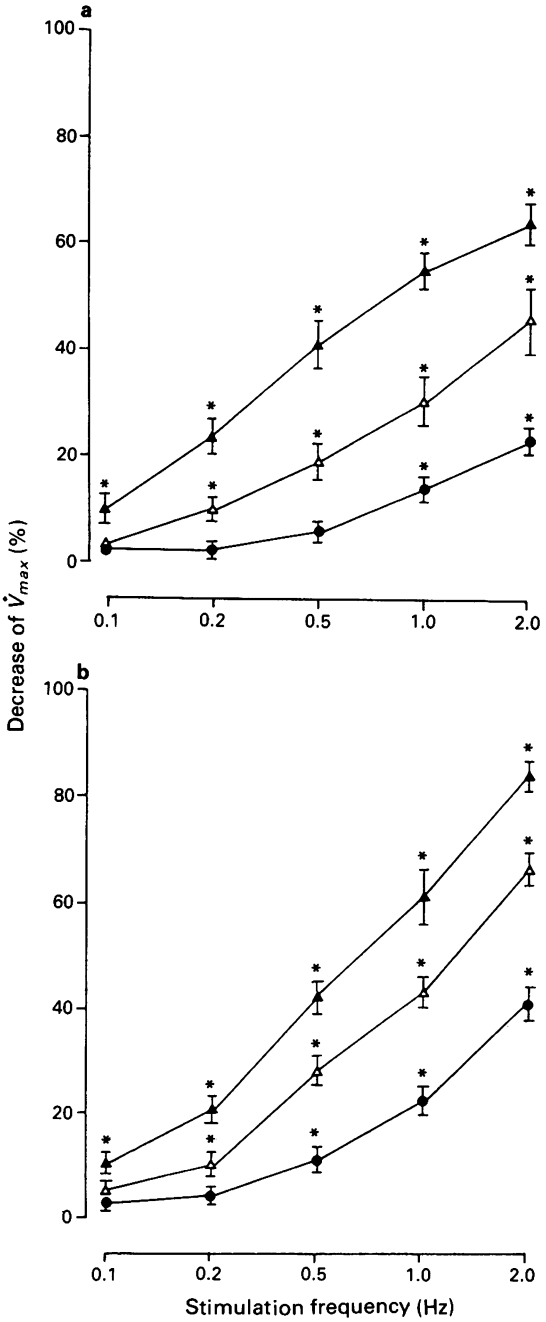

Figure 3 Relationship between stimulation frequency and intensity of the use-dependent block. Ordinate scales: % decrease of maximum upstroke velocity of action potential ($\dot{V}_{max}$) from first action potential of stimulation trains to new steady-state level. Abscissa scales: stimulation frequency. Data were obtained 40 min after application of (a) diprafenone or (b) propafenone at 10^{-6} M (●), 3×10^{-6} M (△) and 10^{-5} M (▲). Values are means and vertical lines show s.e.mean of the five preparations. *Change was statistically significant from control at $P<0.05$.

The recovery of $\dot{V}_{max}$ from the use-dependent block was studied by applying a single test stimulus at various coupling interval following a stimulation train for 60 s at 1.0 Hz. Before the application of the drugs, the $\dot{V}_{max}$ of the test action potential recovered almost completely within 100 ms of the diastolic interval (the interval from the end of the last action potential to the beginning of the test action potential). After treatment with diprafenone (3×10^{-6} M) or with propafenone (3×10^{-6} M), much slower $\dot{V}_{max}$ recovery was observed. Representative results are shown in Figure 4, where fractional reduction of $\dot{V}_{max}$ of test action potentials were plotted against the diastolic interval in a semilogarithmic graph. In the presence of diprafenone, the recovery time course of $\dot{V}_{max}$ with a diastolic interval longer than 100 ms was approximated by a single exponential function. The average time constant (τR) was 15.5 ± 0.9 s ($n = 5$). Similar exponential recovery of $\dot{V}_{max}$ was also observed in papillary muscles treated with propafenone at τR of 8.8 ± 0.2 s ($n = 5$).

Influence of resting membrane potential on the tonic and use-dependent $\dot{V}_{max}$ inhibition

Tonic and use-dependent block of $\dot{V}_{max}$ by diprafenone and propafenone were also examined in partially depolarized papillary muscles under high extracellular K^+ concentrations. In the medium with 8 and 10 mM K^+, RP of the preparation was decreased to -78.2 ± 0.4 mV ($n = 5$) and -69.6 ± 0.8 mV ($n = 5$), respectively. In such preparations, diprafenone (3×10^{-6} M) caused a decrease in $\dot{V}_{max}$ of the first action potential following a long (90 s) rest period by $16.0 \pm 3.6\%$ ($n = 5$) with 8 mM $[K^+]_o$, and $29.1 \pm 5.8\%$ ($n = 5$) with 10 mM $[K^+]_o$. RP was unaffected by the drug treatment. Thus, the tonic block of $\dot{V}_{max}$ by diprafenone was augmented in partially depolarized preparations (Figure 5a). The tonic block of $\dot{V}_{max}$ with propafenone (3×10^{-6} M) was augmented more remarkably by similar decrease of RP under high $[K^+]_o$ (Figure 5a).

The intensity of use-dependent block with diprafenone (3×10^{-6} M) during the stimulation train at 1.0 Hz was increased significantly from $37.3 \pm 3.1\%$ at 4 mM $[K^+]_o$ to $57.6 \pm 3.6\%$ at 8 mM $[K^+]_o$ and to $67.1 \pm 5.4\%$ at 10 mM

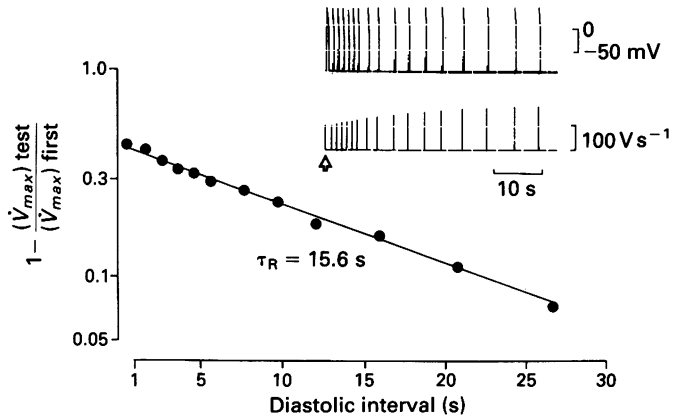

Figure 4 Offset of the use-dependent block of $\dot{V}_{max}$ by diprafenone in papillary muscles. Inset shows records of action potentials (upper trace) and their differentiated upstroke spikes (lower trace) 40 min after application of diprafenone (3×10^{-6} M). The preparation was stimulated at 1.0 Hz for 60 s following a rest period of 90 s. An arrow indicates the last action potential by the stimulation train. After cessation of the train, a single test stimulus was applied with various coupling intervals. The graph indicates recovery process of $\dot{V}_{max}$ of test action potential. Ordinate scale: fractional $\dot{V}_{max}$ reduction of the test action potential as compared with $\dot{V}_{max}$ of the first action potential by the train. Abscissa scale: diastolic interval, which was measured from the end (at 95% repolarization) of the last action potential induced by the stimulation train to the upstroke of the test action potential. The time course was approximated by single exponential function with a time constant (τR) of 15.6 s.

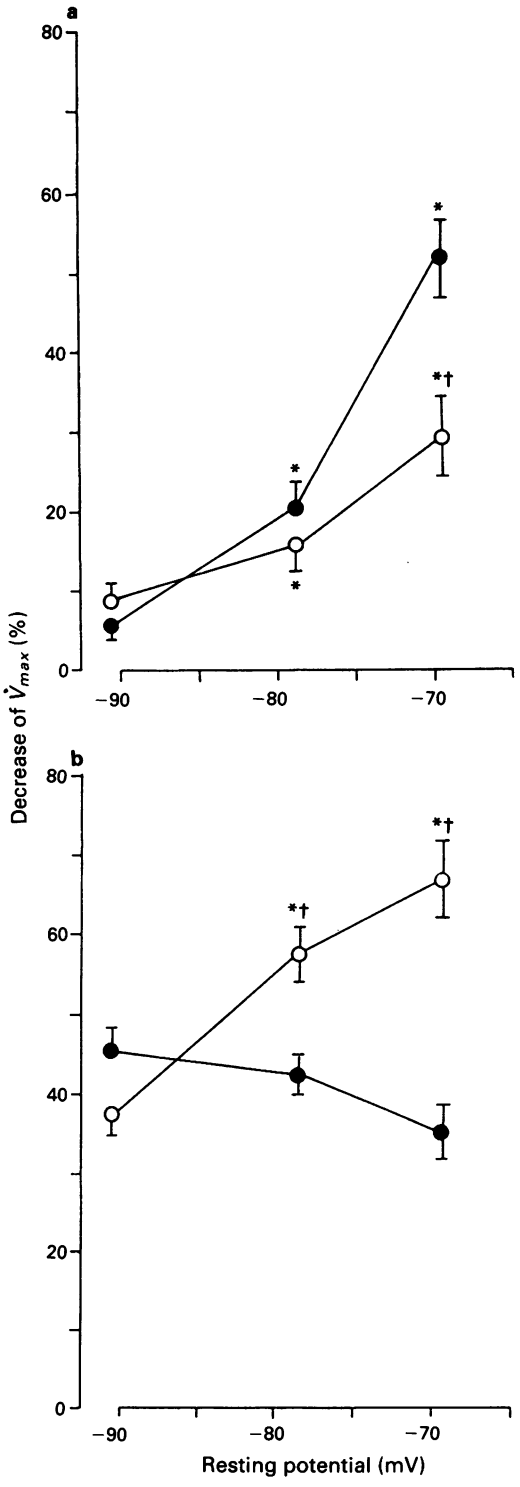

Figure 5 Membrane potential dependence of tonic block (a) and use-dependent block (b). (a) Ordinate scale indicates percentage decrease of $\dot{V}_{max}$ of first action potential following a long (90 s) rest period after application of diprafenone (3×10^{-6} M, ○) or propafenone (3×10^{-6} M, ●) with 4, 8 and 10 mM $[K^+]_o$. Reference level is the $\dot{V}_{max}$ before the drug application with the respective $[K^+]_o$. Abscissa scale shows resting membrane potential. (b) Ordinate scale indicates percentage decrease of $\dot{V}_{max}$ from the first action potential to the new steady-state level during the stimulation train at 1.0 Hz in the presence of diprafenone (3×10^{-6} M, ○) or propafenone (3×10^{-6} M, ●) with 4, 8 and 10 mM $[K^+]_o$. Abscissa scale shows resting membrane potential. In both graphs, values are presented as means ± s.e.mean of five preparations. *Significantly different from the value with 4 mM $[K^+]_o$ at $P<0.05$. †Significantly different from the value for propafenone with the same $[K^+]_o$ at $P<0.05$.

$[K^+]_o$ (Figure 5b). In constrast, there was no significant difference in the intensity of use-dependent block with propafenone (3×10^{-6} M) among the three different $[K^+]_o$. The use-dependent block with diprafenone (3×10^{-6} M) at a resting membrane potential more negative than − 80 mV tended to be less than that with propafenone (3×10^{-6} M). However, the relationship was reversed at a level more positive than − 80 mV; the use-dependent block with diprafenone was significantly greater than that with propafenone.

The recovery kinetics from the use-dependent block with diprafenone (3×10^{-6} M) and propafenone (3×10^{-6} M) showed the opposite change in the higher K^+ media. The recovery time constant (τR) for diprafenone was shortened, while the value for propafenone was lengthened at the less negative RP under the higher $[K^+]_o$ (Figure 6). Consequently, τR for diprafenone, which was significantly longer than propafenone at normal (4 mM) $[K^+]_o$, became significantly shorter than propafenone at the highest (10 mM) $[K^+]_o$.

$\dot{V}_{max}$ *of single ventricular myocytes*

In single ventricular myocytes, the effects of a conditioning clamp pulse on $\dot{V}_{max}$ of the subsequent test action potential were examined in order to determine whether the use-dependence of $\dot{V}_{max}$ inhibition by diprafenone and propafenone is due to the blockade of an activated or an inactivated sodium channel. The baseline characteristics of action potential elicited in the cell at a long interstimulus interval (90 s) were as follows: RP, − 82.6 ± 0.3 mV; $\dot{V}_{max}$, 446 ± 15 Vs^{-1}; APD_{80}, 172 ± 16 ms ($n = 8$). There was no significant difference in any of these parameters between the subgroups of cells exposed to diprafenone and propafenone.

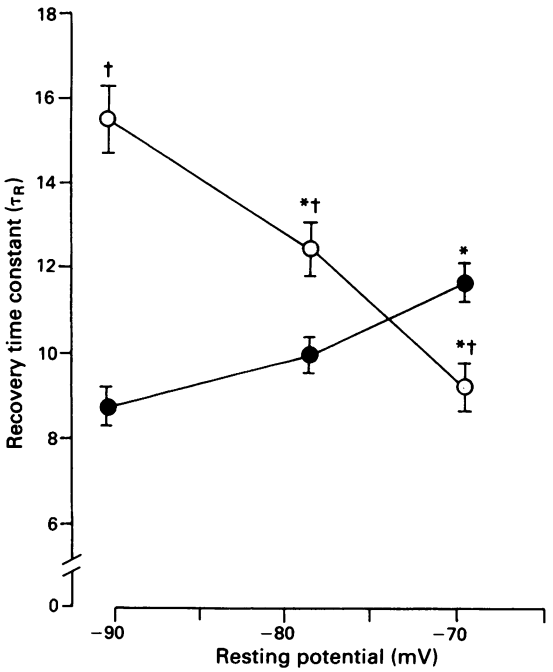

Figure 6 Influence of resting membrane potential on $\dot{V}_{max}$ recovery from the use-dependent block. Ordinate scale: time constant of $\dot{V}_{max}$ recovery from the use-dependent block induced by trains of stimulation at 1.0 Hz with 4, 8 and 10 mM $[K^+]_o$. Abscissa scale: resting membrane potential of the preparation. Data were obtained 40 to 60 min after application of diprafenone (3×10^{-6} M, ○) or propafenone (3×10^{-6} M, ●). Values are means and vertical lines show s.e.mean of the five preparations. *Significantly different from the value with 4 mM $[K^+]_o$ at $P<0.05$. †Significantly different from the value for propafenone with the same $[K^+]_o$ at $P<0.05$.

Representative experiments are shown in Figure 7. Following a rest period of 90 s, the membrane potential was clamped up from the resting level (holding potential at −83 mV) to 0 mV for 10 to 2,000 ms. At the end of the conditioning clamp pulse, the membrane potential was clamped back to the holding voltage for 100 ms, which is long enough for a drug-free channel to reactivate fully (Carmeliet & Vereecke, 1979; Ebihara & Johnson, 1980), but short enough so that only partial dissociation of the drug from the blocked channel occurs (Grant et al., 1984). The voltage-clamp was then released, and a stimulus was applied to elicit a test action potential.

In untreated control myocytes, such a clamp pulse with a duration less than 500 ms had no significant effect on the $\dot{V}_{max}$ of the test action potential. However, further prolongation of the clamp pulse duration resulted in a slight but significant $\dot{V}_{max}$ reduction probably due to slow inactivation of sodium channels. A clamp pulse of 2,000 ms in duration decreased $\dot{V}_{max}$ by 13.0 ± 2.2% ($n = 4$) from the value of the action potential without a conditioning clamp pulse (reference level).

Treatment of the myocytes with diprafenone (10^{-5} M) or with propafenone (10^{-5} M) for 10 to 20 min did not affect the RP. $\dot{V}_{max}$ of the reference action potential was decreased by 9.0 ± 2.2% with diprafenone ($n = 4$, $P < 0.05$) and by 8.1 ± 1.5% with propafenone ($n = 4$, $P < 0.05$) from the values before the drug application. In such myocytes, a conditioning clamp pulse caused a greater $\dot{V}_{max}$ reduction of test action potential. In the presence of diprafenone, the shortest clamp pulse (10 ms) decreased $\dot{V}_{max}$ by 10.1 ± 0.6% ($n = 4$) from the reference level. When the clamp pulse duration was prolonged, the $\dot{V}_{max}$ reduction was enhanced, and reached 32.3 ± 2.1% ($n = 4$) at 2,000 ms. In the myocytes treated with propafenone, the shortest (10 ms) clamp pulse caused $\dot{V}_{max}$ reduction by 6.8 ± 0.3% ($n = 4$). The values were significantly less than those for diprafenone. However, further prolongation of the clamp pulse duration resulted in a greater $\dot{V}_{max}$ reduction, and it reached 43.1 ± 4.9% ($n = 4$) at 2,000 ms. The values were significantly larger than those for diprafenone.

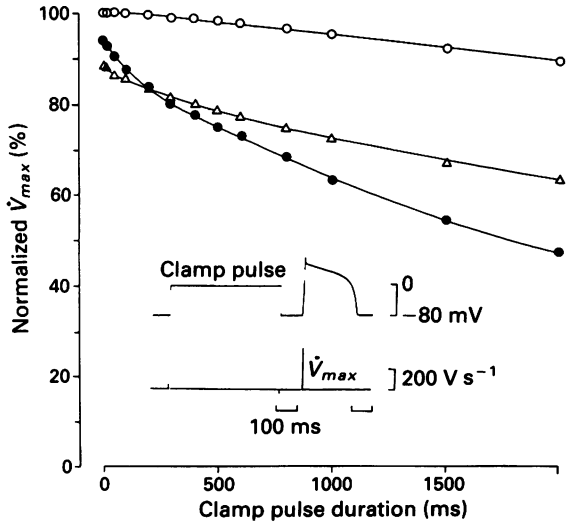

Figure 7 Effects of 0 mV conditioning clamp pulse on the $\dot{V}_{max}$ inhibition induced by diprafenone and propafenone. Ordinate scale: $\dot{V}_{max}$ of test action potential normalized by the value of action potential without clamp pulse (referenced at the tonic block subtracted value). Abscissa scale: duration of the conditioning 0 mV clamp. Data were obtained before (○) and 10 to 20 min after application of diprafenone 10^{-5} M (△) or propafenone 10^{-5} M (●).

Discussion

The present results have revealed that both diprafenone and propafenone above 10^{-6} M cause a concentration-dependent decrease in $\dot{V}_{max}$ of papillary muscles. APD was prolonged a little at lower concentrations around 3×10^{-7} M–10^{-6} M, while shortened significantly at the higher concentrations. The data for propafenone are more or less in agreement with those obtained by previous investigators in papillary muscle experiments (Kohlhardt & Seifert, 1980; 1983; 1985) and in single ventricular cell experiments (Honjo et al., 1989) of guinea-pigs. Kohlhardt & Fichtner (1988a) also showed that diprafenone (10^{-5} M) caused $\dot{V}_{max}$ inhibition and APD shortening in guinea-pig papillary muscles.

Since the entire tissue was excited simultaneously and there was no conduction within the preparation under the present experimental conditions, the decrease in $\dot{V}_{max}$ by diprafenone or propafenone without any accompanying change in RP reflects an inhibitory effect of these drugs on the fast sodium inward current, I_{Na} (Gintant et al., 1983; Grant et al., 1984). The probable convex-shaped non-linear relationship between $\dot{V}_{max}$ and the peak sodium inward current (I_{Na}) in cardiac cells (Cohen et al., 1984; Sheets et al., 1988) might introduce variable errors in estimating the precise amount of sodium channel block. Nevertheless, such a limitation does not invalidate $\dot{V}_{max}$ as a qualitative index, since voltage-clamp techniques currently available for I_{Na} measurement require more artificial experimental conditions (low temperature and low extracellular sodium concentration) than those for $\dot{V}_{max}$ measurement.

The potency of $\dot{V}_{max}$ inhibition by diprafenone in guinea-pig papillary muscles constantly driven at 1.0 Hz ($IC_{20} = 3.1 \times 10^{-6}$ M) is slightly lower than that of propafenone ($IC_{20} = 1.6 \times 10^{-6}$ M), but still higher than those for quinidine and disopyramide (Campbell, 1983a).

The inhibition of $\dot{V}_{max}$ by diprafenone was, like propafenone, enhanced at the higher stimulation frequency. Thus, in normally polarized papillary muscles, the two drugs at concentrations ranging from 10^{-6} to 10^{-5} M caused minimal or modest tonic $\dot{V}_{max}$ inhibition despite their marked use-dependent $\dot{V}_{max}$ inhibition. Such frequency-dependent or use-dependent $\dot{V}_{max}$ inhibition can be interpreted within the framework of the 'modulated receptor' hypothesis (Hondeghem & Katzung, 1977) or 'guarded receptor' hypothesis (Starmer et al., 1984) to explain the interaction between local anaesthetic type (Class I) antiarrhythmic drugs and cardiac sodium channels. According to these hypotheses, the reduction of I_{Na} is due to the accumulation of drug-associated non-conducting channels (blocked channels). Both diprafenone and propafenone, like most other Class-I antiarrhythmic drugs, may bind to the sodium channel receptors mainly during activated and/or inactivated states. This characteristic would lead to an accumulation of blocked channels during stimulation trains above certain rates.

The use-dependent block of $\dot{V}_{max}$ by diprafenone and propafenone was observed during stimulation trains at rates $\geqslant 0.1$ Hz. The present data for the onset rate of use-dependent block with propafenone (Table 2) is comparable to those in previous reports by Kohlhardt & Seifert (1985) in guinea-pig papillary muscles (0.25 AP^{-1} at 10^{-6} M, 1.0 Hz) and by Honjo et al. (1989) in single guinea-pig ventricular cells (0.33 AP^{-1} at 3×10^{-6} M, 1.0 Hz). Our experiments have revealed that the onset kinetics of use-dependent block by diprafenone are significantly slower than propafenone (Table 2).

The offset kinetics of use-dependent block by diprafenone were also very slow; the average time constant of $\dot{V}_{max}$ recovery (τR) was 15.5 s. The τR value is appreciably longer than those for propafenone in the present experiments (8.8 s) as well as in previous studies by Kohlhardt & Seifert (1985) (4.5 s) and by Honjo et al. (1989) (4.8 s). These facts may indicate that diprafenone belongs to slow kinetic Class-I drugs like flecainide, encainide and lorcainide (Campbell, 1980; 1983a,b).

Tonic block of $\dot{V}_{max}$ with propafenone was more remarkable at less negative membrane potentials. This agrees with a previous report by Kohlhardt & Seifert (1980) and may suggest substantial binding of the drug to the inactivated state of sodium channels (Honjo *et al.*, 1989). The tonic block with diprafenone was also enhanced by a decrease in RP, but the voltage-dependence was appreciably less than that with propafenone (Figure 5a), suggesting less binding of diprafenone to the inactivated state of channels.

Such different state-dependence of sodium channel block by diprafenone and propafenone was also suggested from the present experiments on single ventricular myocytes. Methods and protocols were essentially the same as in our previous study (Kodama *et al.*, 1990). In the presence of diprafenone (10^{-5} M), the shortest (10 ms) conditioning clamp to 0 mV caused a substantial $\dot{V}_{max}$ reduction of the test action potential. The $\dot{V}_{max}$ reduction was enhanced modestly by further prolongation of the conditioning clamp pulse duration. The results obtained for propafenone are consistent with our previous paper (Honjo *et al.*, 1989). Thus, a small $\dot{V}_{max}$ reduction by the shortest (10 ms) clamp pulse was followed by an additional large $\dot{V}_{max}$ inhibition with further prolongation of the clamp pulse duration. These findings may indicate that although diprafenone and propafenone block the sodium channel in both the activated and inactivated states, the relative contribution of inactivation block is less important for diprafenone than propafenone.

The approximate extent of activated channel block (ACB), which was defined by a percentage of $\dot{V}_{max}$ by a 10 ms clamp pulse from the reference level (tonic block subtracted value) was estimated to be 10.1% for diprafenone (10^{-5} M) and 6.8% for propafenone (10^{-5} M) on average. The approximate extent of inactivated channel block (ICB), which was defined by an additional percentage decrease in $\dot{V}_{max}$ when the clamp pulse duration was prolonged from 10 ms to 500 ms was 12.8% for diprafenone (10^{-5} M) and 21.1% for propafenone (10^{-5} M). The ratio of ICB/ACB was 1.27 with diprafenone and 3.10 with propafenone. The value for diprafenone is less than half that for propafenone, and comparable to that for quinidine and disopyramide (Kodama *et al.*, 1990).

In our preliminary experiments using single guinea-pig ventricular cells, we compared the decrease in $\dot{V}_{max}$ of the test action potential induced by a single prolonged clamp pulse (500 ms × 1) and by multiple short clamp pulses (10 ms × 10, 9 Hz) by using the same protocol as employed in our previous study (Kodama *et al.*, 1990). The average ratio of multiple/single $\dot{V}_{max}$ reduction was 1.38 for propafenone (10^{-5} M, $n = 3$), and 1.90 for diprafenone (10^{-5} M, $n = 3$). This finding may also indicate the lesser contribution of inactivation block for diprafenone action than that for propafenone.

A change of RP of papillary muscles, which was produced by an alteration of extracellular K^+ concentration, had different effects on the use-dependent block of $\dot{V}_{max}$ with diprafenone and propafenone. First, the intensity of use-dependent block with diprafenone was enhanced at a less negative RP, whereas that with propafenone was virtually unchanged. Second, the time constant of $\dot{V}_{max}$ recovery (τR) from the use-dependent block with diprafenone was shortened by the depolarization, while that with propafenone was unaffected or rather prolonged. This could be explained by their different binding and unbinding characteristics to the sodium channels.

$\dot{V}_{max}$ recovery from the use-dependent block with lignocaine and mexiletine is known to be accelerated at more negative membrane potential (hyperpolarization). This has been attributed to their higher affinity for the inactivated state of the sodium channel than the resting state. Under such conditions, hyperpolarization would enhance drug dissociation from channel receptors by increasing the resting fraction of drug-associated channels at the expense of their inactivated fraction (Hondeghem & Katzung, 1984; Grant *et al.*, 1984). The voltage-dependence of $\dot{V}_{max}$ recovery for diprafenone is opposite to these drugs, and similar to those reported for penticainide (Carmeliet, 1988) and disopyramide (Gruber & Carmeliet, 1989). They proposed the idea of 'activation trapping' to interpret the reverse voltage-dependence of $\dot{V}_{max}$ recovery. According to this idea, the drug molecules, which bind to and unbind from sodium channels primarily during the activated state, are trapped in the channel when it returns from the activated to the resting state. Since the probability of having the activation gate in the open position will be smaller the more negative the membrane potential, dissociation of the drugs from channel receptors would be accelerated by depolarization.

Dissociation (unbinding) of drugs from the sodium channels during the activated state (activation unblock) is enhanced by hyperpolarization of the resting membrane potential (Anno & Hondeghem, 1990), leading to an attenuation of the extent of block during the stimulation trains. In fact, like the present data for diprafenone, the extent of use-dependent block of $\dot{V}_{max}$ of I_{Na} with penticainide, disopyramide and flecainide was shown to be attenuated by hyperpolarization, while enhanced by depolarization of membrane potential within the range of -90 to -70 mV (Carmeliet, 1988; Gruber & Carmeliet, 1989; Anno & Hondeghem, 1990).

In the case of propafenone, such an influence of 'activation trapping' or 'activation unblock' on the use-dependent $\dot{V}_{max}$ inhibition is considered to be minimal, because it binds to the sodium channel during both the activated and inactivated states. Nevertheless, further experimental studies to estimate the activation unblock more directly, will be required to substantiate such an interpretation.

We cannot rule out other possible mechanisms for the different voltage-dependence of the use-dependent sodium channel inhibition with diprafenone and propafenone. For instance, Kohlhardt & Fichtner (1988b) have demonstrated in their cell-attached patch clamp experiments recording single Na^+ channel currents that diprafenone, unlike propafenone, shortens the lifetime of unblocked channels as an additional effect, apart from its blocking action. Such modification is expected to have a complex influence on the effects of the drug on the net sodium inward current and $\dot{V}_{max}$ of action potential.

The use-dependent block of $\dot{V}_{max}$ with diprafenone was significantly less than that with propafenone in normally polarized papillary muscles, but *vice versa* in partially depolarized muscles (Figure 5b). This may endow diprafenone with a potential advantage over propafenone as a local anaesthetic-type (Class-I) antiarrhythmic drug. Tachyarrhythmias, which jeopardize haemodynamic conditions, are frequently associated with a decrease in the resting membrane potential (depolarization) of cardiac tissues. The excitability and conductivity of these abnormal arrhythmogenic tissues may be effectively suppressed in a frequency-dependent manner by diprafenone at concentrations causing less interference with the electrical activity in normally polarized tissues. The higher antiarrhythmic potency of diprafenone than propafenone could be attributed, at least in part, to such a highly selective inhibitory action of the former compound against the abnormal cardiac tissues.

Approximate plasma levels of diprafenone and propafenone in patients with the drugs at therapeutic doses were reported to be 0.1–2.0 $\mu g\ ml^{-1}$ (Trenk *et al.*, 1989; Kunze *et al.*, 1989) and 0.5–3.0 $\mu g\ ml^{-1}$ (Siddoway *et al.*, 1984; Greenberg *et al.*, 1989) respectively. These ranges correspond roughly to 3×10^{-7} M–5×10^{-6} M diprafenone and $1-8 \times 10^{-6}$ M propafenone. The present *in vitro* data cover such clinical concentrations of the two drugs.

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Angiotensin converting enzyme binding sites in human heart and lung: comparison with rat tissues

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1 Angiotensin converting enzyme (ACE), a dipeptidyl carboxypeptidase which catalyzes the final activation step in the formation of angiotensin II, was identified by radioligand studies in rat heart and lung. In this work we identified ACE binding sites in human left ventricle and lung by radioligand binding using the ACE inhibitor [^{3}H]-ramiprilat, and compared its binding characteristics in human and rat tissues.

2 Binding of [^{3}H]-ramiprilat in all tissues tested was saturable, temperature and zinc-dependent, and inhibited by EDTA. In human left ventricle homogenate we found a density of binding sites of 121 ± 15 fmol mg^{-1} protein ($n = 4$) with an affinity (K_d) of 850 ± 55 pM, whereas in rat left ventricle the same values were 23 ± 4 fmol mg^{-1} protein and 315 ± 30 pM, ($n = 4$), respectively.

3 [^{3}H]-ramiprilat binding to rat ($n = 4$) and human lung ($n = 4$) showed a binding site density of 2132 ± 155 and 1085 ± 51 fmol mg^{-1} protein respectively with an affinity of 639 ± 54 and 325 ± 22 pM. The lung:heart ratio of ACE binding site density was about 9:1 in man and 100:1 in rat.

4 The binding affinities of 13 ACE inhibitors were evaluated on human heart and lung: the drugs tested showed a wide range of affinities for the ACE binding sites in both tissues, and the affinity for lung was significantly greater than for heart for most of the drugs.

5 The greater potency of some ACE inhibitors in displacing [^{3}H]-ramiprilat in human lung compared with the heart indicates differences between ACE binding sites in these tissues and suggests the possibility of a selective organ-targeted therapeutic approach.

Keywords: Angiotensin converting enzyme; binding sites; tissue converting enzyme inhibition

Introduction

Angiotensin-converting enzyme (ACE; EC 3.4.15.1), a carboxy dipeptidase, catalyzes generation of angiotensin II from the precursor angiotensin I as well as cleavage of bradykinin (Yang *et al.*, 1970) and other molecules (Erdös & Skidgel, 1986; Erdös, 1990). The enzyme is widely distributed throughout the body. It has been identified in the luminal aspect of vascular endothelial cells in lung, where it plays an important role in the process of fluid extravasation in the context of the 'neurogenic inflammation' (Piedimonte *et al.*, 1991), and in other areas, where it is implicated in vascular permeability changes (Caldwell *et al.*, 1976). Membrane-bound ACE is also found in renal proximal tubules (Oshima *et al.*, 1974; Schulz *et al.*, 1988), in the gastrointestinal brush border (Ward *et al.*, 1980), in brain structures (choroid plexuses, circumventricular structures) (Igic *et al.*, 1977; Defendini *et al.*, 1983), and in the testis and epididymis (Srittmaster & Snyder, 1984).

A complete renin-angiotensin system at the level of a single organ has been demonstrated in animals. Studies have been conducted mainly in rat heart, leading to the detection in this site of renin and angiotensinogen mRNA, renin, ACE, angiotensin II and angiotensin II receptors (Baker *et al.*, 1984; Dzau *et al.*, 1987; Saito *et al.*, 1987; Lindpaintner & Gauten, 1991). The physiological roles of myocardial angiotensin II include paracrine effects on cardiac contractility and excitability and on coronary vasomotion (Goodfriend, 1986; Lindpaintner & Gauten, 1991). Angiotensin II is also capable of inducing the synthesis of contractile protein in cultured vascular smooth muscle cells (Naftilan *et al.*, 1989a,b; Turla *et al.*, 1991) and is involved in the process of smooth muscle and myocardial hypertrophy (Hori *et al.*, 1989; Katoh *et al.*, 1989; Naftilan *et al.*, 1989b; Aceto & Baker, 1990; Baker & Aceto, 1990). Receptors for angiotensin II have also been recently demonstrated in normal and failing human heart (Urata *et al.*, 1989). The involvement of ACE in the control of cardiac growth is emphasized by the recent finding that treatment with ACE inhibitors prompts regression of myocardial hypertrophy in hypertensive rats (Kromer & Riegger, 1988; Linz *et al.*, 1989; Baker *et al.*, 1990; Fröhlich & Sasaki, 1990; Fröhlich & Orinaka, 1991) and dogs (Julius *et al.*, 1991) in the absence of a significant pressor effect.

Studies of ACE binding sites and ACE inhibitors have been conducted mostly in animal models, such as the rat. Studies on human heart ACE previously addressed the enzymatic activity of ACE and were confounded by the high levels of non ACE-dependent angiotensin I degrading activity present in the heart (Lanzillo *et al.*, 1986; Urata *et al.*, 1990).

We have now evaluated the characteristics of ACE binding sites (density and affinity) as measured by the radiolabelled ACE inhibitor [^{3}H]-ramiprilat in the lung and in the left ventricle of man and rat. Furthermore, in an analogy to that discovered by comparing the binding characteristics of different ligands on other binding sites (Weiland & Molinoff, 1981; Kenakin & Boselli, 1989; Kenakin, 1990), we tested for possible differences in the relative potencies of a series of ACE inhibitors in heart with respect to lung, in order to establish whether the binding sites in these tissues have the same characteristics or might be differentiated into subtypes.

Methods

Heart and lung tissue sources

Fifteen to 20 grams of the left ventricular anterior wall, adjacent to the apex, were excised from two prospective

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organ donors and two traffic victims (3 males, 1 female, age 35, 37, 41 and 42 years) whose hearts were obtained at autopsy within 3 h of death after being judged as not suitable for heart transplant. On anamnesis the organ donors were medication-free. Human lung tissue was obtained from seven patients operated on for pulmonary adenocarcinoma; the healthy part of the excised lobe was used. Left ventricular and pulmonary tissue was also obtained immediately after death from 250 g male Sprague-Dawley rats, which were killed by cervical dislocation.

Plasma membrane preparation

Heart and lung tissues were freed of connective tissue and fat, trimmed with scissors, and pulverized at the temperature of liquid nitrogen by a tissue dismembrating device (Mikro-Dismembrater II, B. Braun, Melsungen, Germany). The pulverized tissue was resuspended in buffer A (composition, mM: Tris-HCl 20, NaCl 125, KCl 10, sodium acetate 10, D-glucose 5, zinc sulphate 50 μM, pH 7.4 at 20°C). In some experiments the zinc-dependency of binding was evaluated in plasma membranes prepared in the same buffer without zinc sulphate (buffer B). The homogenate was centrifuged at 600 *g* for 10 min at 4°C; the supernatant was recovered and centrifuged at 48,000 *g* for 35 min at 4°C. The pellet was resuspended in the same buffer and used immediately for the assay, or stored in liquid nitrogen.

Binding assay

Cardiac homogenate (100 μg/tube) or lung homogenate (10 μg/tube) was incubated in polypropylene tubes containing 0.15–10 nM [^{3}H]-ramiprilat in buffer A or B, in a volume of 250 μl, at 37°C for 60 min. Nonspecific binding was determined in the presence of 1 μM quinaprilat. The reaction was stopped by addition of 3 ml of ice-cold Buffer A and quick filtration of samples on Whatman GF/C filters (Whatman Int Ltd., Maidstone, England), which were then quickly rinsed with 2 × 10 ml Buffer A. The filter-bound radioactivity was evaluated by liquid scintillation spectrometry.

The kinetics of [^{3}H]-ramiprilat binding were determined by incubating plasma membranes resuspended in buffer A with 0.35 nM (lung) or 0.8 nM (heart) [^{3}H]-ramiprilat, in the presence or absence of 1 μM quinaprilat, at 4°C and 37°C for variable times between 5 and 120 min. Dissociation was tested after 65 min by addition to the reaction mixture of 1 μM quinaprilat, and the dissociation was monitored for 120 min. The membrane-bound radioactivity was determined as described above.

Competition experiments were performed on heart and lung plasma membranes (respectively 100 and 10 μg/tube) by incubation at 37°C for 60 min with 0.35 nM [^{3}H]-ramiprilat and the competing ligands at various concentrations, in the appropriate range for each drug (see also Results). After incubation, the radioactivity bound to plasma membranes was evaluated as above.

Binding data

Scatchard (1949) plots of concentration-dependent [^{3}H]-ramiprilat binding were linear and were analyzed by linear regression analysis. Competition isotherms of [^{3}H]-ramiprilat in the presence of various ACE-inhibitors were analyzed with a computer-assisted modelling technique as previously reported (Vago *et al.*, 1989).

Drugs

[^{3}H]-ramiprilat (specific activity 55.3 Ci mmol^{-1}, radiochemical purity 96%) was obtained from Hoechst AG, Frankfurt-am-Main, Germany. The following ACE inhibitors were tested: enalapril, enalaprilat and lisinopril (obtained from Merck Sharp & Dohme, Rahway, NY, U.S.A.); captopril (from Squibb, Paris la Defense, France); benazeprilat (from Ciba-Geigy, Origgio, Italy); quinapril and quinaprilat (from Parke-Davis, Lainate, Italy); spirapril and spiraprilat (from Schering-Plough, Comazzo, Italy); perindopril and perindoprilat (from Servier, Fleury-les-Aubrais, France); cilazaprilat (from Roche, Welwyn Garden City, England). Other chemicals were analytical grade reagents purchased from usual suppliers.

Statistical analysis

Binding data are expressed as the mean ± s.d. of n experiments. Comparison between binding data (B_{max}, K_d, pK_i values) was made by Student's unpaired t test.

Results

Saturation analysis

As shown in Figure 1, the binding of [^{3}H]-ramiprilat to human heart homogenate was saturable and disclosed a binding site density of 121 ± 15 fmol mg^{-1} protein, with a K_d of 850 ± 55 pM (n = 4). The binding was completely inhibited by 0.1 mM EDTA or in zinc-free buffer (data not shown). In rat heart the binding site density was 23 ± 4 fmol mg^{-1} protein and the K_d was 315 ± 30 pM (n = 4). Both B_{max} *and* K_d values were significantly lower in rat than in man ($P < 0.001$). The binding of [^{3}H]-ramiprilat to rat and human lung homogenate was also saturable (Figure 2) and disclosed a site density of 1085 ± 51 fmol mg^{-1} protein (Figure 2) with a K_d = 325 ± 22 pM in man (n = 4) and a density of 2132 ± 155 fmol mg^{-1} protein with a K_d of 639 ± 54 pM in rat (n = 4).

Kinetic analysis

The binding of [^{3}H]-ramiprilat at 37°C to human heart homogenate was rapid and plateaued at 60 min (Figure 3). The addition of 1 μM quinaprilat prompted a fast dissociation, which was maximal within 120 min. Association and dissociation were much slower at 4°C. The kinetics of the binding of [^{3}H]-ramiprilat to human lung were similar to

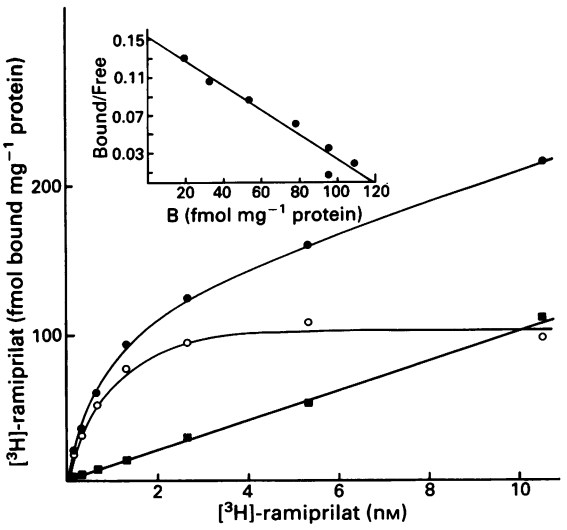

Figure 1 Equilibrium binding of [^{3}H]-ramiprilat on human heart plasma membrane preparation; data from a representative experiment replicated 4 times. Nonspecific binding has been evaluated in the presence of 1 μM quinaprilat: (●) total; (○) specific; (■) non specific. Inset shows the Scatchard plot from the same data (K_d = 781 pM; B_{max} = 119 fmol mg^{-1} protein).

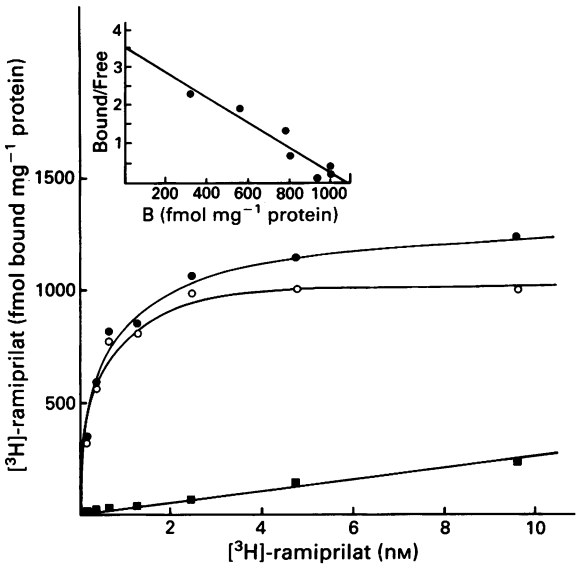

Figure 2 Equilibrium binding of [^{3}H]-ramiprilat on human lung plasma membrane preparation; data from a representative experiment replicated 4 times. Nonspecific binding was determined in the presence of 1 μM quinaprilat: (●) total; (○) specific; (■) non specific. Inset shows the Scatchard plot from the same data (K_d = 305 pM; B_{max} = 1074 fmol mg^{-1} protein).

those observed in heart (Figure 4). The K_d values calculated from kinetics at 37°C were similar to those obtained from equilibrium binding (458 pM on heart and 470 pM on lung respectively).

Competition analysis

Figures 5 and 6 and Table 1 show the observed competition curves and pK_i values for 13 ACE inhibitors. The pro-drugs tested (quinapril, enalapril, perindopril and spirapril) were about 100 to 1000 fold less potent than the respective active compounds, both on heart and lung. When the relative potencies of the same drug on heart and lung were evaluated, we found that in most cases the drugs tested had higher affinity for pulmonary than cardiac binding sites, and displayed a relative potency (lungs vs. heart) ranging from 2 to

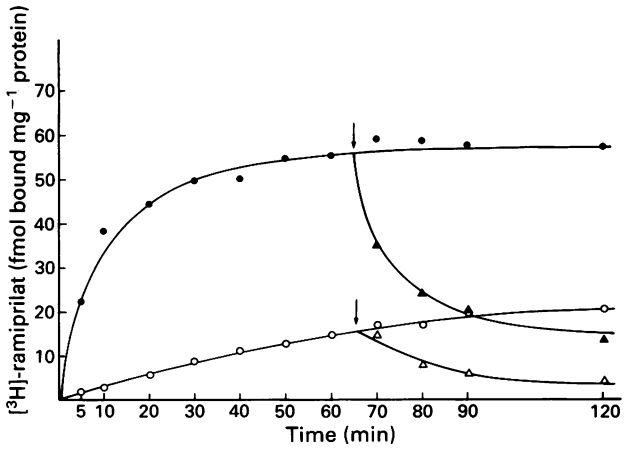

Figure 3 Kinetics of association and dissociation of [^{3}H]-ramiprilat binding to human heart plasma membranes at 37°C and 4°C. Note that saturation is apparently not reached at 4°C after 120 min. Association: (●) at 37°C; (○) at 4°C; dissociation: (▲) at 37°C; (△) at 4°C.

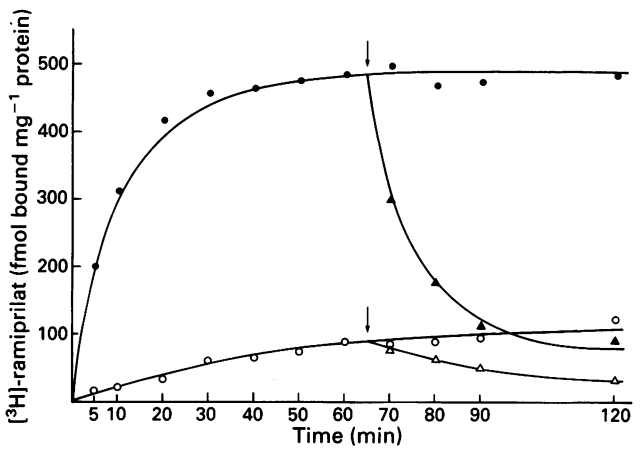

Figure 4 Kinetics of association and dissociation of [^{3}H]-ramiprilat binding to human lung plasma membranes at 37°C and 4°C. As in heart (Figure 3), equilibrium is not reached at 4°C after 120 min. Association: (●) at 37°C; (○) at 4°C; dissociation: (▲) at 37°C; (△) at 4°C.

7.41. Enalapril, a pro-drug, had a relative potency in lung vs. heart of 12.3, whereas quinapril (a pro-drug) and captopril (active) were equally effective in both tissues (relative potencies of 1.62 and 1.7 respectively; difference not significant).

Discussion and Conclusions

The binding sites identified by [^{3}H]-ramiprilat binding have consistently been proven to be genuine ACE binding sites, in that these sites show a binding of high affinity, reversible and with an absolute requirement for zinc and calcium. Moreover, a number of ACE inhibitors displaced in a competitive manner the specific binding of [^{3}H]-ramiprilat.

The first of two main findings of this work is the identification of striking differences in ACE binding site density in tissues of human origin compared to rat. In human left ventricle we found a 5 fold higher density of ACE binding sites, and in lung a density about half that found in rat. The

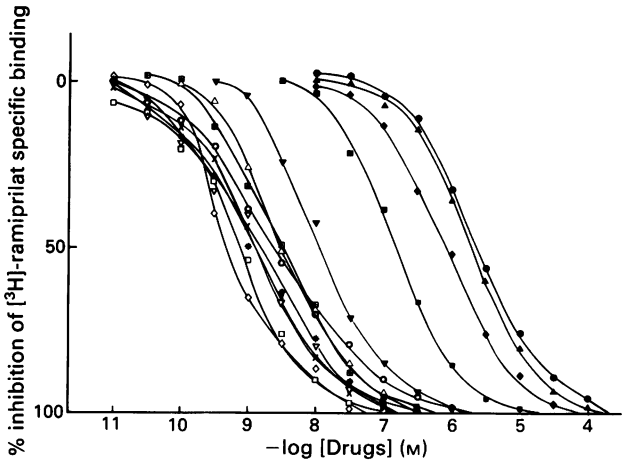

Figure 5 Competition isotherms of 13 angiotensin converting enzyme inhibitors on [^{3}H]-ramiprilat binding in human heart plasma membranes. Corresponding pK_i values for each drug are given in Table 1. (□) Quinaprilat; (■) quinapril; (△) enalaprilat; (▲) enalapril; (○) perindoprilat; (●) perindopril; (◇) spiraprilat; (◆) spirapril; (▼) captopril; (×) benazeprilat; (▽) cilazaprilat; (*) ramiprilat; (⊡) lisinopril.

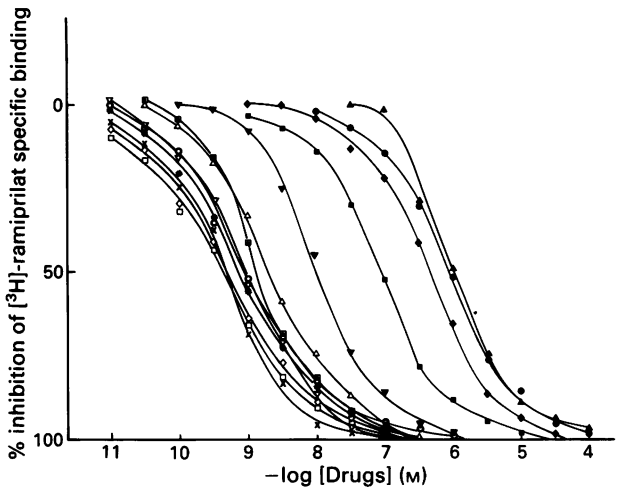

Figure 6 Competition isotherms of 13 angiotensin converting enzyme inhibitors on [^{3}H]-ramiprilat binding in human lung plasma membranes. Corresponding pK_i values for each drug are given in Table 1. (□) Quinaprilat; (■) quinapril; (△) enalaprilat; (▲) enalapril; (○) perindoprilat; (●) perindopril; (◇) spiraprilat; (◆) spirapril; (▽) captopril; (×) benazeprilat; (▽) cilazaprilat; (*) ramiprilat; (⊡) lisinopril.

Table 1 Relative potencies of 13 angiotensin converting enzyme (ACE) inhibitors on human heart and lung plasma membrane preparations, expressed as the ratio between mean K_i values on lung and on heart

	pK_i ($n = 4$)		*Relative potency*	
Drug	*Heart*	*Lung*	(lung vs. heart)	
Quinapril	7.24 ± 0.141	7.45 ± 0.110	1.62	NS
Quinaprilat	9.25 ± 0.074	9.92 ± 0.136	4.68	0.0003
Enalapril	5.63 ± 0.189	6.72 ± 0.118	12.3	0.0002
Enalaprilat	8.56 ± 0.177	9.23 ± 0.095	4.68	0.0012
Perindopril	6.00 ± 0.101	6.48 ± 0.099	3.02	0.0011
Perindoprilat	8.48 ± 0.142	9.35 ± 0.083	7.41	0.0001
Spirapril	6.16 ± 0.073	6.82 ± 0.041	4.57	0.00001
Spiraprilat	9.35 ± 0.090	9.71 ± 0.084	2.29	0.0024
Benazeprilat	9.33 ± 0.104	9.81 ± 0.094	3.02	0.0011
Cilazaprilat	9.25 ± 0.070	9.55 ± 0.083	2.00	0.0031
Lisinopril	8.77 ± 0.133	9.45 ± 0.089	4.79	0.0003
Captopril	8.30 ± 0.164	8.53 ± 0.053	1.70	NS
Ramiprilat	9.08 ± 0.044	9.44 ± 0.098	2.29	0.0012

P values from unpaired *t* test on pK_i. NS = not significant. Values are mean ± s.d.

rat is normally used as a model for *in vitro* and *in vivo* studies on the specific physiological role of ACE in various tissues. Our findings demonstrate that man has important differences in the quantitative distribution of ACE in heart and lung compared to the rat, with a ratio between ACE densities in lung and heart of 9:1 in man and 100:1 in rat. Such differences between ACE binding sites in man and rat support the hypothesis of different functional roles of this enzyme in the two species. Moreover, in view of these differences, data on rat cannot be easily transposed to man, and the rat, therefore, seems a questionable model for pharmacological research on ACE inhibitors with a human therapeutic purpose.

The second main finding of this work is the different affinity of several ACE inhibitors for human lung and heart. In fact, we found a higher affinity of [^{3}H]-ramiprilat for binding sites in human lung than in heart. Moreover, all the ACE inhibitors tested (currently used clinically in man) disclosed some differences in binding affinity on heart compared to lung. For most of the drugs tested we found a greater affinity for ACE in lung than in heart, with relative potencies ranging from 2 to 12.3, with the exception of captopril and quinapril (the latter is a pro-drug). Thus, some ACE inhibitors may display a tissue specificity. Similar conclusions were drawn by Jackson *et al.* (1986a,b), who differentiated the binding affinities of the ACE inhibitor [^{125}I]-MK-351A in various rat tissues and rat plasma. Such data were partially contradicted by Grima *et al.* (1991), who used the same ligand as we did but did not detect significant binding differences in ACE solubilized from different tissues by Triton X-100. The discrepancy between our data and those reported by Grima *et al.* may be explained by species differences (rat versus man) or may be the consequence of the solubilization procedure. Our hypothesis points to the possibility that ACE displays changes in affinity for inhibitors and possibly for the natural substrate(s), depending on factors strictly related to the specific organ or tissue where it is expressed. The assumption that a single gene encoding for ACE has been identified does not rule out, however, the possibility that multiple forms of the enzyme can be generated through initiation of mRNA transcription at different sites. In fact, this has been shown to occur for mRNA of testis- and lung-derived ACE in rat (Sen *et al.*, 1990) and rabbit (Kumar *et al.*, 1991), where the level of ACE mRNA expression as well as the isoenzyme expressed are regulated in a tissue-specific manner. Several variables, such as different amounts of enzyme syalylation (Erdös, 1990), can also act at the posttranslational level to modify enzymatic activity. Finally we should consider the possibility of an interaction between ACE and other membrane-associated structures that could directly influence enzymatic activity. This kind of interaction is lost and therefore cannot be studied if detergents are used for enzyme extraction.

In conclusion, our data point to a strikingly different density of ACE binding sites in rat and human heart. The rat model is therefore less suitable for studies of cardiac ACE directed to therapeutic applications. ACE inhibitors show intrinsic differences regarding relative affinity and selectivity for lung and heart ACE in man, thus defining tissue-specific differences of this enzyme. Further research in this field might lead to the synthesis of ACE inhibitors with selective tissue specificity of potential therapeutic usefullness, i.e. ACE inhibitors with relatively greater affinity for cardiac tissue than for peripheral vessels or lung tissue.

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Endotoxin impairs the response of rabbit mesenteric artery to electrical stimulation via a prejunctional mechanism

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1 The effect of *E. coli* lipopolysaccharide (LPS) on sympathetic neuro-effector transmission was studied in the rabbit mesenteric artery. The experiments were performed on artery rings isolated 5 or 20 h after intravenous treatment with LPS or saline as well as on artery rings isolated from non-treated rabbits (for assessment of the effect of *in vitro* preincubation with LPS). In most experiments, neural elements in the arteries were stimulated electrically (10 V, 2 ms, 1–32 Hz).

2 Preincubation with LPS (10 μg ml^{-1}) for 5 or 20 h had no effect on the contraction responses of endothelium-intact artery rings to electrical stimulation. In contrast, *in vivo* intravenous pretreatment with LPS (10 μg) led to an inhibition of the contraction; LPS elicited this effect when injected 20 h, but not 5 h, before the experiment. The effect of LPS was eliminated in artery rings isolated from animals receiving an inhibitor of protein synthesis (actinomycin D or cycloheximide) before treatment with LPS. LPS (injected 20 h before the experiment) had no effect on the concentration-response curves for exogenous noradrenaline and tyramine in endothelium-intact artery rings.

3 The inhibition of electrically induced contractions produced by LPS treatment in endothelium-intact artery rings was attenuated by atropine and yohimbine, but not by phentolamine. Yohimbine plus atropine restored the depressed contraction to the normal level. Clonidine and acetylcholine mimicked the effect of LPS in endothelium-intact artery rings isolated from saline-treated animals.

4 When steady-state contractions were induced by 5 min of stimulation at 16 Hz, acetylcholine or clonidine reduced the contraction in endothelium-denuded artery rings from both saline-treated rabbits and animals receiving LPS 20 h before the experiment. The reduction produced by acetylcholine or clonidine of the contraction in artery rings from LPS-treated rabbits was significantly greater than in artery rings from saline-treated animals.

5 These results suggest that treatment of rabbits with LPS inhibits noradrenaline release from sympathetic nerve endings via increased sensitivity of both prejunctional inhibitory muscarinic receptors and α_2-adrenoceptors in mesenteric arteries. They also suggest that the effect of LPS is independent of endothelial cells but linked to protein synthesis.

Keywords: Endotoxin; nerve stimulation; sympathetic nerve endings; prejunctional muscarinic receptors; prejunctional α_2-adrenoceptors; endothelium; rabbit mesenteric artery

Introduction

Endotoxins are lipopolysaccharides located in the outer membrane of gram-negative bacteria. Gram-negative endotoxic shock is associated with a wide variety of haemodynamic disturbances. Most commonly, blood pressure falls, cardiac output is redistributed and blood pools in the venous system (Gilbert, 1960; Brackett *et al.*, 1985). Although the aetiology of endotoxic shock has been extensively studied, the exact sequence of events that leads to hypotension is not well defined. Diminished peripheral resistance is thought to play a key role in the fall in blood pressure. Loss of responsiveness to vasoconstrictors is a hallmark of endotoxic shock in humans. Decreased responsiveness to α-adrenoceptor agonists has been documented *in vivo* during endotoxic shock in rats (Parratt, 1973; Fink *et al.*, 1985; Auclair *et al.*, 1986) and *in vitro* in blood vessels isolated from rats during endotoxin-induced shock (Pomerantz *et al.*, 1982; Wakabayashi *et al.*, 1987). Desensitization of α-adrenoceptors has been suggested as a possible mechanism (Zhou & Jones, 1990). In agreement with this suggestion, a decrease in postjunctional α_1-adrenoceptors has been reported (Carcillo *et al.*, 1988).

Endotoxin is also known to impair vascular responsiveness to sympathetic nerve stimulation (Tomikawa *et al.*, 1989; Gray *et al.*, 1990). Although this might be explained by a decreased sensitivity of postjunctional α_1-adrenoceptors (Auclair *et al.*, 1986; Carcillo *et al.*, 1988; Bigaud *et al.*, 1990), an inhibition of neurotransmitter, i.e. noradrenaline (NA), release is an alternative possibility. The release of NA is known to be controlled, inter alia, by prejunctional α_2-adrenoceptors (see Starke, 1977; Westfall, 1980; Langer, 1981) and muscarinic receptors (Lindmar *et al.*, 1968; see Muscholl, 1979; 1980; Vanhoutte & Levy, 1980). Both kinds of prejunctional receptor occur in vascular nerves of the rabbit (Steinsland *et al.*, 1973; Allen *et al.*, 1975; Endo *et al.*, 1977). In this paper, we show that treatment of rabbits with endotoxin inhibits the release of NA from sympathetic terminal axons in the mesenteric artery, apparently by activation or sensitization of prejunctional α_2-adrenoceptors and muscarinic receptors.

Methods

General

All experiments were performed in male albino (New Zealand) rabbits (2.0–2.5 kg). Some rabbits were given intravenous injections (in an ear vein) of a lipopolysaccharide (LPS) extracted from *E. coli* (10 μg dissolved in 1.0 ml of 0.9% NaCl) 5 or 20 h before the experiment. Saline-control animals were injected with 1.0 ml of 0.9% NaCl.

Vessel preparation, isometric tension recording, and stimulation

In accordance with our institutional Animal Care Committee guidelines, mesenteric arteries were taken from the animals

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after exsanguination during anaesthesia with diethyl ether. Fat and other nonvascular tissue was gently removed from the blood vessels, which were cut into rings (2 mm width), without disturbing the intimal layer, after immersion in ice-cold modified Krebs-Ringer solution (mM: indomethacin 0.05 to prevent electrical stimulation-induced release of vasoactive prostanoids from endothelium, NaCl 118.0, KCl 4.7, $MgSO_4$ 1.18, $CaCl_2$ 2.5, KH_2PO_4 1.18, $NaHCO_3$ 25.0 and glucose 5.5, aerated with 95% O_2–5% CO_2; pH 7.2–7.3). The endothelium was removed from some rings by gently rubbing the luminal surface with a wooden implement. Rings with and without endothelium were prepared from adjacent segments of the same vessel.

The rings were suspended in a 10 ml water-jacketed tissue bath (37°C) with one end tied to a fixed point and the other to a force transducer (Nihon Kohden JB-612T, Tokyo, Japan); changes in isometric force were recorded with an amplifier (Nihon Kohden AP-601G, Tokyo, Japan) attached to a recorder (Nihon Kohden RM-6000, Tokyo, Japan). Before the start of the experiment, the rings were allowed to equilibrate for 45 min in Krebs-Ringer solution which was changed at 15 min intervals. During this time, the rings were stretched to a passive tension of 15.3 mN (1.5 g).

The tissue preparations were placed between a pair of rectangular platinum electrodes (8 × 8 mm; 0.5 mm thick). The gap between the preparation and the electrodes was wide enough to allow undisturbed contractions, and yet sufficiently narrow to permit effective stimulation of intramural nerve terminals. The ring preparations were stimulated by trains of 2 ms square pulses of supramaximal intensity at frequencies of 1, 2, 4, 8, 16, or 32 Hz, 10 V (Greenberg *et al.*, 1981), provided by a direct current power supply and a switching transistor triggered by a stimulator (Nihon Kohden SEN-3201, Tokyo, Japan). The responses of the ring preparations to electrical stimulation were measured for a time long enough to assure equilibrium responses. Each frequency increment was initiated on return of force to prestimulation values after cessation of stimulation. In some experiments, electrical stimulation (10 V, 2 ms, 8 or 16 Hz) of intramural nerves was carried out for 5 min.

Further details are given in the Results section.

In vitro *preincubation with lipopolysaccharide*

In order to determine the direct effect of LPS *in vitro* on electrical stimulation-induced responses, the endothelium-intact ring preparations isolated from non-treated animals were preincubated at 4°C with LPS (10 μg ml^{-1} Krebs-Ringer solution) for 5 or 20 h before the start of the experiment. Time-matched control rings were incubated in the absence of LPS.

Drugs

The LPS used in the present study was *E. coli* 0127:B8 (Difco, Detroit, MI, U.S.A.) prepared by the Westphal method (Westphal & Jann, 1965). Sources of other drugs were: indomethacin (Sigma), phentolamine mesylate (Ciba-Geigy), (−)-noradrenaline hydrochloride (Sigma), tyramine hydrochloride (Sigma), clonidine hydrochloride (Sigma), yohimbine hydrochloride (Sigma), acetylcholine chloride (Sigma), atropine sulphate (Tokyo Kasei, Tokyo, Japan), cycloheximide (Sigma), and actinomycin D (Banyu, Tokyo, Japan). All agents except LPS, indomethacin, cycloheximide and actinomycin D were dissolved in distilled water and diluted in Krebs-Ringer solution before being added to the tissue bath. Indomethacin stock solution was prepared by dissolving three parts of indomethacin and one part of sodium bicarbonate in distilled water. Cycloheximide or actinomycin D dissolved in pyrogen-free pure water was injected intravenously into rabbits 5 h before the injection of LPS. LPS was dissolved in Krebs-Ringer solution when the effect of *in vitro* preincubation was studied.

Statistical analysis

Means and s.e.mean are given throughout. Two sets of statistical comparisons were made. Student's *t* test for paired samples was used when two populations were compared. Comparisons of subsequent intervention to controls were made by one-way analysis of variance, followed by Duncan's multiple-range test (Walpole, 1974). Differences were considered significant when $P < 0.05$.

Results

Prejunctional effects of acetylcholine and clonidine

In order to assess whether acetylcholine (ACh) and clonidine exert a prejunctional inhibitory effect on adrenergic neurotransmission in the mesenteric artery ring preparations, it is necessary to allow endothelium-denuded vessels to be contracted by electrical stimulation for a fairly long time. The final condition chosen was 5 min of stimulation at 8 Hz. At steady-state (equilibrium) contraction induced by electrical stimulation, addition of ACh (10^{-5} M) produced an atropine (10^{-7} M)-sensitive reduction of the contraction: ACh had no effect on NA (10^{-6} M)-induced contractions (Figure 1a). Under the same conditions, the α_2-adrenoceptor agonst clonidine (10^{-5} M) produced a yohimbine (10^{-7} M)-sensitive reduction of the contraction induced by electrical stimulation, and clonidine did not significantly alter the NA-induced contraction (Figure 1b). The observed effects of the agonists and antagonists were concentration-dependent (Figure 2), suggesting that activation of prejunctional inhibitory muscarinic receptors and α_2-adrenoceptors can in fact reduce electrical stimulation-induced contractions.

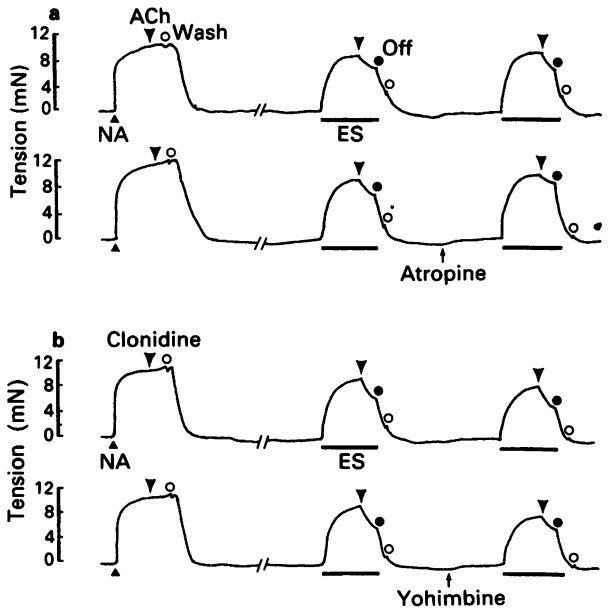

Figure 1 Recordings showing the effect of acetylcholine (ACh, 10^{-5} M) and clonidine (10^{-5} M) on noradrenaline (NA, 10^{-6} M)- and electrical stimulation (ES; 8 Hz, 5 min)-induced contractions of endothelium-denuded ring preparations isolated from non-treated rabbits. Duration of stimulation is illustrated below each response. (a) Shows typical examples of the effect of ACh (upper record) and atropine (lower record) in two rings from the same animal, and (b) examples of the effect of clonidine (upper record) and yohimbine (lower record) in two rings from another animal. Atropine (10^{-7} M) or yohimbine (10^{-7} M) was added 5 min before 8 Hz stimulation.

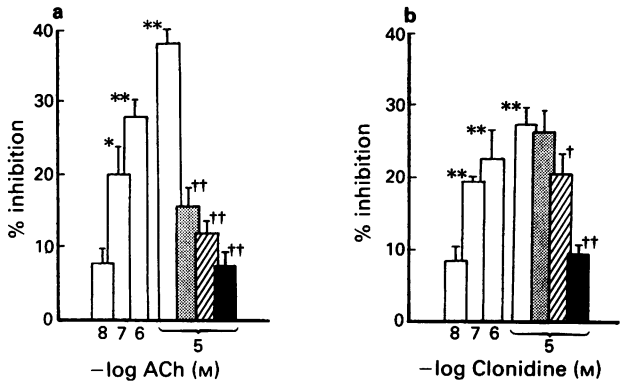

Figure 2 Effects of acetylcholine (ACh) and clonidine on stimulation-induced (8 Hz) contractions of endothelium-denuded ring preparations isolated from non-treated rabbits, and effect of atropine and yohimbine. The preparations were electrically stimulated according to the protocol of Figure 1. (a) Shows the effect of ACh, and (b) the effect of clonidine. Four ring preparations (for 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M of the agonists; open columns) obtained from the same vessel were studied in parallel, and one concentration of atropine (a; stippled column, 10^{-8} M; hatched column, 10^{-7} M; solid column, 10^{-6} M) or yohimbine (b; stippled column, 10^{-7} M; hatched column, 10^{-6} M; solid column, 10^{-5} M) was tested against 10^{-5} M ACh or clonidine per ring preparation. Atropine or yohimbine was added 5 min before 8 Hz stimulation. Data are expressed as percentage inhibition of the stimulation-evoked contraction and shown as the mean, vertical lines indicate s.e.mean ($n = 5$); n refers to the number of rabbits from which the mesenteric artery was taken. $*P<0.05$; $**P<0.01$: significantly different from corresponding value for 10^{-8} M ACh or clonidine; $\dagger P<0.05$; $\dagger\dagger P<0.01$: significantly different from corresponding value for 10^{-5} M ACh or clonidine alone.

Effect of lipopolysaccharide in endothelium-intact ring preparations

Figure 3 compares the effect of *in vitro* preincubation with LPS (Figure 3a) and intravenous injection of LPS (Figure 3b) on the response to electrical stimulation. Preincubation with LPS for 5 or 20 h did not change the response to electrical stimulation. In contrast, LPS injection 20 h, but not 5 h, before the experiment led to the appearance of an inhibitory effect. LPS (injected 20 h before the experiment) had no effect on the concentration-response curves for exogenous NA (Figure 4a) or tyramine (Figure 4b).

If the prejunctional cholinergic and α_2-adrenergic modulation of NA release is involved in the effect of LPS (injected 20 h before the experiment), the effect of LPS should be attenuated by muscarinic receptor antagonists and α_2-adrenoceptor antagonists, respectively; furthermore, muscarinic receptor agonists or α_2-adrenoceptor agonists should mimic the effect of LPS. As indicated in Figure 5, contractions depressed by pretreatment with LPS were increased by atropine (10^{-7} M) and yohimbine (10^{-6} M) but not by phentolamine (10^{-6} M); yohimbine plus atropine restored the depressed contraction to the normal level. Moreover, clonidine (10^{-5} M) and ACh (10^{-7} M) mimicked the effect of LPS in rings isolated from saline-treated control animals.

Interaction of lipopolysaccharide with acetylcholine and clonidine in endothelium-denuded ring preparations

It is difficult to evaluate the prejunctional effect of ACh in endothelium-intact rings because of its ability to induce the formation of endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980). Therefore, responses of endothelium-denuded rings were also studied under experimental conditions similar to those described in Figure 1 except that the vessel rings were prepared from animals treated with LPS or saline, and the ring preparations were stimulated at frequency of 16 Hz.

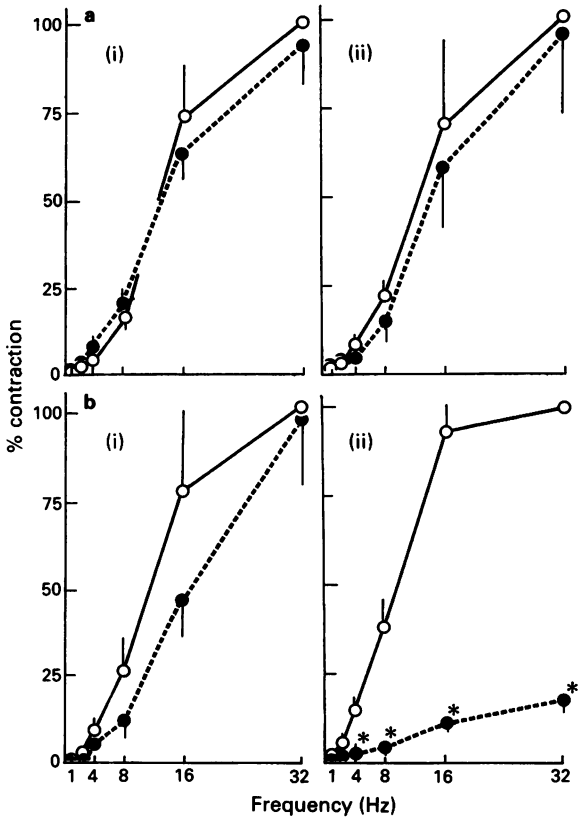

Figure 3 Comparison of the effect of *in vitro* preincubation with lipopolysaccharide (LPS, 10 μg ml^{-1} Krebs-Ringer solution) and intravenous injection of LPS (10 μg dissolved in 1 ml of saline) on the response of endothelium-intact ring preparations to electrical stimulation. Mesenteric arteries were isolated from non-treated rabbits or were isolated 5 or 20 h after LPS or saline (1 ml) injection. Each artery was stimulated electrically (10 V, 2 ms, 1 to 32 Hz). (a) Shows the effect of *in vitro* preincubation for 5 (i) or 20 h (ii): (○) time-matched control; (●) LPS. (b) Shows responses of ring preparations isolated 5 (i) or 20 h (ii) after LPS injection: (○) saline-control; (●) LPS injection. Responses to 32 Hz stimulation in time-matched control or saline-control are taken as 100%; these responses averaged 17.3 ± 2.6 mN ($n = 6$; a(i)), 13.9 ± 1.5 mN ($n = 7$; a(ii)), 19.6 ± 1.8 mN ($n = 6$; b(i)) and 21.1 ± 0.9 mN ($n = 22$; b(ii)). Each point represents the mean, and vertical lines show s.e.mean ($n = 5$–22); n refers to the number of rabbits from which the mesenteric artery was taken. $*P<0.01$: significantly different from corresponding value for saline-control.

The results are shown in Figures 6 and 7. At steady-state contraction induced by electrical stimulation but not by NA, the addition of ACh (10^{-5} M) or clonidine (10^{-5} M) caused a reduction of the contraction both in rings from saline-treated and in rings from LPS-treated animals (Figure 6). The percentage inhibition induced by ACh or clonidine in rings from LPS-treated animals was significantly greater than that in saline-controls (Figure 7).

Effect of protein synthesis inhibitors

To test whether the effect of LPS (injected 20 h before the experiment) depends on protein synthesis, responses to electrical stimulation of endothelium-intact ring preparations isolated from animals treated with actinomycin D (80 μg kg^{-1}, i.v.; Pickering & Fink, 1975; Sugihara *et al.*, 1991) or cycloheximide (312 μg kg^{-1}, i.v.; Deblois *et al.*, 1988; Sugihara *et al.*, 1991) 5 h before the injection of LPS or saline were determined.

Actinomycin D (Figure 8) or cycloheximide (Figure 9) by itself had no significant effect on the frequency-response curves. The depression induced by treatment with LPS however, was greatly attenuated by the protein synthesis inhibitors.

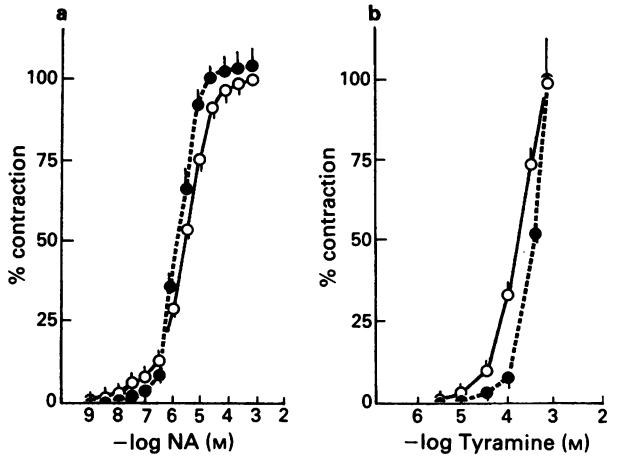

Figure 4 Concentration-response curves to noradrenaline (NA) (a) and tyramine (b) in endothelium-intact ring preparations isolated from lipopolysaccharide (LPS)-treated rabbits. The experiments were performed on artery rings isolated 20 h after intravenous treatment with LPS (●, 10 μg dissolved in 1 ml of saline) or saline (○, 1 ml). Concentration-response curves were determined by stepwise cumulative addition of NA or tyramine. Responses to 10^{-3} M NA and 10^{-3} M tyramine in saline-control are taken as 100%; these responses averaged 22.1 ± 0.7 mN ($n = 10$) and 9.3 ± 0.9 mN ($n = 15$), respectively. Each point represents the mean, and vertical lines show s.e.mean ($n = 10-15$); n refers to the number of rabbits from which the mesenteric artery was taken.

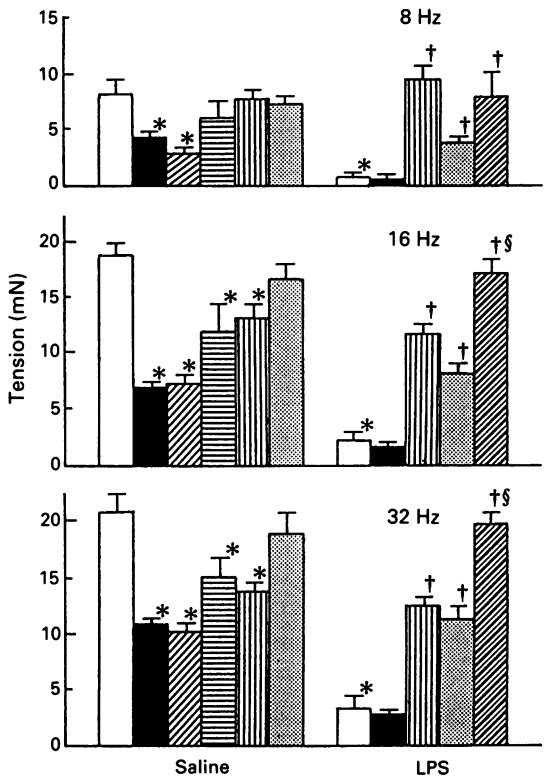

Figure 5 Response of endothelium-intact ring preparations isolated from lipopolysaccharide (LPS)-treated rabbits to electrical stimulation, and effects of agonists and antagonists at α_2-adrenoceptors and muscarinic receptors. Mesenteric arteries were isolated 20 h after intravenous LPS (10 μg dissolved in 1 ml of saline) or saline (1 ml) injection. Six or five preparations (for 'saline-no drug', 10^{-6} M phentolamine, 10^{-5} M clonidine, 10^{-7} M acetylcholine, 10^{-6} M yohimbine, and 10^{-7} M atropine in artery rings from saline-treated control; and for 'LPS-no drug', 10^{-6} M phentolamine, 10^{-6} M yohimbine, 10^{-7} M atropine and a mixture of yohimbine and atropine in artery rings from LPS-treated animals, respectively) obtained from the same vessel were studied in parallel, and one frequency-response curve (10 V, 2 ms, 8 to 32 Hz) in the presence or absence of drugs was determined per ring preparation. Either no drug (□) or phentolamine (■), clonidine (▨), acetylcholine (▤), yohimbine (▥), atropine (▩), or a mixture of yohimbine and atropine (▨) was added 5 min before stimulation started. Each column represents the mean, and vertical lines show s.e.mean ($n = 6$); n refers to the number of rabbits from which the mesenteric artery was taken. *$P < 0.05$: significantly different from 'saline-no drug'; †$P < 0.05$: significantly different from 'LPS-no drug'; §$P < 0.05$: significantly different from 'LPS-atropine'.

Discussion

α_2-Adrenoceptor and muscarinic receptor agonists inhibit NA release via a prejunctional mechanism in many species and tissues (Starke, 1977; Westfall, 1980; Langer, 1981). The present study demonstrates that appropriate treatment of rabbits with LPS leads to inhibition of NA release in the rabbit mesenteric artery, and that activation or sensitization of prejunctional α_2-adrenoceptors and muscarinic receptors is involved in this effect. This postulate is based on the following significant observations: (1) the response of endothelium-intact mesenteric artery to electrical stimulation, but not to exogenously added NA or tyramine, was markedly depressed when artery rings were prepared from the animals that received LPS 20 h before the experiment (Figures 3 and 4); (2) the depression was attenuated by the addition of the muscarinic receptor antagonist atropine (at a concentration which had no effect on the contraction induced by electrical stimulation in rings isolated from saline-treated rabbits) as well as of the α_2-adrenoceptor antagonists yohimbine, and yohimbine plus atropine restored the depressed contraction to the normal level (Figure 5); and (3) the muscarinic agonist, ACh and the selective α_2-adrenoceptor agonist, clonidine mimicked the effect of LPS (Figure 5).

It has been proposed that postjunctional α_2-adrenoceptors occur in the vicinity of the postganglionic sympathetic varicosities in the vasculature in the pithed rabbit (McGrath *et al.*, 1982; Bulloch *et al.*, 1987). However, vasoconstrictor responses of the rabbit ileocolic artery, which is a branch of the mesenteric artery, to exogenous NA are mediated exclusively by α_1-adrenoceptors, since they are abolished by relatively low concentrations of prazosin but not attenuated by yohimbine (von Kügelgen & Starke, 1985; Bulloch & Starke, 1990). Therefore, in light of the present findings, it is suggested that the attenuation by yohimbine of the effect of LPS (see LPS group in Figure 5) and the inhibitory effect of clonidine (see 'Saline' in Figure 5) are elicited prejunctionally and not due to postjunctional α_2-adrenoceptor blockade and activation, respectively. Yohimbine, like the nonselective α_1- and α_2-adrenoceptor antagonist, phentolamine ('Saline' in Figure 5), inhibited electrically induced contraction of ring preparations from saline-treated rabbits ('Saline' in Figure 5). One explanation is that yohimbine 10^{-6} M will block postjunctional α_1-adrenoceptors to a considerable degree: its pA_2 value at rabbit vascular α_1-adrenoceptors is 6.56 (Weitzell *et al.*, 1979).

EDRF plays a role in the vascular effects of many endogenous and exogenous substances. In the present study, we designed experiments to exclude a role of the endothelium. When endothelium-denuded ring preparations isolated from non-treated animals were used, the inhibition by ACh and clonidine of neurogenic contractions was prevented by atropine and yohimbine, respectively, in a concentration-dependent manner (Figures 1 and 2). Moreover, the inhibition produced by ACh or clonidine in artery rings from LPS-treated animals was significantly greater than in rings from saline-treated controls (Figures 6 and 7). LPS (injected 20 h before the experiment) had no detectable effect on the relaxation produced in endothelium-intact ring preparations by endothelium-dependent vasodilators (10^{-5} M ACh, 10^{-7} M bradykinin, and 10^{-7} M of the calcium ionophore, A23187)

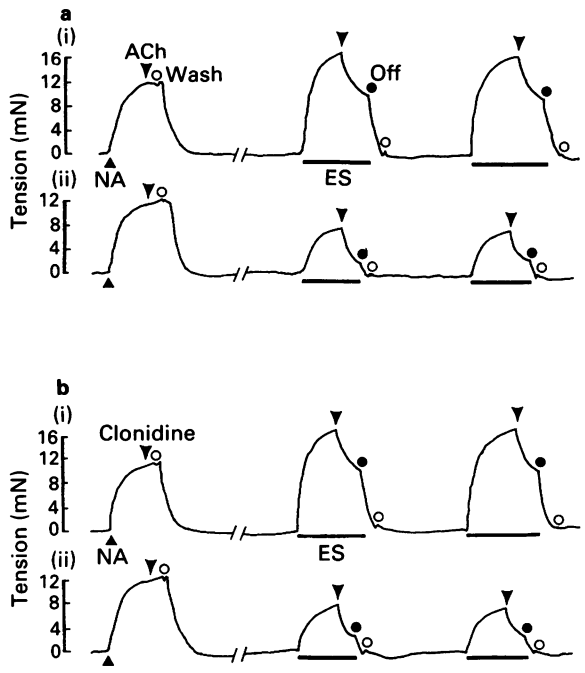

Figure 6 Recordings showing the effect of acetylcholine (ACh, 10^{-5} M) and clonidine (10^{-5} M) (b) on noradrenaline (NA, 10^{-6} M)- and electrical stimulation (ES; 16 Hz, 5 min)-induced contractions of endothelium-denuded ring preparations isolated from lipopolysaccharide (LPS)-treated rabbits. Mesenteric arteries were isolated 20 h after intravenous treatment with saline (1 ml) (i) or LPS (10 μg dissolved in 1 ml of saline) (ii). Duration of stimulation is illustrated below each response. (a(i)) and (b(i)) represent experiments on two ring preparations from the same vessel of a saline-treated animal; (a(ii)) and (b(ii)) represent experiments on two ring preparations from the same vessel of a LPS-treated animal.

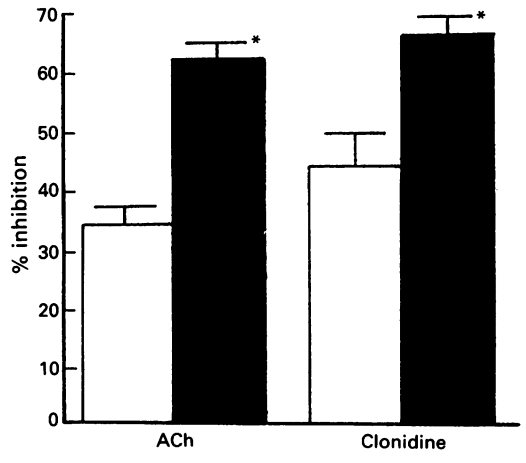

Figure 7 Effects of acetylcholine (ACh, 10^{-5} M) and clonidine (10^{-5} M) on stimulation-induced (16 Hz, 5 min) contractions of endothelium-denuded ring preparations isolated from lipopolysaccharide (LPS)-treated rabbits. Mesenteric arteries were isolated 20 h after intravenous treatment with saline (1 ml, open columns) or LPS (10 μg dissolved in 1 ml of saline, solid columns). Experimental conditions as in Figure 6. Data are expressed as percentage inhibition of the stimulation-evoked contraction and shown as the mean, vertical lines show s.e.mean ($n = 5$); n refers to the number of rabbits from which the mesenteric artery was taken. $^*P < 0.05$: significantly different from corresponding value for saline-control.

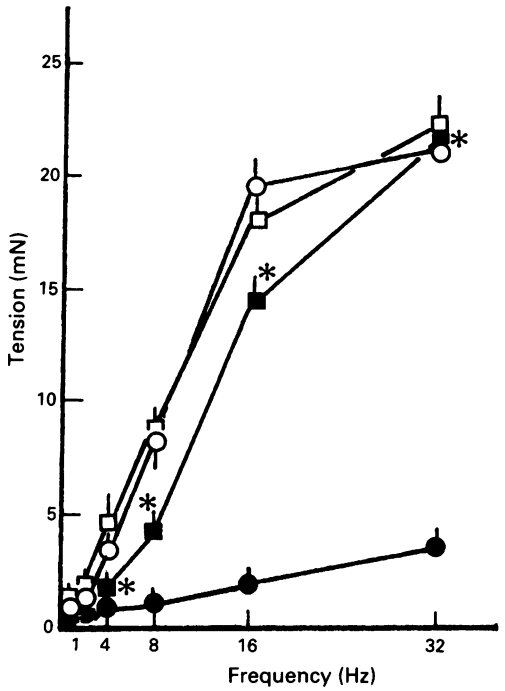

Figure 8 Effect of actinomycin D on the response to electrical stimulation (10 V, 2 ms, 1 to 32 Hz) of endothelium-intact ring preparations isolated from lipopolysaccharide (LPS)-treated rabbits. The experiments were performed on artery rings isolated 20 h after intravenous treatment with LPS (10 μg dissolved in 1 ml of saline) or saline (1 ml). Actinomycin D (80 μg kg^{-1}) was injected intravenously 5 h before LPS or saline. Each point represents the mean, and vertical lines show s.e.mean ($n = 5$); n refers to the number of rabbits from which the mesenteric artery was taken: (○) saline; (●) LPS; (□) actinomycin D + saline; (■) actinomycin D + LPS. $^*P < 0.05$: significantly different from corresponding value for LPS alone.

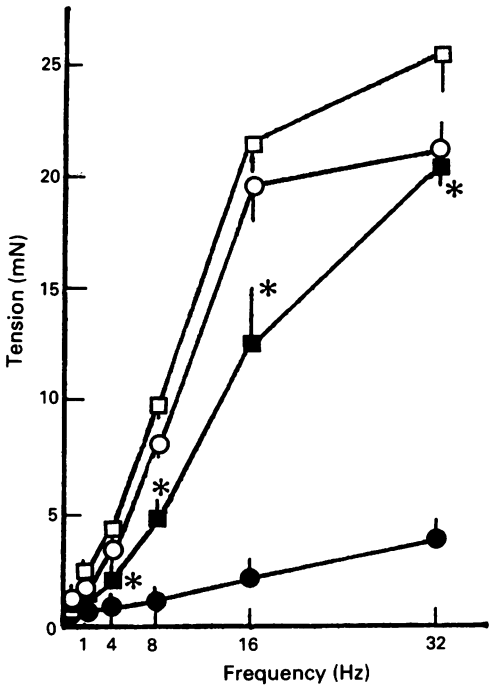

Figure 9 Effect of cycloheximide on the reponse to electrical stimulation (10 V, 2 ms, 1 to 32 Hz) of endothelium-intact ring preparations isolated from lipopolysaccharide (LPS)-treated rabbits. The experiments were performed on artery rings isolated 20 h after intravenous treatment with LPS (10 μg dissolved in 1 ml of saline) or saline (1 ml). Cycloheximide (312 μg kg^{-1}) was injected intravenously 5 h before LPS or saline. Each point represents the mean, and vertical lines show s.e.mean ($n = 5$); n refers to the number of rabbits from which the mesenteric artery was taken: (○) saline; (●) LPS; (□) cycloheximide + saline; (■) cycloheximide + LPS. $^*P < 0.05$: significantly different from corresponding value for LPS alone.

(data not shown). Thus, the observed effect of LPS is not endothelium-dependent.

The effect of LPS (injected 20 h before the experiment) was greatly reduced by actinomycin D and cycloheximide (Figures 8 and 9), two inhibitors of protein synthesis acting at different levels. Actinomycin D binds to double-helical DNA and thereby blocks the transcription of DNA (Craig, 1973). Cylcoheximide may exert its effect on protein synthesis after amino acid activation or transfer of activated amino acids to tRNA (Ennis & Lubin, 1964; Wettstein *et al.*, 1964). These inhibitors of protein synthesis did not affect stimulation-evoked contractions of ring preparations isolated from saline-treated controls. Therefore, our results are consistent with a requirement for sequential transcriptional and post-transcriptional events in the effect of LPS on the sensitivity of prejunctional muscarinic receptors and α_2-adrenoceptors, although this conclusion must be regarded as speculative at this time.

In addition to the classical transmitters NA and ACh, co-transmitters have been identified in perivascular nerves, such as adenosine 5'-triphosphate (ATP), neuropeptide Y and 5-hydroxytryptamine in sympathetic nerves and vasoactive intestinal peptide in parasympathetic neurones (Kupfermann, 1991). In the rabbit mesenteric artery, von Kügelgen & Starke (1985) suggested that ATP, in addition to NA, mediates sympathetic neuro-effector transmission. We cannot rule out the possibility that pretreatment with LPS reduced the release of both NA and ATP. Our observation perhaps indicates that it is the release NA which is decreasd predominantly: phentolamine and yohimbine reduced neurogenic contractions of rings from saline-treated animals (Figure 5), indicating a marked adrenergic component, whereas phentolamine did not change and yohimbine increased neurogenic contractions after treatment with LPS, indicating that the adrenergic component had almost disappeared.

In conclusion, the results presented here raise the possibility that treatment of rabbits with LPS inhibits NA release from adrenergic nerve endings via increased sensitivity of both prejunctional inhibitory muscarinic receptors and α_2-adrenoceptors in mesenteric arteries. The effect of LPS was observed when the artery rings were prepared from animals receiving LPS 20 h, but not 5 h, before the experiment. The effect of LPS was apparently independent of endothelial cells but linked to protein synthesis. Clearly, our results raise the question of the LPS-induced biological sequence of actinomycin D-sensitive, and cycloheximide-sensitive steps that lead to the activation or sensitization of the prejunctional receptors.

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Changes in purinergic responses of the rabbit isolated central ear artery after chronic electrical stimulation *in vivo*

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1 The effect of chronic (4–16 days) electrical stimulation (5 Hz, 0.3 ms, 4–10 V) of the great auricular nerve *in vivo* on sympathetic cotransmission in the rabbit isolated central ear artery was examined.

2 Chronic stimulation had no significant effect on frequency-dependent (4–60 Hz) neurogenic contractions or contractile responses induced by exogenous noradrenaline (0.1–300 μM).

3 In contrast, contractions induced by exogenous α,β-methylene ATP (10.0 μM) were significantly decreased in preparations from 16-day stimulated animals in comparison with sham-operated, 4-day and 8-day chronically stimulated animal groups.

4 It is concluded that chronic electrical stimulation of nerves supplying the ear artery may lead to the selective alteration of postjunctional P_{2x}-purinoceptor mechanisms, while the effects mediated by postjunctional α_1-adrenoceptors remain unchanged.

Keywords: Rabbit ear artery; chronic electrical stimulation; noradrenaline; sympathetic neurotransmission; purinergic transmission

Introduction

Chronic electrical nerve stimulation is used clinically in the treatment of intractable pain, eye and ear disorders, activation of paralysed skeletal muscles, and more recently for stimulating autonomic nerves to correct bladder control and impotency (Loeb, 1989). Few studies have investigated the effects of chronic electrical stimulation *in vivo* on the local control of the vasculature which is important in terms of how best the procedure can be used to alleviate disorders such as peripheral vascular disease (Tallis *et al.*, 1983). It is known, however, that prolonged electrical stimulation increases tissue blood flow and induces structural changes in the vasculature, including neovascularization (Brown *et al.*, 1976; Cameron *et al.*, 1989; Hudlická *et al.*, 1991). The rabbit isolated central ear artery is a useful model since the great auricular nerve which supplies the artery (Feldberg, 1926) is easily accessible for stimulation *in vivo*. The preparation is also known to show structural and functional changes after chronic activity (Bevan & Tsuru, 1979; Fronek & Alexander, 1986; Tsuru, 1990).

In the present study, changes in the neurogenic contractions and contractions of the ear artery induced by noradrenaline (NA) and α,β-methylene ATP (α,β-MeATP), a stable and potent analogue of ATP which acts as a cotransmitter with NA in this preparation (Kennedy *et al.*, 1986; Saville & Burnstock, 1988), were compared following 4, 8 and 16 days of electrical stimulation of the great auricular nerve and in sham-operated animals.

Methods

Experiments were performed on adult (4–6 months) male New Zealand White rabbits (2.5–3.0 kg).

Procedure for chronic electrical stimulation

Animals were anaesthetized with Hypnorm (fentanyl citrate (0.09 mg kg^{-1}) and fluanisone (3 mg kg^{-1}), injected i.p. collectively, Janssen Pharmaceuticals, U.K., 0.3 mg kg^{-1}, i.p.) and Hypnovel (midazolam chloride, up to 2 mg kg^{-1}, i.p., Roche Products, U.K.). Additional intramuscular injections of 0.4 mg Hypnorm were given every 30–40 min as necessary.

Two multistranded, stainless steel electrodes were sutured 2–3 mm from either side of, and parallel to, the right great auricular nerve. The length of the insulation-free part of the electrodes was approximately 1–2 mm. The teflon-coated, silicone-insulated stainless steel wires were run subcutaneously and attached to a silicone-coated external plug which was sutured onto the back of the animal. All animals were given approximately 3 mg kg^{-1} gentamycin intramuscularly and allowed to recover.

Stimulation began not earlier than 48 h after postoperative recovery. Continuous electrical stimulation parameters of 5 Hz, 0.3 ms pulse width, 4–10 V with a Grass SD9 stimulator were given for 6–12 h each day for 4, 8 and 16 days with rest periods at weekends. Animals had freedom of movement, were given food and water *ad libitum* and monitored hourly to ensure that stimulation parameters were not inducing undue stress during periods of stimulation. Upon the initial onset of stimulation, most animals either intermittently shook their heads from side-to-side or stroked the stimulated ear for approximately 1–2 min. Thereafter the animals appeared unconcerned. A few animals initially attempted to disconnect themselves but they subsequently adapted to the chronic stimulation. None of the animals showed signs of discomfort during the course of the experiments. Conditions of these experiments were examined at depth and applied under the current Animals (Scientific Procedures) Act, 1986, UK. Sham-operated control animals had electrodes implanted and were attached to the stimulator, but not stimulated, for an 8-day period.

Animals were killed the day after the last period of stimulation with an overdose of sodium pentobarbitone followed by exsanguination. The ipsilateral (stimulated) and

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contralateral (non-stimulated) proximal segments of the rabbit central ear artery were isolated, cleaned of connective tissue and prepared for *in vitro* organ bath pharmacological investigations as previously described (Bevan & Osher, 1972).

Pharmacology

Experiments were conducted as outlined by Maynard *et al.* (1991). Briefly, proximal isolated ring segments (4 mm), two rings from each ear of the rabbit (i.e. ipsi- and contralateral ears), were bathed in modified Krebs solution in organ baths under isometric conditions with a resting tension of 0.5–1.0 g for at least 1 h. The Krebs solution was composed of the following (mM): NaCl 133, KCl 4.7, $NaHPO_4$ 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, $CaCl_2$ 2.52 and glucose 7.8 and was gassed with 95% O_2 and 5% CO_2 and maintained at 37°C. Electrical field stimulation was provided by a Grass SD9 stimulator and carried out with two platinum wire electrodes placed parallel to, and on either side of, the rabbit ear artery rings. The stimulator was set at supramaximal voltage (60 V), 0.3 ms pulse width for 1 s train duration at 4, 8, 16, 30 and 60 Hz. Responses to electrical field stimulation were elicited and recorded at 2 min intervals, and were abolished in the presence of tetrodotoxin (1 μM) which confirms that the contractions elicited were neurogenic and not due to direct muscle stimulation. Cumulative concentration-response curves were established for noradrenaline (0.1–300 μM), whilst α,β-MeATP (0.5, 1.0 and 10 μM) was added as single applications 45 min apart to avoid desensitization of the P_{2x}-purinoceptors. The latter drug was washed out once a maximum response was established; consequently exposure time was approximately 1–2 min. Each rabbit central ear artery ring was investigated by electrical field stimulation to obtain frequency-response data and responses to exogenous noradrenaline and α,β-MeATP in that order. There was a minimum of 20 min and three changes of Krebs solution between each series of contractions (e.g., between neurogenic contractions and noradrenaline-induced contractions). Potassium chloride (120 mM) was added to each preparation at the end of the experimental protocol as a control for the contractile capacity of the vascular smooth muscle in each preparation.

Drugs

Salts for Krebs solution, (−)-noradrenaline bitartrate (NA), α,β-MeATP and tetrodotoxin were obtained from Sigma Chemical Company, Poole, Dorset. NA (0.1 M) in 100 μM ascorbic acid was made up each day and diluted in distilled water. All other drugs were salts made in aqueous solution.

Expression of results and statistical methods

Throughout this study n refers to the number of animals investigated. Data from each animal ($n = 1$) were calculated as the average from two preparations per animal. All results are expressed as mean ± standard error of the mean (s.e.mean). All contractile responses are expressed as a percentage of the response induced by KCl (120 mM) in the same preparation. pD_2 values were calculated as $-\log[NA]$ which produced 50% of the maximal response. Statistical significance was determined by analysis of variance followed by Tukey's statistic where appropriate, using the Crunch Statistical Package (Crunch Software Corporation, California, 1987). A probability level of $P < 0.05$ was considered significant.

Results

KCl- (120 mM) induced contractions did not differ either between groups of animals, or between the ipsi- versus contralateral side within any one animal group. In sham-operated (control) animals. KCl (120 mM)-induced contractions amounted to 2.7 ± 0.4 g ($n = 4$). Since data obtained from ipsi- and contralateral preparations from sham-operated animals were not significantly different, the values were pooled (Tables 1, 2; Figure 1).

Frequency-response data

Electrical field stimulation of the perivascular nerves of the rabbit central ear artery preparations at basal tone elicited frequency-dependent (4–60 Hz) contractions which were blocked by tetrodotoxin (1 μM). There were no significant differences either across all the animal groups, or between ipsilateral and contralateral sides within each group investigated (Table 1).

Responses to exogenous noradrenaline

Cumulative application of exogenous NA (0.1–300 μM) induced concentration-dependent contractile responses in isolated ear artery preparations. These responses were not significantly different in their pD_2 values or maximal responsiveness, either across all the groups or between ipsi- and contralateral sides within each group investigated (Table 2).

Table 1 Neurogenic contractions of the rabbit central ear artery induced by electrical field stimulation (60 V, 0.3 ms pulse width, 1.0 s train duration)

	Frequency (Hz)				
	4	8	16	30	60
Sham	8.0 ± 3.5	14.7 ± 3.8	39.5 ± 8.8 (4)	70.0 ± 6.2	98.9 ± 11.1
4 Days ipsilateral	0.6 ± 0.6	7.4 ± 1.0	29.0 ± 4.4 (4)	64.3 ± 13.6	97.9 ± 19.6
Contralateral	0.2 ± 0.2	6.2 ± 2.7	24.6 ± 7.3	54.0 ± 6.4	84.4 ± 11.2
8 Days ipsilateral	3.9 ± 1.9	14.7 ± 5.3	29.3 ± 8.2 (6)	45.9 ± 10.0	65.3 ± 11.4
Contralateral	8.6 ± 4.1	17.9 ± 7.8	37.1 ± 10.5	57.5 ± 9.9	78.8 ± 9.7
16 Days ipsilateral	1.4 ± 1.4	17.2 ± 6.2	40.0 ± 8.9 (4)	60.5 ± 10.4	72.4 ± 11.6
Contralateral	3.3 ± 1.1	23.4 ± 4.3	48.4 ± 7.2	72.5 ± 8.9	87.8 ± 9.5

Preparations from sham-operated animals and animals subjected to chronic electrical stimulation of the great auricular nerve were examined. Unit of measurement is % of the response to KCl (120 mM) and data are expressed as the mean ± s.e.mean. There is no significant difference either across all the groups at each frequency examined, or between ipsi- (stimulated) and contralateral (not stimulated) sides within each group. The number in parentheses refers to the number of animals studied.

Table 2 pD_2 values and maximum contractile responses induced by exogenous noradrenaline (0.1–300 μM) added cumulatively to rabbit isolated ear artery preparations

	pD_2 values	(n)	*Noradrenaline maximum response* (% of responses to 120 mM KCl)
Sham	6.4 ± 0.2	4	161.5 ± 4.6
4 Days ipsilateral	6.5 ± 0.3	3	157.3 ± 4.6
Contralateral	6.3 ± 0.2	3	183.3 ± 36.5
8 Days ipsilateral	6.2 ± 0.2	6	165.2 ± 15.9
Contralateral	6.6 ± 0.1	6	155.9 ± 6.0
16 Days ipsilateral	6.9 ± 0.4	4	140.6 ± 9.1
Contralateral	6.9 ± 0.2	4	144.3 ± 9.6

Preparations from sham-operated animals and animals subjected to chronic electrical stimulation of the great auricular nerve were examined. Values represent mean ± s.e.mean, where *n* is the number of animals. There was no statistical significance either across all the groups investigated using either parameter, or between ipsi- and contralateral segments within each group.

Responses to exogenous α,β-methylene ATP

A single addition of α,β-MeATP (0.5, 0.1 or 10 μM) to preparations at resting tone produced a rapid concentration-dependent contraction that was not maintained. There was a tendency towards an increase in contractile responses to exogenous α,β-MeATP at all three concentrations examined in preparations from animals 4 and 8 days after chronic electrical stimulation *in vivo*, in comparison with sham-operated animals. These increases, however, were not significantly different. There were also no significant differences between ipsilateral and contralateral sides across any of the animal groups. There was, however, a significant reduction in the contractile responses induced by α,β-MeATP (10 μM) in preparations fom 16-day chronically stimulated animals. Contractile responses in both ipsilateral and contralateral segments in these animals were significantly reduced in comparison with preparations from corrersponding sides of 8-day chronically stimulated animals. In addition, contractile responses induced by α,β-MeATP (10 μM) on the contralateral preparations of 16-day chronically stimulated animals were also significantly attenuated in comparison with the comparable side from 4-day stimulated animals and sham-operated animals (Figure 1).

Discussion

It is evident from this study that chronic electrical stimulation of the great auricular nerve, which supplies the rabbit central ear artery with sympathetic perivascular nerves (Feldberg, 1926), does not induce changes in frequency-dependent neurogenic contractions in rabbit isolated ear artery ring preparations. This suggests that there were no changes in the prejunctional and/or postjunctional events during sympathetic cotransmission. Since, however, neurogenic contractions in the ear artery have both noradrenergic and purinergic components (Kennedy *et al.*, 1986; Saville & Burnstock, 1988), responses to exogenous NA and α,β-MeATP were also examined to see whether there were any postjunctional receptor-related changes.

The findings from exogenous cumulative concentration-response curves to NA indicate that there were no changes in either the sensitivity, represented by the pD_2 values, or the maximal responsiveness to exogenous NA. We therefore concluded that postjunctinal α_1-adrenoceptors, known to mediate the noradrenergic component of vasoconstriction in the rabbit central ear artery (Kennedy *et al.*, 1986) were unaffected by chronic electrical stimulation.

Although there were significant differences in the responses to exogenous α,β-MeATP only at the highest concentration, these trends were apparent at all the concentrations

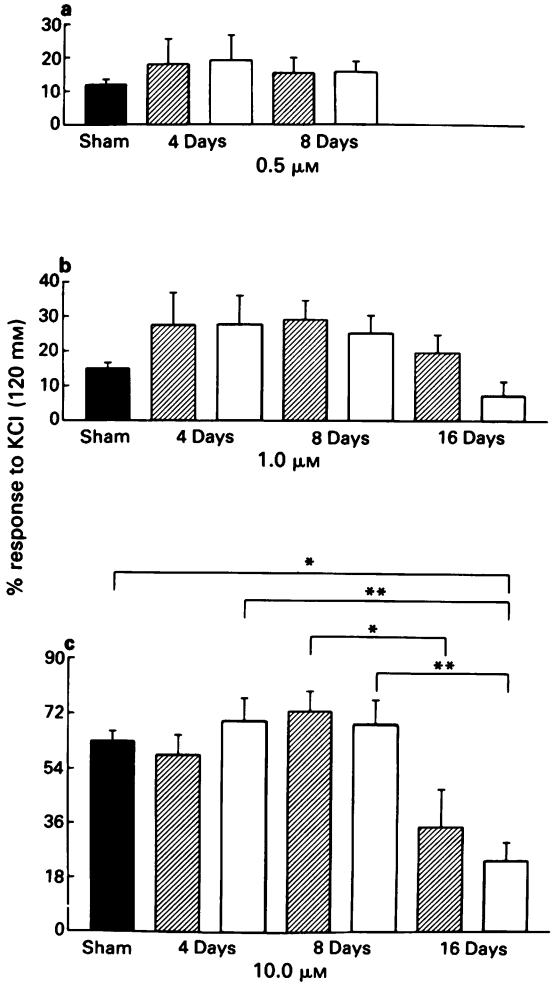

Figure 1 Contractions induced by α,β-methylene ATP (0.5 (a), 1.0 (b) and 10.0 μM (c)) on rabbit isolated central ear artery preparations from sham-operated animals ($n = 4$) and animals subjected to chronic electrical stimulation of the great auricular nerve for 4 ($n = 4$), 8 ($n = 6$) and 16 ($n = 4$) days. Both ipsilateral (stimulated preparations represented by hatched columns) and contralateral (unstimulated preparations represented by open columns) sides are compared with sham-operated control animals (solid columns). Responses were calculated as a percentage of the response to KCl (120 mM) for each preparation and are represented as the mean (± s.e.mean, vertical bars). Statistical analysis was performed using analysis of variance with Tukey's statistic where necessary. $*P < 0.05$; $**P < 0.01$.

examined. The reduction in responses after 16 days of electrical stimulation in comparison with all other groups suggests that postjunctional P_{2x}-purinoceptors may have been down-regulated in this group of animals. Down-regulation is likely to have occurred due to the increased sympathetic acivity (Finberg & Kopin, 1986) and release of sympathetic cotransmitters NA and ATP produced by chronic electrical nerve stimulation. An alternative explanation for the reduction in responses to exogenous α,β-MeATP is that there may have been changes in the P_{2x}-purinoceptor pharmacomechanical coupling mechanism.

Why postjunctional P_{2x}-purinoceptors appear to be selectively affected in this study is unclear. It has been reported however, that repetitive ionophoretic application of ATP results in a decrease in the amplitude of the ATP response in the rabbit ear artery (Suzuki, 1985). In addition, purine nucleosides and nucleotides interact with normal and subsensitive α-adrenoceptors to promote a more rapid rate of α-adrenoceptor resensitization (Holck & Marks, 1978). Taken together, these findings might explain why, particularly after chronic electrical stimulation, contractions to exogenous α,β-MeATP were reduced, yet neurogenic contractile responses, predominantly mediated by α_1-adrenoceptors except at low frequencies (Kennedy *et al.*, 1986), were unchanged in our study.

The reduction of α,β-MeATP-induced responses in the unstimulated contralateral as well as the stimulated ipsilateral ear preparations may have occurred since stimulation of the great auricular nerve is likely to have increased neural activity both ortho- and antidromically in sensory and motor nerves comprising the great auricular nerve (Feldberg, 1926). Consequently, the increased activity may have been transmitted centrally. Indeed, during electrical stimulation, both ipsi- and contralateral ears of some rabbits were occasionally seen to exhibit motor responses consistent with the frequency of stimulation.

We have previously shown that when sympthetic cotransmission is impaired, such as after a single massive dose of x-irradiation (Stewart-Lee *et al.*, 1991) or chronic acrylamide treatment (Maynard *et al.*, 1991), responses to exogenous ATP are usually increased. In this procedure, the opposite effect is observed, i.e. there is increased sympathetic activity which appears to attenuate responses to exogenous ATP. Whether there is a basic underlying mechanism involving the purinergic and noradrenergic components of sympathetic cotransmission after manipulation or injury requires further investigation. Regardless, this study supports the hypothesis that postjunctionally mediated responses to exogenous ATP are more sensitive and selectively affected initially, in contrast to noradrenergic responses which may be affected later under pathophysiological conditions (p.11 of Stjärne, 1989; Burnstock, 1990; Maynard *et al.*, 1991; Stewart-Lee *et al.*, 1991).

It is reported that transcutaneous electrical nerve stimulation reduces sympathetic tone in man (Owens *et al.*, 1979), and it has been suggested that increased physiological nerve impulses might result in a selective desensitization of α_1-postjunctional receptors (Finberg & Kopin, 1986). Our results show that chronic electrical stimulation selectively impairs postjunctional P_{2x}-purinoceptor-mediated responses. The mechanism by which this occurs has yet to be determined.

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A possible role of the L-arginine-nitric oxide pathway in the modulation of cholinergic transmission in the guinea-pig taenia coli

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1 The role of the L-arginine-nitric oxide (NO) pathway for non-adrenergic, non-cholinergic (NANC) relaxation of the guinea-pig taenia coli was studied by recording isometric tension in response to transmural field stimulation (TMS).

2 In preparations precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10^{-6} M), TMS induced frequency-dependent responses of the muscle strips which could be abolished by tetrodotoxin (10^{-6} M). N^G-nitro-L-arginine (L-NNA, 10^{-4} M), an L-arginine analogue, and potent inhibitor of NO synthesis, stereospecifically inhibited maximum relaxations, but did not shift the frequency-response curve. Preincubation with N^G-nitro-D-arginine (D-NNA, 10^{-4} M), atropine (10^{-6} M) plus L-NNA (10^{-4} M), or atropine (10^{-6} M) alone, had no influence on the frequency-response characteristics.

3 L-NNA (10^{-7}–10^{-4} M) concentration-dependently inhibited relaxations in $PGF_{2\alpha}$ (10^{-6} M) precontracted strips in response to TMS, but did not abolish relaxations. Preincubation with L-arginine (10^{-4} M) inhibited these effects of L-NNA. L-NNA (10^{-4} M) had no effect on the inhibitory response during TMS in strips preincubated with atropine (10^{-6} M).

4 The relaxation induced by sodium nitroprusside and forskolin (10^{-9}–10^{-4} M) was not influenced by L-NNA (10^{-4} M) preincubation as expressed by identical pD_2 and E_{max} values.

5 Contractions induced by $PGF_{2\alpha}$ (10^{-9}–10^{-4} M) and carbachol (10^{-9}–10^{-4} M) were not affected by pretreatment with L-NNA (10^{-4} M), as expressed by identical pD_2 and E_{max} values.

6 In conclusion, the L-arginine-NO pathway seems to play a role in the NANC innervation of the guinea-pig taenia coli. The inhibitory effect of NO or a NO-like compound depends on the integrity of the cholinergic pathways and it is proposed that this compound exerts its effects prejunctionally on cholinergic nerves, by inhibiting the release of acetylcholine.

Keywords: NANC nerves; ATP; nitric oxide; guinea-pig taenia coli; cholinergic nerves; prejunctional inhibition; smooth muscle relaxation; L-arginine metabolism

Introduction

The guinea-pig taenia coli has a prominent non-adrenergic, non-cholinergic (NANC) inhibitory innervation (Burnstock *et al.*, 1966). Although the NANC inhibitory transmitter in the taenia coli has not been identified with certainty, a substantial amount of evidence seems to favour adenosine 5'-triphosphate (ATP) as a candidate, while neuropeptides such as vasoactive intestinal polypeptide (VIP) appear less likely (for review, see White, 1988). Recent studies have established that nitric oxide (NO) or a NO-like substance could be the major or the sole mediator of NANC-mediated inhibition in certain tissues (Gillespie *et al.*, 1989; Li & Rand, 1989; Kundsen *et al.*, 1991; Tøttrup *et al.*, 1991a,b; 1992), but the exact role of NO in the innervation of the guinea-pig taenia coli is unknown.

NO is synthesized from L-arginine, a process that is inhibited by N^G-nitro-L-arginine (L-NNA) and other L-arginine analogues (Hobbs & Gibson, 1990). Interestingly, it has been suggested that NO modulates cholinergic transmission in the guinea-pig trachea (Belvisi *et al.*, 1990). Thus, the present study was designed to investigate the possible role of the L-arginine-NO pathway for neurogenic responses in the guinea-pig taenia coli.

Methods

Guinea-pigs of either sex weighing 450–1270 g ($n = 18$) were killed by cervical dislocation. the taenia coli was dissected from the caecum, and six to eight muscle strips from each animal measuring 5–6 mm in length and 1–2 mm in diameter were prepared. Silk ligatures were tied to each end of the strips with a distance of 2–3 mm between the knots, and these were then transferred to thermostatically controlled (37.0 ± 0.5°C) 5 ml organ baths containing Krebs solution (for composition, see below). The pH of the organ bath fluid was kept constant at 7.40 ± 0.05 by bubbling the Krebs solution with 95% O_2 and 5% CO_2. The strips were mounted between two small L-shaped hooks of which one was connected to a force transducer (Grass FT.03) for measurement of isometric tension, and the other to a sledge, which enabled adjustments of the length of the strips. The distance between the two hooks was gradually increased over a period of about 20 min until a resting tension of 2 mN was reached. This resulted in an elongation of approximately 150% of resting length. Tension was registered on a six-channel Grass Polygraph (model 7C). During the subsequent equilibration period lasting at least 1 h, the muscle strips developed a certain amount of phasic activity varying substantially from strip to strip, but generally declining with time, and after a single exposure to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10^{-6} M). Between each experimental series, the Krebs solution was renewed every 20 min.

Response to field stimulation (TMS)

After the equilibration period, $PGF_{2\alpha}$ (10^{-6} M) was added to the baths to induce contraction of the muscle strips. Strips which could not sustain a tonic contraction after exposure to $PGF_{2\alpha}$ (10^{-6} M), were excluded from further study. Trans-

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mural field stimulation (TMS; Grass S48 stimulator) was applied to the strips through platinum wire electrodes placed parallel on each side of the strip at a distance of 1 mm from the muscle. Five second trains of impulses were sent every 2 min (0.4 ms impulse duration, frequency of 10 Hz, and supramaximal voltage). Transmural field stimulation (TMS) induced rapid relaxations beginning shortly after the start of stimulation, and lasting the duration of the stimulus. After cessation of field stimulation, the level of tonic tension was promptly regained. The effects of drugs, N^G-nitro-L-arginine (L-NNA, 10^{-7}–10^{-4} M), and of L-NNA (10^{-7}–10^{-4} M) either preincubated with atropine (10^{-6} M) or L-arginine (10^{-4} M) on the TMS-induced relaxations were registered. In other experiments, using 5 s trains of impulses (0.4 ms impulse duration, and supra-maximal voltage), the response to variations in the stimulus frequency (0.1–40 Hz, alternating between high and low frequency) was examined. The following drugs were applied: L-NNA (10^{-4} M); N^G-nitro-D-arginine (D-NNA, 10^{-4} M); atropine (10^{-6} M); and atropine + L-NNA (10^{-6} M and 10^{-4} M, respectively). Passive tension level was determined after each experiment by adding either sodium nitroprusside (SNP) 10^{-5} M to the baths, or by replacing the ordinary Krebs solution with a calcium-free solution.

Response to drugs

Drugs i.e. L-NNA, sodium nitroprusside (SNP), carbamoylcholine chloride (carbachol) and forskolin were administered to the baths in cumulatively increasing concentrations (all concentrations given are final bath concentrations). After at least 10 min, and when the responses to TMS were stable (<5% variation between three successive stimualtions), the effects of the drug were considered to be optimal and the next dose of the drug was then added to the baths. The following investigations were performed: (1) Effects of drugs in response to TMS (at 10 Hz, and at varying frequency), as described above; (2) drug concentration-response on precontracted muscle strips (forskolin and SNP (10^{-9}–10^{-4} M), with and without preincubation with L-NNA, 10^{-4} M); and (3) drug concentration-response in resting muscle strips ($PGF_{2\alpha}$ and carbachol, 10^{-9}–10^{-4} M, with and without preincubation with L-NNA, 10^{-4} M).

Calculations of pD_2 and EC_{50}

In experiments, where the maximum effect (E_{max}) of the drug studied could be determined, the concentration (EC_{50}) producing half-maximum response ($\frac{1}{2}E_{max}$) was assessed by linear interpolation on the semilogarithmic concentration-response curve and was expressed as $pD_2 = -\log(EC_{50})$. E_{max} is expressed as the maximum response of a drug in each muscle strip, and is given as the percentage of the $PGF_{2\alpha}$-induced tonic contraction.

Calculations of log (F_{50}) and F_{50} values

In experiments, where maximum relaxation (E_{max}) to TMS was determinable, calculation of the frequency (F_{50} (Hz)) inducing half-maximum response ($\frac{1}{2}E_{max}$) was made by linear interpolation on the semilogarithmic frequency-response curve, and was expressed as log (F_{50}). E_{max} expresses the maximum relaxation during TMS in each muscle strip, and is given as a percentage of the $PGF_{2\alpha}$-induced tonic contraction.

Composition of solutions (mM)

Krebs solution: NaCl 119, KCl 4.6, $NaHCO_3$ 15, $CaCl_2$ 1.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2 and glucose 11. Calcium-free solution: NaCl 119, KCl 4.6, $NaHCO_3$ 15, $MgCl_2$ 1.2, NaH_2PO_4 1.2, glucose 11, and 0.01 ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetracetate (EGTA).

Drugs

N^G-nitro-L-arginine (L-NNA; Sigma); N^G-nitro-D-arginine (D-NNA; Sigma); sodium nitroprusside (SNP; Sigma); forskolin (Sigma); carbamoylcholine chloride (carbachol; Sigma); tetrodotoxin (TTX; Sigma); atropine sulphate (atropine; Danish Pharmacy Laboratories, DAK), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Upjohn) were used. Forskolin was initially dissolved in ethanol to give a stock solution of 10^{-3} M. Subsequent dilutions of forskolin and all other dilutions were made in 0.9% NaCl containing 1.0 mM ascorbic acid.

Statistical analyses

Amplitudes of the responses to TMS were measured as the difference between the level of tonic contraction to the peak of either the relaxation or the contraction. In frequency-response experiments and in concentration-response experiments where TMS was applied, control responses were the responses elicited before incubation with drugs. In concentration-response experiments where TMS was not applied, the control strips were time matched preparations. Values are given as a percentage of the $PGF_{2\alpha}$-induced tonic contraction. All data are expressed as mean ± s.e. Differences between mean values were compared by one way analysis of variance (ANOVA), and subsequently, P values were determined by an unpaired Student's t test corrected for multiple comparisons by Bonferroni's method. In experiments where maximum effects of the drugs could not be determined, the areas under the curves were measured (values are given in square (sq) units), and differences in mean values were compared using an unpaired Student's t test (Matthews *et al.*, 1990). The threshold level for statistical significance was 0.05.

Results

$PGF_{2\alpha}$ (10^{-6} M) was added to the baths to induce contraction of the muscle strips, as described previously. $PGF_{2\alpha}$ (10^{-6} M) and also carbachol (10^{-6} M) induced a rapid phasic contraction of the muscle strips followed by a tonic contraction that lasted until the Krebs solution was renewed.

Effects of TMS

Transmural field stimulation (TMS) induced frequency-dependent responses of the muscle strips. These responses were abolished by TTX (10^{-6} M; 74.2 ± 13.0% before and 0% after TTX, respectively, 10 Hz; $n = 3$). Maximum relaxation was reached at 8 Hz (76.6 ± 4.2%, $n = 7$). Subsequently, responses gradually declined, and at 40 Hz, contractions were seen in 4 out of 7 muscle strips (Figure 1). L-NNA (10^{-4} M) inhibited maximum relaxations (E_{max}), but did not shift the frequency-response curve as indicated by identical log F_{50} values (Table 1). Two out of 6 strips preincubated with L-NNA (10^{-4} M) contracted at 40 Hz. Preincubation with D-NNA (10^{-4} M), atropine (10^{-6} M) + L-NNA (10^{-4} M), or atropine (10^{-6} M) alone, had no influence on maximum relaxations compared to the control strips, and there was no shift of the frequency-response curves in any of the cases, as illustrated by identical log F_{50} and E_{max} values (Table 1). Contractions were not elicited at any frequency in the presence of atropine (Figure 1).

Effects of drugs

L-NNA inhibited the relaxation in response to TMS (10 Hz, 0.4 ms impulse duration, supramaximal voltage) in a concentration-dependent manner, from 73.7 ± 3.9% to 20.5 ± 6.8% ($n = 18$) with the highest concentration of L-NNA (10^{-4} M; $P<0.0001$; Figures 2 and 3). The threshold concentration for this effect was 3×10^{-6} M. The substrate for the L-arginine-NO pathway, L-arginine (10^{-4} M) inhibited the

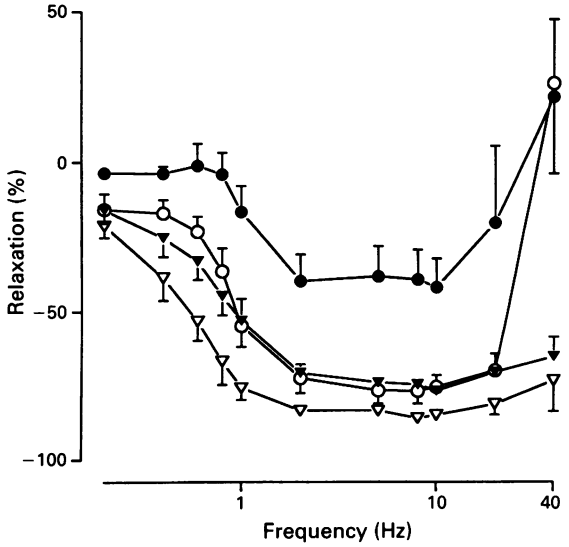

Figure 1 Effect of N^G-nitro-L-arginine (L-NNA, 10^{-4} M; ●), atropine (10^{-6} M; ▽), L-NNA + atropine (10^{-4} M and 10^{-6} M, respectively; ▼), and control (○) on response to transmural field stimulation (TMS) in the guinea-pig taenia coli. Five second trains of impulses (0.4 ms pulse duration; supramaximal voltage) were sent every 2 min with varying frequency. Relaxations are given as a percentage of the level of tonic contraction induced by prostaglandin $F_{2\alpha}$ (10^{-6} M), and values are means with s.e.mean (vertical bars) indicated. $n = 6-7$. Note that the x-scale is logarithmic.

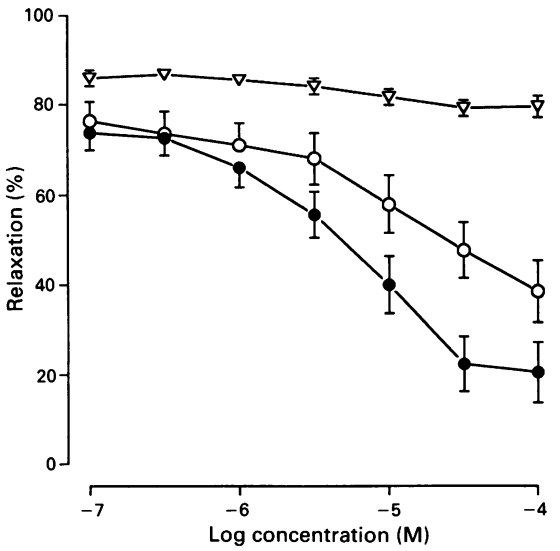

Figure 2 Effect of N^G-nitro-L-arginine (L-NNA, $10^{-7}-10^{-4}$ M; ●), L-NNA ($10^{-7}-10^{-4}$ M) in strips preincubated with L-arginine (10^{-4} M; ○), and L-NNA ($10^{-7}-10^{-4}$ M) in strips preincubated with atropine (10^{-6} M; ▽) on response to transmural field stimulation (TMS) in the guinea-pig taenia coli. Five second trains of impulses (0.4 ms impulse duration; 10 Hz; supramaximal voltage) were sent every 2 min. Relaxations are given as a percentage of the level of tonic contraction induced by prostaglandin $F_{2\alpha}$ (10^{-6} M) and values are means with s.e. mean (vertical bars) indicated. $n = 9-18$.

effect of L-NNA as indicated by an increase in the area under the curve from 50.3 ± 8.4 sq units ($n = 18$) to 79.6 ± 8.8 sq units ($n = 9$; $P < 0.02$) in strips preincubated with L-arginine (Figure 2). The threshold concentration for this effect was 3×10^{-6} M. L-NNA did not influence relaxations when strips were preincubated with atropine (10^{-6} M; $85.9 \pm 1.8\%$ and $79.7 \pm 2.4\%$ ($n = 9$) before and after L-NNA 10^{-4} M; Figures 2 and 3). Sodium nitroprusside (SNP) elicited complete, and practically instantaneous relaxation of the muscle strips at concentrations $> 10^{-6}$ M, and L-NNA preincubation had no effect on this response as expressed by identical E_{max} and pD_2 values (Table 2). Forskolin also caused complete relaxation of the muscle strips, albeit this took place gradually over a period of time, i.e. a new stable value was typically reached 10 min after increasing drug concentration. The E_{max} and pD_2 values for the concentration-response curve were unaffected by the presence of L-NNA (Table 2). Carbachol and $PGF_{2\alpha}$ concentration-dependently produced a tonic contraction of the muscle strips. L-NNA preincubation had no influence on the carbachol- and $PGF_{2\alpha}$-induced tone, as expressed by identical E_{max} and pD_2 values (Table 2). $PGF_{2\alpha}$ 10^{-6} M resulted in a tonic contraction of approximately 68% of the maximal response to this drug.

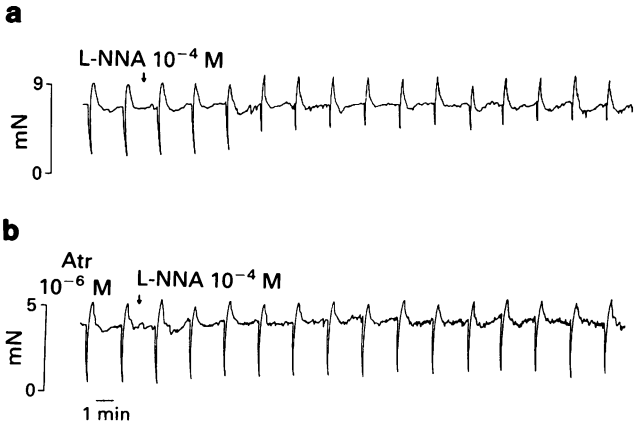

Figure 3 Effect of N^G-nitro-L-arginine (L-NNA, 10^{-4} M; a), and L-NNA (10^{-4} M) preincubated with atropine (10^{-6} M; b) on responses to transmural field stimulation (TMS) in the guinea-pig taenia coli. Five second trains of impulses (0.4 ms impulse duration; 10 Hz; supramaximal voltage) were applied every 2 min.

Table 1 Responses to N^G-nitro-L-arginine (L-NNA), N^G-nitro-D-arginine (D-NNA), atropine and atropine + L-NNA during transmural field stimulation (TMS)

	n	E_{max} (%)	log (F_{50})	F_{50} (Hz)
Control	7	79.9 ± 3.4	-0.10 ± 0.05	0.84
L-NNA[a]	6	45.8 ± 8.1*	0.11 ± 0.05	1.35
D-NNA[a]	4	69.2 ± 8.6	0.13 ± 0.08	1.42
Atropine[b]	6	87.6 ± 2.0	-0.31 ± 0.06	0.52
Atropine[b] + L-NNA[a]	7	77.4 ± 1.9	-0.23 ± 0.12	0.71

[a]: 10^{-4} M; [b]: 10^{-6} M; *$P < 0.001$.

The responses of the control strips were the responses elicited before incubation with drugs. E_{max} expresses the maximal relaxation during TMS in each muscle strip, and is given as a percentage of prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$, 10^{-6} M)-induced tonic contraction. Calculation of the frequency (F_{50} (Hz)) inducing half-maximum response ($\frac{1}{2}E_{max}$) was made by linear interpolation on the semilogarithmic frequency-response curve, and was expressed as log (F_{50}).

Table 2 Responses to sodium nitroprusside (SNP), forskolin, carbachol, and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)

	n	E_{max} (%)	pD_2	EC_{50} (M)
SNP				
Control	7	100 ± 0	7.50 ± 0.17	4.71×10^{-8}
L-NNA (10^{-4} M)	7	100 ± 0	7.31 ± 0.10	5.34×10^{-8}
Forskolin				
Control	7	100 ± 0	6.78 ± 0.09	1.87×10^{-7}
L-NNA (10^{-4} M)	7	100 ± 0	6.90 ± 0.09	1.40×10^{-7}
Carbachol				
Control	11	244 ± 21	6.80 ± 0.20	3.37×10^{-7}
L-NNA (10^{-4} M)	9	296 ± 36	6.60 ± 0.10	2.86×10^{-7}
$PGF_{2\alpha}$				
Control	9	109 ± 10	6.4 ± 0.1	6.7×10^{-7}
L-NNA (10^{-4} M)	9	131 ± 13	6.4 ± 0.1	6.2×10^{-7}

The control strips were time-matched preparations. E_{max} expresses the maximal response of the drug in each muscle strip, and is given as a percentage of $PGF_{2\alpha}$-induced tonic contraction. The concentration (EC_{50}) inducing half-maximum response ($\frac{1}{2}E_{max}$) was assessed by linear interpolation on the semilogarithmic concentration-response curve and is expressed as $pD_2 = -\log(EC_{50})$.

Discussion

A substantial amount of evidence has been accumulating favouring ATP as the principal NANC inhibitory neurotransmitter in the guinea-pig taenia coli (Burnstock, 1972; Cocks & Burnstock, 1979; Burnstock, 1979; Mackenzie & Burnstock, 1980; White, 1983; Costa *et al.*, 1986). The electrical- and mechanical response elicited by exogenous ATP and field stimulation are often mutually indistinguishable. Thus, in both cases a hyperpolarization of the smooth muscle membrane is induced together with a rapid, transient relaxation (Bennett *et al.*, 1966; Burnstock, 1979; Cocks & Burnstock, 1979; Hills *et al.*, 1983). The bee venom apamin, a presumed potassium-channel blocker, has been shown to reverse the hyperpolarization evoked by exogenously applied ATP to a depolarization in the taenia coli (Maas & den Hertog, 1979). As the hyperpolarization caused by field stimulation was not blocked completely by apamin (Maas & den Hertog, 1979), the residual hyperpolarization could be due to the existence of an additional neurotransmitter other than ATP. VIP has also been suggested as a neurotransmitter in the guinea-pig taenia coli (Grider *et al.*, 1985), and could be responsible for the apamin-insensitive part of the response to field stimulation (Mackenzie & Burnstock, 1980; Hills *et al.*, 1983; Costa *et al.*, 1986). However, evidence speaks against VIP, as the proteolytic enzyme, chymotrypsin, abolished the responses to exogenous VIP, while the responses to field stimulation and exogenous ATP were unaffected (Mackenzie & Burnstock, 1980).

NO or a NO-like substance seems to be solely responsible for the NANC-mediated neural responses in certain tissues as the responses were blocked by L-arginine analogues (Gillespie *et al.*, 1989; Li & Rand, 1989; Tøttrup *et al.*, 1991a,b; Tøttrup *et al.*, 1992). The possible role of NO in the inhibitory response in the guinea-pig taenia coli has not yet been investigated. The present experiments showed that L-NNA inhibited relaxations in precontracted taenia coli strips in response to field stimulation. Moreover, relaxant responses to forskolin and SNP, stimulators of adenylate and guanylate cyclase, respectively (Seamon *et al.*, 1981; Rapoport & Murad, 1983), were unaffected by L-NNA preincubation, indicating that the inhibitory effect of L-NNA was not due to a direct effect on these enzymes. In this particular tissue, however, we found that the L-arginine-NO pathway apparently plays a less prominent role for relaxation, as L-NNA in the applied concentrations had a more pronounced effect on NANC-mediated relaxations in some other tissues studied (Li & Rand, 1989; Hobbs & Gibson, 1990: Tøttrup *et al.*, 1991a,b; Tøttrup *et al.*, 1992). Further support is added to these considerations, as L-NNA did not shift the frequency-response curve, implying a non-competitive nature of L-NNA on inhibitory responses, and thus, suggesting the action of at least one other inhibitory NANC mediator.

Field stimulation applied to guinea-pig taenia coli is known to excite both inhibitory and excitatory nerves (Burnstock, 1972). Correspondingly, the present results showed that the mechanical response of the smooth muscle to stimulation differed in the presence of atropine as compared to control responses. Thus, in addition to the NANC inhibitory nerves, excitatory cholinergic nerves were stimulated, although contractile responses were only seen at higher frequencies. Interestingly, we found that the inhibitory effect of L-NNA on relaxations was dependent on the integrity of cholinergic transmission, as L-NNA had no effect on relaxations in the presence of atropine. To interpret these results, and a possible mode of action of the NO-like material, the response to field stimulation could be conceived as the sum of actions of the released mediators on the smooth muscle. In terms of relative transmitter influence, L-NNA augmented the effect of the cholinergic transmitter, since relaxations were smaller after L-NNA. This could indicate that the product of the L-arginine-NO pathway normally acts prejunctionally to inhibit acetylcholine release. Alternatively, the release of the NO-like compound could depend on muscarinic receptor stimulation. We found that muscarinic receptor stimulation with carbachol was unaffected by preincubation with L-NNA indicating that muscarinic receptors are not involved in the release of the NO-like compound. This speaks in favour of a prejunctional action of the NO-like substance inhibiting acetylcholine release, in line with a previous suggestion from studies of the trachea (Belvisi *et al.*, 1991). Also, the NO-like substance appears to exert a marked inhibitory influence on acetylcholine release, since addition of atropine alone did not significantly affect the relaxation responses to TMS.

In conclusion, the L-arginine-NO pathway seems to play a role in the complex innervation of the guinea-pig taenia coli. The inhibitory effect of NO is dependent on the integrity of cholinergic transmission, and it is suggested that NO or a NO-like compound exerts its effects prejunctionally by inhibiting the release of acetylcholine. The predominant part of the inhibitory NANC response is not mediated by a NO-like compound synthesized from L-arginine, in contrast to some other NANC innervated tissues.

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Iron-sulphur cluster nitrosyls, a novel class of nitric oxide generator: mechanism of vasodilator action on rat isolated tail artery

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1 Two iron-sulphur cluster nitrosyls have been investigated as potential nitric oxide (NO·) donor drugs (A: tetranitrosyltetra-μ3-sulphidotetrahedro-tetrairon; and B: heptanitrosyltri-μ3-thioxotetraferrate(1-)). Both compounds are shown to dilate precontracted, internally-perfused rat tail arteries.

2 Bolus injections (10 μl) of compound A or B generate two kinds of vasodilator response. Doses below a critical threshold concentration (D_T) evoke transient (or *T*-type) responses, which resemble those seen with conventional nitrovasodilators. Doses $> D_T$ produce sustained (or *S*-type) responses, comprising an initial, rapid drop of pressure, followed by incomplete recovery, resulting in a plateau of reduced tone which can persist for several hours.

3 *T*- and *S*-type responses are attenuated by ferrohaemoglobin (Hb) and by methylene blue (MB), but not by inhibitors of endothelial NO· synthase. Addition of either Hb or MB to the internal perfusate can restore agonist-induced tone when administered during the plateau phase of an *S*-type response. Moreover, subsequent removal of Hb causes the artery to re-dilate fully.

4 We conclude that *T*- and *S*-type responses are both mediated by NO·. It is postulated that *S*-type responses represent the sum of two vasodilator components: a reversible component, superimposed upon a non-recoverable component. The former is attributed to free NO·, preformed in solution at the time of injection; and the latter to NO· generated by gradual decomposition of a 'store' of iron-sulphur-nitrosyl complexes within the tissue. This hypothesis is supported by histochemical studies which show that both clusters accumulate in endothelial cells.

Keywords: Nitric oxide donors; iron-sulphur-nitrosyls; vasodilator responses; nitrovasodilators; endothelium

Introduction

Endothelial cells release a labile factor which relaxes vascular smooth muscle (Furchgott & Zawadski, 1980). Endothelium-derived relaxing factor (or EDRF) has recently been identified as either nitric oxide (NO·; Palmer *et al.*, 1987; Ignarro *et al.*, 1988); a labile nitrosothiol, possibly S-nitroso-cysteine (Myers *et al.*, 1990); or a nitrosyl-iron complex with thiol ligands (Vanin, 1991). NO· is formed from the terminal guanidino nitrogen atom of L-arginine by a citrulline-forming enzyme, referred to as NO synthase (Palmer *et al.*, 1988a,b). Endothelial NO synthase (NOS) is NADPH- (Palmer & Moncada, 1989) and calmodulin-dependent (Bredt & Snyder, 1990). It can be inhibited by several L- (but not D-) analogues of arginine (Rees *et al.*, 1990).

Haemodynamic studies using stereospecific NOS inhibitors have firmly established the importance of NO· in controlling peripheral resistance *in vivo* (Vallance *et al.*, 1989; Aisaka *et al.*, 1989; Rees *et al.*, 1989; Gardiner *et al.*, 1990; Chu *et al.*, 1991). Endothelium-dependent relaxations of vascular smooth muscle are attenuated in some diseased states, notably in hypertension (Winquist *et al.*, 1984; Luscher & Vanhoutte, 1986; Otsuka *et al.*, 1988; Tesfamariam & Halpern, 1988; Sunano *et al.*, 1989) and in atherosclerosis (Harrison *et al.*, 1987; Chappell *et al.*, 1987; Henry *et al.*, 1987; Forstermann *et al.*, 1988, Guerra *et al.*, 1989), though it remains unclear whether these conditions are necessarily associated with impaired synthesis and/or release of NO· (Van der Voorde & Leusen, 1986; Hoeffner & Vanhoutte, 1989; Chester *et al.*, 1990; Jacobs *et al.*, 1990; Fozard & Part, 1991).

Clinical 'nitrovasodilators', including amyl nitrite, glyceryl trinitrate, nitroprusside (NP) and molsidomine, act independently of the endothelium, augmenting its function temporarily by releasing NO· *in vivo* (Waldman & Murad, 1984; Feelisch & Noak, 1987). Their hypotensive actions are generally of short duration and continuous drug infusions are necessary to effect sustained responses (Kreye, 1980). Here we show that two tetrairon-sulphur cluster nitrosyls (compound A: tetranitrosyl-tetra-μ3-sulphidotetrahedro-tetrairon; and compound B: heptanitrosyl-tri-μ3-thioxotetraferrate(1-), also known as Roussin's Black Salt; Roussin, 1858) are able to generate unusually protracted vasodilator responses when tested on rat isolated, internally-perfused tail artery preparations. The structures of both clusters, based on X-ray diffraction studies (Chu & Dahl, 1977; Chu *et al.*, 1982), are shown in Figure 1a,b. The vasodilator actions of A and B are blocked by haemoglobin and also by methylene blue, but not by agents which suppress NOS. Histochemical studies reveal that both clusters are able to penetrate cell membranes with extraordinary ease and that they accumulate inside the endothelium, forming a molecular 'store' of NO·. Our results suggest that their gradual decomposition from sites within the endothelium generates free NO· and that its slow release serves to sustain their vasodilator actions.

Preliminary accounts of some aspects of this work have appeared elsewhere (Flitney *et al.*, 1990; 1992)

Methods

Preparation

Experiments were performed on segments of tail artery taken from normotensive adult Wistar rats (250–460 g). Animals

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were killed by cervical dislocation. A length of artery (0.8–1.5 cm) was dissected free, cannulated (Portex cannula) and transferred to a Perspex bath.

Apparatus

The apparatus is shown in Figure 1. The cannula (C) formed part of a constant flow perfusion circuit, driven by a peristaltic pump (P1; Gilson Minipuls). The vessel (V) was perfused internally (flow rate: 2 ml min^{-1}) with solution pre-warmed by passage through a heat exchanger. Drugs were introduced into the lumen of the artery by bolus injection through a side tube (I). The outer surface of the vessel was superfused continuously with solution driven by a second peristaltic pump (P2). The temperature of the chamber was held at 30–35°C by adjustment of the flow rate in the external circuit (ca. 8 ml min^{-1}).

Light from an Argon ion laser (L; Spectra Physics Ltd., type 168-09) could be made to irradiate the artery directly, so as to induce photorelaxation of vascular smooth muscle (Furchgott *et al.*, 1961). The beam ($\lambda = 514.5$ nm; output intensity 1 mW) was reflected onto the preparation by means of a front-silvered mirror (M; beam diam. at preparation: approx 2 cm).

A differential pressure transducer (T: Sensym type SCX 150NC; Farnell Electronic Components, Leeds) detected changes in back pressure due to changes in arterial tone. Responses were displayed on a chart recorder.

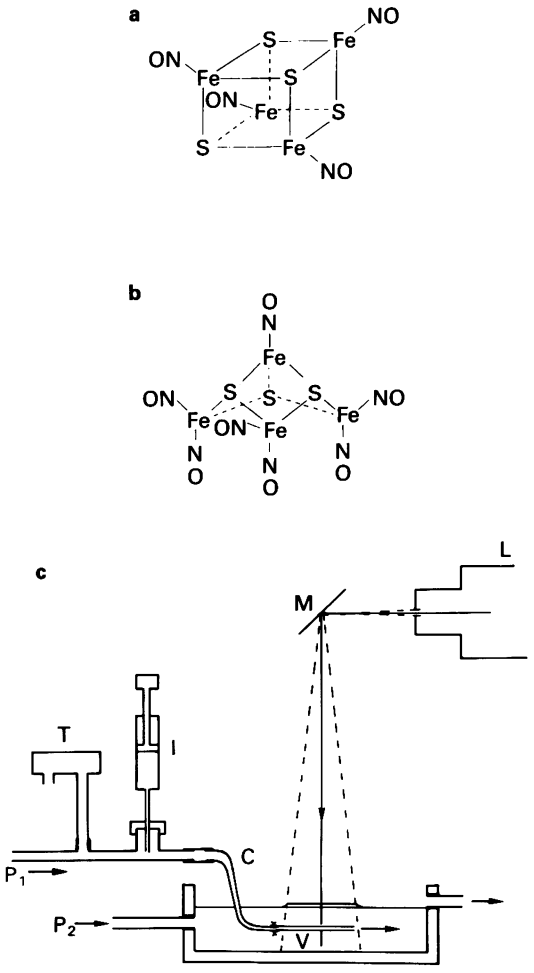


Figure 1 (a, b) Molecular structures of clusters A and B, as determined by X-ray diffraction studies. Compound A contains four and B seven ligated NO· groups per molecule. (c) Apparatus used for perfusing isolated segments of rat tail artery and for irradiating preparations with laser light. See text for full description and explanation of lettering.

Experimental protocol

Arteries were perfused internally with oxygenated Krebs solution (composition, mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, NaH_2PO_4 1.15, $CaCl_2$ 2.5, $MgCl_2$ 1.1, glucose 5.6, gassed with 95% O_2/5% CO_2 to maintain pH 7.4), initially at a low flow rate which was increased gradually over the next 10–20 min to reach a final value of 2 ml min^{-1}. The preparation was allowed to stabilize for 20–30 min, after which it was precontracted with Krebs plus phenylephrine HCl (= Krebs-+PE: mean (± s.e.) [PE] = 6.5 ± 0.5 μM. Mean (± s.e.) agonist-induced perfusion pressure = 101 ± 3.5mmHg).

Experiments were made to establish the mode of action of iron-sulphur cluster nitrosyls. Responses to bolus injections (10 μl) of A and B were compared with those evoked by (i) carbachol (CCh), an endothelium-dependent vasodilator; and (ii) either nitroprusside (NP), S-nitroso-N-acetylpenicillamine (SNAP) or exposure to laser light (L) all of which relax vascular smooth muscle independently of the endothelium.

The effects of adding the following to the internal perfusate were examined: (i) ferro-haemoglobin (Hb) a nitric oxide 'scavenger'; (ii) methylene blue (MB); and (iii) either N^G-monomethyl-L-arginine (L-NMMA) or N-nitro-L-arginine methyl ester (L-NAME), both stereospecific inhibitors of NOS. Comparisons were made with responses evoked by NP or SNAP.

Drugs: commercial sources and synthetic procedures

L-phenylephrine HCl (mol. wt. 203.7; Sigma Chemicals) was used at concentrations ranging from $2–12 \times 10^{-6}$M. Stock solutions (10^{-2}M) of NP (mol. wt. 298; BDH Ltd., 'Analar' grade) were made and the dose required adjusted by serial dilution immediately prior to use. SNAP (mol. wt. 220) was synthesized by reacting N-acetylpenicillamine with sodium nitrite (Field *et al.*, 1978). Compound B was prepared by reacting iron(II) sulphate heptahydrate, sodium nitrite and sodium sulphide in hot aqeous solution under nitrogen gas (Brauer, 1960). Compound A was prepared by addition of elemental sulphur to a solution of B dissolved in re-distilled toluene and refluxed overnight (16 h) under dry nitrogen gas (Gall *et al.*, 1974).

Purities of iron sulphur nitrosyl compounds were checked by (a) infra red spectroscopy (A had a single IR absorption maximum at wave number 1790 cm^{-1}; B had three maxima, at 1795 cm^{-1}, 1747 cm^{-1} and at 1707 cm^{-1}) and (b) ^{14}N-nuclear magnetic resonance (NMR) spectroscopy.

Solutions of B (mol. wt. 553) were made up in Krebs solution, immediately before use. Compound A (mol. wt. 472) is not soluble in water and so working solutions were made by serial dilution of 5 mg A dissolved in 1 ml dimethyl sulphoxide (ca. 10^{-2}M).

An important note concerning experimental procedures

All experiments were conducted in a darkened laboratory with a red safelight (60W) as the sole means of illumination. There were two reasons for this. First, the ambient lighting under normal laboratory conditions is sufficient to induce photorelaxation of precontracted vascular smooth muscle, causing a progressive loss of vessel tone. Second, NP, A and B are photosensitive and decompose when exposed to light, releasing free NO· (Wolfe & Swinehart, 1975; Flitney & Kennovin, 1988; Flitney *et al.*, unpublished results). Drug solutions were therefore made up in the dark and stored in glass vials covered with aluminium foil prior to use.

Results

Bolus injections of either NP or SNAP invariably produce fully-reversible (transient or *T*-type) vasodilator responses: an

initial, rapid drop of pressure, followed by complete recovery, often with some positive 'overshoot'. Compounds A and B produced *T*-type responses only at doses below a critical threshold concentration (D_T). Doses$>D_T$ generate long-lasting (sustained or *S*-type) responses, comprising an initial, rapid decrease of pressure, followed either by partial recovery only, or no recovery at all. The hallmark of the *S*-type response is a remarkably stable plateau of reduced vessel tone which can persist for several hours.

Mediation of T-*type responses by NO·*

Figure 2 compares vasodilator responses to carbachol (CCh), nitroprusside (NP) and laser light (L; = 514.5 nm; 1 mW intensity) with *T*-type responses produced by A and B. Control responses are shown in Figure 2a. The responses to A and B were blocked by either methylene blue (b; 75 μM) or by ferro-haemoglobin (c; 15 μM), a NO· scavenger. However, neither L-NMMA (d; 100 μM) nor L-NAME (not illustrated), both of which suppress endothelial NOS (Rees *et al.*, 1990), inhibited responses to A or B. Indeed, it was usual to find that treatment with either L-NMMA or L-NAME increased the responsiveness of arteries to A and B.

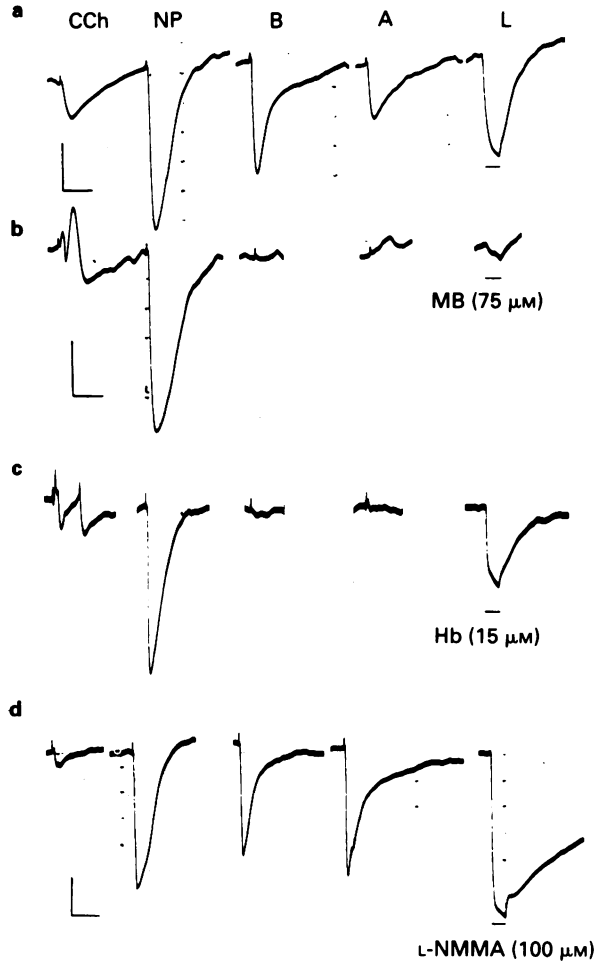


Figure 2 (a) Vasodilator responses produced by microinjection (10 μl) of test doses of carbachol (CCh: 10^{-2} M) and nitroprusside (NP; 2.5×10^{-4} M) and by irradiation with laser light (L; λ = 514.5 nm; 1 mW; 2 min), compared with *T*-type responses produced by clusters A (10^{-4} M) and B (5×10^{-4} M). Artery pre-contracted with 5×10^{-6} M phenylephrine (agonist-induced pressure = 135 mmHg). Female rat, 274 g. Temp., 32°C. (b) Suppression of responses to A and B and to laser light by treatment with methylene blue (MB 7.5×10^{-5} M). Details as above. (c) Inhibition of responses to compound A and B by haemoglobin (Hb, 15×10^{-6} M). Hb increased agonist-induced pressure to 1.8x control level (after ca. 6 min). Artery pre-contracted with 4×10^{-6} M phenylephrine (agonist-induced pressure = 114 mmHg). Female rat, 373 g. Temp. 32°C. (d) The NO synthase (NOS) inhibitor, N^G-monomethyl-L-arginine (L-NMMA, 1×10^{-4} M) increased agonist-induced tone to around 1.28x the control level. Drugs tested 20 min after beginning perfusion with L-NMMA. Responses to carbachol attenuated by L-NMMA, but not those produced by nitroprusside (NP), A, B or laser light. Details as for (c) above. Calibration bars (all recordings): vertical, 20% agonist-induced tone (0.2Y; see Figure 3a); horizontal, 4 min.

S-*type responses produced by sequential injections of increasing doses of A or B*

Pressure recordings made by injection of increasing doses of NP (Figure 3c) or SNAP (not illustrated) confirm that both compounds produce *T*-type responses only. Similar experiments with A or B show that *T*-type responses give way to *S*-type responses when the injected dose exceeds D_T: both the rate and extent of the recovery following successive injections become progressively reduced, resulting in a stepwise and persistent loss of vessel tone (Figure 3a,b).

Three parameters have been measured (X, Y and Z; see Figure 3a) to enable quantitative comparisons to be drawn between NP and SNAP and either A or B. Figure 4 shows perfusion pressure minima (filled circles) immediately following injection of each drug (X) and the steady-state pressures attained after recovery (Z; open circles), both expressed as a fraction of the initial agonist-induced pressure (Y) and plotted as a function of $\log_{10}$ injected dose. The concentrations of SNAP, NP, A and B (μM) required to produce half-maximal

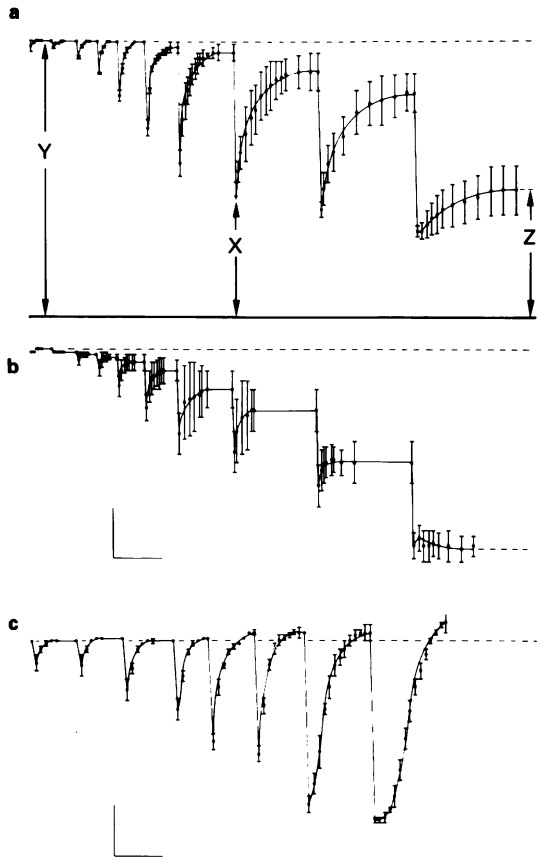
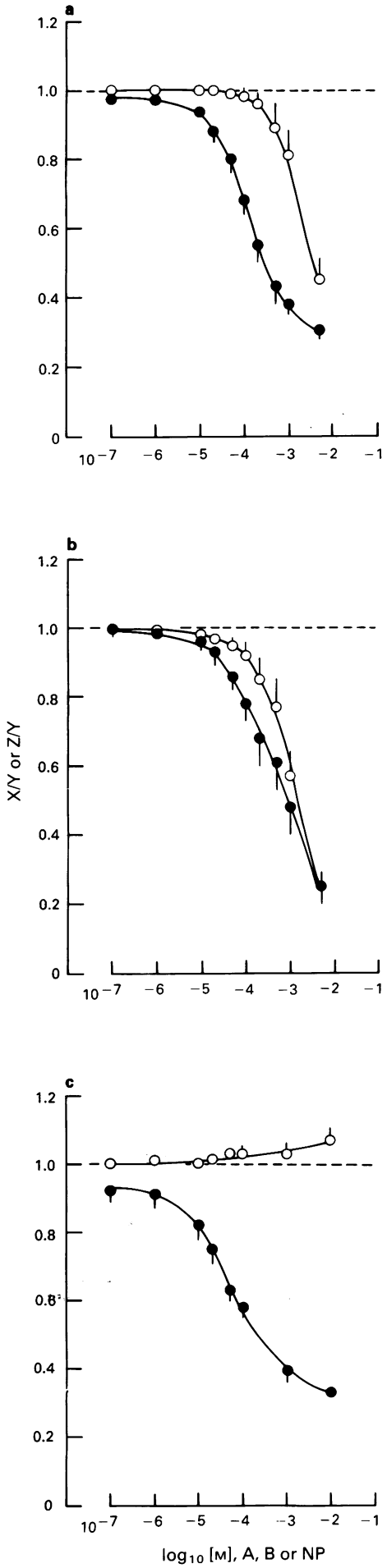


Figure 3 Averaged pressure recordings obtained by successive injections of increasing doses of compound A, B or nitroprusside (NP) (graphs a, b and c, respectively). Mean values (± s.e., vertical bars) are plotted ($n = 6$ preparations for a, and $n = 5$ for both b and c). Note that injection doses of A or B which exceed D_T (see text) produce a sustained drop in vessel tone (*S*-type response), whereas responses to NP are fully reversible (*T*-type response). In (a), Y = agonist-induced pressure; X = pressure minimum following each injection; and Z = steady-state (plateau) pressure attained after recovery (see also Figure 4). Calibration bars: vertical = 0.2Y; horizontal = 20 min (A and B) or 10 min (C).



decreases of X/Y (EC_{50} values) are 36, 43, 126 and 399, respectively. The corresponding curves for Z/Y reflect the tendency for A and B (but not NP) to generate S-type responses. The effect is more pronounced for B than for A, as shown by the greater proximity of the X/Y and Z/Y curves. D_T is estimated to be around 1–10 μM for B and 20–100 μM for A.

Abolition of the 'plateau' phase of the S*-type response by Hb or by MB*

The *S*-type response was remarkable in that even prolonged perfusion ('wash-out') with Krebs+PE solution alone (up to 5 h) failed to induce any further vasoconstriction, once the plateau phase was established. However, the addition of either Hb (15 μM) or MB (>10 μM; not illustrated) to the internal perfusate initiated a prompt and complete restoration of all agonist-induced tone; indeed, as Figure 5 clearly shows, the perfusion pressure rises well above the control

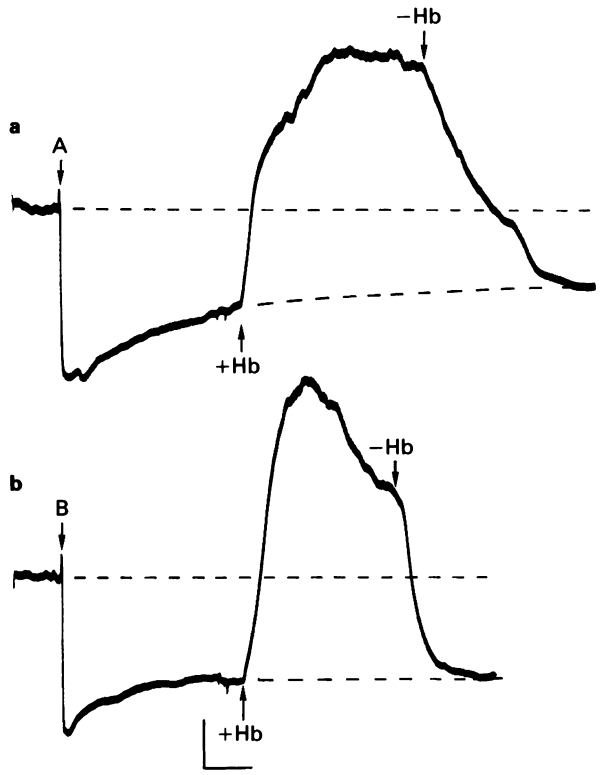


Figure 5(a, b) Pressure recordings from two preparations showing responses to ferro-haemoglobin (Hb, 31.5×10^{-5} M) added to, and then later removed from, the internal perfusate during the plateau phase of an *S*-type response. *S*-type responses were produced by a single bolus injection of 5×10^{-3} M A or B (upper and lower recordings respectively). The addition of Hb (upward arrows) elicits a prompt vasoconstriction which causes the perfusion pressure to rise above the control (pre-injection) value. Removal of Hb produces a vasodilatation which returns the pressure to the plateau value (curved dashed lines). Similar responses to these have been recorded when the time delay between the injection of A or B and treatment with Hb was increased to 6 h. Calibration bars: vertical = 0.2Y; horizontal = 4 min.

Figure 4 Mean (+ or − s.e., vertical lines values for X/Y (●) and Z/Y (○; see Figure 3a) plotted as a function of $\log_{10}$ injected dose for A (a), B (b) and nitroprusside (NP) (c). Responses to NP show complete recovery, with some pressure overshoot at the higher doses (Z/Y > 1.0). S-nitroso-N-acetylpenicillamine (SNAP) (data not shown) gives results which are qualitatively similar to those seen with NP. The values of Z/Y for both clusters (measured 20 min after each injection) are <1.0 at doses which exceed D_T (by definition; see text), reflecting the tendency for A and B to evoke *S*-type responses. Data obtained from same pressure recordings used to construct Figure 3 (a-c).

level (Y), here to around 1.6Y (A) and 1.8Y (B). Even more striking, the subsequent removal of Hb (but not of MB) causes arteries to re-dilate fully (Figure 5a,b). These observations lead us to conclude that extremely brief exposure of arteries to A or B (the 'transit' time for a 10 µl bolus passing through the vessel is ca. 0.3 s) can suffice to establish a durable source of NO· within the tissue.

Histochemical studies showing that iron sulphur cluster nitrosyls penetrate endothelial cells and accumulate there

The existence of a long-lasting source (store) of NO· in vessels exposed to either cluster is supported by microscopic studies of treated arteries. Solutions of A and B are intensely black and visual inspection of segments of artery subjected to repeated bolus injections (experiments of the type used to construct Figure 3) revealed some discolouration of the vessel lining. Microscopic examination of freshly-frozen, unfixed sections of artery exposed to cluster B (5 x 10^{-3}M, 5 min continuous perfusion, followed by 15 min washout with Krebs+PE to remove excess drug from the lumen of the artery) showed that the discolouration was confined to the endothelium (Figure 6a). Unfixed, frozen sections which had been stained with either bathophenanthroline (Figure 6b) or ferrocyanide (not shown), to detect iron(II) derived from the clusters (Pearse, 1972), showed a similar distribution of reaction product, located predominantly in endothelial cells, but sometimes with some faint staining of adjacent smooth muscle cells also. Control (untreated) arteries did not react positively for iron.

Discussion

This paper establishes iron-sulphur cluster nitrosyls as a novel class of NO· donor drug with unusual vasodilator properties. The compounds investigated here are able to generate two distinct kinds of response, designated *T*-and *S*-type responses. In this respect they differ from either NP or SNAP, both of which give fully-reversible responses only (Figures 3 and 4). The ability of Hb and of MB, but not NOS inhibitors, to block *T*- and *S*-type responses (Figure 2) shows that they are mediated by NO· derived from each cluster, and not by enhanced EDRF production through stimulation of the endothelial L-arginine:NO· pathway.

Composite nature of the S-type response

Our results lead us to postulate that the S-type response represents the sum of two, distinct vasodilator components: a reversible component, attributable to free NO· generated by the spontaneous decomposition of clusters in solution and present at the time of injection; superimposed on a 'non-recoverable' component, caused by the delayed release of NO· from clusters which have become trapped within the endothelium. This hypothesis is supported by histochemical studies (Figure 6), which revealed the presence of Fe(II) within endothelial cells of treated (but not control) arteries, and by our observation that the plateau phase of the *S*-type response can be abolished in a reversible manner by perfusing preparations with Hb (Figure 5).

Vanin and co-workers (Vanin, 1991) have shown that iron-dinitrosyls ($Fe(NO)_2$) complexed with low molecular weight thiols (e.g. cysteine or glutathione) can vasodilate isolated blood vessels and also inhibit platelet aggregation. Interestingly, the vasodilator action exhibits two-phase kinetics when tested on intact animals, resembling the *S*-type response described in the present study: that is, an initial, rapid decrease of arterial pressure, followed by a persistent (several hours) hypotensive action. The rapid and sustained components of the response are attributed to NO· released from (i) iron-dinitrosyl low-molecular thiol complexes (= rapid phase) and

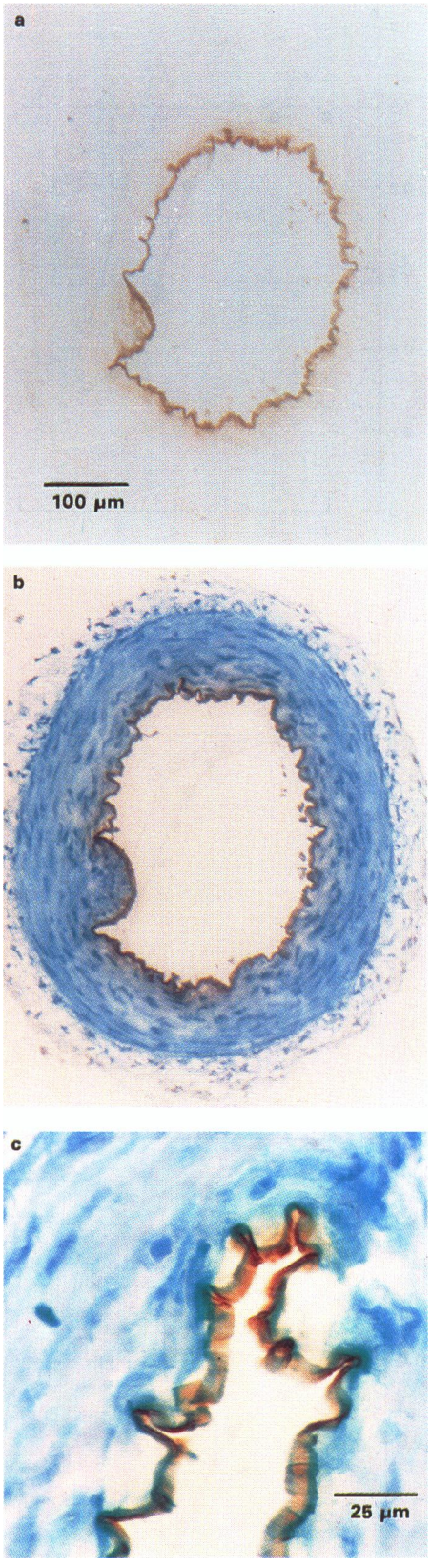


Figure 6 Transverse sections (20 µm) of a freshly-frozen artery perfused with B (5 mM, 2 ml min^{-1}) for 5 min, followed by 15 min perfusion (wash-out) with Krebs+PE solution only. (a) Unstained, unfixed section showing discolouration of the vessel lining. (b) Freshly-frozen, unfixed section after staining for 7 h with bathophenanthroline, followed by brief (4 min) counterstaining with methylene blue (scale as in (a)). Endothelial cells stain positively (red-brown colouration) for iron(ii) derived from the cluster. (c) Unfixed section stained for 4 min with methylene blue to show up smooth muscle cells. Note that brown colouration is confined to the endothelial lining of the vessel.

(ii) from iron-dinitrosyl protein-thiol complexes (= sustained phase) formed within the tissue. The latter are thought to result from the transfer of $Fe(NO)_2$ groups from low molecular weight to protein-borne ligands, forming a long-lasting, molecular 'store' of NO· in the vascular bed.

Suppression of endogenous EDRF production did not occur during the plateau phase of S-*type responses*

The effects of Hb administered during established *S*-type responses lead us to conclude that the formation of endogenous NO· from L-arginine is not suppressed by either cluster and that its continued release from endothelial cells helps to sustain the plateau phase. Thus, perfusing vessels with Hb does not merely re-establish the control (pre-injection) perfusion pressure, but instead drives it to a level substantially greater than this (Figure 5). The inference to be drawn is that Hb scavenges NO· from both exogenous (cluster-derived) and endothelial sources.

The nature of the intracellular mechanism(s) underlying the vasodilator actions of iron-sulphur nitrosyls remains to be established before meaningful comparisons can be made with other nitrovasodilators. The fact that Hb and MB can block responses to either cluster (and also to SNAP) but not those evoked by NP (Figure 2) clearly implies different cellular mechanisms of action. This has not been investigated in the present study. However, there is evidence to suggest that NP may relax vascular smooth muscle through both guanosine 3':5'-cyclic monophosphate (cyclic GMP)-dependent and cyclic GMP-independent pathways (Otsuka *et al.*, 1988). A component of the relaxant effect of glyceryltrinitrate may also be mediated by a mechanism which does not involve elevated cyclic GMP levels (Diamond & Chu, 1983).

Physicochemical properties of iron-sulphur cluster nitrosyls

The physicochemical properties of iron sulphur cluster nitrosyls (Butler *et al.*, 1988) provide some insight into their vasodilator actions. Both compounds are potentially able to transport large quantities of NO·, with 4 and 7 mol. of ligated NO· per mol. of A and B, respectively. Their selective retention and gradual decomposition from within the endothelium can evidently generate physiologically significant amounts of NO· at this most relevant of sites. The mechanism by which NO· is released from clusters trapped inside the endothelium (whether spontaneous or enzymatic) is not known. Extended Hückel molecular orbital calculations show that A and B are electron precise (Sung *et al.*, 1985): this means that addition of an electron to A, and either the addition or removal of an electron in the case of B, will weaken the cage bonding and cause the iron sulphur framework to disintegrate, releasing free NO·. The oxidative status of the endothelial cell may therefore prove to be an important factor in determining the rate of NO· release *in vivo*.

The apparent ease with which both clusters are able to penetrate the endothelial cell membrane is probably related to their high solubility in non-polar solvents (Butler *et al.*, 1988). Compound B is especially interesting in this regard because it is ionic (A is neutral) and therefore soluble in polar solvents too. Curiously, though, the marked contrast in the intensity of staining as seen between endothelial cells and overlying smooth muscle cells (see Figure 6) shows that neither compound can traverse the endothelial cell layer readily. The faint reaction product which is sometimes discernible in smooth muscle cells immediately adjacent to the endothelium is probably a diffusion artefact of the staining procedure, because neither cluster survives conventional fixation methods (including vapour fixation) and so histochemical observations were of necessity made on freshly-frozen sections. This explanation is supported by preliminary X-ray microprobe studies of rapidly-quenched arteries following treatment with A or B, which reveal elemental Fe and S within endothelial cells, but no evidence for either in nearby smooth muscle cells (unpublished observations: Elder, Pediani, Megson & Flitney).

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Nitric oxide synthase in ferret brain: localization and characterization

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1 In the present study, we have investigated the distribution of nitric oxide synthase in the ferret brain. Nitric oxide snythase was determined biochemically and immunochemically.

2 In the rat brain, the highest nitric oxide snythase activity has been detected in the cerebellum. However, in the ferret brain, the highest activity was found in the striatum and the lowest in the cerebellum and cerebral cortex. The enzymatic activity was localized predominantly in the cytosolic fractions, it was dependent on NADPH and Ca^{2+}, and inhibited by N^G-nitro-L-arginine or N^G-methyl-L-arginine.

3 Western blot analysis revealed that all regions of the ferret brain contained a 160 kD protein crossreacting with an antibody to nitric oxide synthase purified from the rat cerebellum, and the levels of relative intensity of staining by the antibody correlated with the distribution of nitric oxide synthase activity.

4 These results indicate that the ferret brain contains a nitric oxide snythase similar to the rat brain, but the distribution of enzymatic activity in the ferret brain differs markedly from the rat brain.

Keywords: Nitric oxide; nitric oxide snythase; arginine; citrulline; guanylyl cyclase; cyclic GMP

Introduction

It has been recognized that nitric oxide (NO) is formed in the central nervous system (Garthwaite *et al.*, 1988; Knowles *et al.*, 1989) as well as in many other cell types (Ishii *et al.*, 1989; Förstermann *et al.*, 1991b) and may play an important role in neuronal signal transduction (Garthwaite, 1991; Bredt & Snyder, 1992). Recently, NO synthase has been purified from the rat cerebellum (Bredt & Snyder, 1990; Schmidt *et al.*, 1991), and the regional distribution of the enzymatic activity in the rat brain has been shown (Förstermann *et al.*, 1990). In the rat brain, the highest NO synthase activity was found in the cerebellum. Furthermore, immunohistochemical mapping of NO synthase in the rat brain has recently been reported (Bredt *et al.*, 1991). These facts suggest that NO synthase exists in neuronal cells in the brain and may be closely related to their physiological functions. Therefore, it seems important to know whether the distribution of NO synthase in the brain is the same in other mammals. Here we show that there is a different regional distribution of NO synthase activity in the ferret brain.

Methods

Dissection of ferret brain

Male ferrets (1.1–1.5 kg) were killed by intraperitoneal injection of an overdose of sodium pentobarbitone (more than 60 mg kg^{-1}). The brains were removed immediately and dissected on ice into seven different regions. The regions were as follows: olfactory bulb, medulla oblongata (including pons), cerebellum, hippocampus, midbrain (including thalamus and hypothalamus), striatum and cerebral cortex. The brain parts were frozen in liquid nitrogen and weighed. Subsequently, they were homogenized in five volumes (w/v) of homogenization buffer (Tris/HCl 50 mM, pH 7.4) containing EDTA (0.1 mM), EGTA (0.1 mM), β-mercaptoethanol (12 mM), leupeptin (1 μM), pepstatin A (1 μM), phenylmethylsulphonyl fluoride (1 mM). Protein concentrations were determined according to Bradford (Bradford, 1976) with bovine serum albumin used as a standard.

Measurement of NO synthase activity

NO synthase activity was determined in crude homogenates of different regions by measuring the formation of [^{3}H]-L-citrulline from [^{3}H]-L-arginine as previously described (Bredt & Snyder, 1990; Schmidt *et al.*, 1991). Briefly, crude homogenates of each region (approximately 100 μg protein) were incubated in the presence of [^{3}H]-L-arginine (10 μM, 5 GBq $mmol^{-1}$), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (BH_4, 3 μM) and Ca^{2+} (2 mM) in a total volume of 100 μl. After 20 min incubation at 25°C, the reaction was stopped by the addition of 1 ml of HEPES buffer (20 mM, pH 5.5) containing EDTA (2 mM) and EGTA (2 mM). The incubations were then applied to 1 ml Dowex AG 50WX-8 columns (Na^+ form, Bio-Rad) and the eluted [^{3}H]-L-citrulline was measured by liquid scintillation counting. In some experiments, crude homogenates were incubated in the absence of NADPH, in the presence of EGTA (1 mM; without Ca^{2+}) or in the presence of N^G-nitro-L-arginine (L-NNA, 100 μM) or N^G-methyl-L-arginine (L-NMA, 100 μM).

In further experiments, NO synthase activity in subcellular fractions was analyzed. Crude homogenates were centrifuged (150,000 *g*, 1 h, 4°C), and cytosolic and particulate fractions were collected. The particulate fractions were incubated with 1 M KCl in homogenization buffer for 5 min at 4°C to remove loosely bound cytosolic proteins, and after centrifugation (150,000 *g*, 30 min, 4°C), the particulate fractions were resuspended in the homogenization buffer, as described previously (Förstermann *et al.*, 1991a). NO synthase activity in the cytosolic and KCl-washed particulate fractions was then determined by the [^{3}H]-L-citrulline formation assay.

NO synthase activity in the cytosolic fractions was also determined by its stimulating effect on soluble guanylyl cyclase of cultured rat foetal lung fibroblasts (RFL-6 cells). Because contamination of the cytosolic fractions with haemo-

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globin prevents the detection of NO synthase activity, the cytosolic fractions were treated with 10% (v/v) phosphocellulose (in potassium phosphate 2 mM, pH 6.7, containing 100 μM EDTA and 10% glycerol) for 30 min at 4°C to remove haemoglobin, as previously described (Förstermann *et al.*, 1990). After the incubation, the cytosolic fractions were separated from phosphocellulose by centrifugation, and used in the subsequent assay. The accumulation of guanosine 3',5'-cyclic monophosphate (cyclic GMP) in RFL-6 cells was used to measure NO synthase activity, as described previously (Ishii *et al.*, 1989; 1991). Briefly, RFL-6 cells were preincubated for 20 min at 37°C with Locke solution (composition, mM: NaCl 154, KCl 5.6, $CaCl_2$ 2.0, $MgCl_2$ 1.0, $NaHCO_3$ 3.6, glucose 5.6 and HEPES 10, pH 7.4) containing 3-isobutyl-1-methylxanthine (300 μM) and superoxide dismutase (SOD; 20 u ml^{-1}). The cytosolic fractions were then added to the RFL-6 cells together with L-arginine (100 μM), NADPH (100 μM), calmodulin (30 nM) and BH_4 (3 μM). After incubation for 3 min at 37°C, the reaction was stopped by the removal of medium, the addition of ice cold sodium acetate (50 mM, pH 4.0) and the rapid freezing of the samples with liquid nitrogen. Cyclic GMP content in each sample was determined by radioimmunoassay (Ishii *et al.*, 1991). In some cases, incubations were performed in the absence of SOD or the presence of haemoglobin (1 μM).

Western blot analysis

Western blot analysis of the different regions of ferret brain was performed as described previously (Schmidt *et al.*, 1992). Briefly, equal amounts of protein in each brain region were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 7.5% w/w gel) and transferred to a nitrocellulose membrane. All procedures were done in Tris buffer (40 mM, pH 7.55) containing 0.3 M NaCl and 0.3% Tween 20. The membrane was blocked with dried milk (6% w/v), and subsequently incubated with specific polyclonal rabbit antibody prepared against rat cerebellar NO synthase and a horseradish peroxidase-conjugate of affinity purified goat antibody to rabbit IgG. The immune-complexes were detected on a photographic film by H_2O_2/luminol chemiluminescence and the bands were scanned with a laser scanner.

Materials

L-[2,3,4,5-^{3}H]-arginine monohydrochloride was purchased from Amersham (Arlington Heights, IL, U.S.A.). L-Arginine and N^G-nitro-L-arginine (L-NNA) were purchased from Sigma (St. Louis, MO, U.S.A.). N^G-methyl-L-arginine (L-NMA) was the kind gift of Dr J.F. Kerwin Jr. (Abbott Laboratories, IL, U.S.A.). Tetrahydrobiopterin (BH_4) was obtained from Dr B. Schirks Laboratories (Jona, Switzerland). All other reagents were of the highest grade available.

Statistics

All values represent means ± s.e.mean from *n* experiments. Statistical difference between groups was assessed by Student's *t*-test for unpaired data and a *P* value of less than 0.05 taken as significant.

Results

Distribution of NO synthase activity in different regions of ferret brain

Western blot analysis of crude homogenates from different brain regions revealed that all regions contained a 160 kD protein crossreacting with anti-rat NO synthase antiserum, but the levels of relative intensity of staining by the antibody differed among these regions. The highest levels were found in the striatum and olfactory bulb and the lowest in the cerebellum (Figure 1a). When the specific NO synthase activity in crude homogenates of each region were assayed, marked differences among these regions were detected. As shown in Figure 1b, the highest specific activity of NO synthase was found in the striatum and the lowest in the cerebellum and cerebral cortex. A relatively high specific activity was found in the olfactory bulb of the ferret, which is similar to rat brain (Bredt *et al.*, 1991). The levels of relative intensity of staining by the antibody were nearly identical to the distribution of specific NO synthase activity determined by the [^{3}H]-L-citrulline formation assay. The highest total activity was found in the cerebral cortex owing to the tissue mass (Figure 1c).

Characteristics of NO synthase in ferret brain

As shown in Figure 2, the specific activity of NO synthase in crude homogenates of each region was greatly reduced when NADPH or Ca^{2+} were omitted from the incubations converting L-arginine to L-citrulline. Figure 2 also shows the inhibition of NO synthase by L-NNA or L-NMA in all regions. The enzymatic activity was localized predominantly in the cytosolic fractions of all regions, while negligible enzymatic activity remained in KCl-washed particulate fractions (data not shown). In addition, when the cytosolic fractions of each region were added to RFL-6 cells, increases in the cyclic GMP content in these cells were detected (Figure 3). Similar to the L-citrulline formation assay, the highest increase in cyclic GMP was found in the striatal preparation followed by the midbrain and the hippocampus. Accumulation of cyclic GMP in RFL-6 cells was markedly reduced when SOD was omitted from the incubation buffer (Figure 3) or in the presence of haemoglobin (data not shown).

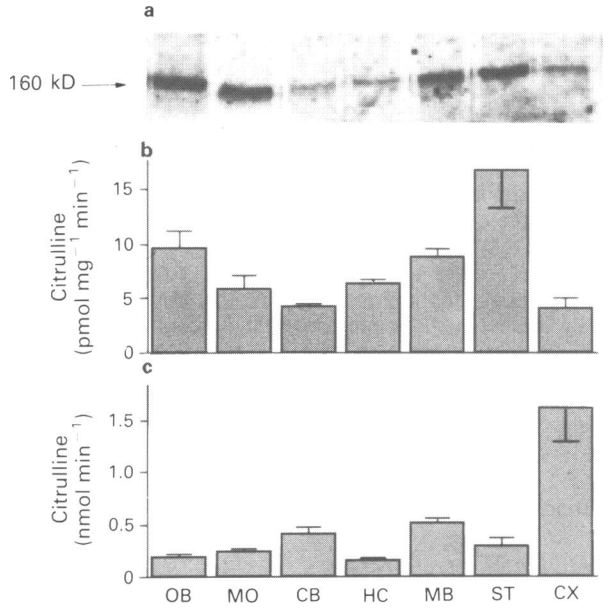

Figure 1 Distribution of NO synthase activity in the ferret brain. Representative Western blot analysis (a) of crude homogenates of each region (equal amounts of protein) with anti-rat NO synthase antiserum. Proteins were separated by SDS-PAGE (7.5%), transferred to a nitrocellulose membrane and probed with the antibody to rat NO synthase. Immune-complexes were visualized with a horseradish peroxidase-linked secondary antibody on a photographic film and scanned. Specific (b) and total (c) activity of NO synthase in crude homogenates of each region. The enzymatic activities were assayed by the formation of [^{3}H]-L-citrulline from [^{3}H]-L-arginine (10 μM) in the presence of NADPH (1 mM), Ca^{2+} (2 mM), calmodulin (30 nM) and BH_4 (3 μM). Data are means (± s.e.mean, vertical bars) from 4 experiments (b and c). Abbreviations: OB, olfactory bulb; MO, medulla oblongata (including pons); CB, cerebellum; HC, hippocampus; MB, midbrain (including thalamus and hypothalamus); ST, striatum; CX, cerebral cortex.

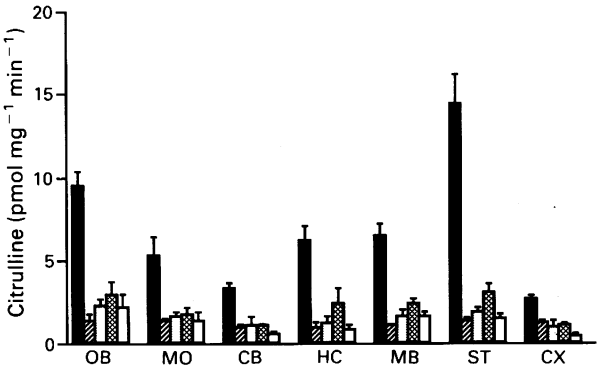

Figure 2 Effect of removal of cofactors or addition of inhibitors on NO synthase activity in crude homogenates of different regions of the ferret brain. The enzymatic activity was determined by the [^{3}H]-L-citrulline formation assay. Control activity (solid column) was measured in the presence of L-arginine (10 μM), NADPH (1 mM), Ca^{2+} (2 mM), calmodulin (30 nM) and BH_4 (3 μM). In the absence of NADPH (hatched columns) or in the presence of EGTA (1 mM) without Ca^{2+} (stippled columns), the enzymatic activity was reduced. When 100 μM N^G-methyl-L-arginine (checkered columns) or 100 μM N^G-nitro-L-arginine (open columns) was added to the preparation, the enzymatic activity was inhibited. Data are means ($\pm$ s.e.mean, vertical bars) from 4–6 experiments. Abbreviations are the same as in Figure 1.

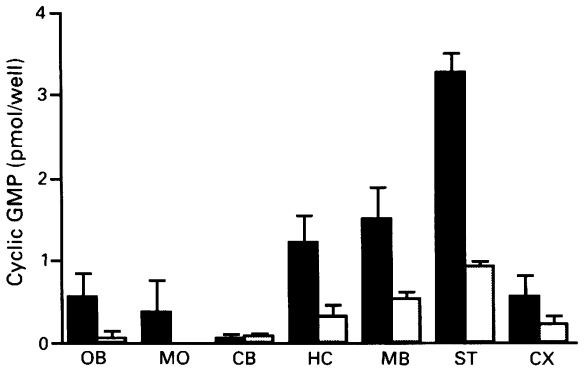

Figure 3 Effect of the absence of superoxide dismutase (SOD) on NO snythase activity in the cytosolic fractions of different regions of the ferret brain (after removal of contaminating haemoglobin with phosphocellulose). Control activity (solid columns) was determined by the accumulation of cyclic GMP in RFL-6 detector cells in the presence of L-arginine (100 μM), NADPH (100 μM), calmodulin (30 nM) and BH_4 (3 μM) in Locke solution containing 3-isobutyl-1-methylxanthine (300 μM) and SOD (20 u ml^{-1}). In the absence of SOD (stippled columns), soluble guanylyl cyclase stimulation was reduced. Data are means $\pm$ (s.e.mean, vertical bars) from 3–4 experiments. Abbreviations are the same as in Figure 1.

Discussion

Several isoforms of NO synthase have been described and at least three isoforms have been purified and well characterized (Förstermann *et al.*, 1991b). The rat brain NO synthase has been described as cytosolic and Ca^{2+}-dependent, and localized with the highest enzymatic activity in rat cerebellum (Förstermann *et al.*, 1990). Our present results show that although the ferret brain has a similar type of NO synthase to the rat brain, the highest NO synthase activity is localized in the striatum and the activity is very low in the cerebellum and cerebral cortex of this species. The possibility that different concentrations of residual haemoglobin in crude homogenates of each region were responsible for the measured differences in NO synthase activity can be excluded by the fact that there was a good correlation between the formation of L-citrulline (the detection of which is not affected by haemoglobin) and NO production as determined by cyclic GMP accumulation in RFL-6 detector cells.

Several species of animals have been found to possess NO synthase in the brain (Schmidt *et al.*, 1989; Mayer *et al.*, 1990; Bredt *et al.*, 1991; Salter *et al.*, 1991), and in the monkey brain, NO synthase seems to exist in neuronal cells as in the rat brain (Bredt *et al.*, 1991). Human NO synthase has recently been purified from the cerebellum and shown to crossreact with anti-rat cerebellar NO synthase antiserum (Schmidt & Murad, 1991). Our present results suggest that there are differences in the distribution of brain NO synthase among species. According to a recent report, the cerebellum of the bovine brain does not contain the highest NO synthase activity (Ohshima *et al.*, 1992).

Although the link of glutamatergic neurotransmission, especially via N-methyl-D-aspartic acid (NMDA) receptors, with NO synthase has been suggested, recent studies indicate that in the rat brain the regional distribution of NMDA receptors does not match that of NO synthase (Garthwaite, 1991). The regional distribution of glutamate has been reported in rat brain (Palkovits *et al.*, 1986). Although high glutamate levels were reported in the cerebellum of the rat, glutamate distribution in other brain areas does not correlate with that of NO synthase. There is no information thus far about the distribution of glutamate or NMDA receptors in the ferret brain.

Little is known about the function of NO in the brain, but the marked differences in distribution of brain NO synthase between species may be indicative of differences in its function.

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Blockade of 5-HT_3 receptor-mediated currents in dissociated frog sensory neurones by benzoxazine derivative, Y-25130

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1 The effect of Y-25130, ((±)-N-(1-azabicyclo[2.2.2]oct-3-yl)-6-chloro-4-methyl-3-oxo-3,4-dihydro-2H-1,4-benzoxazine-8-carboxamide hydrochloride), a high affinity 5-hydroxytryptamine$_3$ (5-HT_3) receptor ligand, was examined on the 5-HT-induced response in dissociated frog dorsal root ganglion (DRG) neurones by use of the extremely rapid concentration-jump ('concentration-clamp') and the conventional whole-cell patch-clamp techniques.

2 5-HT induced a rapid transient inward current associated with an increase in membrane conductance at a holding potential of -70 mV. The current amplitude increased sigmoidally as 5-HT concentration increased. The half-maximum value (K_a) and the Hill coefficient estimated from the concentration-response curve were 1.7×10^{-5} M and 1.7, respectively.

3 The current-voltage ($I-V$) relationship of 5-HT-induced current ($I_{5\text{-HT}}$) showed inward rectification at potentials more positive than -40 mV. The reversal potential ($E_{5\text{-HT}}$) was -11 mV. The $E_{5\text{-HT}}$ value was unaffected by total replacement of intracellular K^+ by Cs^+, indicating that the 5-HT-gated channels might be large cation channels.

4 Both the activation and inactivation phases of $I_{5\text{-HT}}$ were single exponentials. The time constants of activation and inactivation (τ_a and τ_i) decreased with increasing 5-HT concentration.

5 The 5-HT response was mimicked by a selective 5-HT_3 receptor agonist, 2-methyl-5-HT, but the maximum response induced was approximately 25% that of 5-HT. The 5-HT response was reversibly antagonized by the 5-HT_3 receptor antagonists, ICS 205-930, metoclopramide and Y-25130, but not by a 5-HT_{1A} receptor antagonist, spiperone, and a 5-HT_2 receptor antagonist, ketanserin. The half-inhibition concentrations (IC_{50}) were 4.9×10^{-10} M for Y-25130, 4.8×10^{-10} M for ICS 205-930 and 8.6×10^{-9} M for metoclopramide.

6 Y-25130 (5×10^{-10} M) caused a rightward shift of the concentration-response curve for 5-HT while decreasing the maximum response.

7 The results suggest that Y-25130 is a potent antagonist of the 5-HT_3 receptor-channel complex.

Keywords: Frog dorsal root ganglion neurone; 5-HT_3 receptor-mediated response; benzoxazine derivative; Y-25130

Introduction

5-Hydroxytryptamine (5-HT) induces a rapid depolarization accompanied by conductance increase in sensory neurones (Todorovic & Anderson, 1990) and sympathetic and parasympathetic neurones (Higashi, 1977; Akasu *et al.*, 1987; Wallis & Dun, 1988). In cultured neuroblastoma cells and cultured neurones, voltage-clamp studies demonstrated that 5-HT-induced transient current is mediated by 5-HT_3 receptors (Neijt *et al.*, 1988a; Yakel & Jackson, 1988; Derkach *et al.*, 1989; Lambert *et al.*, 1989). These transient depolarizing responses and/or inward currents evoked by 5-HT were inhibited by selective 5-HT_3 receptor antagonists, such as ICS 205-930 (Neijt *et al.*, 1988a; Derkach *et al.*, 1989; Robertson & Bevan, 1991) and GR38032F (Lambert *et al.*, 1989), at picomolar concentrations and mimicked by a selective 5-HT_3 receptor agonist, 2-methyl-5-HT. Since 5-HT_3 receptor-induced responses characteristically exhibit rapid activation and subsequent inactivation, a rapid application technique is necessary to study the kinetics and pharmacological properties of 5-HT_3 receptor-induced responses. Recently, an extremely rapid concentration-jump method, termed the 'concentration-clamp' technique was developed (Akaike *et al.*, 1986; Inoue *et al.*, 1986). This technique combines intracellular perfusion (Akaike *et al.*, 1978; Hattori *et al.*, 1984) and rapid exchange (within 2 ms) of external solution (Krishtal *et al.*, 1983), using single-electrode voltage-clamp, and allows detailed analysis of the kinetics of ligand-gated ionic currents in various neurones. In the present study, we have investigated the physiological and pharmacological properties of the 5-HT-induced current in dissociated frog dorsal root ganglion (DRG) neurones by the use of the 'concentration-clamp' technique. In addition, the blocking action of 5-HT_3 receptor-induced current by a newly synthesized benzoxazine derivative, Y-25130, which is effective against emesis induced by cytotoxic drugs or total body x-radiation (Fukuda *et al.*, 1991), was also studied.

Methods

Preparation

The experimental methods used were as described previously (Hattori *et al.*, 1984; Ishizuka *et al.*, 1984; Akaike *et al.*, 1986). In brief, isolated lumbar dorsal root ganglia of bullfrog (*Rana catesbiana*) were digested in normal Ringer solution containing 0.3% collagenase and 0.05% trypsin at pH 7.4 for 15 to 20 min at 37°C. During the enzyme treatment, the preparation was gently agitated by bubbling the bathing medium with 95% O_2 plus 5% CO_2. Then, neurones were mechanically dissociated from the ganglia with finely polished pins under a binocular microscope. The isolated neurones were stored in a solution containing equal amounts of Ringer solution and Eagle's minimum essential medium (Nissui, Tokyo) at room temperature for a minimum of 3 h.

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Solutions

Dissociated DRG neurones were perfused with external and internal solutions, the ionic compositions of which were (in mM): external, NaCl 115, KCl 2.5, $CaCl_2$ 2, glucose 5 and N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid 10 (HEPES); internal, NaCl 5, KCl 55, K-aspartate 70, ethylene glycol-*bis*-(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) 1 and HEPES 10. Current-voltage ($I-V$) relationships were determined in modified external and internal solutions in which K^+ was replaced with equimolar Cs^+. The pH of external and internal solutions was adjusted to 7.4 and 7.2, respectively, with tris (hydroxymethyl) aminomethane-base (Tris-OH).

Rapid drug application using a concentration-clamp technique

The concentration-clamp technique was used for rapid application of external test solutions within 2 ms (Akaike *et al.*, 1986). The cell-attached tip of the suction-pipette was inserted into a plastic tube through a hole (500 μm diameter). The lower end of the tube could be exposed directly to the external test solution by moving up and down a stage on which dishes containing the drug were placed. A negative pressure (about − 30 cmHg) was applied to the upper end of the tube. The exchange speed and amount of external solution were controlled by adjusting both the negative pressure and the opening speed of the electromagnetic valve driven by 24 V d.c. The power supply was switched on for the desired duration by a stimulator (Nihon Kohden, SEN-7103).

Electrical measurements

Membrane potential was measured through an Ag-AgCl wire in a Ringer-agar plug mounted on a suction-pipette holder. The reference electrode was also an Ag-AgCl wire connected to the bathing medium through a Ringer-agar plug mounted on a rapid solution-change tube. The resistance between the suction pipette (tip diameter 7 μm) and the reference electrode in Ringer solution was 200–300 kΩ. Both electrodes were connected to a voltage-clamp circuit for single-electrode recording (Ishizuka *et al.*, 1984). Both current and voltage were monitored on a digital storage oscilloscope (National, VP-5730A) and simultaneously recorded on a pen recorder (Graftic, SR-6335). Data were also stored on an FM data recorder (Teac, MR30) for computer analysis.

Drugs

Collagenase and metoclopramide hydrochloride (Sigma), trypsin (Difco) and 5-hydroxytryptamine creatinine sulphate (Merck) were purchased. Y-25130 (Figure 1), ICS 205-930 ((3α-tropanyl)-1*H*-indole-3-carboxylic acid ester), ketanserin hydrochloride, spiperone and 2-methyl-5-HT hydrobromide were synthesized by our research laboratories. Y-25130, ICS 205-930, metoclopramide, ketanserin and spiperone were initially dissolved in dimethyl sulphoxide (DMSO) and diluted with the external test solution just before use. DMSO at final concentrations (0.2% or less) did not affect the 5-HT response. All experiments were carried out at room temperature (20–24°C).

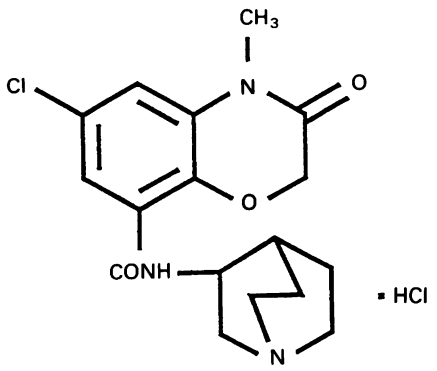

Figure 1 Chemical structure of Y-25130, ((±)-N-(1-azabicyclo [2.2.2] oct-3-yl) -6-chloro-4-methyl-3-oxo-3,4-dihydro-2H-1, 4-benzoxazine-8-carboxamide hydrochloride).

Results

Concentration-dependence of 5-HT-induced currents ($I_{5\text{-}HT}$)

5-HT (2×10^{-5} M) elicited a transient depolarization under current-clamp conditions. The depolarizing response was accompanied by an apparent decrease in membrane input resistance (Figure 2a). Figure 2b (inset) shows the 5-HT-induced inward currents at a holding potential (V_H) of − 70 mV under voltage-clamp, which underlies the 5-HT-induced depolarization under current-clamp. In 32% of DRG neurones examined ($n = 172$), 5-HT elicited a transient inward current at a V_H of − 70 mV. The remaining 68% of the neurones sampled had no discernible response to 5-HT. The 5-HT-induced currents ($I_{5\text{-}HT}$) activated rapidly to peak amplitude and then completely inactivated within 2 s at all

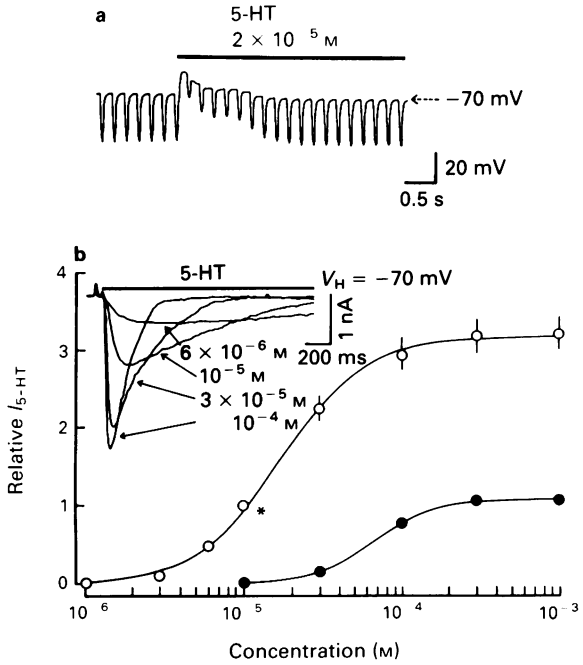

Figure 2 Response of frog DRG neurone to 5-hydroxytryptamine (5-HT). (a), 5-HT-induced depolarization under current-clamp. 5-HT was applied for the period shown by a solid line above the voltage trace. The response was accompanied by an apparent increase in membrane conductance, this being reflected in a decrease in amplitude of the electrotonic potentials. The hyperpolarizing current pulses (0.5 nA, 50 ms) were applied through the pipette at 5 Hz. (b) Inset: transient inward currents induced by 5-HT at various concentrations under voltage-clamp. The superimposed recordings of 5-HT-induced current ($I_{5\text{-}HT}$) were obtained from the same neurone. V_H was − 70 mV. With increasing 5-HT concentration the peak amplitude of the $I_{5\text{-}HT}$ increased and the kinetics became more rapid. (b) Concentration-response curves for 5-HT (○) and 2-methyl-5-HT (●) at a V_H of − 70 mV. All responses were normalized to the peak current amplitude (*) evoked by 10^{-5} M 5-HT alone. Theoretical curves were drawn using K_a (1.7×10^{-5} M) and n (1.7) for 5-HT, and K_a (6.6×10^{-5} M) and n (2.2) for 2-methyl-5-HT. Each point is the mean of values from 4–6 neurones and bars indicate ± s.e.mean.

concentrations used, indicating receptor desensitization. In the present study, 5-HT was applied every 3 min, at which interval a constant 5-HT response could be repeatedly evoked for 1 h or more. Figure 2b shows the peak amplitude of $I_{5\text{-HT}}$ plotted as a function of 5-HT concentration. In the figure all $I_{5\text{-HT}}$ were normalized to the peak current amplitude induced by 10^{-5} M 5-HT (*). The current amplitude increased in a sigmoidal fashion as the 5-HT concentration increased. The threshold concentration of 5-HT was 3×10^{-6} M, and the half-maximum concentration (K_a) was 1.7×10^{-5} M. A nearly maximum response was observed at 3×10^{-4} M. The concentration-response relationship for $I_{5\text{-HT}}$ was in accordance with the conventional expression:

$$I = I_{max} \frac{C^n}{C^n + K_a^n} \quad (1)$$

where I is the observed $I_{5\text{-HT}}$, I_{max} is the maximum current, C is the 5-HT concentration, K_a is the 5-HT concentration that evokes the half-maximal response, and n is the Hill coefficient. When a continuous line was drawn according to equation (1) with n (1.7) and K_a (1.7×10^{-5} M), all experimental points fitted the theoretical curve well. Although a selective 5-HT$_3$ receptor agonist, 2-methyl-5-HT, elicited similar inward currents, maximum current amplitude was approximately 25% of that obtained with 5-HT, indicating that 2-methyl-5-HT is a partial agonist at this 5-HT receptor. The K_a value and the Hill coefficient for 2-methyl-5-HT were 6.6×10^{-5} M and 2.2, respectively.

Voltage-dependence of $I_{5\text{-HT}}$

As seen in Figure 3, the current-voltage (I–V) relationship for $I_{5\text{-HT}}$ showed inward rectification at membrane potentials more positive than – 40 mV, indicating a voltage-dependency of the 5-HT response. The reversal potential for 5-HT ($E_{5\text{-HT}}$) estimated from the I–V relationship was – 11 mV, which was close to the reversal potential (0 mV) of a non-selective large cation channel (E_{Na+K} or E_{Na+Cs}) calculated from the Nernst values for an external solution containing 115 mM Na^+ and 2.5 mM K^+ or Cs^+ and an internal solution of 5 mM Na^+ and 125 mM K^+ or Cs^+. The results indicate that the 5-HT-gated channels are almost equally permeable to Na^+, K^+ and Cs^+. In addition, $E_{5\text{-HT}}$ was not affected by changing external Ca^{2+} and Cl^- concentrations (data not shown).

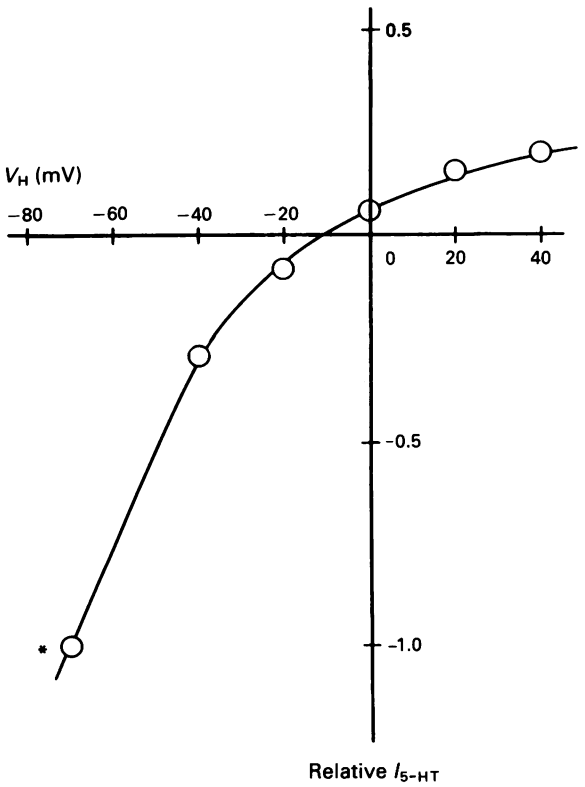

Figure 3 Current-voltage relationship of 5-hydroxytryptamine (5-HT)-induced currents ($I_{5\text{-HT}}$) in a neurone. All responses were normalized to the peak current amplitude (*) evoked by 2×10^{-5} M 5-HT alone at a V_H of – 70 mV. The equilibrium potential for 5-HT ($E_{5\text{-HT}}$) was – 11 mV. Similar results were obtained from another two neurones.

Kinetics of activation and inactivation of $I_{5\text{-HT}}$

The activation and inactivation phases of $I_{5\text{-HT}}$ at a V_H of – 70 mV each consisted of a single exponential. Figure 4 shows the activation and inactivation time constants (τ_a and τ_i) of the currents induced by 5-HT at various concentrations. Both τ_a and τ_i plotted as a function of 5-HT concentration decreased with increasing 5-HT concentration, indicating that the time constants have a definite concentration-dependence. However, τ_a and τ_i of $I_{5\text{-HT}}$ did not show any voltage-dependence (data not shown).

Pharmacological characteristics of $I_{5\text{-HT}}$

To identify the receptor specificity of $I_{5\text{-HT}}$, the effects of 5-HT$_{1A}$, 5-HT$_2$ and 5-HT$_3$ receptor antagonists were examined. The inhibitory effects of the antagonists developed time-dependently. The pretreatment time of the 5-HT$_3$ antagonists for 1 min was enough to produce a steady-state inhibition of the 5-HT$_3$ response because a much longer pretreatment time of 3 min made no difference to the inhibitory action of antagonists. In the following experiments, the preparations were pretreated for 1 min with each antagonist; thereafter simultaneous application of one of antagonists and 5-HT was made. The inward current induced by 2×10^{-5} M 5-HT was inhibited by the selective 5-HT$_3$ receptor antagonist (ICS 205-930) and the non-selective 5-HT$_3$ receptor antagonist (metoclopramide) with half inhibitory concentrations (IC_{50} values) of 4.8×10^{-10} M and 8.6×10^{-9} M, respectively. These inhibitions of $I_{5\text{-HT}}$ by ICS 205-930 and metoclopramide were completely reversible. Y-25130 also reversibly inhibited the $I_{5\text{-HT}}$ in a concentration-dependent manner. The IC_{50} value of Y-25130 was 4.9×10^{-10} M (Figure 5). Neither a 5-HT$_{1A}$ receptor antagonist (spiperone, 10^{-6} M) nor a selective 5-HT$_2$ receptor antagonist (ketanserin, 10^{-6} M) had any inhibitory effects on the $I_{5\text{-HT}}$ in frog DRG neurones. The results indicate that $I_{5\text{-HT}}$ is mediated by 5-HT$_3$ receptor and that Y-25130 is a potent antagonist at this receptor.

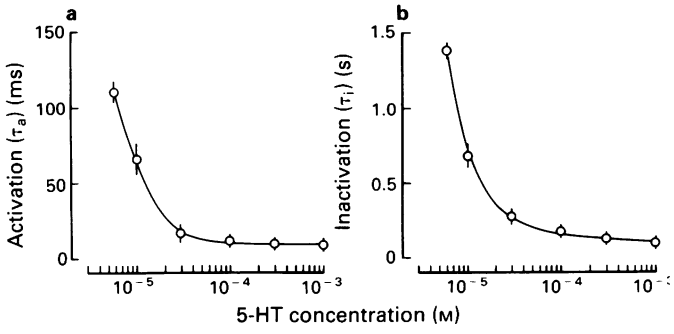

Figure 4 Concentration-dependence of activation and inactivation kinetics of 5-hydroxytryptamine (5-HT)-induced currents ($I_{5\text{-HT}}$) at a V_H of – 70 mV. The time constants of both activation (a) and inactivation (b) decreased with increasing concentration of 5-HT. Each point is the mean from 6 neurones and bars indicate ± s.e.mean.

Figure 6 shows the concentration-response curves for 5-HT with or without ICS 205-930, metoclopramide or Y-25130. The K_a values and Hill coefficients estimated from the 5-HT concentration-response curves were 1.7×10^{-5} M and 1.7 for control, 2.1×10^{-5} M and 1.2 in the presence of 5×10^{-10} M ICS 205-930, 3.8×10^{-5} M and 0.8 in the presence of 10^{-8} M metoclopramide, and 2.8×10^{-5} M and 1.0 in the presence of 5×10^{-10} M Y-25130, respectively. When all responses were normalized to the peak current amplitude induced by 10^{-5} M 5-HT (*), maximum response changed from 3.2 for control to 1.5, 2.2 and 1.8 in the presence of ICS 205-930, metoclopramide and Y-25130, respectively. Blockade of $I_{5\text{-HT}}$ by these compounds was apparently non-competitive, as indicated by the shift to the right of the concentration-response curves with decreasing maximum response.

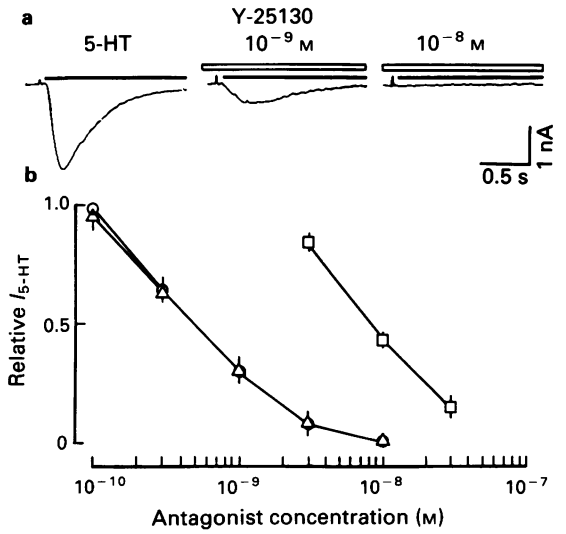

Figure 5 Effects of antagonists on 5-hydroxytryptamine (5-HT)-induced currents ($I_{5\text{-HT}}$) at a V_H of -70 mV. (a), Inhibition of $I_{5\text{-HT}}$ by Y-25130. (b), Dose-dependent inhibition of $I_{5\text{-HT}}$ by ICS 205-930 (Δ), metoclopramide (□) and Y-25130 (○). Each antagonist was applied for 1 min before the simultaneous application of 5-HT (2×10^{-5} M). All responses were normalized to the peak response evoked by 2×10^{-5} M 5-HT. Each point is the mean from 4 neurones and bars indicate ± s.e.mean.

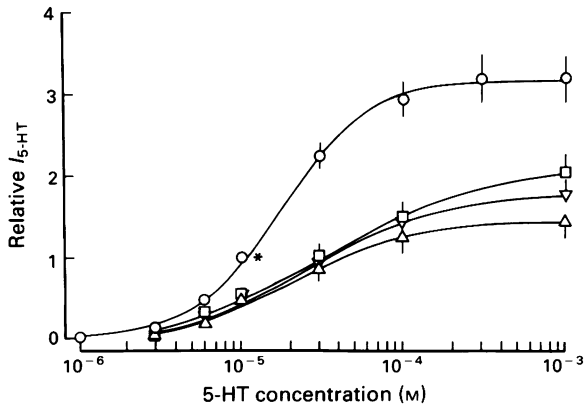

Figure 6 Concentration-response curves for 5-hydroxytryptamine (5-HT) in the presence of 5-HT_3 receptor antagonists. All responses were normalized to the peak response evoked by 10^{-5} M 5-HT (*). Each point is the mean from 4 neurones and bars indicate ± s.e. mean. V_H was -70 mV. Each antagonist was perfused for 1 min before application of 5-HT at various concentrations. Theoretical curves were drawn using K_a (1.7×10^{-5}M) and n (1.6) for control (○), K_a (2.2×10^{-5}M) and n (0.8) for 5×10^{-10}M ICS 205-930 (Δ), K_a (3.8×10^{-5}M) and n (1.2) for 10^{-8} M metoclopramide (□) and K_a (2.8×10^{-5} M) and n (1.0) for 5×10^{-10} M Y-25130 (▽).

Discussion

The results indicate that $I_{5\text{-HT}}$ in frog DRG neurones is mediated by a 5-HT_3 receptor, based on the potent blockade of $I_{5\text{-HT}}$ by ICS 205-930 and metoclopramide and the mimetic action of the 5-HT_3 receptor agonist, 2-methyl-5-HT. Blockade of $I_{5\text{-HT}}$ by Y-25130 (IC_{50} 4.9×10^{-10} M) indicates that this new compound is also a potent antagonist at the 5-HT_3 receptor. In addition, the 5-HT_3 antagonists behaved in our experiments as non-competitive antagonists at the receptor. The possibility that the non-competitive action of the antagonists is open-channel blockade is conceivable. However, as shown in Figure 5a, this can be ruled out because the antagonists did not induce an increase in the rate of desensitization of the 5-HT_3 response. Radioligand binding studies on membranes prepared from NG108-15 cells indicated that the interactions of agonist and antagonist with [^{3}H]-ICS 205-930 recognition sites were competitive in nature, suggesting binding to the same recognition site (Neijt *et al.*, 1988b). Whether these discrepancies between electrophysiological and binding studies reflect subtle variations in the properties of 5-HT_3 receptors between cell types or simply differences in experimental protocol requires further investigation.

The concentration-response curve suggests that the 5-HT_3 receptor of frog DRG neurones has a K_a value of 1.7×10^{-5} M and a Hill coefficient of 1.7. In the present study, inactivation had a time constant of approximately 100 ms. With a drug application time of 2 ms, it is unlikely that there was significant response attenuation caused by desensitization. Therefore, the Hill coefficient of 1.7 for $I_{5\text{-HT}}$ suggests that the 5-HT_3 receptor of frog DRG neurones has at least two binding sites for the agonist. The maximum current evoked by the 5-HT_3 receptor agonist, 2-methyl-5-HT, was approximately 25% of that of 5-HT in the same neurone. The result indicates that 2-methyl-5-HT is a partial agonist at the 5-HT_3 receptor. Similar results were obtained for the 5-HT response in N1E-115 and NG108-15 cells (Yakel & Jackson, 1988; Sepulveda *et al.*, 1991).

As in NCB-20 cells (Lambert *et al.*, 1989), N1E-115 cells (Lambert *et al.*, 1989) and rat DRG neurones (Robertson & Bevan, 1991), the $I-V$ relationship for 5-HT in frog isolated DRG neurones also showed an inward rectification at potentials more positive than -40 mV. The $E_{5\text{-HT}}$ was close to the theoretical value calculated from extra- and intracellular Na^+ and K^+ or Cs^+ concentrations. These results suggest that $I_{5\text{-HT}}$ is generated by an increase in conductance to monovalent cations such as Na^+, K^+ and Cs^+ and that this channel could be classified as relatively non-selective, cation-specific pore. Such a cation selectivity of the 5-HT_3 receptor-channel complex was also reported for NG108-15 cells (Yakel *et al.*, 1990), NCB-20, N1E-115 cells (Lambert *et al.*, 1989) and rat DRG neurones (Robertson & Bevan, 1991).

In the present experiments, the activation and inactivation time constants (τ_a and τ_i) of $I_{5\text{-HT}}$ were single exponentials. The τ_a value is rapid enough to suggest that the 5-HT_3 receptor, unlike other 5-HT receptors, is directly coupled to an ion channel. The kinetics of $I_{5\text{-HT}}$ mediated by the 5-HT_3 receptor in frog DRG neurones were similar to those of responses induced by activation of the nicotinic acetylcholine receptor (cholinoceptor) in frog sympathetic ganglion neurones (Akaike *et al.*, 1989). Similarities include the reversal potential under standard ionic conditions, ion permeabilities, rapid activation and inactivation, sensitivity to curare (Higashi & Nishi, 1982; Akaike *et al.*, 1989; Peters *et al.*, 1990), and modulation of inactivation by forskolin (Seamon *et al.*, 1981; Yakel *et al.*, 1988).These numerous functional similarities between 5-HT_3 and nicotinic cholinoceptors may be explained by molecular biological evidence that the 5-HT_3 receptor gene is structurally related to the nicotinic cholinoceptor. A complementary DNA clone containing the coding sequence of the 5-HT_3 receptor-channel complex has been isolated by screening a neuroblastoma expression lib-

rary for funtional expression of 5-HT-induced currents in *Xenopus* oocytes (Maricq *et al.*, 1991). These studies indicate that the 5-HT$_3$ receptor exhibits sequence similarity to the α subunit of *Torpedo californica* nicotinic cholinoceptor.

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Endothelin ET_A and ET_B receptors mediate vascular smooth muscle contraction

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1 We have investigated the receptors mediating endothelin-induced contraction of rabbit isolated jugular vein (RJV) and rat isolated thoracic aorta (RTA).

2 Endothelin-1 (ET-1) and endothelin-3 (ET-3) contracted RJV preparations with similar potency (EC_{50} values $\sim$1 nM), whereas, ET-1 (EC_{50}:4.5 nM) was $\sim$80 fold more potent than ET-3 in contracting RTA. In addition, the ET_B receptor-selective agonist [$Ala^{1,3,11,15}$]ET-1 contracted RJV (EC_{50}:2.1 nM) but not RTA.

3 The ET_A receptor antagonist, BQ123, competitively antagonized (pA_2 6.93) the contraction of RTA produced by ET-1, but had no effect (at 10 μM) on the contractile effects of either ET-1, ET-3 or [$Ala^{1,3,11,15}$]ET-1 in RJV.

4 These data suggest that both ET_A and ET_B receptors can mediate vascular smooth muscle contraction.

Keywords: Endothelin; receptor subtypes; vascular smooth muscle contraction; ET_A antagonist; BQ123

Introduction

The discovery of endothelin-1 (ET-1; Yanagisawa *et al.*, 1988) and its isopeptides, endothelin-2 (ET-2) and endothelin-3 (ET-3; Inoue *et al.*, 1989) has stimulated considerable interest. The differential potencies of ET-1, ET-3 and [$Ala^{1,3,11,15}$]ET-1 ([Ala_4]ET-1) suggests that subtypes of the endothelin receptor occur (Masaki *et al.*, 1991; Saeki *et al.*, 1991). Indeed, two subtypes have been cloned and sequenced (Arai *et al.*, 1990; Sakurai *et al.*, 1990) and denoted ET_A, which shows selectivity for ET-1 over ET-3 and mediates contraction, and ET_B at which ET-1 and ET-3 are equipotent, and mediate vasorelaxation, possibly via the release of endothelium-derived relaxant substances (Masaki *et al.*, 1991). Recently, a cyclic pentapeptide antagonist (BQ123; D-Val, Leu, D-Trp, D-Asp, Pro) has been described with a marked selectivity for the ET_A receptor, both *in vitro* and *in vivo* (Ihara *et al.*, 1991; 1992). In this study, we have used BQ123 to characterize the endothelin receptors mediating contraction of rabbit isolated jugular vein (RJV) and rat isolated thoracic aorta (RTA) preparations.

Methods

Ring preparations (2–3 mm) from RJV and RTA were mounted in organ baths for isometric tension measurement. Tissues were equilibrated at 37°C in a gassed (95% O_2, 5% CO_2) Krebs solution (composition, mM: NaCl 118, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 0.6, D-glucose 11, $CaCl_2$ 1.3) at a resting tension of 0.5 g (RJV) or 2 g (RTA). Following 50 mM KCl (RTA only), preparations were contracted with either histamine (100 μM, RJV) or with the stable thromboxane A_2-mimetic, U-46619 (10 nM, RTA). The endothelium of RTA preparations was mechanically removed. This could not be undertaken in the more fragile RJV, therefore these preparations were treated with L-N^G-nitroarginine methyl ester (L-NAME, 100 μM) and indomethacin (3 μM) to inhibit, endothelium-derived, nitric oxide and prostanoid formation, respectively. In tissues with induced tone, carbachol (1 μM, RJV) or acetylcholine (1 μM, RTA) were used to assess endothelial cell function. Tissues were then washed prior to a cumulative concentration-effect curve to an endothelin peptide. In experiments with BQ123 tissues were pretreated for 30 min.

Values are the arithmetic mean ($\pm$ s.e.mean) or geometric mean (with 95% confidence intervals; for agonist EC_{50} and concentration-ratio (CR) values) from n animals. The EC_{50} value is defined as the concentration of agonist producing 50% of its own maximum response. Agonist CR values were calculated by dividing the EC_{50} obtained in the presence of the antagonist by that obtained in its absence, and subjected to Schild analysis (Arunlakshana & Schild, 1959). $P < 0.05$ was taken to reflect a significant difference (unpaired Student's t test).

Drugs

Endothelins were obtained from Peninsula and carbachol from BDH. Acetylcholine chloride, L-N^G-nitroarginine methyl ester (L-NAME), bacitracin, leupeptin, phosphoramidon and histamine dihydrochloride were obtained from Sigma. All the above were prepared in Krebs solution. U-46619 (11,9-epoxymethano-prostaglandin H_2), [Ala_4]ET-1 and BQ123 (D-Val, Leu, D-Trp, D-Asp, Pro) (Glaxo Group Research), were dissolved in 1% (w/v) $NaHCO_3$, 0.1% (w/v) ammonium acetate and distilled water, respectively.

Results

Agonist potencies

ET-1 and ET-3 produced well-sustained, concentration-dependent contractions of the RJV and RTA preparations. ET-1 and ET-3 (0.1–100 nM) showed equivalent potencies (EC_{50} values $\sim$1 nM) on the RJV. In contrast, ET-3 was some $\sim$80 times less potent than ET-1 on the RTA preparation, although it behaved as a full agonist (Table 1). Interestingly, [Ala_4]ET-1 contracted the RJV (Table 1), but was neither an agonist nor an antagonist (versus ET-1) at concentrations up to 1 μM in the RTA. Protease inhibitors (50 μg ml^{-1} bacitracin, 5 μg ml^{-1} leupeptin and 100 μM phosphoramidon) did not affect the potency of ET-1 or ET-3 on

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Table 1 Potencies of endothelin-1 (ET-1), ET-3 and [Ala$_4$]ET-1 as contractile agonists in preparations of rabbit isolated jugular vein (RJV) and rat isolated thoracic aorta (RTA)

	RJV		*RTA*	
Isopeptide	EC_{50} (nM)	*Max response* (% histamine)	EC_{50} (nM)	*Max response* (% U-46619)
ET-1	0.7 (0.4–1.1)	131 (± 5)	4.5 (3.6–5.6)	131 (± 5)
ET-3	0.9 (0.5–1.8)	111* (± 4)	370 (230–580)	121 (± 6)
[Ala$_4$]ET-1	2.1 (0.6–8.0)	106* (± 8)	--NSE-- at 1 μM	

Cumulative concentration-effect curves to endothelin-1 (ET-1), endothelin-3 (ET-3) and [Ala^{1,3,11,15}]ET-1 ([Ala$_4$]ET-1) were produced for contraction of RJV and RTA. Values are arithmetic means (± s.e.mean) or geometric means (95% confidence limits) from 6–32 experiments. Maximum responses are expressed relative to the contraction produced by either histamine (100 μM, RJV, mean 0.92 ± 0.1 g) or U-46619 (10 nM, RTA, mean 1.9 ± 0.1 g).
*Significantly different ($P < 0.05$) from ET-1. NSE: no significant effect.

either preparation. It is unlikely that nitric oxide or prostanoid formation in the RJV influenced the contractile activity to the endothelins, as L-NAME and indomethacin, did not affect either the EC_{50} or the maximum contraction to ET-1 or ET-3 (data not shown).

Antagonist potencies

BQ123 (0.3–3 μM) produced a concentration-dependent, rightward parallel displacement of the ET-1 concentration-effect curve in RTA, with no suppression of the maximum response (Figure 1a). Schild analysis yielded a mean pA_2 value for BQ123 of 6.93 ± 0.06 and slope of 1.00 ± 0.06 ($n = 5$). BQ123 also antagonized the contractile effects of ET-3 on this preparation. The weak agonist potency of ET-3 precluded the determination of a full pA_2 value but using a single concentration (30 nM) of BQ123, a mean pK_B value of 8.3 ± 0.1 ($n = 4$) was estimated. In addition, BQ123 (3 μM) also antagonized the contractile effects of ET-1 (CR = 26.6 [8.76–81.8], $n = 4$) in endothelium-intact RTA preparations exposed to L-NAME (100 μM) and indomethacin (3 μM). In contrast, BQ123 even at 10 μM, had no effect on the contraction of RJV to ET-1 (CR = 1.0 [0.3–3.5], $n = 5$; Figure 1b), ET-3 (CR = 0.7 [0.2–1.7], $n = 5$) or [Ala$_4$]ET-1 (CR = 1.3 [0.7–2.3], $n = 3$). Consistent with its ET receptor specificity, BQ123 (10 μM) did not change either the resting tension or the contractile response to either histamine (100 μM, RJV) or to U-46619 (10 nM, RTA).

Discussion

This study demonstrates that at least two receptor subtypes mediate endothelin-induced vascular smooth muscle contraction and can be distinguished on the basis of both agonist and antagonist selectivities. One type of receptor, as found in the RTA, can be classified as ET_A in that (1) ET-1 was more potent than ET-3, whilst [Ala$_4$]ET-1 was inactive, and (2) the actions of ET-1 were competitively antagonized by BQ123, the pA_2 value of 6.93 being comparable to that reported elsewhere (Ihara *et al.*, 1992). In contrast, a second endothelin receptor, as found in the RJV, resembles the ET_B receptor, since the endothelins ET-1, ET-3 and [Ala$_4$]ET-1 showed comparable agonist potencies which were importantly not antagonized by BQ123.

On the RTA, ET-3-induced contractions were also antagonized by BQ123 but with an estimated potency ($pK_B = 8.3$) greater than the pA_2 value of 6.9 obtained against ET-1 on this preparation. The reason(s) for this is not clear. However, the weak agonist potency of ET-3 precluded its use at the very high concentrations required to produce full concentration-effect curves in the presence of BQ123. Consequently, the potency estimates for BQ123 against ET-3-induced contractions may be of limited value. It is possible, however, that ET-3 may act through a different receptor from ET-1 which is more sensitive to BQ123.

The data from our study suggest that endothelin-induced vascular smooth muscle contraction can be mediated via both ET_A and ET_B reeptors. Alternatively, there may be

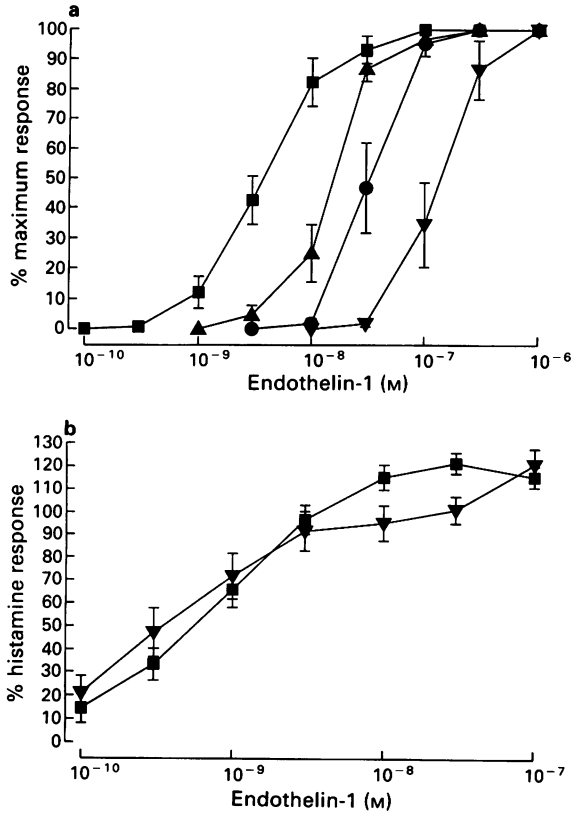

Figure 1 Effects of BQ123 on endothelin-1 (ET-1)-induced contraction of rat isolated thoracic aorta (RTA) and rabbit isolated jugular vein (RJV) ring preparations. (a) RTA rings were pretreated for 30 min with either vehicle (controls, ■) or BQ123 at 0.3 μM (▲), 1 μM (●), or 3 μM (▼), before exposure to endothelin-1. Cumulative concentration-effect curves were constructed and EC_{50} values determined. Agonist concentration-ratios were calculated and used to obtain pA_2 and slope values from Schild plots. The data are means (with s.e.mean shown by the vertical bars) from 5 determinations, yielding mean pA_2 and slope values of 6.93 (± 0.06) and 1.00 (± 0.06) respectively. (b) RJV rings were pretreated for 30 min with either vehicle (control, ■) or BQ123 (10 μM, ▼) before exposure to ET-1. Data are means (with s.e.mean shown by the vertical bars) from 5 determinations, yielding a mean agonist concentration-ratio for ET-1 of 1.0 [0.3–3.5].

subtypes of the ET_A contractile receptor in which one type exhibits an agonist profile (the potency of ET-1 = ET-3) comparable to that observed at ET_B receptors. Thus, the concept of a smooth muscle ET_A receptor mediating vasoconstriction and an endothelial cell ET_B receptor mediating vasodilatation (Masaki *et al.*, 1991) needs reconsideration. Consistent with our proposal are the very recent data from Moreland *et al.* (1992), using a variety of isolated arterial and venous smooth muscle preparations from several species, which indicate that ET_A-like contractile receptors predominate on arterial preparations and ET_B-like contractile receptors on venous smooth muscle. Similarly, Fukuroda and colleagues (1992) noted that in porcine isolated coronary and pulmonary blood vessels part of the ET-1-induced contractile responses was resistant to antagonism with the cyclic pentapeptide ET_A antagonist, BQ153.

It remains to be firmly established which type of ET receptor mediates the vasorelaxant effects of the endothelins. For example, an ET_C receptor, (potency of ET-3 $\gg$ ET-1), has been described in bovine (Emori *et al.*, 1990), but not in human (White *et al.*, 1992) cultured endothelial cells. In addition, ET-1 and ET-3 have been reported to produce relaxation of endothelium-intact porcine pulmonary artery vessels which is unaffected by BQ123, in keeping with an ET_B-mediated response (Fukuroda *et al.*, 1992). However, the ET_B-selective agonist sarafotoxin 6c does not elicit a relaxation response in rabbit carotid arteries with an intact endothelium (Moreland *et al.*, 1992).

In conclusion, the present study has demonstrated that vascular smooth muscle contraction evoked by the endothelins can be mediated by at least two receptor subtypes, which can be differentiated on the basis of the relative potencies of ET-1, ET-3 and [Ala_4]ET-1 and by susceptibility to antagonism by the ET_A receptor antagonist, BQ123.

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Role of nitric oxide and guanosine 3′,5′-cyclic monophosphate in mediating nonadrenergic, noncholinergic relaxation in guinea-pig pulmonary arteries

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1 Nonadrenergic, noncholinergic (NANC) nerves mediate vasodilatation in guinea-pig pulmonary artery (PA) by both endothelium-dependent and endothelium-independent mechanisms. The transmitter(s) involved in the endothelium-independent pathway have not yet been identified. We have therefore investigated the possibility that nitric oxide (NO) and guanosine 3′,5′-cyclic monophosphate (cyclic GMP) may mediate this neural vasodilator response in guinea-pig branch PA rings denuded of endothelium.

2 Electric field stimulation (EFS, 50 V, 0.2 ms) induced a frequency-dependent (1–24 Hz), tetrodotoxin-sensitive relaxation of the U44069-precontracted PA rings in the presence of adrenergic and cholinergic blockade.

3 The NO synthase inhibitors N^G-monomethyl L-arginine (L-NMMA, 100 μM) and N^G-nitro L-arginine methyl ester (L-NAME, 30 μM), and the guanylyl cyclase inhibitor methylene blue (5 μM) inhibited the EFS (16 Hz)-induced relaxation by 53 ± 5, 74 ± 9 and 82 ± 9% respectively ($n = 5–7$, $P < 0.01$, compared with control rings).

4 Excess concentrations of L-, but not D-arginine (300 μM) completely reversed the inhibitory effect of L-NMMA.

5 The EFS-elicited relaxation (4 Hz) was potentiated by 1 μM zaprinast, a type V phosphodiesterase inhibitor which inhibits guanosine 3′:5′-cyclic monophosphate (cyclic GMP) degradation, but was unaffected by 0.1 μM zardaverine, a type III/IV phosphodiesterase inhibitor which inhibits cyclic AMP degradation.

6 EFS (50 V, 0.2 ms, 16 Hz) induced a 3 fold increase in tissue cyclic GMP content, an action which was inhibited by L-NMMA (100 μM).

7 Pyrogallol (100 μM), a superoxide anion generator, also inhibited the EFS-induced relaxation by 53 ± 9%, and this effect was prevented by superoxide dismutase.

8 Chemical sympathetic denervation with 6-hydroxydopamine had no effect on the relaxant response to EFS in the endothelium-denuded PA rings.

9 In endothelium-denuded branch PA rings at resting tone, L-NMMA (100 μM) significantly augmented the adrenergic contractile response, an effect which was completely reversed by L-arginine, but not by D-arginine. In the same groups of vessel rings, L-NMMA had no significant effect on the matched contractile response to exogenous noradrenaline.

10 These results suggest that NO may be released from intramural nerve endings other than adrenergic nerves (probably NANC nerves), and this leads to vasodilatation via activation of guanylyl cyclase.

Keywords: Innervation; NANC nerves; pulmonary artery; nitric oxide; electrical field stimulation; 6-hydroxydopamine; guanylyl cyclase; cyclic GMP; vasodilatation

Introduction

In a previous study we have shown that electrical field stimulation (EFS) of precontracted guinea-pig branch pulmonary artery (PA) rings causes a nonadrenergic, noncholinergic (NANC) relaxation, which is mediated by two pathways, one of which is endothelium-dependent and one of which is endothelium-independent (Liu *et al.*, 1992). The endothelium-dependent component appears to be mediated by adenosine 5′-triphosphate (ATP), but the transmitters responsible for the endothelium-independent component of this relaxation remain unknown.

Recently, evidence has accumulated for nitric oxide (NO) as a NANC transmitter in both vascular and nonvascular tissues (for review see Gillespie *et al.*, 1990; Moncada *et al.*, 1991; Rand, 1992). NO causes vasorelaxation by activating soluble guanylyl cyclase, resulting in an increase in intracellular guanosine 3′,5′-cyclic monophosphate (cyclic GMP) (Ignarro *et al.*, 1989; Moncada *et al.*, 1991). Endogenous NO also modulates pulmonary adrenergic vasoconstriction (Liu *et al.*, 1991b) and hypoxic pulmonary vasoconstriction (Liu *et al.*, 1991a). However, the NO could be released from NANC nerves as a transmitter, from adrenergic nerve endings as a cotransmitter, or from both.

In the present study, we tested the possibility that NO and cyclic GMP may mediate the endothelium-independent component of this neural relaxation. We studied the effect of EFS on tissue cyclic GMP content. Since cyclic GMP is broken down by phosphodiesterase (PDE), we also investigated the role of cyclic GMP in this neural relaxation using a type V PDE inhibitor which inhibits cyclic GMP degradation and a type III/IV PDE inhibitor which inhibits cyclic AMP breakdown (Nicholson *et al.*, 1991). Additionally, we studied the effect of chemical sympathetic denervation on the neural relaxant response to EFS in order to ascertain whether adrenergic nerves also release NO.

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Methods

Tissue preparation

Branch PA rings from male Dunkin-Hartley guinea-pigs (300–350 g) were prepared and mounted in organ baths for tension recording as described previously (Liu *et al.*, 1992). All the vessel rings were allowed to equilibrate at their optimal resting tension (700 mg) in the baths for at least 60 min and washed with fresh Krebs-Henseleit (K-H) solution every 20 min during the equilibration period. The K-H solution is composed of (mM): NaCl 118, KCl 5.9, $MgSO_4.7H_2O$ 1.2, $CaCl_2.6H_2O$ 2.5, NaH_2PO_4 1.2, glucose 5.6 and $NaHCO_3$ 25.5. Endothelium was removed from all vessel rings by gently rubbing their intimal surfaces with a piece of fine abrasive paper as previously described (Liu *et al.*, 1992). Removal of endothelium was confirmed by loss of the relaxant response to substance P prior to the experiment in all vessel rings (Bolton & Clapp, 1986; Maggi *et al.*, 1990) and by histological examination at the end of the experiment in 12 of these rings.

Nerve stimulation

Electrical field stimulation (EFS, 50 V, 0.2 ms, for 15 s at 4 min interval) was applied via two platinum wire electrodes positioned at each end of the vessel ring and connected to a Grass S88 stimulator (Grass Instruments, Quincy, U.S.A.). The vessel rings were preincubated with phentolamine, atropine and propranolol (all 1 μM) for 20 min and precontracted with 3 μM U44069 (9,11-dideoxy-9α, 11α-epoxymethano-prostaglandin $F_{2\alpha}$). After a stable contraction was obtained, frequency-response relationships (1–24 Hz) were constructed in the chemical adrenergic denervation study. In other studies, the vessel rings were stimulated with fixed EFS stimuli (50 V, 0.2 ms, 16 Hz, for 15 s). After 3–4 reproducible responses had been obtained, the vessel rings were incubated with N^G-monomethyl L-arginine (L-NMMA), N^G-nitro L-arginine methyl ester (L-NAME), methylene blue or pyrogallol or their respective vehicles for 10 min and a further 4–5 EFS stimulations performed. EFS was continued until the effect of these inhibitory agents was maximal. The effect of an increase in vascular tone on the NANC relaxation was evaluated by comparing the EFS (16 Hz)-induced relaxation before and after the vascular tone had been raised further by 5-hydroxytryptamine (5-HT) (10 μM) in a group of U44069-precontracted PA rings. In the reversibility studies, L-arginine or superoxide dismutase were added to the organ baths at the peak of L-NMMA or pyrogallol effects. The effects of two PDE inhibitors, zaprinast (1 μM) and zardaverine (0.1 μM) on EFS-induced relaxation were evaluated in 1 μM U44069-precontracted PA rings. Similarly, 3–4 reproducible relaxant responses to EFS were recorded before and after the addition of the two inhibitors. In two groups of non-precontracted PA rings, 3–4 reproducible contractile responses to exogenous noradrenaline (NA) were recorded before and after treatment of the vessel rings with 100 μM L-NMMA. To match the NA-induced contraction with EFS-induced contraction, variable concentrations (20–30 nM) of NA were used.

Concentration-response curve

Paired PA rings were precontracted with 3 μM U44069 and concentration-response curves to zaprinast in the absence and presence of 100 μM pyrogallol were obtained.

6-Hydroxydopamine pretreatment

To achieve chemical sympathetic denervation, a group of guinea-pigs (300 g) were injected with 6-hyroxydopamine (6-OHDA 20 mg kg^{-1}), intraperitoneally, every 12 h, on 4 occasions. A batch matched control group received 4 intraperitoneal injections of the same volume of vehicle for 6-OHDA (ascorbic acid, 1 mg ml^{-1}) at the same intervals. On the third day after the first 6-OHDA (or vehicle) injection, the animals were killed and pulmonary arteries dissected for use. The effectiveness of this 6-OHDA treatment protocol in destroying the adrenergic nerve endings has been proved previously by Tranzer & Thoenen (1968) using ultrastructural examination.

Cyclic GMP determination

Tissue cyclic GMP levels were determined in endothelium-denuded branch PA rings that had been equilibrated under optimal resting tension and subjected to precontraction (3 μM U44069) as performed in other rings. Tension was monitored and EFS (50 V, 0.2 ms, 16 Hz, for 15 s) applied, whereupon the tissue was quickly frozen in liquid nitrogen. Samples were extracted and assayed for cyclic GMP by radioimmunoassay as previously described (Crawley *et al.*, 1992).

Histological examination

At the end of the functional studies, 12 paired branch PA rings (6 rubbed and 6 unrubbed) from 6 animals were chosen in a randomized manner and fixed in 10% formal saline for histological examination. Three μM sections were cut and stained with haematoxylin and eosin. Sections were then examined under a light microscope from which it was possible to confirm the presence or absence of an endothelial lining.

Drugs

The following drugs were used: noradrenaline hydrochloride, substance P, tetrodotoxin, phentolamine hydrochloride, pyrogallol, atropine sulphate, propranolol hydrochloride, 5-hydroxytryptamine, methylene blue, U44069 (9,11-dideoxy-9α,11α-epoxymethano-prostaglandin $F_{2\alpha}$), L-arginine hydrochloride, D-arginine hydrochloride, superoxide dismutase, N^G-monomethyl L-arginine, N^G-nitro L-arginine methyl ester, 6-hydroxydopamine (all from Sigma, Poole, U.K.), zaprinast (Rhone-Poulenc, Dagenham, U.K.) and zardaverine (Byk-Gulden Pharmaceutics, Koustanz, Germany).

Analysis of results

Contraction is presented as absolute tension (mg), and relaxation as a percentage of the U44069-induced contraction. The contractions induced by EFS and NA after L-NMMA or its vehicle treatment were compared with those before treatment and calculated as percentage augmentations. Likewise, vasorelaxant responses to EFS in the presence of various inhibitors or their vehicles were compared with EFS responses before adding these inhibitors or vehicles, and expressed as percentage inhibition. Mean values at each concentration or frequency point were used to compare the difference between two frequency-response or concentration-response curves. Values are presented as mean ± s.e. and n indicates the number of animals from which the tissues were dissected. Statistical analysis of results was performed by use of paired and unpaired Student's t test or by one way analysis of variance followed by Bonferroni corrected t test, when multiple comparisons were made. A P value < 0.05 was considered to be significant.

Results

Histological examination

Light microscopic examination revealed that an intact endothelial lining was consistently present in the unrubbed

and absent in the rubbed rings, confirming the successful removal of vascular endothelium.

Effects of L-NMMA and L-NAME

In the presence of adrenergic and cholinergic blockade, EFS induced a transient, frequency-dependent relaxation of the U44069-precontracted, endothelium-denuded branch PA rings, which was abolished by 1 μM tetrodotoxin. EFS induced a reduction of the U44069-induced vascular tone of 3 ± 1, 10 ± 2.5, 18.6 ± 2.7, 26.9 ± 3.9, 31 ± 3.5 and 29.7 ± 3.8% at 1, 2, 4, 8, 16 and 24 Hz respectively. Treatment with L-NMMA (100 μM) or L-NAME (30 μM) had no effect on baseline or U44069-generated tension, but markedly inhibited EFS-elicited relaxation (Figure 1). The inhibitory effect of L-NMMA was completely reversed by 300 μM L-arginine but was unaffected by an identical concentration of D-arginine (Figure 2).

Effects of methylene blue and zaprinast

U44069-induced contraction was 725 ± 72 and 693 ± 48 mg for control and methylene blue-treated rings respectively ($P > 0.05$, $n = 7$). In the presence of phentolamine, atropine and propranolol (all 1 μM), EFS (16 Hz) induced a reproducible relaxation of U44069-precontracted vessel rings. Methylene blue (5 μM) treatment caused a further increase in vascular tension of 192 ± 50 mg ($n = 7$) and a significant ($P < 0.001$) inhibition of the NANC relaxation (Figure 1). To determine whether the tension increase by methylene blue was contributory to the inhibition of the NANC relaxation, the vascular tone of precontracted rings was elevated further with 5-HT (10 μM). Increase of the vascular tone by 5-HT (225 ± 48 mg) had no significant effect on the relaxant response to EFS (Figure 1). We also evaluated the effects of zaprinast (1 μM) and zardaverine (0.1 μM), which are type V and type III/IV specific PDE inhibitors respectively, on the relaxant response to EFS. To minimize the effect of functional antagonism on the possible potentiation of the EFS-induced relaxation by these inhibitors, vessel rings were precontracted with 1 μM U44069 and stimulated at 4 Hz. U44069-generated vascular tone was 623 ± 123, 589 ± 88 and 551 ± 104 mg for control, zaprinast and zardaverine-treated rings respectively ($P > 0.05$, $n = 5$). Zaprinast (1 μM) significantly potentiated the EFS-induced relaxation, but zardaverine had no effect (Figure 3).

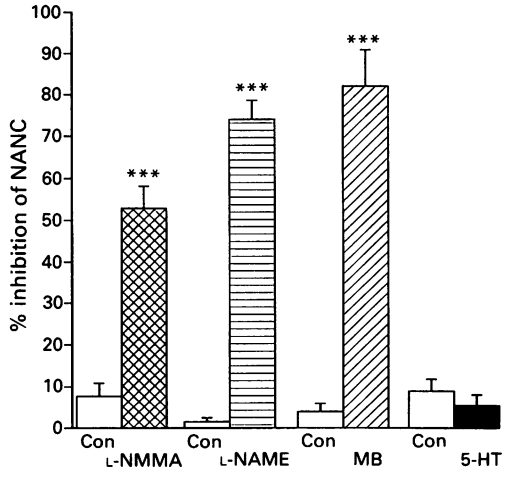

Figure 1 Inhibition of the nonadrenergic noncholinergic (NANC) relaxant response to electrical field stimulation (EFS, 50 V, 0.2 ms, 16 Hz, for 15 s) in U44069 (3 μM)-precontracted endothelium-denuded branch pulmonary artery (PA) rings by N^G-monomethyl L-arginine (L-NMMA, 100 μM), N^G-nitro L-arginine methyl ester (L-NAME, 30 μM) and methylene blue (MB, 5 μM), and the effect of increase in vascular tension by 5-hydroxytryptamine (5-HT, 10 μM) on NANC relaxation. MB further increased the vascular tension by 192 ± 50 mg and greatly inhibited the NANC relaxation. 5-HT further increased the vascular tone by 225 ± 48 mg, but had no effect on the NANC relaxation. Con: vehicle controls. ***$P < 0.001$, compared with vehicle control rings, $n = 5$–7.

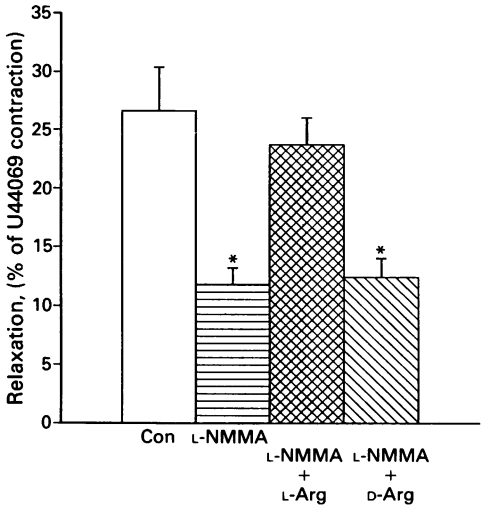

Figure 2 Reversibility of N^G-monomethyl L-arginine (L-NMMA) induced inhibition of nonadrenergic, noncholinergic (50 V, 0.2 ms, 16 Hz, for 15 s) vasodilator response in endothelium-denuded branch PA rings precontracted with U44069 (3 μM) by L-arginine (L-Arg) and D-arginine (D-Arg). 300 μM L-Arg, but not 300 μM D-Arg completely reversed the inhibitory effect of 100 μM L-NMMA on NANC relaxation. Relaxation was expressed as a percentage of the U44069-generated contraction. *$P < 0.05$, compared with vehicle control (Con) and L-NMMA plus L-Arg-treated rings, $n = 5$.

EFS-induced cyclic GMP formation

Tissue cyclic GMP content was determined in 3 groups ($n = 6$, in each group) of endothelium-denuded branch PA rings precontracted with 3 μM U44069. EFS (50 V, 0.2 ms, 16 Hz, for 15 s) caused a 3 fold increase in tissue cyclic GMP level. The EFS-induced cyclic GMP accumulation was significantly inhibited by pretreatment with 100 μM L-NMMA (Figure 4).

Effects of pyrogallol

U44069-generated contractions were 562 ± 42 and 512 ± 47 mg in control and pyrogallol-treated rings, respectively ($P > 0.05$, $n = 6$). EFS (16 Hz) induced a reduction of 18.7 ± 3.7% of the U44069-induced tone. Pyrogallol (100 μM) markedly inhibited the relaxant response to EFS (Figure 5), but had no effect on zaprinast-induced relaxation. Zaprinast-evoked relaxations were 11 ± 4, 21 ± 6, 35 ± 9 and 64 ± 8% in control rings and 12 ± 3, 21 ± 5, 36 ± 7 and 78 ± 2% in pyrogallol (100 μM)-treated rings at concentrations of 3, 10, 30 and 100 μM respectively ($n = 5$, $P > 0.05$). The relaxation to EFS was fully restored by adding superoxide dismutase (100 u ml^{-1}) at the peak of pyrogallol-induced inhibition (Figure 5).

Effect of L-NMMA on the contractile responses to EFS and noradrenaline

In endothelium-denuded PA rings at resting tension, EFS (50 V, 0.2 ms, 4 Hz) caused a concentration of 46 ± 5 mg, which was blocked by phentolamine (1 μM) and tetrodotoxin (1 μM). Treatment of the vessel rings with L-NMMA (100 μM) significantly enhanced the contractile response to

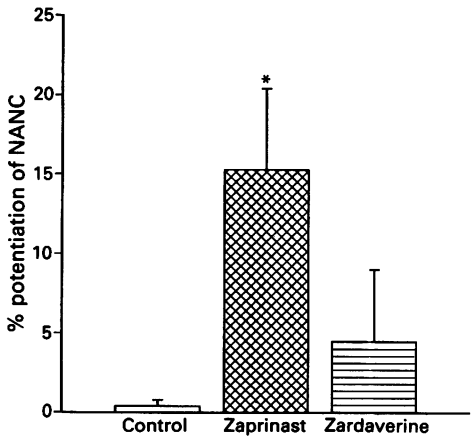

Figure 3 Potentiation of the nonadrenergic, noncholinergic (NANC) vasodilator response to electrical field stimulation (50 V, 0.2 ms, 4 Hz for 15 s) in the U44069 (1 μM) precontracted endothelium-denuded branch pulmonary artery (PA) rings by the cyclic GMP specific phosphodiesterase (PDE) inhibitor, zaprinast (1 μM), but not by the cyclic AMP specific PDE inhibitor, zardaverine (0.1 μM). $*P<0.05$, compared with vehicle control rings, $n=5$.

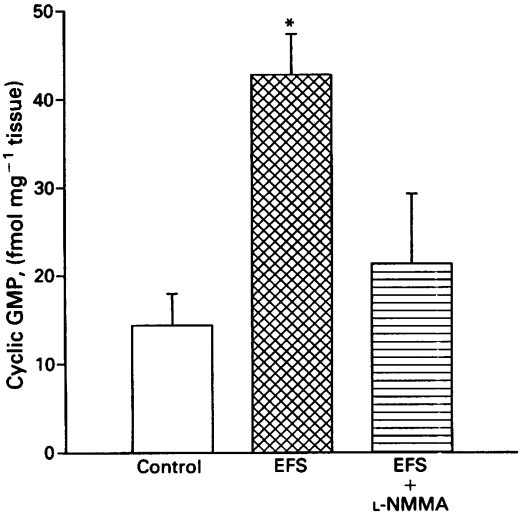

Figure 4 Stimulation of cyclic GMP formation by electrical field stimulation (EFS, 50 V, 0.2 ms, 16 Hz, for 15 s) in the endothelium-denuded branch pulmonary artery rings precontracted with 3 μM U44069. Control rings were subjected to precontraction and quick frozen. EFS stimulated rings, and rings treated with 100 μM N^G-monomethyl L-arginine (L-NMMA), were precontracted and quick frozen at 15 s after the onset of EFS. $*P<0.05$, compared with both control and L-NMMA-treated rings, $n=6$.

EFS ($n=9$, $P<0.01$, compared with control rings), but had no significant effect on the matched contractile response to NA (Figure 6). This effect of L-NMMA on the adrenergic contractions was completely reversed by 300 μM L-arginine, whereas an identical concentration of D-arginine was ineffective (Figure 7).

Effect of sympathetic denervation on NANC relaxation

In the presence of atropine (1 μM), U44069 (3 μM) contracted the endothelium-denuded PA rings from both vehicle and 6-OHDA pretreated animals by 1162 ± 152 and 1256 ± 153 mg respectively ($n=5$, $P>0.05$). EFS reduced U44069-generated vascular tensions by 2 ± 1, 12 ± 2, 25 ± 3, 34 ± 4, 41 ± 5 and 41 ± 5% in PA rings from vehicle-treated animals, and by 6 ± 2, 14 ± 4, 26 ± 5, 30 ± 5, 32 ± 4 and

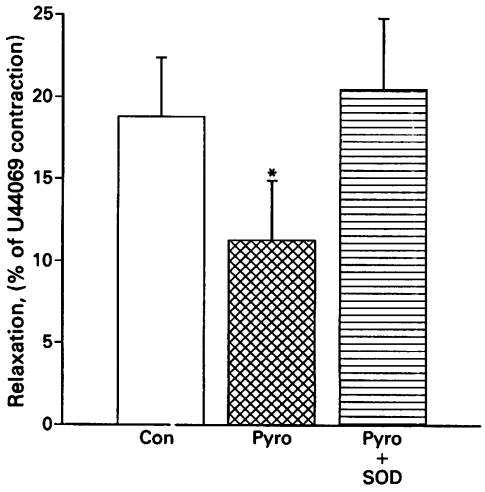

Figure 5 Inhibition of the nonadrenergic, noncholinergic vasodilator response to electrical field stimulation (50 V, 0.2 ms, 16 Hz, for 15 s) by pyrogallol (Pyro, 100 μM) and reversal of this inhibition by superoxide dismutase (SOD, 100 u ml^{-1}) in the endothelium-denuded branch pulmonary artery (PA) rings. Relaxation was expressed as a percentage of U44069-induced contraction. Con: control rings. $*P<0.05$, $n=6$.

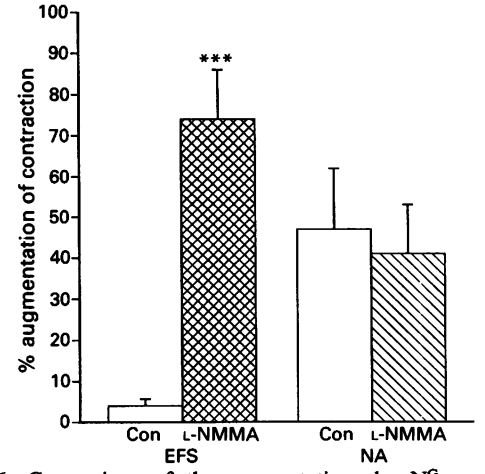

Figure 6 Comparison of the augmentations by N^G-monomethyl L-arginine (L-NMMA, 100 μM) of the matched contractile responses to electrical field stimulation (EFS, 50 V, 0.2 ms, 4 Hz, for 15 s) and exogenous noradrenaline (NA, 20–30 nM). Con: vehicle control rings. $***P<0.001$, compared with control rings, $n=6$.

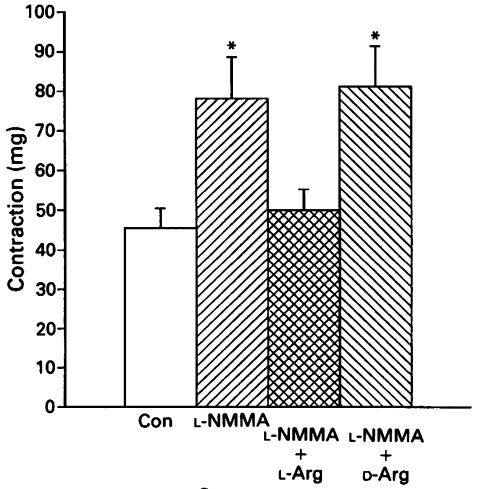

Figure 7 Reversibility of N^G-monomethyl L-arginine (L-NMMA)-induced augmentation of the contractile response to electrical field stimulation (EFS, 50 V, 0.2 ms, 4 Hz, for 15 s). L-Arginine (L-Arg, 300 μM), but not D-arginine (D-Arg, 300 μM) reversed the augmenting effect of L-NMMA (100 μM). $*P<0.05$, compared with control (Con) and, L-NMMA plus L-Arg group rings, $n=6$.

28 ± 4% in the PA rings from 6-OHDA-treated animals, at a frequency of 1, 2, 4, 8, 16 and 24 Hz respectively ($P > 0.05$, $n = 5$), indicating that chemical adrenergic denervation by 6-OHDA did not affect the NO-mediated neural relaxation. The effectiveness of adrenergic denervation was confirmed by the abolition of the adrenergic contractile response to EFS in the PA rings from 6-OHDA-treated animals, whereas EFS caused a frequency-dependent adrenergic contraction in the rings from vehicle-treated animals (data not shown).

Discussion

In previous studies we demonstrated that, in the presence of adrenergic and cholinergic blockade, stimulation of the intramural nerves of the guinea-pig branch PA by EFS induced a tetrodotoxin-sensitive relaxation. This relaxation was reduced but not abolished after endothelial denudation, indicating that both endothelium-dependent and endothelium-independent mechanisms were involved (Liu *et al.*, 1992). We also demonstrated that the endothelium-dependent component may be mediated by ATP. Here, we expand our previous study by showing that the endothelium-independent component of NANC relaxation is mediated predominantly through NO and cyclic GMP formation. We have also provided evidence against NO as a cotransmitter of sympathetic nerves.

NO is synthesized from the semi-essential amino acid L-arginine (Palmer *et al.*, 1988a,b; Schmidt *et al.*, 1988; Moncada *et al.*, 1991) by NO synthase. L-NMMA and L-NAME are specific inhibitors of NO synthesis (Palmer *et al.*, 1988b; Johns *et al.*, 1990; Moore *et al.*, 1990; Moncada *et al.*, 1991). NO exerts its effect via the activation of guanylyl cyclase and elevation of intracellular cyclic GMP concentration (Katsuki *et al.*, 1977; Ignarro, 1989). Methylene blue is a specific inhibitor of this enzyme (Martin *et al.*, 1985). Pyrogallol also inhibits NO action by generating superoxide anions that inactivate NO (Moncada *et al.*, 1986). Our results showed that L-NMMA and L-NAME markedly inhibited the endothelium-independent component of the NANC relaxation. The inhibitory effects of L-NMMA on the NANC relaxation were completely reversed by the NO precursor L-arginine, whereas D-arginine was ineffective. Additionally, pyrogallol also significantly reduced the NANC relaxation, whereas it had no effect on zaprinast-induced relaxation, indicating that its effect is not due to nonspecific inhibition of smooth muscle relaxation. Furthermore, the inhibitory action of pyrogallol was completely reversed by adding superoxide dismutase. All these results are consistent with the hypothesis that NO mediates the NANC relaxation. This NANC relaxation is highly sensitive to methylene blue (82% inhibition at 5 μM) and is significantly potentiated by zaprinast, a type V PDE inhibitor which prevents cyclic GMP degradation. By contrast, zardaverine, a type III/IV PDE inhibitor which prevents cyclic AMP degradation, had no significant effect on the NANC relaxation. Moreover, EFS induced a 3 fold increase in tissue cyclic GMP concentration. The EFS-induced elevation in tissue cyclic GMP concentration was markedly inhibited by L-NMMA. These results indicate that cyclic GMP is a key component in the mechanism underlying this neural relaxation in the guinea-pig branch PA. Thus, the endothelium-independent component of the NANC relaxation in this vessel is mediated through NO and cyclic GMP generation.

Although a large body of evidence supports a role for NO in the mediation of NANC relaxation, the possibility that other nerves may also release NO has not been explored. Moreover, NO may be released as a sympathetic nerve cotransmitter rather than as a primary transmitter from separate NANC nerves. In most reported studies, either adrenoceptor blockade or catecholamine depletion was employed to exclude the adrenergic response during EFS. Because catecholamine depletion does not necessarily mean the destruction of adrenergic nerve endings, such studies cannot preclude the possibility that adrenergic nerves may release NO. 6-Hydroxydopamine is neurotoxic agent that selectively destroys sympathetic nerves (Tranzer & Thoenen, 1968; Bennett *et al.*, 1970). The failure of 6-OHDA treatment to affect the relaxant response to EFS in precontracted branch PA rings indicates that the NO mediating this neural relaxation in this vessel is not released by, or related to, adrenergic nerve activation. We could not rule out the possibility that NO is released from parasympathetic nerves as a cotransmitter with acetylcholine, but NO may also be released by separate NANC nerves. The possibility that NO may be released from cholinergic nerves cannot be further investigated until it is possible to deplete these nerves selectively.

If the hypothesis that NO is a NANC transmitter is correct, we reasoned that inhibition of NO synthesis or release should augment adrenergic contraction. Application of EFS activates both adrenergic and NANC nerves, and NO released from NANC nerves will exert a functional antagonism on adrenergic contraction, which is mediated by noradrenaline acting on α-adrenoceptors (Liu *et al.*, 1991b). Our demonstration that L-NMMA induced an L-arginine-reversible augmentation of adrenergic contraction in the endothelium-denuded PA rings provides further evidence for NO as a NANC neurotransmitter.

In the endothelium-denuded guinea-pig PA rings, both L-NMMA and L-NAME had no effects on either the basal or U44069-generated tension, suggesting that there may be no smooth muscle-derived NO production in this tissue, which is in contrast to the endothelium-denuded bovine pulmonary arteries where both basal and stimulated release of smooth muscle-derived NO was demonstrated (Wood *et al.*, 1990). The ability of methylene blue to elevate further U44069-generated tension indicates that there is an intrinsic NO-independent generation of cyclic GMP, which may also modulate the intrinsic smooth tone. Similar findings have been made in bovine pulmonary arteries (Ignarro *et al.*, 1987).

Based on the equal enhancement of the contractile response to both EFS and the matched contraction to exogenous NA, we concluded that L-NMMA augments adrenergic contraction by a postjunctional mechanism in the endothelium intact guinea-pig branch PA rings (Liu *et al.*, 1991b). In the present study, L-NMMA potentiated the contractile response to adrenergic nerve stimulation but had not effect on the matched contraction induced by exogenous NA, suggesting that L-NMMA augments adrenergic contraction via a prejunctional mechanism in the endothelium-denuded PA rings. The presence or absence of endothelium could explain this difference. In intact rings, 100 μM L-NMMA enhanced the adrenergic contraction by 341%, whereas it caused only 74% augmentation of the contraction in endothelium-denuded rings. These results suggest that L-NMMA enhances adrenergic contraction mainly through the inhibition of endothelially-derived NO, although inhibition of neurally-released NO is also contributory.

In summary, together with our previous studies, our results indicate that in the guinea-pig branch pulmonary artery, EFS induces a NANC vasodilator response. This neural relaxation is mediated via two pathways, an endothelium-dependent, and an endothelium-independent pathway. The former pathway is at least partially mediated by ATP, which causes vasodilatation through the activation of endothelial P_{2y}-purinoceptors and NO release. The endothelium-independent pathway is mediated predominantly by NO and cyclic GMP.

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Protection against the effects of anticholinesterases on the latencies of action potentials in mouse skeletal muscles

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1 Adult male albino mice were injected subcutaneously with an organophosphorous anticholinesterase to initiate excessive variability in the latency of indirectly elicited muscle action potentials (jitter) when assessed 5 days later.

2 Pretreatment of the mice with a single dose of pyridostigmine prevented the development of jitter after subsequent dosing with an organophosphate.

3 Treatment with one dose of pralidoxime (2PAM) prevented the development of jitter if given less than 1 h after treatment with ecothiopate, a reactivatable inhibitor of cholinesterase. Similar treatment with 2PAM after a non-reactivatable inhibitor did not prevent the development of jitter. The repeated administration of 2PAM over 12 h did ameliorate jitter.

4 Pretreatment of mice orally with α-tocopherol and N-acetylcysteine, known to prevent ecothiopate-induced myopathy, did not prevent the development of jitter after ecothiopate.

5 It is concluded that the development of jitter was a consequence of the inhibition of acetylcholinesterase, and although jitter did not develop acutely, the potential for the full development of jitter was achieved about 1 h after intoxication with ecothiopate. The development of jitter did not involve the generation of free radicals. Reduction of the early effects of intoxication with anticholinesterases by pyridostigmine or 2PAM prevented the development of jitter.

Keywords: Organophosphorous anticholinesterase; skeletal muscle; action potential conduction; pralidoxime; antioxidants

Introduction

It has been reported (Kelly *et al.*, 1990) that the organophosphate (OP) anticholinesterases, ecothiopate (ECO), pinacolyl *S*-(2-trimethylaminoethyl) methylphosphonothioate (BOS), and diisopropylfluorophosphate (DFP) affected the latencies of indirectly-evoked muscle action potentials (APs). A single dose of any of these OPs increased the variability of the latencies of a train of indirectly-evoked muscle APs, i.e. increased muscle AP jitter. The jitter was manifest as the increased latency which occurred at the beginning of the train and the increased variation of the latency of consecutive APs later in the train. These effects of ECO, BOS or DFP were dose-dependent and lasted for several days after the acetylcholinesterase (AChE) activities of muscle homogenates had returned to control values. This increased jitter was present after doses of OPs which also caused damage to muscle originating at the endplate (Townsend, 1988), and it was also seen after smaller, non-necrotizing doses. Although the increased jitter was a consequence of the inhibition of AChE, the size of the increase did not appear to be related directly either to the maximum inhibition of the enzyme or to its inhibition at the time when APs were recorded.

Protection against the lethal effects of large doses of OPs may be afforded by prophylaxis with pyridostigmine (Leadbeater *et al.*, 1985); or, provided that the enzyme-inhibitor complex has not 'aged', by treatment with oximes to reactivate the AChE. In previous experiments done in this laboratory, protection against the myopathy induced by ECO could be provided by prophylaxis with pyridostigmine, or by treatment with pralidoxime (2PAM) (Townsend, 1988), or by prophylaxis with vitamin E and other antioxidants (Das, 1989).

The amelioration by drugs of the toxic effects of OPs gives rise to the following questions which are addressed in the study described here: (1) Does prophylaxis with pyridostigmine or with vitamin E ameliorate the increased jitter produced by ECO? (2) Does treatment with 2PAM prevent development of increased jitter, and is there a period for optimal treatment? (3) Does 2PAM affect the increase in jitter after BOS, an OP which produces a non-reactivatable inhibition of AChE?

Methods

Male albino mice aged 6–7 months were used in all experiments. The OP anticholinesterases, ECO 0.5 mol kg^{-1}, or BOS 8 mol kg^{-1}, were given with atropine 0.7 mol kg^{-1} by subcutaneous injection in the scruff of the neck. At various times after intoxication with the OP, animals were killed by section of the spinal cord in the neck. ECO and BOS are quaternary organophosphates selected because these water-soluble compounds do not penetrate the CNS and exert effects only peripherally (Koelle & Steiner, 1956). The inclusion of atropine in the injection prevents muscarinic effects so that the main action of these drugs is at the neuromuscular junction. The acute toxic effect of a subcutaneous injection of a mixture of atropine sulphate and the quaternary organophosphates ECO and BOS used in these experiments was fasciculation which began about 20 min after the injection. With ECO, the fasciculations lasted 1–2 h and during this period animals were able to move around, but usually remained quiet with reduced exploratory behaviour. By 3 h after ECO, behaviour was undistinguishable from normal. Dosing was in the morning so that the reduced mobility did not coincide with the normal feeding/activity pattern. With BOS, the onset and time course of drug action was slower, but mobility had returned to normal by 10–12 h. None of the many human studies refer to any association of pain with fasciculation after organophosphate poisoning.

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Electrophysiology

The left hemidiaphragm and phrenic nerve was rapidly removed from each animal and pinned to Sylgard 184 (Dow Corning) in a Perspex bath through which flowed physiological saline of the following composition (mM): NaCl 137, $NaHCO_3$ 12, NaH_2PO_4 1, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1 and glucose 25. The saline was gassed with a mixture of 95% O_2 and 5% CO_2 and the temperature was 37.0 ± 0.5°C. The phrenic nerve was stimulated via a suction electrode by supramaximal pulses of 0.05 ms duration at 30 Hz.

Glass capillary microelectrodes filled with 3 M KCl were used intracellularly to record resting membrane potentials (RMPs) and action potentials (APs). When recording action potentials, many pins were used to fix the muscle to the Sylgard and, if necessary, some muscle fibres were cut to minimize movement of muscle in response to nerve stimulation.

Action potentials recorded at the ends of uncut muscle fibres near the tendon were displayed on an oscilloscope and recorded on an FM tape recorder (Racal Store 4) with a tape speed of 30 inches per second (ips). These recordings were then replayed at 15/16 ips and analysed with analogue-to-digital (AD) converter in a PDP 11/03 minicomputer. With an AD sampling rate of 20 kHz and a record/replay ratio of 32, the effective sampling rate of the recordings was 640 kHz. Trains of 30 APs were recorded from each muscle fibre and approximately 10 fibres were sampled from each muscle. Data from a fibre were rejected if, during the train, the RMP fell by more than 5 mV or the amplitude of the AP fell by more than 10%.

Analysis of AP recordings Computer programmes were devised which measured the amplitude, time course, and latency of each AP as the interval between the stimulus and a point on the AP's rising phase at 10% of peak amplitude. The latency of the first 10 APs in the train usually increased gradually and subsequent APs showed about the same latency, but with some variability. The data relating to individual APs were used to calculate the Mean Consecutive Difference (MCD) of latencies of APs 11–30 (i.e. at the plateau) and the individual latencies of the first 16 APs with respect to the first AP of the train. The formula used to calculate the MCD was:

$$MCD = [|L11-L12| + |L12-L13| + \ldots\ldots |L29-L30|]/19$$

Where Ln is the latency of AP number *n*.

Use of the MCD as a measure of variability is considered to reduce the effects of any long-term drifts in latency, but might hide any initial changes at the beginning of trains of APs (Ekstedt *et al.*, 1974). In our experiments, the initial change in latency is measured as the 'delay'of the sixteenth AP relative to the first AP (i.e. latency of sixteenth AP minus latency of first AP). Using a pulse generator accurate to 1 in 10^6 (Digitmer) to simulate APs at 10 Hz, the value obtained for MCD was 2.4 μs, which represents the intrinsic error of the process of record/replay/analysis.

Measurement of cholinesterase activity

To estimate the cholinesterase activity of muscle, strips of the junctional region were made by cutting across the hemidiaphragms approximately 2 mm either side of the visible intramuscular nerve. The muscle was then weighed and homogenized in 5 ml of 0.1 M sodium phosphate buffer (pH 8.0). The homogenate was incubated for 30 min with 50 mM ethopropazine, a selective inhibitor of butyrylcholinesterase (Bayliss & Todrick, 1953), then sonicated and centrifuged at 1500 *g* for 15 min at 4°C. The clear supernatant was assayed for cholinesterase activity using the method of Ellman *et al.* (1961). Cholinesterase activity was expressed in nmol acetylthiocholine hydrolysed per minute per milligram of muscle and is considered to be due substantially to the acetylcholinesterase activity of the endplate (Das, 1989).

The anticholinesterases used were: ecothiopate, (S-(2-trimethylammoniumethyl) phosphorothioate iodide) or BOS (pinacolyl *S*-(2-trimethylaminoethyl) methylphosphonothioate).

Ecothiopate (ECO) was made up from Phospholine Eyedrops (Ayerst) which contained 12.5 mg ECO and a potassium acetate/boric acid buffer. The amount of potassium injected in the standard dose of ECO was negligible (approximately 6.0 μmol kg^{-1}). Solutions of BOS for injection were made up shortly before use.

Administration of possible protective agents

Single doses of pralidoxime methiodide (Sigma), 110 μmol kg^{-1} (2PAM), were given subcutaneously to mice at the first appearance of fasciculations i.e. at 10–15 min after the injection of ECO or of BOS. Two protocols were used for multiple dosing with 2PAM after BOS. The shorter protocol (7 × 2PAM) involved seven injections over a period of 12 h; the first was 110 μmol kg^{-1}, the next 5 injections were of 55 μmol kg^{-1} at 2 h intervals, with a final injection of 110 μmol kg^{-1} 12 h after the first. The longer protocol (12 × 2PAM) began as the shorter protocol, plus further injections of 110 μmol kg^{-1} at 24, 30, 36, 42 and 48 h after the first.

In experiments with pyridostigmine, a single injection (0.38 μmol kg^{-1}) of pyridostigmine bromide (Mestinon; Roche) was given 30 min before 0.5 mol kg^{-1} ECO.

In experiments with antioxidants, mice were dosed by gavage with vitamin E (Sigma) and/or with N-acetylcysteine solution (Parvolex; Duncan Flockhart). Both were given by gavage once daily for 7 days, vitamin E (VitE, 1.2 mmol kg^{-1}) dissolved in 0.1 ml corn oil; N-acetylcysteine (NAC, 6.2 mmol kg^{-1}). Some mice received both at different times in the day. ECO (0.5 mol kg^{-1} or 0.4 mol kg^{-1}) was injected on the seventh day. In another experiment, the same dose of vitamin E alone was given for 12 days, with ECO (0.5 mol kg^{-1}) being injected on the seventh day.

Statistical analysis

Unless otherwise stated, all results are expressed as mean ± 1 s.d. of values from 2 to 5 animals, with the number of muscle fibres in parentheses. To test for significances of differences between groups of data, non-parametric tests were used and differences were taken to be significant if $P < 0.05$ (2-tail). For data on jitter i.e. delay and MCD, usually the Kolmogorov-Smirnov test was used as this is more sensitive to changes in the skewness of the distributions under test. Because reciprocal transformation of MCD values normalizes its distribution (Baker *et al.*, 1987), the Mann-Whitney test was also applied to groups of MCD data thus transformed. Groups of data of cholinesterase activity were compared by the Mann-Whitney test.

Results

The effect of treatment on acetylcholinesterase activity

Two series of experiments were carried out, one to study the effectiveness of drugs which protect AChE against inhibition by anticholinesterases or which may reactivate the inhibited enzyme, the other series was to study the effect of an antioxidant to chelate oxygen-derived free radicals. In the control experiments of both series, almost 90% of the acetylcholinesterase (AChE) activity of muscle homogenates was inhibited (Table 1) 3 h after injection *in vivo* of either ECO (0.5 μmol kg^{-1}) or BOS (8.0 μmol kg^{-1}). Pretreatment with pyridostigmine (PYR) before ECO or treatment with a single dose of pralidoxime (2PAM) soon after ECO almost doubled the amount of uninhibited AChE. Prophylaxis with PYR

Table 1 Effect of protective agents on acetylcholinesterase (AChE) activity in homogenates of junctional strips of diaphragm muscle 3 h after injection of anticholinesterase *in vivo*

	Drug dose (μmol kg^{-1})	*Muscle cholinesterase activity* (nmol min^{-1} mg^{-1})	*% inhibition*
Series 1	Untreated	1.34 ± 0.43 (38)†	0
	ECO (0.5)	0.17 ± 0.04 (8)*	87
	BOS (8.0)	0.18 ± 0.05 (11)*	87
	ECO + 2PAM	0.35 ± 0.08 (17)*†	74
	BOS + 2PAM	0.20 ± 0.02 (5)*	85
	PYR + ECO	0.34 ± 0.09 (4)*†	75
Series 2	Untreated	1.42 ± 0.25 (55)†	0
	ECO (0.5) + H_2O	0.14 ± 0.03 (4)*	89
	VitE + ECO	0.14 ± 0.01 (4)*	89
	NAC + ECO	0.15 ± 0.03 (8)*	89
	NAC + VitE + ECO	0.15 ± 0.05 (5)*	89

Series 1:- the effect of ECO or BOS, with 2PAM, 110 μmol kg^{-1} injected when fasciculations were seen, or PYR 0.38 μmol kg^{-1} injected 30 min before ECO.
Series 2:- the effect of antioxidants VitE 1.2 mmol kg^{-1} and/or NAC 6.2 mmol kg^{-1} daily for 7 days before ECO. For abbreviations, see text.
Values are mean ± 1 s.d., with the number of preparations in parentheses.
*Indicates significant difference from untreated preparations; †indicates significant difference from preparations after ECO.

prevented the appearance of fasciculations after ECO, and treatment with 2PAM abolished them within 10–15 min. Treatment with a single dose of 2PAM after BOS did not affect the inhibition produced by this non-reactivatable inhibitor.

In the second series of experiments, Vitamin E or NAC given singly or together had no effect on the inhibition of AChE measured 3 h after ECO.

It is concluded that the administration of pyridostigmine or of 2PAM diminished the inhibition of AChE 3 h after ECO, but 2PAM had no effect on the inhibition due to BOS, and that the inhibition of AChE after ECO was unaffected by the antioxidants VitE and NAC.

The effect of treatment on electrophysiological characteristics

None of the treatments described above in Methods, with or without challenge by ECO had any effect on resting membrane potential or on the amplitude or the time to peak of APs.

Pyridostigmine Five days after the injection of ECO, both MCD and delay were increased (Figure 1). Pretreatment with PYR completely prevented the increase in delay and in MCD. In control experiments in which the PYR alone was given without challenge by ECO, there was no effect on jitter.

Pralidoxime The reactivator 2PAM given alone had no effect on jitter. In experiments with 2PAM given after ECO (Figure 1), it was found that there was an optimal time for 2PAM to be effective in preventing or reducing the effects of ECO on MCD and delay. Giving 2PAM 10–20 min after ECO i.e. when fasciculations were first seen, resulted in values for delay and MCD indistinguishable from the untreated controls. When 2PAM was given 1 h after ECO, it reduced the increase in delay and abolished the increase in MCD. 2PAM given at 2 h or 6 h after ECO, reduced the increase in the delay to that with 2PAM given 1 h after ECO, but had no effect on the increase in the MCD which was not different from that after ECO alone. 2PAM given 24 h after ECO resulted in a smaller increase in the delay than after ECO alone, but no reduction of the increase in MCD.

It is concluded that prevention of the effects of ECO can be effected by prophylaxis with pyridostigmine or by early treatment with 2PAM. If treatment was delayed for more than 1 h after intoxication with ECO, then 2PAM was only partially effective in reducing the increased delay and ineffective on MCD.

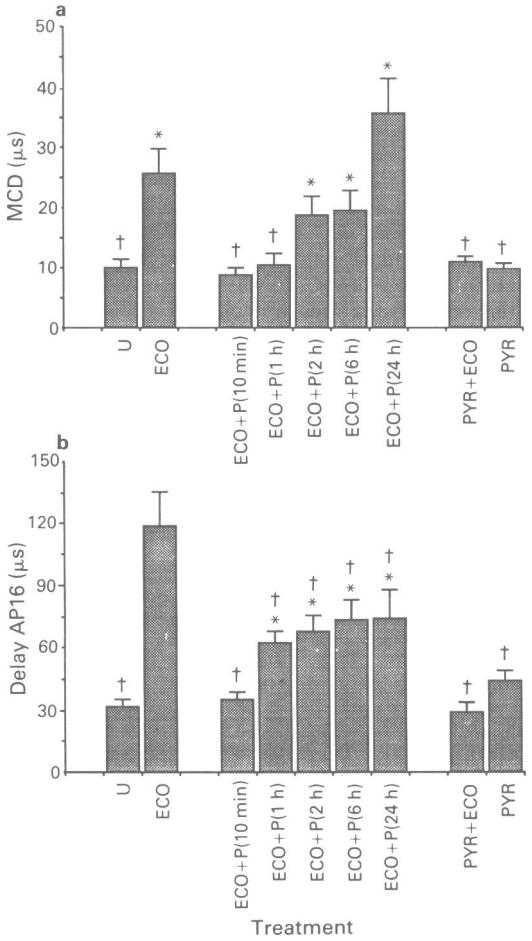

Figure 1 The effects of a single dose either of pralidoxime (2PAM) at various times after ecothiopate (ECO) or of pyridostigmine (PYR) 30 min before ECO on the mean consecutive difference (MCD) (a) and delay (b) of action potentials at 30 Hz 5 days after ECO was injected *in vivo*. Treatments are indicated by: U (untreated); ECO (ECO only); ECO + P (ECO followed by 2PAM, at times in parentheses); PYR + ECO (pyridostigmine before ECO); and PYR (pryidostigmine only). Values are mean ± 1 s.e. of data from 2 to 5 animals. *Indicates significant difference from untreated preparations; †indicates significant difference from preparations after ECO.

Antioxidants Although pretreatment with antioxidants ameliorates damage of diaphragm muscle after ECO, neither VitE nor NAC given as pretreatment, either alone or together, had any effect on the increase in MCD and delay measured 5 days after ECO. Furthermore, prolonging treatment with VitE after the 7 days pretreatment before ECO to include the 5 days before the determination of jitter, gave no additional protection (pVITE in Table 2).

BOS and pralidoxime Whilst early treatment with a single dose of 2PAM was able to prevent the increased jitter after ECO, such treatment was unable to prevent the increases in delay and MCD after BOS (Table 3). This result was not unexpected as 2PAM did not reactivate AChE after BOS (Table 1). However, 2PAM has a short half-life compared to BOS, 1–1.5 h in man (Sidell & Groff, 1971), so its availability was continued by repetitive dosing. When 7 injections of 2PAM were given during the first 12 h after BOS, a smaller increase in jitter resulted measured 5 days later. A more protracted dosing with 2PAM for up to 3 days was ineffective in reducing the increased delay and MCD. This protracted dosing with 2PAM had no effect on normal jitter.

Thus there was an optimal period of 12 h of repeated dosing with 2PAM which ameliorated the effects of BOS on jitter.

Table 3 The effects of single or multiple doses of pralidoxime (2PAM) on the delay and mean consecutive difference (MCD) of action potentials at 30 Hz, 5 days after BOS was injected *in vivo*

Drug (μmol kg^{-1})	*Delay* (μs)	*MCD* (μs)
Untreated	32 ± 22 (42)†	9.9 ± 3.6 (42)†
BOS (8.0)	162 ± 181 (59)*	53.3 ± 86.2 (59)*
BOS (8.0) + 1 × 2PAM	109 ± 69 (21)*$^{\&}$	34.9 ± 38.3 (21)*$^{\$@}$
BOS (8.0) + 7 × 2PAM	85 ± 86 (23)*†$^{\$@}$	22.2 ± 22.1 (23)*†$^{\$\#}$
BOS (8.0) + 12 × 2PAM	126 ± 79 (33)*$^{\&@}$	41.2 ± 24.4 (33)*$^{@\#}$
12 × 2PAM	32 ± 23 (29)†	9.0 ± 3.1 (29)†
7 × 2PAM	32 ± 33 (19)†	9.3 ± 3.0 (19)†

Details of dosage and experimental protocol in Methods. Values are mean ± 1 s.d. of data from 2 to 5 animals, with the number of muscle fibres in parentheses. For abbreviations, see text.
*Indicates significant difference from untreated preparations; † indicates significant difference from values after BOS alone.
$^{\#,@}$ Indicate that values so marked are significantly different.
$^{\&}$ Indicate that values so marked are not different.

Discussion

Effects of protecting or reactivating acetylcholinesterase

The acetylcholinesterase activity of diaphragm homogenates was inhibited rapidly after the injection of ECO and its recovery was evident 1 day later (Townsend, 1988; Bamforth, 1989) and was complete by 5 days (Kelly *et al.*, 1990). The increased jitter appeared 1 day after ECO at a maximal value which persisted until day 5. Kelly *et al.* concluded that although inhibition of AChE was necessary for the onset of jitter, the relationship was not clear.

Previous workers studying large doses of OPs have attributed the protection against the lethal effects afforded by PYR and by 2PAM to the preservation or reactivation of a small, essential component of AChE activity in muscle (Dirnhuber & Green, 1978; French *et al.*, 1979; Leadbeater *et al.*, 1985). In our experiments, protection by PYR of some of the AChE against inhibition of ECO or the early reactivation of some of the ECO-inhibited enzyme with 2PAM, was associated with prevention of increased MCD or delay. There was doubling of the AChE activity remaining, for approximately 75% of the enzyme was inhibited 3 h after ECO instead of the usual 87%. Considering these results in isolation, it might seem that initiation of increased jitter requires inhibition of more than 75% of the AChE. However, Kelly *et al.* (1990) showed that ECO 0.3 mol kg^{-1} caused 87% inhibition at 3 h, but only an increase in delay at 5 days, and 1 mol kg^{-1} BOS increased MCD but caused only 37% inhibition of acetylcholinesterase. Thus there appears to be no simple relationship between increased jitter and the maximum inhibition of the AChE of diaphragm homogenates at 3 h after injection of organophosphate. It may be that some of the acetylcholinesterase activity measured may not be 'functional' i.e. involved in the modulation of transmitter action. The amount of synaptic AChE, which must include the functional enzyme, may be only about 60% of the total AChE activity of homogenates, and ECO inhibits both synaptic and extrasynaptic AChE located both intra- and extra-cellularly (Newman *et al.*, 1984). Thus it may not be reasonable to expect a strict correlation between total AChE activity and jitter.

The interval between giving ECO and treatment with 2PAM to prevent or reduce the increase in jitter is also of interest. The administration of 2PAM at onset of fasciculations, about 10–20 min after ECO, completely prevented the increase in both MCD and delay, whereas postponing the injection of 2PAM until 1 h after ECO prevented the increase in MCD but merely reduced the increase in delay. Injection of 2PAM at later times (up to 24 h) after ECO did not ameliorate the increase in MCD but reduced the increase in delay. Thus it appears that the increased delay requires a shorter duration of inhibition for its appearance, but because the increase in delay can be reduced by 2PAM up to 24 h after injection of ECO, the full development of increased delay depends upon prolonged inhibition of AChE. In contrast, the appearance of increased MCD requires a longer duration of inhibition, about 1–2 h, but the full development of increased MCD comes on soon after this. These observations confirm the suspicions that delay and MCD have different aetiologies.

Table 2 The effects of antioxidants on the delay and mean consecutive difference (MCD) of action potentials at 30 Hz, 5 days after ecothiopate (ECO) was injected *in vivo*

Drug (μmol kg^{-1})	*Delay* (μs)	*MCD* (μs)
Untreated	32 ± 22 (42)†	9.9 ± 3.6 (42)†
ECO (0.5)	119 ± 104 (40)*	25.6 ± 28.0 (39)*
ECO (0.5 + VitE	94 ± 44 (23)*	17.4 ± 7.9 (23)*
ECO (0.5) + NAC	132 ± 77 (33)*$^{@}$	31.5 ± 22.8 (22)*$^{@}$
ECO (0.5) + VitE + NAC	90 ± 36 (29)*	19.6 ± 14.5 (29)*
ECO (0.5) + pVitE	85 ± 43 (31)*$^{@}$	19.3 ± 16.6 (32)*$^{@}$

Data from 2 to 5 animals, with the number of muscle fibres in parentheses. VitE, NAC indicate treatment for 7 days before ECO. pVitE indicates treatment for 7 days before ECO and 5 days after. For abbreviations, see text. All values in each column not significantly different from each other except:-
*indicates significant difference from untreated preparations; † indicates significant difference from preparations after ECO. @ indicates that values so marked are significantly different.

Effects of 2PAM after BOS

A single dose of 2PAM given at the start of fasciculation did not affect the increased jitter produced by BOS and did not

produce any reactivation of the inhibited AChE, perhaps because of the rapid aging of the phosphorylated enzyme complex (Harris *et al.*, 1971). However, seven injections of 2PAM given over a 12 h period after the BOS reduced the effects of BOS on both MCD and delay. This indicates that the beneficial effects of 2PAM are not due to reactivation of the enzyme and may be due to another antidotal action of oximes (Smith & Muir, 1977; Smith *et al.*, 1981). One such action may be to reduce the mean open time of frog endplate channels (Alkondon *et al.*, 1988), although such an action has not been confirmed in murine muscle (Tattersall, personal communication). However, an effect of 2PAM on the endplate channels of mouse diaphragm is indicated by the reduction by 2PAM of the half decay time of extracellularly-recorded miniature endplate currents. This reduction in half decay time was seen in untreated mouse diaphragm muscles but the reduction was even more pronounced in muscles from mice pretreated with BOS (Bamforth, 1990). Perhaps such an action may contribute to the ability of multiple doses of 2PAM to reduce the effects of BOS on jitter.

Increasing the duration of treatment with 2PAM removed rather than increased its beneficial effects. The administration of 2PAM alone did not increase jitter, so it appears that the prolonged treatment negates the effects of the shorter multiple-dose treatment. The multiple actions of oximes at the neuromuscular junction, and their possible interactions, make it difficult to find a plausible mechanism for this observation.

Antioxidants and jitter after ECO

The administration of antioxidants such as VitE for 7 days before ECO ameliorated the damage to mouse diaphragm, perhaps by chelation of free radicals (Das, 1989). Thus if the increased jitter after ECO was closely associated with necrosis, e.g. occurred in fibres just short of necrosis or in regenerating fibres, it would be expected that antioxidants might reduce to prevent the increased jitter. However, neither vitamin E nor NAC, alone or in combination, in dosage regimes which ameliorated the myopathy, had any effect on the increase in MCD and delay produced by ECO, even after prolonging the daily dosing with Vitamin E to cover the period after ECO exposure until the day when the jitter was measured. The failure of treatment with antioxidants to prevent the increased jitter together with the observation that non-necrotizing doses of ECO can produce increased MCD and delay (Kelly *et al.*, 1990), is evidence that increased jitter is not a consequence of necrosis. Thus the common steps in the aetiology of necrosis and jitter after ECO precede the generation and action of free radicals.

It may be that the toxic effects of the inhibition of AChE are consequences of the entry of calcium into the muscle cell. The prolonged action of ACh at the neuromuscular junction promotes the entry of Ca^{2+} to the muscle cell at the endplate (Evans, 1974; Miledi *et al.*, 1977; Burd *et al.*, 1989). An increased amount of calcium at the endplate has been found to be one of the earliest effects of ECO given *in vivo* (Townsend, 1988) and could initiate myopathy by a number of processes including generation of free radicals. Whilst the ineffectiveness of treatment with antioxidants excludes free radicals as agents of increased jitter, the possibility remains that increased jitter may be a consequence of an increased cytosolic calcium due to loading of fibres with Ca^{2+} or to interference with its intracellular buffering. In this regard, ECO caused gross swelling of the sarcoplasmic reticulum after administration *in vitro* (Ferry & Cullen, 1991), and *in vivo* (Townsend, 1988). Such damage decreased from a peak at 24 h, which suggests that excessive jitter may not require damage sufficiently great to be evident in electronmicrographs.

The treatments with PYR or with 2PAM which successfully prevent the appearance of jitter after ECO, also prevent the induction of gross myopathy and diminish the loading with calcium (Das, 1989), which is consistent with the hypothesis of Ca-loading as a mechanism in common for the induction of jitter and of necrosis of the endplate region of muscle. Perhaps an increased cytosolic $[Ca^{2+}]$, which was moderate in extent because of effective buffering, would initiate events which culminate in increased jitter 1–5 days later. A larger increase in $[Ca^{2+}]$ might overwhelm the Ca-buffers and might then initiate further mechanisms involving generation of free radicals resulting in necrosis within 3–12 h.

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Inhibition of the haemodynamic effects of angiotensin II in conscious rats by AT_2-receptor antagonists given after the AT_1-receptor antagonist, EXP 3174

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1 Conscious, Long Evans rats ($n = 10$), chronically instrumented for the measurement of regional haemodynamics, were studied on 3 consecutive experimental days to assess responses to angiotensin II (AII) (125 pmol kg^{-1}, i.v.) and noradrenaline (1 nmol kg^{-1}, i.v.) in the absence and presence of the AT_2-receptor antagonist, PD 123319 (10 mg kg^{-1}, i.v.) (day 1), the AT_1-receptor antagonist, EXP 3174 (1 mg kg^{-1}, i.v.) (day 2), and PD 123319 (10 mg kg^{-1}, i.v.) given 24 h after EXP 3174 (day 3).

2 In naive rats (day 1), PD 123319 did not antagonize the haemodynamic effects of AII or noradrenaline. EXP 3174 (day 2) caused a marked, prolonged blockade of the haemodynamic effects of AII but not those of noradrenaline. Twenty four h after administration of EXP 3174 (day 3) there was still significant attenuation of the haemodynamic effects of AII. However, administration of PD 123319 at this time caused a further inhibition (lasting 1 h) of the effects of AII but not those of noradrenaline.

3 An identical 3 day protocol was used in a separate group of rats ($n = 6$) in which the AT_2-receptor antagonist, PD 123177, was given instead of PD 123319, and the results were essentially the same, i.e., PD 123177 significantly attenuated the haemodynamic effects of AII but only when given 24 h after EXP 3174.

4 In a separate group of rats ($n = 4$), a low dose of EXP 3174 (60 $\mu g\ kg^{-1}$ i.v.) was given to naive rats in order to simulate the degree of inhibition of the effects of AII seen after administration of AT_2-receptor antagonists in animals pretreated with EXP 3174. This low dose of EXP 3174 did not produce a sustained inhibition of the effects of AII and the time course of recovery of AII responses was similar to that seen with PD 123319 or PD 123177 given after the high dose of EXP 3174.

5 The apparent inhibition of the effects of AII by the AT_2-receptor antagonists, PD 123319 and PD 123177, when these were administered 24 h after the AT_1-receptor antagonist, EXP 3174, may have been due to the functional activation of AT_2-receptors and/or loss of AT_2-receptor antagonist selectivity, and/or the displacement of nonspecifically bound EXP 3174 by AT_2-receptor antagonists. While the latter explanation seems the most likely, these results raise the possibility that nonpeptide, AII-receptor antagonists that act at both AT_1- and AT_2-receptors may have therapeutic advantages over selective AT_1-receptor antagonists.

Keywords: Angiotensin II; EXP 3174; PD 123319; PD 123177; conscious rats; haemodynamic effects

Introduction

Recently, the development of several nonpeptide angiotensin II (AII) antagonists, and their application in radioreceptor assays has provided convincing evidence for the existence of two subtypes of the AII receptor (Chiu *et al.*, 1989; Whitebread *et al.*, 1989). Based on studies using nonpeptide antagonists such as DuP 753 (Losartan) (Chiu *et al.*, 1990; Wong *et al.*, 1990b), PD 123177 (Chiu *et al.*, 1989; Wong *et al.*, 1990a) and PD 123319 (Dudley *et al.*, 1990), a new receptor classification for AII has been proposed (Bumpus *et al.*, 1991). The AII receptor subtype sensitive to either DuP 753, or its active metabolite, EXP 3174 (Wong *et al.*, 1990c), has been designated AT_1, while the receptor subtype sensitive to either PD 123319 or PD 123177 has been designated AT_2.

DuP 753 is approximately 10,000 fold more selective for the AT_1-, than the AT_2-receptor, while PD 123177 displays about a 3500 fold selectivity for AT_2-receptors (Timmermans *et al.*, 1991). AT_1- and AT_2-receptors are widely distributed in the brain and peripheral tissues; however, the majority of AII receptors in vascular tissue and in central cardiovascular control regions sensitive to AII are of the AT_1 subtype (Whitebread *et al.*, 1989; Gehlert *et al.*, 1991). Furthermore, all the cardiovascular and fluid homeostatic effects of AII appear to be mediated through stimulation of AT_1-receptors. As yet, there has been no functional role identified for the AT_2-receptor (Dudley *et al.*, 1990; Wong *et al.*, 1990a), although a novel signal transduction mechanism has recently been identified (Bottari *et al.*, 1992).

The possibility exists that AT_2-receptors are clearance receptors, analogous to those for atrial natriuretic peptide (Maack *et al.*, 1987). However, Wong *et al.* (1990a) found that pretreatment with AT_2-receptor antagonists did not augment responses to AII acutely, so this possibility seems unlikely. Furthermore, the ability of AT_1-receptor antagonists to abolish all known effects of AII indicates that AT_2-receptors are not involved in the effects of AII under normal, acute circumstances. Nevertheless, considering the long duration of action of peripherally-administered AT_1-receptor antagonists it seemed feasible that in the period following administration of AT_1-receptor antagonists when responses to AII were recovering, a functional role for AT_2-receptors might be discernible. To assess this possibility, we gave the non-competitive AT_1-receptor antagonist, EXP 3174 (Wong *et al.*, 1990c) to conscious rats, and determined whether or not the haemodynamic effects of AII were sensitive to the subsequent administration of the AT_2-receptor antagonists, PD 123319 or PD 123177. A preliminary account of this study has been published (Widdop *et al.*, 1992).

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Methods

Male, Long Evans rats (350 to 450 g) were anaesthetized (sodium methohexitone, 60 mg kg^{-1}, i.p. supplemented as required) and had pulsed Doppler flow probes implanted to monitor changes in renal, mesenteric and hindquarters blood flows (Gardiner *et al.*, 1990a). At least 7 days later, animals were briefly anaesthetized (sodium methohexitone, 40 mg kg^{-1}, i.p.) and had 3 catheters implanted in the right jugular vein and a catheter inserted into the distal aorta, via the caudal artery. After a further 24 h recovery, experiments were begun in conscious, unrestrained animals with free access to food and water.

Continuous recordings were made of mean and phasic intra-arterial blood pressures and instantaneous heart rate, and the mean and phasic renal, mesenteric and hindquarters Doppler shift signals with a modified (Gardiner *et al.*, 1990b) pulsed Doppler system (Crystal Biotech, Holliston, U.S.A.) Regional vascular conductances were calculated by dividing the appropriate mean Doppler shift by mean arterial blood pressure (Gardiner *et al.*, 1990a).

Experiment 1

This experiment lasted for 3 days. On day 1, animals ($n = 8$) received randomized bolus i.v. injections of AII (125 pmol kg^{-1}) and noradrenaline (1 nmol kg^{-1}) at least 1 h before, and at approximately 15 min and 1 and 2 h after PD 123319 (10 mg kg^{-1}). On day 2, bolus i.v. injections of AII and noradrenaline were given at least 1 h before and at approximately 15 min, and 1, 2 and 6 h after administration of EXP 3174 (1 mg kg^{-1}). On day 3, AII and noradrenaline were given at least 1 h before (i.e. 24 h after EXP 3174) and at approximately 15 min, and 1 and 2 h after PD 123319 (10 mg kg^{-1}).

To avoid any possible interaction arising from the repeated administration of PD 123319, only the latter 2 days of the above protocol were run in 2 additional animals. Since these data provided essentially identical results to the full, 3 day protocol, these data have been included for days 2 and 3 (i.e. $n = 10$).

In 6 of these animals, EXP 3174 (1 mg kg^{-1}) was given 2 h after PD 123319 (10 mg kg^{-1}) on day 3 and responses to AII were assessed 15 min later.

Experiment 2

The protocol for this experiment was identical to Experiment 1 except that animals ($n = 6$) received PD 123177 (10 mg kg^{-1}) instead of PD 123319, to ensure the results with these AT_2-receptor antagonists were similar.

Experiment 3.

It became apparent in Experiments 1 and 2 that both AT_2-receptor antagonists, PD 123319 and PD 123177, attenuated the haemodynamic effects of AII (by 40–60%), but only when tested 24 h after the administration of the AT_1-receptor antagonist EXP 3174 (see Results). Moreover, under these circumstances, the effects of PD 123319 and PD 123177 were relatively short-lasting (see Results). Therefore, in order to assess the time course of recovery from the effects of a dose of EXP 3174 that inhibited responses to AII to an extent similar to that seen after administration of AT_2-receptor antagonists in animals pretreated with EXP 3174, naive rats ($n = 4$) were given a lower dose of EXP 3174 (60 μg kg^{-1}) and the responses to AII (125 pmol kg^{-1} i.v.) were assessed before and 15 min, and 1 and 2 h after this compound.

The 60 μg kg^{-1} dose of EXP 3174 was determined from pilot experiments, and agrees well with the IC_{50} value of EXP 3174 against the pressor effects of AII in conscious normotensive rats studied by Wong *et al.* (1990c).

Data analysis

The effects of the nonpeptide antagonists against either AII or noradrenaline on each experimental day were subjected to an initial analysis of variance (ANOVA) with repeated measures using a commercially available statistical package (CLR ANOVA on a Macintosh SE). Additionally, the effects of antagonists relative to baseline were assessed in a similar manner. A P value <0.05 was taken as significant.

Drugs

Angiotensin II (Bachem), noradrenaline bitartrate (Sigma), PD 123177 (1-[(4-amino-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazol[4, 5-c]pyridine-6-carboxylic acid), and PD 123319 ((S)-1-[[4-(di-methylamino)-3-methylphenyl]methyl]- 5-(diphenylacetyl)-4, 5, 6, 7-tetrahydro-1H-imidazol[4,5-c]pyridine-6-carboxylic acid) were dissolved in isotonic saline, while EXP 3174 ((2-n-butyl-4-chloro-1-[(2'-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole-5-carboxylic acid) was dissolved in equal parts of 5% $NaHCO_3$ and 5% dextrose. EXP 3174 and PD 123177 were obtained from Dr R. Smith (DuPont, U.S.A.) and PD 123319 was obtained from Dr J. Keiser (Parke-Davis, U.S.A.). AII and the nonpeptide antagonists were injected via separate i.v. catheters and flushed in with 0.1 ml saline (that being the catheter dead space).

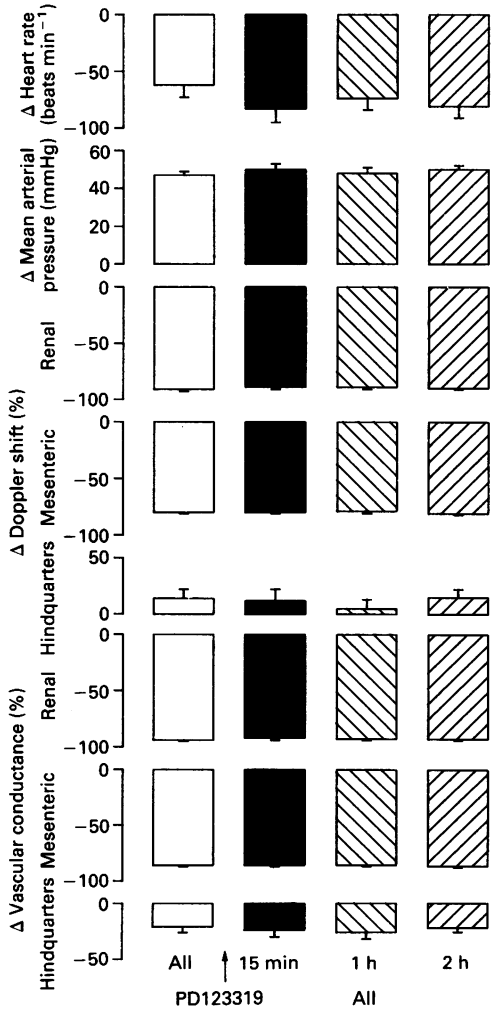

Figure 1 Cardiovascular responses to angiotensin II (AII) (125 pmol kg^{-1}, i.v.) before and 15 min and 1 and 2 h after administration of the AT_2-receptor antagonist, PD 123319 (10 mg kg^{-1}, i.v.) in conscious, Long Evans rats ($n = 8$) on the first experimental day. Values are mean with s.e.mean shown by vertical lines.

Results

Experiment 1

Day 1 AII (125 pmol kg^{-1}, i.v.) caused marked pressor and bradycardic responses, accompanied by pronounced reductions in renal and mesenteric flow, but smaller changes in hindquarters flow (see Figures 1 and 2). Hence, there were marked reductions in renal and mesenteric conductance, as well as a small reduction in hindquarters conductance. This haemodynamic pattern of marked renal and mesenteric vasoconstriction, with a negligible hindquarters effect in response to AII has been reported previously from our laboratory (Gardiner *et al.*, 1988).

Noradrenaline (1 nmol kg^{-1}, i.v.) evoked changes similar to, but less marked, than those seen with AII; they consisted of increases in blood pressure (34 ± 2 mmHg) and decreases in heart rate (−55 ± 8 beats min^{-1}), renal (−35 ± 4%), mesenteric (−65 ± 2%) and hindquarters (−5 ± 5%) flows, and decreases in renal (−50 ± 3%), mesenteric (−73 ± 2%) and hindquarters (−27 ± 3%) conductances.

PD 123319 (10 mg kg^{-1}, i.v., $n = 8$), itself, caused a transient tachycardia (at 1 min, 29 ± 7 beats min^{-1}, $P<0.05$), together with transient increases in renal flow (at 1 min, 10 ± 2%, $P<0.01$), mesenteric flow (1–2 min, maximum = 15 ± 2%, $P<0.01$), renal conductance (at 1 min, 11 ± 2%, $P<0.01$), and mesenteric conductance (1–3 min, maximum = 15 ± 2%, $P<0.05$). PD 123319 did not significantly alter the haemodynamic effects of AII (Figure 1) or noradrenaline (data not shown), when these were tested over the following 2 h.

Day 2 The control haemodynamic effects of AII (and noradrenaline) were similar to those obtained on day 1 (Figure 3). EXP 3174 (1 mg kg^{-1} i.v., $n = 10$), itself, caused a modest change in haemodynamic status, with the maximum effects occurring within 15 min after injection; there was a small fall in blood pressure (4–15 min, maximum = − 7 ± 2 mmHg, $P<0.01$) and increases in heart rate (1–15 min, maximum = 46 ± 11 beats min^{-1}, $P<0.01$), renal flow (1–15 min, maximum = 17 ± 5%, $P<0.01$), mesenteric flow (1–15 min, maximum = 15 ± 4%, $P<0.05$), renal conductance (1–15 min, maximum = 22 ± 6%, $P<0.01$), mesenteric conductance (1–15 min, maximum = 22 ± 5%, $P<0.01$) and hindquarters conductance (at 15 min, 13 ± 7%, $P<0.05$).

The effects of AII were abolished when it was administered 15 min after EXP 3174, and even 6 h after the latter compound the effects of AII were still substantially inhibited (Figures 2 and 3). However, hindquarters flow was increased by AII to a similar extent throughout the 6 h period. As a consequence, when the pressor response to AII was abolished by EXP 3174, this resulted in an increase in hindquarters conductance. In contrast to AII, the effects of noradrenaline were not significantly altered by EXP 3174 (data not shown).

Day 3 Twenty four h after the administration of EXP 3174, the haemodynamic effects of AII were still significantly attenuated compared with the effects of AII given before EXP 3174 (Figure 4). Administration of PD 123319 (10 mg kg^{-1}, i.v., $n = 10$) at this time again caused transient increases in heart rate (1–3 min, maximum = 68 ± 1 beats min^{-1}, $P<0.01$), renal flow (1–4 min, maximum = 10 ± 2%, $P<0.05$) and mesenteric flow (1–2 min, maximum = 13 ± 3%, $P<0.05$). Additionally, there was a small, but significant, pressor response (1–2 min, maximum = 6 ± 2 mmHg, $P<0.05$), which was not seen on day 1.

Fifteen min after the administration of PD 123319 on day

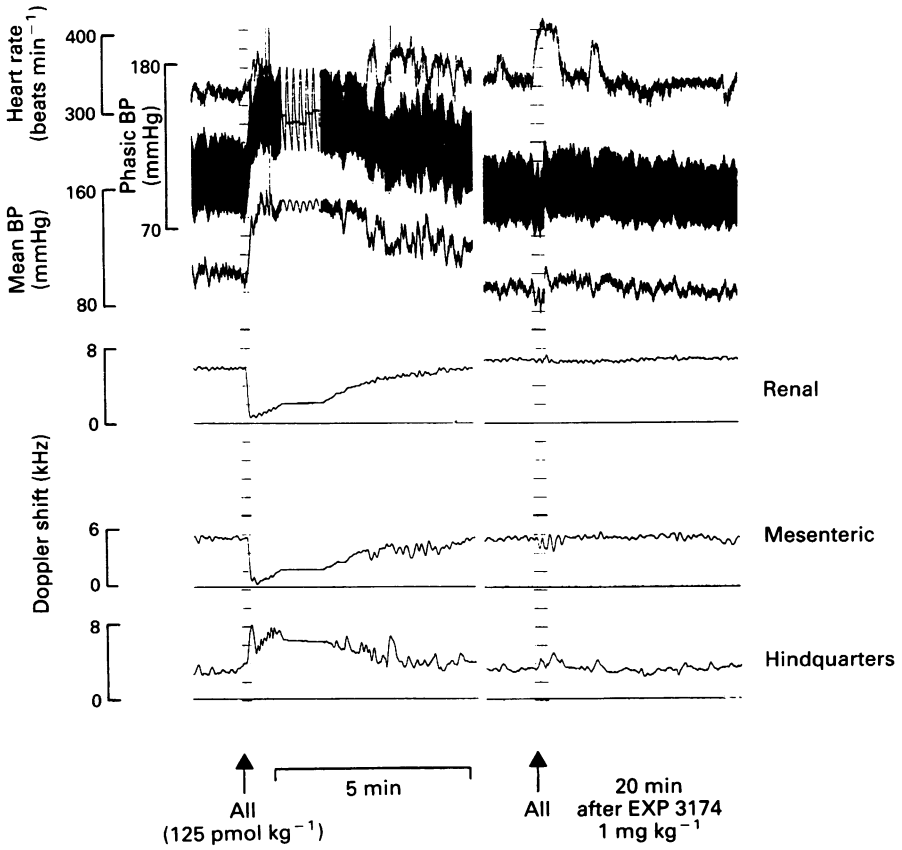

Figure 2 Cardiovascular responses to angiotensin II (AII) (125 pmol kg^{-1}, i.v.) in a conscious Long Evans rat before and approximately 20 min after the AT_1-receptor antagonist, EXP 3174 (1 mg kg^{-1}, i.v.), on the second experimental day.

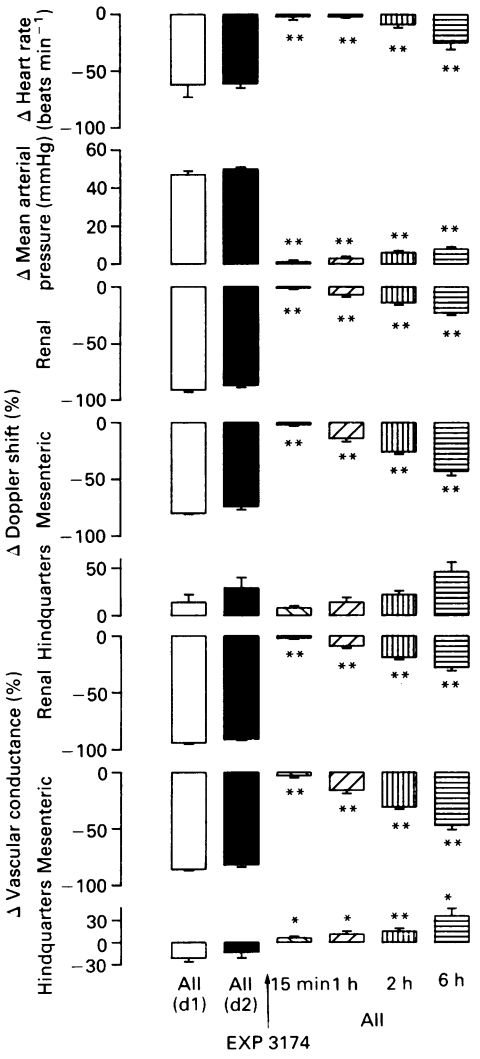

Figure 3 Cardiovascular responses to angiotensin II (AII) (125 pmol kg^{-1}, i.v.) on the first experimental day (d 1) (open columns) and on the second experimental day (d 2) (solid columns), and then at the times indicated after administration of the AT_1-receptor antagonist, EXP 3174 (1 mg kg^{-1}, i.v.) on day 2 in conscious, Long Evans rats ($n = 10$). Values are mean with s.e.mean shown by vertical lines; $*P<0.05$, $**P<0.01$ versus day 2 control value (ANOVA).

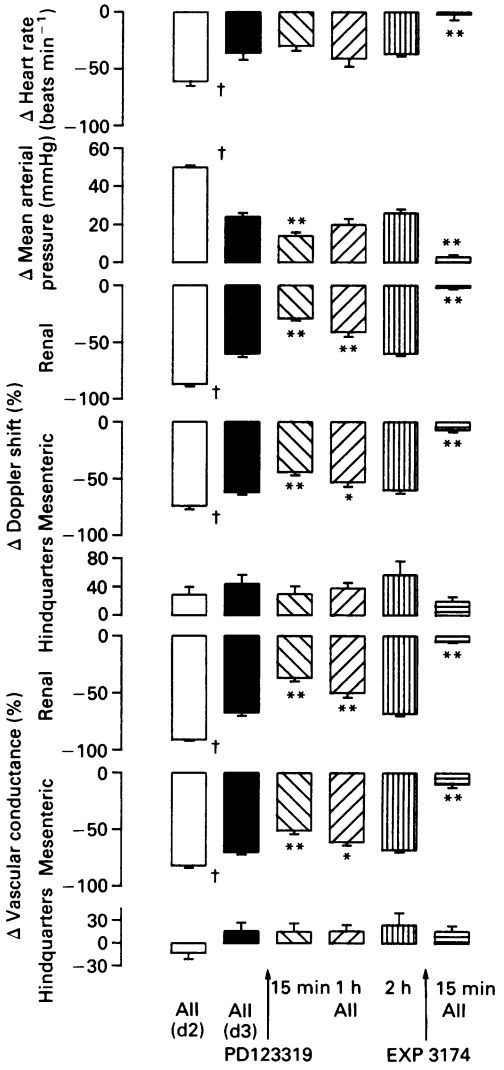

Figure 4 Cardiovascular responses to angiotensin II (AII) (125 pmol kg^{-1}, i.v.) on the second experimental day (d 2) (open columns) and 24 h after EXP 3174, i.e., on the third experimental day (d 3) (solid columns), and then at the times indicated after administration of the AT_2-receptor antagonist, PD 123319 (10 mg kg^{-1}, i.v. $n = 10$) on day 3. Finally, EXP 3174 (1 mg kg^{-1}, i.v., $n = 6$) was given approximately 2 h after PD 123319 and the response to AII was tested 15 min later. Values are mean with s.e.mean shown by vertical lines.
†Between the 2 left-hand histograms indicate significant differences between day 2 and day 3 ($P<0.01$); asterisks over subsequent histograms ($*P<0.05$; $**P<0.01$) show significant differences versus day 3 control values (ANOVA).

3, the pressor effect of AII was significantly attenuated relative to the control response of that day. Furthermore, the reductions in renal and mesenteric flows and conductances evoked by AII were attentuated 15 and 60 min after PD 123319 (Figure 4). However, 2 h after PD 123319, the effects of AII were not significantly different from the control responses of that day (Figure 4). In addition, the effects of noradrenaline were not significantly altered by PD 123319 at any time (data not shown).

EXP 3174 (1 mg kg^{-1}, i.v.), given 2 h after the administration of PD 123319 on day 3, abolished the response to AII when tested 15 min later ($n = 6$) (Figure 4).

Experiment 2

Day 1 PD 123177 (10 mg kg^{-1}, i.v., $n = 4$), itself, caused no significant changes in baseline haemodynamic variables, except for a significant tachycardia (1–3 min, maximum = 49 ± 5 beats min^{-1}, $P<0.01$). Moreover, PD 123177 had no significant effect on the haemodynamic responses to either AII or noradrenaline when tested 15 min, and 1 and 2 h later (data not shown).

Day 2 EXP 3174 (1 mg kg^{-1}, i.v., $n = 6$) abolished the effects of AII but not noradrenaline, as described in Experiment 1 (data not shown).

Day 3 Twenty four h after EXP 3174, the response to AII was still significantly attenuated, as observed in Experiment 1 (Figure 6). PD 123177 (10 mg kg^{-1}, i.v., $n = 6$), itself, caused a transient tachycardia (1–3 min, maximum = 32 ± 5 beats min^{-1}, $P<0.01$) and transient increases in mesenteric flow (at 1 min, 13 ± 3%, $P<0.01$) and conductance (1–5 min, maximum = 17 ± 3%, $P<0.01$); all other variables did not change significantly from baseline.

Following the administration of PD 123177, responses to AII were inhibited in a manner similar to that already described for PD 123319 (Figures 5 and 6). Responses to noradrenaline were unaffected by PD 123177 (data not shown).

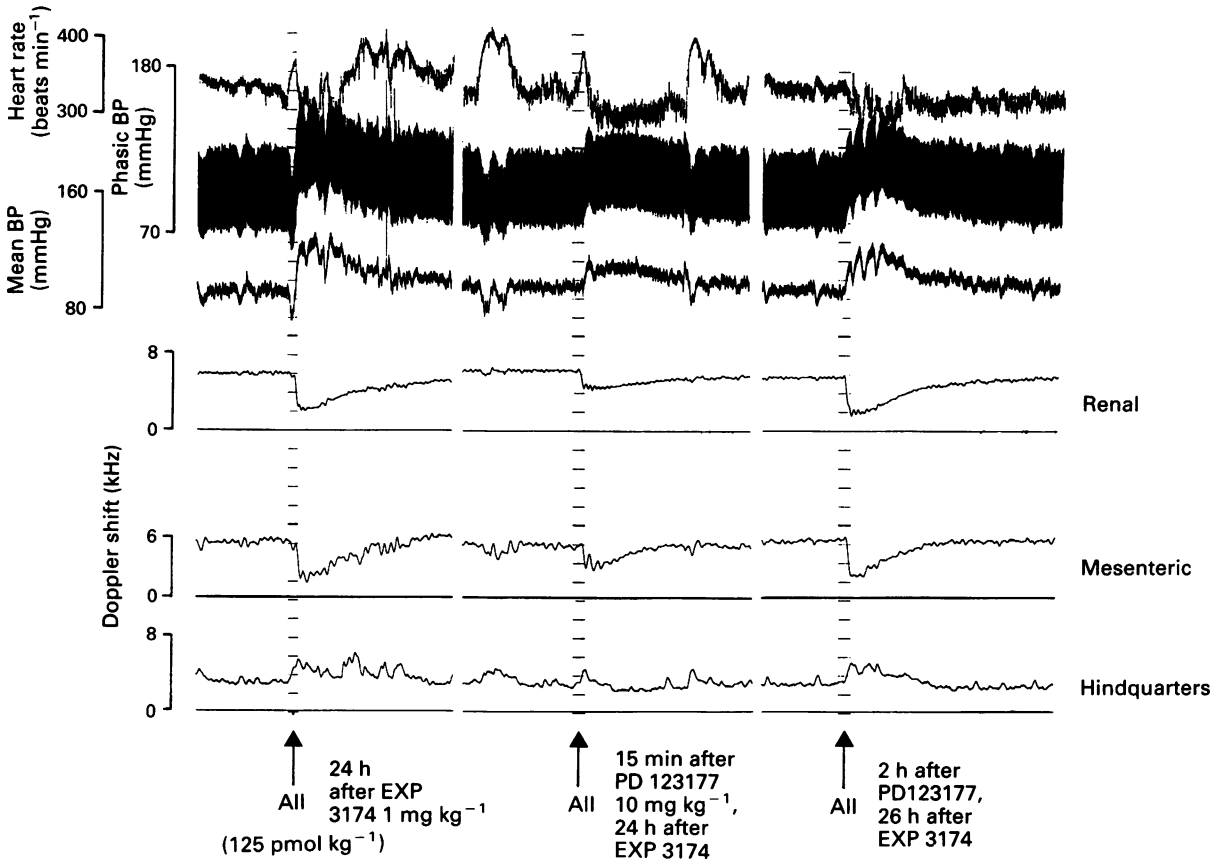

Figure 5 Cardiovascular responses to angiotensin II (AII) (125 pmol kg^{-1}, i.v.) on day 3, in the same, conscious, Long Evans rat as shown in Figure 2. AII was tested before and at the times indicated after the AT_2-receptor antagonist, PD 123177 (10 mg kg^{-1}, i.v.).

EXP 3174 (1 mg kg^{-1}, i.v.), given 2 h after PD 123177 abolished all responses to AII when this was given 15 min later ($n = 3$).

Experiment 3

EXP 3174 (60 μg kg^{-1}, i.v., $n = 4$) caused an increase in renal flow (1–15 min, maximum = 23 ± 4%, $P<0.01$) and conductance (1–15 min, maximum = 27 ± 6%, $P<0.01$) and a decrease in hindquarters flow (at 10 min, −13 ± 3%, $P<0.05$); all other variables did not change significantly from baseline.

The haemodynamic effects of AII were significantly attenuated (by approximately 40 to 60%) 15 min after the administration of EXP 3174 (60 μg kg^{-1}, i.v., $n = 4$) in naive rats (Figure 7). The degree of inhibition of the effects of AII caused by this dose of EXP 3174 was similar to that observed in Experiments 1 and 2, when AII was given 15 min after either PD 123319 or PD 123177 on day 3, i.e. 24 h after administration of EXP 3174 (1 mg kg^{-1}). Furthermore, 2 h after EXP 3174 (60 μg kg^{-1}), the mesenteric responses to AII had fully recovered, while the AII-induced pressor response and reductions in renal flow and conductance were still significantly impaired (Figure 7).

Discussion

The major finding from the present study is that the selective, AT_2-receptor antagonists, PD 123319 and PD 123177, attenuated the regional haemodynamic effects of AII, but only after the prior administration of the AT_1-receptor antagonist, EXP 3174. This finding represents the first demonstration of any effects of AT_2-receptor antagonists on cardiovascular responses to AII. Moreover, the fact that the haemodynamic responses to noradrenaline were unaffected by any of the treatment schedules also indicates that the AT_2-receptor antagonists were not causing a non-specific reduction in cardiovascular reactivity.

We can think of 3 possible explanations of these observations: (1) There was up-regulation of AT_2-receptors following administration of EXP 3174; (2) Following EXP 3174, the relative selectivity of the AT_2-receptor antagonists was lost; (3) There was displacement of non-specific binding of EXP 3174 (to tissue and plasma proteins) by both PD 123319 and PD 123177, sufficient to cause blockade of functional AT_1-receptors.

Since the present work did not provide unequivocal evidence for one or other of these possibilities, we shall consider the pros and cons for each.

(1) *Functional activation of AT_2-receptors*

It is conceivable that, following the administration of EXP 3174, there as an up-regulation of AT_2-receptors and/or an enhanced coupling of AT_2-receptors to effector mechanisms. Although the AT_1-receptor usually predominates in vascular smooth muscle (Whitebread *et al.*, 1989; Chang & Lotti, 1991) it is feasible that an increase in AT_2-receptor number occurred in our experimental conditions. However, a simple increase in receptor number would not necessarily produce the results seen, since, even in the adrenal medulla, which contains approximately 90% AT_2-receptors, AII-induced adrenaline release is mediated via AT_1-receptors (Wong *et al.*, 1990a). Therefore, any up-regulation of AT_2 receptors would probably need to be accompanied by a functional coupling of this binding site to post-receptor effector mechanisms. Interestingly, it has recently been reported that differentiation of

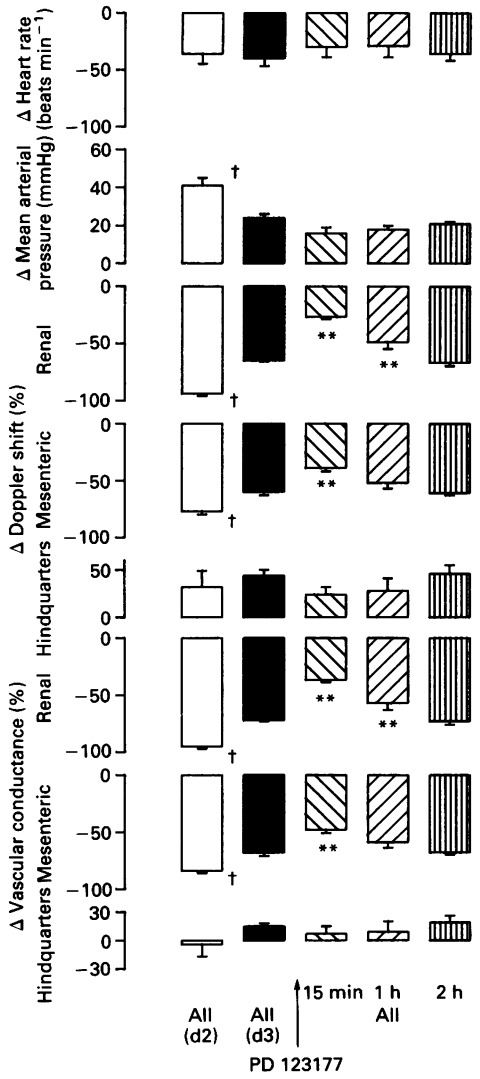

Figure 6 Cardiovascular responses to angiotensin II (AII) (125 pmol kg^{-1}, i.v.) on the second experimental day (d 2) (open columns) and 24 h after EXP 3174, i.e. on day 3 (d 3) (solid columns), and then at the times indicated after administration of the AT_2-receptor antagonist, PD 123177 (10 mg kg^{-1}, i.v.) in conscious, Long Evans rats ($n = 6$). Values are mean with s.e.mean shown by vertical lines.
†Between the 2 left-hand histograms indicate significant differences between day 2 and day 3 ($P<0.01$); asterisks over subsequent histograms ($^{**}P<0.01$) show significant differences versus day 3 control values (ANOVA).

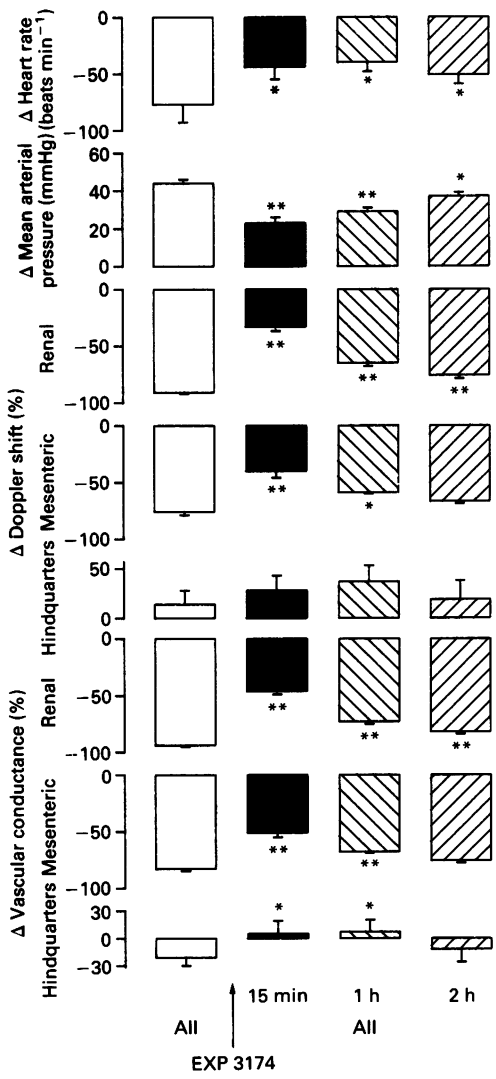

Figure 7 Cardiovascular responses to angiotensin II (AII) (125 pmol kg^{-1}, i.v.) before and 15 min and 1 and 2 h after administration of a low dose of EXP 3174 (60 μg kg^{-1}, i.v.) in conscious, Long Evans rats ($n = 4$). Values are mean with s.e.mean shown by vertical lines.
$^{*}P<0.05$; $^{**}P<0.01$ versus control value (ANOVA).

NG-108-15 cells results in a change from predominantly AT_1- to AT_2-receptor expression, as soon as 2 days later, although a functional response mediated through the AT_2-receptor was not identified (Bryson *et al.*, 1992). In contrast, AT_2-receptors in rat adrenal glomerulosa and PC12W cells stimulate protein tyrosine phosphatase activity which inhibits particulate guanylate cyclase activity (Bottari *et al.*, 1992). However, it remains to be determined if this occurs in vascular smooth muscle.

In the present study, EXP 3174 was also given on day 3, i.e. after the administration of either of the AT_2-receptor antagonists, at a time when the effects of AII were back to control (i.e. day 3) levels. Since EXP 3174 abolished the effects of AII at this stage, it appears that, if up-regulated AT_2-receptors had become functionally coupled, these effects were expressed via an EXP 3174-sensitive mechanism.

(2) *Loss of AT_2-receptor antagonist selectivity following EXP 3174*

It is possible that exposure to EXP 3174 induced a conformational change in the AT_1-binding site which then allowed AT_2-receptor ligands to bind to it. In this context, it is notable that the kinetics of the AT_1-receptor appear to be quite complex, since Wong & Timmermans (1991) found that a competitive AT_1-receptor antagonist reversed the reduced maximum response which occurred with an insurmountable AT_1-receptor antagonist. However, when PD 123177 was added to the rabbit aorta *in vitro* in the presence of DuP 753, it failed to cause any further blockade of the AII-induced contraction of this tissue (Wong *et al.*, 1990a).

The present results might be explained by EXP 3174 reducing the number of available AT_1-receptors to a level where a non-selective antagonism of both AT_1- and AT_2-receptors, by either PD 123177 or PD 123319, was revealed. This proposal would only be tenable if there was normally a large AT_1-receptor reserve, such that AII would occupy only a small proportion of the available AT_1-receptors to evoke its maximum response. Thus, under normal conditions, any apparent 'non-selectivity' of the AT_2-receptor antagonists would

not be observed. However, while the importance of the relationship between receptor occupancy and function is well recognised for adrenoceptor subtypes (see Kenakin, 1984; Ruffolo *et al.*, 1991), there is no information for AII receptor subtypes in this context.

(3) *Displacement of bound EXP 3174 by AT_2-receptor antagonists*

Compounds such as EXP 3174 are highly bound to plasma protein (Chiu *et al.*, 1991). Therefore, in the present work, it is feasible that PD 123319 and PD 123177 displaced previously administered EXP 3174 that was still persistently bound to plasma and/or tissue proteins, and the EXP 3174 then inhibited the effects of AII. If this were so, we reasoned that it should be possible to mimic the effects of the AT_2-receptor antagonists (given 24 h after EXP 3174) by administering a lower dose of EXP 3174 (60 $\mu g\,kg^{-1}$) to naive rats. This dose of EXP 3174, itself, caused hyperaemic renal vasodilatation, while other baseline haemodynamics were not altered. It is difficult to compare these changes with the effects of PD 123319 or PD 123177, given 24 h after the high dose of EXP 3174, since the AT_2-receptor antagonists also exerted some influences on baseline cardiovascular status in naive rats. Nevertheless, selective renal vasodilatation, as occurred with the lower dose of EXP 3174 in naive rats, was not seen with either AT_2-receptor antagonist, given 24 h after the high dose of EXP 3174. However, the degree and time course of inhibition of the effects of AII evoked by the lower dose of EXP 3174 were very similar to the changes seen with the AT_2-receptor antagonists in animals previously exposed to the high dose of EXP 3174. Hence, these findings are, in part, consistent with displacement of bound EXP 3174 by the AT_2-receptor antagonists being responsible for their effects.

It would be interesting to test the selective peptide AT_2-receptor antagonist, CGP 42112A (Whitebread *et al.*, 1989) to determine whether or not a compound structurally dissimilar to either PD 123319 or PD 123177 was an effective displacing agent. Other nonselective peptide antagonists, i.e. analogues of AII, would not be suitable since not only are they partial agonists, but they also have functional antagonistic activity in their own right.

In preliminary experiments ($n = 2$; Widdop *et al.*, unpublished results) we have given PD 123177 at a dose of 100 mg kg^{-1} (i.e. 10 fold higher than here), 24 h after EXP 3174, and have found almost total loss of responses to AII. But this could simply have been due to displacement of a greater amount of EXP 3174 by the higher dose of PD 123177. Against this, there is the additional preliminary finding ($n = 2$; Widdop *et al.*, unpublished results) that PD 123177 (100 mg kg^{-1}) given 48 h after EXP 3174 (1 mg kg^{-1}), when responses to AII were not impaired, caused substantial inhibition of the effects of AII. It seems unlikely this could have been due to displacement of non-specifically bound EXP 3174, unless the latter was present in the absence of any functional signs of antagonism of AII-mediated effects. While this line of investigation may be worth following, additional experiments were not possible due to shortage of AT_2-receptor antagonists.

The fact that PD 123177 is without effect on AII-induced contractions of the rabbit aorta pretreated with DuP 753 *in vitro* (Wong *et al.*, 1990a), could be taken as evidence that the phenomena described here do not occur in the absence of plasma proteins. However, it should be noted those were acute experiments, and that rabbit tissues contain few vascular AT_2-binding sites compared with rat tissues (Chang & Lotti, 1991). Moreover, DuP 753 and EXP 3174 show different behaviour as AT_1-receptor antagonists (Chiu *et al.*, 1990; Wong *et al.*, 1990b,c).

In conclusion, we have shown that the AT_2-selective ligands PD 123319 and PD 123177 caused significant AII antagonism, but only when given 24 h after the AT_1-receptor antagonist, EXP 3174. While we have offered several explanations for these results, there is no unequivocal evidence to support one or other of the hypotheses. However, on balance, the displacement of non-specifically bound EXP 3174 by the AT_2-receptor antagonists seems the most likely explanation.

Regardless of the exact mechanism involved, these novel findings should be borne in mind if EXP 3174, or the precursor compound, DuP 753, are used in the clinical setting. The present results also raise the possibility that nonpeptide, AII-receptor antagonists that act at both AT_1- and AT_2-receptors may have therapeutic advantages over those that are selective for AT_1-receptors, assuming the latter compounds find a clinical application.

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Muscarinic blockade of β-adrenoceptor-stimulated adenylyl cyclase: the role of stimulatory and inhibitory guanine-nucleotide binding regulatory proteins (G_s and G_i)

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1 The functional antagonism that exists between muscarinic and β-adrenoceptor function in guinea-pig tracheal smooth muscle was investigated by assessing G_s and G_i regulated adenylyl cyclase activity in isolated membranes.

2 Membranes from guinea-pig tracheal smooth muscle contain both $G_{i\alpha}$ and $G_{s\alpha}$ as assessed by Western blots with anti-G-protein antibodies.

3 GppNHp, a non-hydrolysable analogue of guanosine 5'-triphosphate (GTP), was shown to stimulate adenylyl cyclase activity at high concentrations (10^{-6}–10^{-4} M). GppNHp also produced a concentration-dependent reduction in pertussis toxin-catalysed adenosine diphosphate (ADP)-ribosylation of $G_{i\alpha}$.

4 Pretreatment of tracheal smooth muscle slices with methacholine (10^{-6} M) provoked a blockade of isoprenaline plus GTP, GppNHp- and GTP-stimulated adenylyl cyclase.

5 Addition of methacholine to membranes did not trigger inhibition of GTP-stimulated adenylyl cyclase activity but did block the isoprenaline-mediated augmentation of GTP-stimulated adenylyl cyclase activity.

6 Pretreatment of tracheal smooth muscle with methacholine (10^{-6} M) provoked a blockade of cholera toxin-catalysed NAD^+-dependent ADP-ribosylation of $G_{s\alpha}$.

7 Phorbol-12-myristate 13-acetate (PMA)-treatment of tracheal smooth muscle slices actually enhanced GppNHp-stimulated adenylyl cyclase activity in subsequently prepared membranes.

8 We suggest that methacholine in addition to inhibiting adenylyl cyclase via a G_i-dependent mechanism induces a functional inactivation of G_s activity. These results together may explain the functional antagonism that exists between increased muscarinic tone and the ability of β-adrenoceptor agonists to provoke excitation-contraction uncoupling.

Keywords: G-protein; protein kinase C; muscarinic agonists; adenylyl cyclase

Introduction

Contractile agonists such as acetylcholine trigger the excitation-contraction coupling of airway smooth muscle via a receptor-mediated stimulation of phospholipase C. This is a guanine nucleotide-binding regulatory protein (G-protein)-mediated event which results in the enzymatic hydrolysis of a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2, Berridge & Irvine, 1984; Nishizuka, 1984; Chilvers *et al.*, 1989). The second messengers produced are Ins(1,4,5)P_3 (which triggers Ca^{2+} mobilization by binding to a sarcoplasmic Ins(1,4,5)P_3 receptor protein) and 1,2-*sn*-diacylglycerol (a protein kinase C activator). The receptor-triggered mobilization of Ca^{2+} leads to the formation of a Ca^{2+}-calmodulin complex and the subsequent activation of myosin light chain kinase (Silver & Stull, 1982). This enzyme triggers excitation-contraction coupling by eliciting the phosphorylation of myosin light chain and thus allowing the myosin to form cross-bridges with actin. The uncoupling events governing excitation-contraction are regulated by agonists which provoke accumulation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Katsuki & Murad, 1977). Thus, β-adrenoceptor agonists provoke relaxation of airway smooth muscle via a receptor-linked G-protein mechanism which results in the stimulation of adenylyl cyclase (Gilman, 1987). The G-protein which allows coupling between β-adrenoceptor and adenylyl cyclase is termed G_s. This protein is a heterotrimer composed of three non-identical sub-units: α (45/52 kDa), β (35/36 kDa) and γ (8 kDa).

Several studies (Torphy *et al.*, 1986; Hall & Hill, 1988) have revealed that functional antagonism occurs between muscarinic, histamine and β-adrenoceptor agonists. Thus, increased muscarinic tone results in a progressive reduction in the ability of β-adrenoceptor agonists to elicit uncoupling of excitation-contraction. Furthermore, the extent of functional antagonism appears to be causally related to the magnitude of the agonist-stimulated Ins(1,4,5)P_3 response (Meurs *et al.*, 1988). We have suggested that this phenomenon may in part be due to 'cross-talk' between the PtdIns(4,5)P_2 signal cascade and the cyclic AMP signalling machinery (Pyne *et al.*, 1991).

In this paper, we demonstrate that methacholine can trigger an alteration in G_s function. The subsequent alteration in G_s function and G_i (inhibitory guanine-nucleotide regulatory binding protein)-mediated pathways may explain the altered responsiveness of airway smooth muscle to β-adrenoceptor agonists.

Methods

Dissection of tracheal smooth muscle

Trachealis was removed from the guinea-pig and rinsed in Krebs-Hensleit solution, pH 7.4, and a longitudinal cut made at the front, opposite the smooth muscle strip. Connec-

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tive tissue was removed and the smooth muscle strip cleaned by dissection under a microscope.

Preparation of tracheal smooth muscle membrane

Tracheal smooth muscle strips were homogenized in 2 ml of buffer A containing (mM): Tris/HCl (pH 7.4) 20, ethylenediaminetetraacetic acid 1, sucrose 250, phenylmethylsulphonylfluoride 0.1 and benzamidine 2, at 4°C, with an ultraturrex homogenizer (4–5 up and down strokes). The resulting homogenate was centrifuged at 50000 *g* for 20 min at 4°C, in a Beckman 55.2 Ti rotor. The supernatant was discarded and the pellet resuspended in 2 ml of buffer A and recentrifuged as before. The final pellet fraction was resuspended with 0.2 ml of buffer A (0.4 mg ml^{-1} of protein). Membranes were used immediately in the adenylyl cyclase assay.

Incubation of trachealis smooth muscle strips

Tracheal smooth muscle strips (3 mg wet weight) were placed into 10 ml of constantly gassed (O_2/CO_2, 95:5%) Krebs-Hensleit, pH 7.4 and allowed to preincubate for 10 min at 37°C. After this time methacholine (10^{-6} M) or phorbol-12-myristate 13-acetate (PMA, 0.16×10^{-6} M) was added to some of the samples and these were allowed to incubate for a further 10–30 min at 37°C. Incubations with atropine (10^{-6} M) involved a 10 min incubation, prior to the addition of methacholine. In all cases, tissue strips were removed and a membrane fraction prepared.

Adenylyl cyclase assay

Adenylyl cyclase activity was assayed according to Salomon *et al.* (1974). The membranes (20–30 μg) were combined with a reaction cocktail containing, final concentrations (mM): Tris/HCl (pH 7.8) 12.5, ATP 0.1, $MgSO_4$ 2.5, cyclic AMP 0.4, creatine phosphate 2.5, creatine kinase 1.5 units, KCl 7.5 and sucrose 30. Routinely, 1 μCi of [α-^{32}P]-ATP was added to each assay. In some instances, additions were made to the incubation and these included: isoprenaline (10^{-6} M), GTP (10^{-4} M), GppNHp (10^{-10}–10^{-4} M) and methacholine (10^{-6} M). The total assay volume was 100 μl and the incubations were performed at 37°C for 30 min and were terminated by the addition of ATP 40 mM, followed by boiling for 2 min. Samples were centrifuged at 15,000 *g* for 10 min and the recovered supernatants 'spiked' with [^{3}H]-cyclic AMP (5000 d.p.m.), which allowed the assessment of recovered [α-^{32}P]-cyclic AMP from the Dowex WH^+ and alumina column chromatography.

Preactivation of G_s by GppNHp

The tracheal smooth muscle membranes (100 μg) were resuspended in final concentrations (mM) Tris/HCl (pH 7.4) 10, $MgCl_2$ 5, dithiothreitol (DTT 1). To this was added an equal volume of GppNHp (final concentration 100 μM), and the samples were incubated at 37°C for 30 min. After this time the samples were centrifuged for 10 min at 15000 *g* at 4°C. The pellet was resuspended in buffer A and taken for cholera toxin-catalysed ADP-ribosylation. Under these conditions, activated ADP-ribosylation factor will accumulate.

Toxin-catalysed ADP-ribosylation of G_i and G_s

Cholera toxin-catalysed ADP-ribosylation of tracheal membrane protein was performed according to Heyworth *et al.* (1985). Membranes (10 μg) were combined with an ADP-ribosylation cocktail containing final concentrations (mM): potassium phosphate, (pH 7.6) 150, DTT 3, $CaCl_2$ 0.005, $MgCl_2$ 1, thymidine 7.5 and to this was added 2 μCi [^{32}P]-NAD^+ 0.02. It is not necessary to buffer the Ca^{2+} in the incubation with EGTA, since isolated membranes are used, and therefore Ca^{2+} is unlikely to vary markedly in the assay. To initiate the incubation, cholera toxin (66 μg ml^{-1}) was added. This was preactivated by combining an equal volume of DTT 50 mM, with cholera toxin and incubating for 1 h at room temperature. Ribosylation incubations were performed at 37°C for 45 min after which they were terminated by the addition of trichloroacetic acid (TCA final concentration 6%, w/v) and deoxycholate (0.01%, w/v). The samples were centrifuged at 15,000 *g* for 10 min at 4°C in a Beckman microfuge. The resulting pellet was resuspended in 10 μl of Tris/HCl 1 M, and 20 μl of the Laemmli buffer and these were boiled for 5 min after which they were subjected to SDS-PAGE (10% acrylamide).

Pertussis toxin-catalysed ADP-ribosylation was performed according to Pyne *et al.* (1989). Membranes (10 μg) were combined with a final concentration (mM): thymidine 30, potasium phosphate 80 (pH 7.5), arginine 20, $MgCl_2$ 1 and to this was added 2 μCi [^{32}P]-NAD^+ 0.02, and these were then combined with preactivated pertussis toxin (0.64 μg/assay) and incubated at 37°C for 30 min. Preactivation of pertussis toxin and the incubation of samples was performed in an identical manner to those treated with cholera toxin.

After electrophoresis, the gels were fixed in 10% TCA (w/v) for 30 min and dried prior to autoradiography at −20°C for 72 h. Location of the ADP-ribosylated peptides was followed by the densitometric analysis of the intensity of the radioactive peptides.

Western blotting

Membranes (200–300 μg protein) were subjected to SDS-PAGE, and transferred to nitrocellulose. After transfer, the sheets were blocked in 3% (w/v) gelatin and buffer B containing Tris/HCl (pH 7.4) 10 mM, NaCl 0.9%, at 37°C for 2 h. After washing, the sheets were incubated with either an anti-G_s antisera or an anti-G_i antisera (SG1), for 12 h at 30°C. Routinely, a 1/200 dilution of antisera was used. After this incubation, the sheets were washed in buffer B containing Tween-20 (0.05% v/v) and then in buffer B alone. The sheets were then incubated with a horse radish peroxidase-linked anti-rabbit antibody (1/200 dilution) for 2 h at room temperature and then washed as before. The sheets were then reacted with 1 ml of O-dianisidine (10 mg ml^{-1}), 10 μl H_2O_2 (30%, v/v) and 40 ml Tris/HCl, pH 7.4 (10 mM) in order to locate the immunoreactive peptide bands.

Materials

All biochemicals such as GppNHp, GTP and ATP were from Boehringer Mannheim (Germany). All chemicals and toxins were from Sigma Chemical Company (England). [^{32}P]-NAD was purchased from Dupont (U.S.A.) and [^{3}H]-cyclic AMP and [α-^{32}P]-ATP were obtained from Amersham International plc (UK). Horse-radish peroxidase linked antibody was a gift from the Scottish antibody production unit (Carluke, Scotland). SG1 was a kind gift from Dr G. Milligan (University of Glasgow). Anti-G_s anti-sera was produced to the C-terminal decapeptide of $G_{s\alpha}$ (RMHLRGYELL).

Results

Immunoblotting of G_i and G_s with peptide-directed antibodies

Western blotting of membranes with anti-$G_{s\alpha}$ anti-sera (which detected recombinant $G_{s\alpha}$, data not shown) revealed a single immunologically reactive peptide with an $M_r = 45$ kDa (Figure 1). This polypeptide co-migrates with the 45 kDa recombinant spliced variant of $G_{s\alpha}$. These data suggest that tracheal smooth muscle membranes contain only the small version of $G_{s\alpha}$, since no immunoreactive polypeptide was identified with a molecular mass of 52 kDa. Furthermore, no immunoreactive polypeptides were identified in the molecular

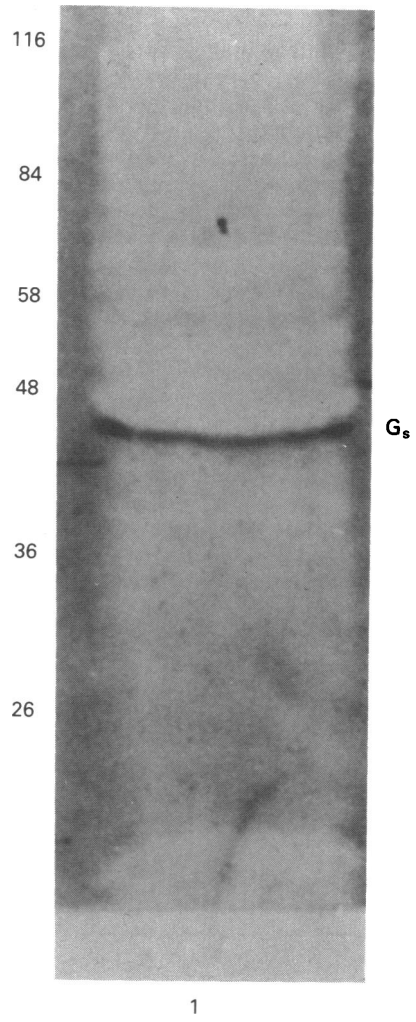

Figure 1 Immunological identification of $G_{s\alpha}$: this figure shows a typical western blot of an SDS PAGE of resolved proteins from tracheal smooth muscle. Blotting was against the anti-G_s antisera. The location of $G_{s\alpha}$ sub-unit is shown. Molecular mass markers are shown. This is a typical result from the experiment performed at least 10 times on different membrane preparations.

mass range of 39–42 kDa corresponding to the α sub-units of G_i, G_0 and G_q.

Western blotting of membranes with SG1 revealed a single immunoreactive peptide with an $M_r = 41$ kDa corresponding to $G_{i\alpha}$ (Figure 2). However, the exact identity of the G_i is unknown, since SG1 immunoreacts with both $G_{i\alpha I}$ and $G_{i\alpha II}$ as both share the common C-terminal decapeptide.

Effect of GppNHp on pertusssis toxin-catalysed ADP-ribosylation of $G_{i\alpha}$ in tracheal smooth muscle

GppNHp produced a concentration-dependent reduction in the ability of pertussis toxin to catalyse ADP-ribosylation of $G_{i\alpha}$ (Figures 3 and 4). The maximal effect of GppNHp upon pertussis toxin-catalysed ADP-ribosylation of $G_{i\alpha}$ was observed at 10^{-6} M GppNHp.

Effect of methacholine-pretreatment of tracheal smooth muscle on isoprenaline and GTP-stimulated adenylyl cyclase

GTP (10^{-4} M) elicited a 2.3 fold stimulation of adenylyl cyclase in isolated membranes (Figure 5). Isoprenaline (10^{-6} M) alone was without effect, but, in combination with GTP elicited a 3.9 fold stimulation of adenylyl cyclase activity. In contrast, methacholine pretreatment of tracheal smooth muscle slices abolished the GTP-stimulated adenylyl cyclase and reduced the ability of isoprenaline to augment GTP-stimulated adenylyl cyclase in isolated membranes to 1.6 fold. Atropine (10^{-6} M) pretreatment of tracheal strips reversed the methacholine-induced blockade of GTP-stimulated adenylyl cyclase activity (data not shown).

Addition of methacholine directly to membranes did not significantly inhibit either basal or GTP-stimulated adenylyl cyclase, but did inhibit the augmentation of GTP-stimulated adenylyl cyclase by isoprenaline (Figure 6).

Effect of methacholine-pretreatment of tracheal smooth muscle upon GppNHp-stimulated adenylyl cyclase

GppNHp (10^{-7}–10^{-4} M) produced a concentration-dependent stimulation of adenylyl cyclase activity (Figure 7) with a 7 fold maximal activation at 10^{-4} M GppNHp. The pretreatment of tracheal smooth muscle with methacholine (10^{-6} M) triggered a blockade of this activity with an approximate 75% reduction in the maximal stimulation induced by GppNHp.

Effect of methacholine-pretreatment upon cholera toxin-catalysed ADP-ribosylation of $G_{s\alpha}$

Methacholine-pretreatment of tracheal smooth muscle slices triggered a 90% reduction in the ability of cholera toxin to

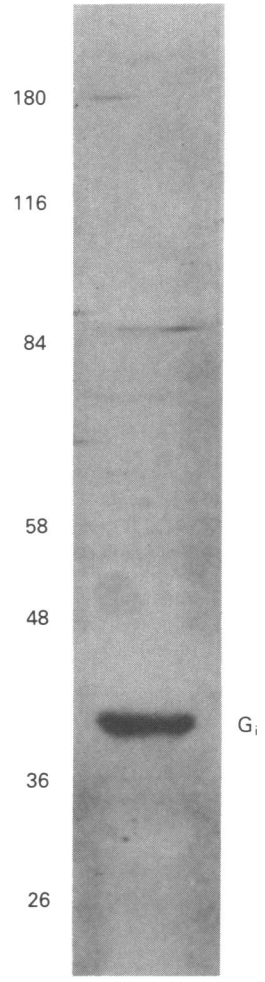

Figure 2 Immunological identification of $G_{i\alpha}$: this figure shows a typical western blot of an SDS PAGE of resolved proteins from tracheal smooth muscle. Blotting was against the anti-G_i antisera. The location of G_i α sub-unit is shown. Molecular mass markers are shown. This is a typical result from the experiment performed at least 10 times on different membrane preparations.

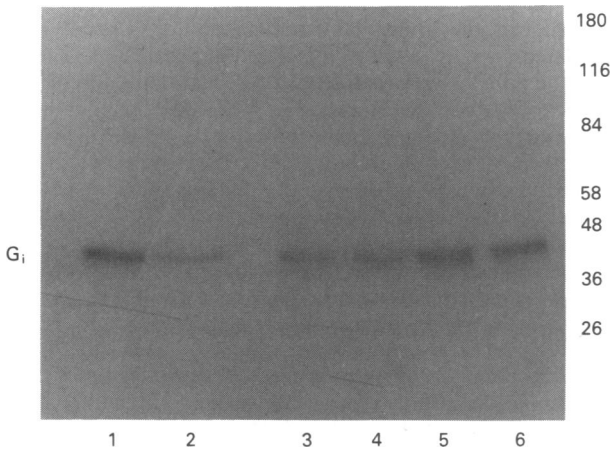

Figure 3 The effect of GppNHp upon pertussis toxin-catalysed ADP-ribosylation of G_i in tracheal smooth muscle. A concentration-dependent effect of GppNHp upon pertussis toxin- catalysed ADP-ribosylation of $G_{i\alpha}$ in isolated tracheal smooth muscle membranes. This is a typical autoradiogram of an SDS PAGE of the resolved [^{32}P]-ADP-ribosylated peptides. Lane 1, no GppNHp; Lane 2, 100 μM GppNHp; Lane 3, 10 μM GppNHp; Lane 4, 1 μM GppNHp; Lane 5, 0.1 μM GppNHp; Lane 6, 0.01 μM GppNHp. The location of G_i α sub-units are shown. Molecular mass markers are shown. This is a typical result from an experiment performed at least 3 times on different membrane preparations.

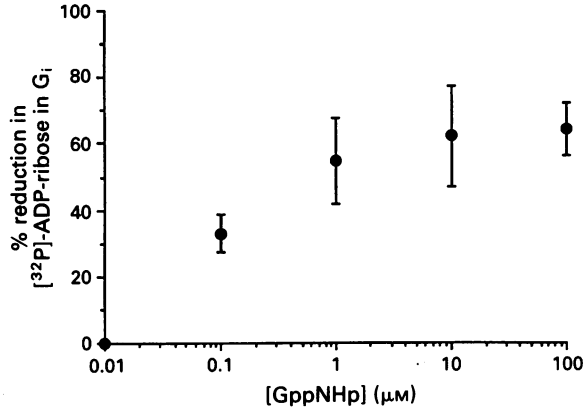

Figure 4 The quantification of the effect of GppNHp upon pertussis toxin-catalysed ADP-ribosylation of G_i in tracheal smooth muscle. A concentration-dependent effect of GppNHp upon pertussis toxin- and catalysed ADP-ribosylation of G_i in isolated tracheal smooth muscle membranes. These are means (± s.e.mean, vertical bars) of the [^{32}P]-ADP-ribose incorporated into G_i at different concentration of GppNHp. These are averaged from results of an experiment performed at least 3 times on different membrane preparations.

catalyse ADP-ribosylation of $G_{s\alpha}$ (Figure 8). GppNHp (100 μM) stimulated the ability of $G_{s\alpha}$ to act as a substrate for cholera toxin-catalysed ADP-ribosylation in membranes from untreated tracheal smooth muscle.

Effect of PMA treatment of tracheal smooth muscle upon adenylyl cyclase activity

Prior treatment of tracheal smooth muscle slices with PMA had no effect upon basal adenylyl cyclase activity. GppNHp (10^{-6} M) triggered a 1.8 fold activation of adenylyl cyclase activity in membranes isolated from untreated smooth muscle slices, yet in membranes from PMA-treated cells GppNHp was more effective at stimulating adenylyl cyclase, yielding a 2.8 fold activation of activity (Table 1).

Discussion

The functional antagonism between muscarinic and β-adrenoceptor agonists, appears to be dependent upon the magnitude of the agonist-stimulated Ins(1,4,5)P_3 (Meurs *et al.*, 1988). In addition to Ins(1,4,5)P_3, muscarinic agonists promote the accumulation of Ca^{2+} and diaclyglycerol, a potent activator of protein kinase C, and it is this enzyme which has been implicated as being responsible for the development of latch-bridges between actin and myosin (Rasmussen *et al.*, 1984) and in the underlying mechanistic antagonism. Protein kinase C has also been shown to phosphorylate and alter the functional activity state of various G-proteins (Katada *et al.*, 1985; Zick *et al.*, 1986; O'Brien *et al.*, 1987; Pyne *et al.*, 1989; 1992a) and adenylyl cyclase (Yoshimasa *et al.*, 1987).

The molecular events that govern the functional antagonism between the muscarinic receptor and the adrenoceptor are unknown. In addressing this question, it is important to note that the protein kinase C-mediated phosphorylation of G_i leads to its inactivation and uncoupling from agonist-bound receptors (Katada *et al.*, 1985; Pyne *et al.*, 1989). We have also previously shown that recombinant $G_{s\alpha}$ is a substrate for

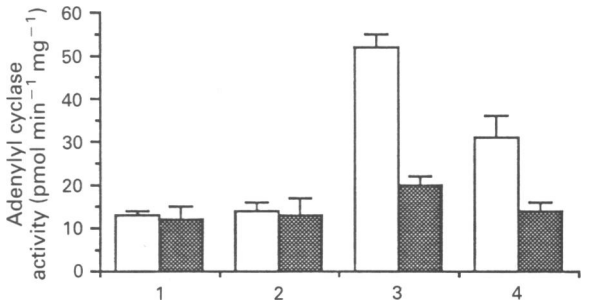

Figure 5 The effect of methacholine pretreatment of tracheal smooth muscle strips upon adenylyl cyclase activity. Histograms showing an assessment of the effect of various agents upon adenylyl cyclase activity in isolated membranes from tracheal smooth muscle strips that were either untreated (open columns) or treated with methacholine (10^{-6} M, stippled columns). Additions to membranes were: (1) no additions; (2) isoprenaline (10^{-6} M); (3) isoprenaline (10^{-6} M) plus GTP (10^{-4} M); (4) GTP (10^{-4} M) alone. These results are expressed as means ± SEM, for 3 different experiments on different membrane preparations. All columns have $P < 0.005$.

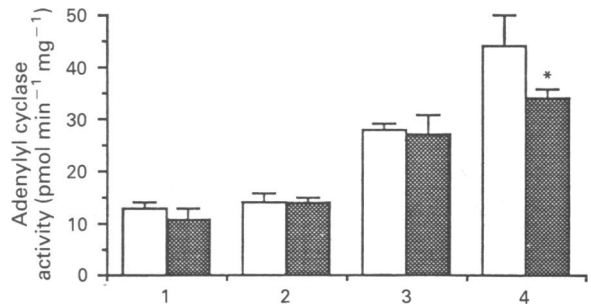

Figure 6 The effect of adding methacholine to membranes: adenylyl cyclase activity. Histograms showing an assessment of the effect of various agents upon adenylyl cyclase activity in isolated membranes from tracheal smooth muscle strips in the presence (stippled columns) and absence (open columns) of methacholine (10^{-6} M). (1) No additions; (2) isoprenaline (10^{-6} M); (3) GTP (10^{-4} M); (4) isoprenaline (10^{-6} M) plus GTP (10^{-4} M). These results are expressed as means with s.e.mean (vertical bars), for 3 different experiments on different membrane preparations.
*Indicates that isoprenaline + GTP stimulated adenylyl cyclase activity in membranes from methacholine-treated smooth muscle is significantly lower than in membranes from untreated tissue, $P < 0.025$ Student's *t* test.

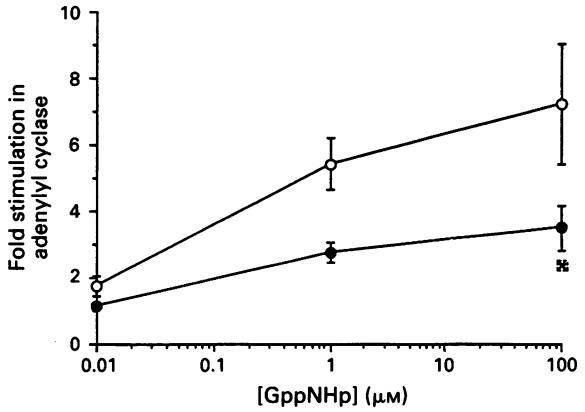

Figure 7 Effect of GppNHp upon adenylyl cyclase activity in membranes from untreated and methacholine-treated smooth muscle strips. A concentration-dependent effect of GppNHp upon adenylyl cyclase in isolated tracheal smooth muscle membranes, from methacholine-treated (10^{-6} M)-treated (●) and untreated (○) tracheal smooth muscle strips. These are means (± s.e.mean, vertical lines), an experiment performed at least 3 times on different membrane preparations.
*Indicates that GppNHp-stimulated adenylyl cyclase activity at 10^{-4} M in membranes from methacholine-treated smooth muscle is significantly lower than in membranes from untreated tissue, $P < 0.025$, Student's t test. All other points have P significance < 0.005.

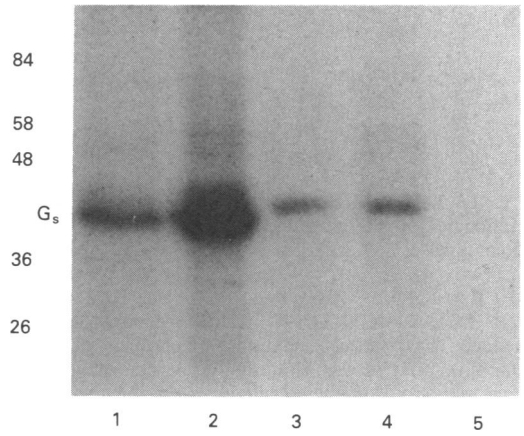

Figure 8 Effect of methacholine upon cholera toxin catalysed-ribosylation. Tracheal smooth muscle strips were either treated with methacholine (10^{-6} M) or untreated. Membranes were then taken for cholera toxin-catalysed ADP-ribosylation and then subjected to SDS PAGE. This is an autoradiogram of the resolved [^{32}P]-ADP-ribosylated peptides. Lane 1, untreated membranes; Lane 2, GppNHp (0.1×10^{-3} M)-treated membranes; Lane 3, membranes from methacholine-treated tissue; Lane 4, GppNHp (0.1×10^{-3} M) treated membranes from methacholine-treated tissue; Lane 5, membranes incubated in the ADP-ribosylation cocktail but without cholera toxin. This is a representative result from an experiment performed at least 3 times. G_s is marked and the molecular wt markers are shown.

protein kinase C- and protein kinase A-catalysed phosphorylation (Pyne *et al.*, 1992a,b). We have shown that PMA-pretreatment of tracheal smooth muscle slices leads to enhanced GppNHp-stimulated adenylyl cyclase activity in subsequently prepared membranes. This might be as a consequence of an enhanced responsiveness of G_s to GppNHp or a reduced responsiveness of G_i. Since basal adenylyl cyclase remains unaltered, it is probable that adenylyl cyclase is not modified by protein kinase C in these cells, although alterations in G-protein and phosphorylated adenylyl cyclase coupling cannot be excluded.

Table 1 Effect of phorbol-12-myristate 13-acetate (PMA)-treatment upon adenylyl cyclase activity

Treatment	*Adenylyl cyclase activity* (pmol min^{-1} mg^{-1})
Untreated smooth muscle	
Basal	13.3 ± 1.3
GppNHp (10^{-6} M)	24 ± 1
PMA-treated	
Basal	11.4 ± 3
GppNHp (10^{-6} M)	37 ± 7

Experiments were performed according to the methods. This is a representative result (mean ± s.e.mean) of an experiment performed at least three times.

Pretreatment of tracheal smooth muscle with methacholine substantially blocked GTP-, isoprenaline and GTP-, and GppNHp-stimulated adenylyl cyclase activity in subsequently prepared membranes. The sustained blockade of adenylyl cyclase activity throughout a procedure involving the disruption of the cells and preparation of membranes was the first indication that this phenomenon might not be entirely due to receptor-mediated G_i activation as this is likely to be a transient process. However, the possibility that the muscarinic receptors remain occupied with methacholine cannot be ignored. An alternative explanation for the inhibitory action of methacholine involves a modification of G_s activity, although this is unlikely to occur via a protein kinase C-mediated phosphorylation of G_s, since methacholine does not mimic PMA. The interpretation that G_s is modified, is based upon the observation that the addition of methacholine directly to isolated membranes does not trigger inhibition of GTP-stimulated adenylyl cyclase activity, whilst in methacholine pretreated tissue, subsequently prepared membranes exhibit markedly inhibited GTP-stimulated adenylyl cyclase activity. Furthermore, G_i appears to be functionally active in isolated membranes, since methacholine added directly to membranes was capable of inhibiting the ability of isoprenaline to augment GTP-stimulated adenylyl cyclase and thus the lack of effect upon GTP-stimulated adenylyl cyclase is not a consequence of an inability to measure G_i function. The selective inhibition of isoprenaline-stimulated adenylyl cyclase activity when methacholine is added directly to membranes is entirely consistent with previous observations (Hildibrandt & Kohnken, 1990) where only hormonal stimulation of adenylyl cyclase appeared susceptible to inhibition by G_i.

Our results also suggest that the α sub-unit of G_i is unlikely to be involved in the inhibition of GppNHp-stimulated adenylyl cyclase at 10^{-4} M GppNHp, since $G_{i\alpha}$ is already maximally activated at this concentration and the equilibrium binding of this guanine nucleotide will be very rapid indeed (Gilman, 1987). This was supported by the observation that GppNHp provoked a reduction in pertussis toxin-catalysed ADP-ribosylation, indicative of GppNHp-stimulated dissociation of the G_i holomer, and this was maximal at 10^{-6} M GppNHp. Our results, therefore, suggest that at the very least, G_i is probably not involved in inhibiting guanine nucleotide-stimulated adenylyl cyclase. Adenosine released in response to methacholine is unlikely to contribute to the observed effects, since adenosine is a bronchodilator acting via A_2 receptors in guinea-pig trachea (Brown & Collis, 1982).

A role for altered G_s function is also indicated from studies using cholera toxin to catalyse ADP-ribosylation of the α sub-unit of this G-protein. In this case, $G_{s\alpha}$ in membranes from methacholine pretreated cells was a markedly poorer substrate for ADP-ribosylation than $G_{s\alpha}$ in untreated cells. The inclusion of GppNHp with membranes from untreated tissue resulted in a marked increase in cholera toxin-catalysed

ADP-ribosylation of $G_{s\alpha}$, and is consistent with the observation of Kahn & Gilman (1986), where GppNHp activates a membrane-bound ADP-ribosyl transferase factor (ARF) which in turn enhances cholera toxin activity.

Free $G_{s\alpha}$ is an exceedingly poor substrate for cholera toxin-catalysed ADP-ribosylation (Graziano *et al.*, 1989), whilst the addition of $\beta\gamma$ sub-units enhances the ability of $G_{s\alpha}$ to act as a substrate for cholera toxin-catalysed ADP-ribosylation. It is possible that $G_{s\alpha}$ is functionally dissociated from the $\beta\gamma$ sub-units in methacholine-treated smooth muscle, and therefore unable to couple to β-adrenoceptors, whilst modification of the free $G_{s\alpha}$-GDP sub-unit may well affect subsequent GTP binding and effector recognition. If this is the case then the reduced amount of ADP-ribosylated $G_{s\alpha}$ may reflect lower amounts of available functionally active G_s. In fact, this is consistent with the reduced maximal activation of adenylyl cyclase by GppNHp, that is observed in membranes from methacholine pretreated smooth muscle. However, the extent of reduced ADP-ribosylation and reduced activation of adenylyl cyclase activity in methacholine-treated cells does not correlate absolutely, although this may be the consequence of the possibility that only a small fraction of G_s may be required for full activation of adenylyl cyclase.

Resolution of the roles of G_i and G_s might be more fully addressed by assessing the effect of pertussis toxin upon the methacholine-induced blockade of isoprenaline stimulated adenylyl cyclase. However, this requires extended incubation times (10–20 h) of tracheal smooth muscle strips with pertussis toxin at 37°C, and after this time the β-adrenoceptor responsiveness of the tissue is severely compromised (data not shown).

Previous studies (Meurs *et al.*, 1987) have shown that protein kinase C can perturb β-adrenoceptor adenylyl cyclase in lymphocytes via an alteration in receptor-G-protein coupling. However, our studies indicate that methacholine treatment of tracheal smooth muscle also perturbs the interaction of G_s with adenylyl cyclase. Sankary *et al.* (1988) have shown that the methacholine-mediated inhibition of adenylyl cyclase activity in canine airway smooth muscle was mediated by G_i. However, the use of pertussis toxin to block this response and the subsequent interpretation has to be speculative, since this toxin can block receptor-mediated activation of phospholipase C (Issakani *et al.*, 1990), and it is unknown whether canine trachea contains a pertussis toxin-insensitive phospholipase C. Our results suggest that G_i may well be involved in inhibiting isoprenaline-stimulated adenylyl cyclase, although G_s inactivation also appears to be an additional mechanism that is important. We suggest, therefore, that the antagonism that exists between increased muscarinic tone and reduced β-adrenoceptor-induced relaxation occurs via a pathway involving modulation of both G_i and G_s.

We are currently investigating whether G_s is a substrate for phosphorylation in primary cultures of tracheal smooth muscle, since this methodology allows more successful incorporation of ^{32}P into the intracellular ATP pool and will enable studies to be performed in the absence of contaminating epithelial cells.

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Ornithine and histidine decarboxylase activities in mice sensitized to endotoxin, interleukin-1 or tumour necrosis factor by D-galactosamine

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1 An injection of D-galactosamine (GalN) into mice together with a lipopolysaccharide (LPS or endotoxin), interleukin-1 (IL-1) or tumour necrosis factor (TNF), sensitized the mice and induced fulminant hepatitis with severe congestion resulting in rapid death. Since LPS and these cytokines induce ornithine decarboxylase (ODC) and histidine decarboxylase (HDC) in the liver and spleen of mice, the effects of GalN on the induction of ODC and HDC in these organs were examined.

2 The induction of ODC by LPS, IL-1 or TNF was suppressed by GalN in the liver, and this suppression preceded the hepatic congestion. There was good agreement between the degree of hepatic congestion and the suppression of ODC induction by various amounts of GalN. The induction of ODC in the spleen was suppressed only at the highest dose of GalN examined.

3 GalN is known to deplete uridine 5'-triphosphate (UTP), resulting in the suppression of RNA and protein synthesis. An injection of uridine, the precursor of UTP, diminished the GalN-induced suppression of ODC induction by LPS and prevented the hepatic congestion and death.

4 LPS-pretreatment before injection of LPS plus GalN prevented the suppression of ODC activity and prevented the hepatic congestion and death.

5 An injection of putrescine, the product of ODC, prolonged survival time and delayed the development of hepatic congestion. However, injection of an ODC inhibitor into the mice given LPS did not produce hepatic congestion.

6 The induction of HDC in the liver by LPS, IL-1 or TNF was not suppressed by GalN and, at high doses, the response to LPS was enhanced. An inhibitor of HDC neither prevented the hepatic congestion nor enhanced the protective effect of putrescine.

7 Although GalN in combination with IL-1α induced a markedly higher HDC activity than was observed when it was combined with TNFα, and suppressed the induction of ODC, the former combination at the doses used did not produce hepatic congestion or death. However, the sensitization to TNFα by GalN was markedly potentiated by IL-1α.

8 These results suggest that suppression of the induction of ODC by GalN may be one cause of the sensitization to LPS, IL-1 or TNF, and that the induction of HDC, i.e. histamine formation, may not be involved in this sensitization.

9 These results are consistent with the hypothesis that both IL-1 and TNF are involved in the sensitization to LPS.

Keywords: Ornithine decarboxylase; polyamines; histamine; histidine decarboxylase; lipopolysaccharide; endotoxin; D-galactosamine; interleukin-l; tumour necrosis factor; hepatitis

Introduction

D-Galactosamine (GalN) is metabolized by enzymes of the galactose pathway which are most abundant in the liver. Since this pathway consumes uridine nucleotides, the administration of GalN rapidly depletes uridine nucleotides, primarily in the liver, and results in a decrease in RNA synthesis (Decker & Keppler, 1974). This agent, within 2 days of its injection into rats, induces hepatic necrosis resembling human viral hepatitis (Decker & Keppler, 1974). In addition, GalN sensitizes various experimental animals to the lethal effect of lipopolysaccharides (LPS) (Galanos *et al.*, 1979). The lethality induced by GalN plus LPS is thought to be due to the hepatic failure that occurs rapidly (within 10 h) after their injection (Galanos *et al.*, 1979; Tiegs *et al.*, 1989).

It has been suggested that the effect of LPS is mediated by the cytokines, interleukin-1 (IL-1) and/or tumour necrosis factor (TNF), because IL-1 and TNF can substitute for LPS, their lethal effects are also augmented by GalN (Lehmann *et al.*, 1987; Wallach *et al.*, 1988; Tiegs *et al.*, 1989) and because their production is stimulated by LPS (Oppenheim *et al.*, 1986; Beutler & Cerami, 1987). However, it is not clear what mechanisms or biochemical events are involved in the sensitization to LPS in the presence of GalN.

Ornithine decarboxylase (ODC) produces putrescine, a precursor of spermidine and spermine, from ornithine. ODC is a rate-limiting enzyme in the synthesis of these polyamines which have been implicated as regualtors of cellular growth and functions, and ODC is induced rapidly by various growth stimuli (Pegg & McCann, 1982). We have shown that ODC is induced in the liver of mice *in vivo* within a few hours after injection of various inflammatory agents (Endo, 1982; 1984; Endo *et al.*, 1985), and that LPS is the most potent inducer among them (Endo, 1984). This action of LPS seems to be mediated by IL-1 and/or TNF, because these cytokines, either by themselves or acting synergistically in combination, induce ODC (Endo *et al.*, 1985; Endo, 1989). IL-1 and TNF have an anti-proliferative action on some tumour cells *in vitro*, and we have shown evidence that suppression of ODC activity in such tumour cells may be involved in the anti-proliferative action of IL-1 or TNF (Endo *et al.*, 1988). Therefore, it is of interest to examine how GalN affects the induction of ODC by LPS, IL-1 or TNF.

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Histamine is produced by histidine decarboxylase (HDC). We have shown that HDC is also induced in the liver of mice by LPS, IL-1 and TNF (Endo, 1982; 1983a; 1989; Endo *et al.*, 1986). Since the GalN-induced sensitization to LPS, IL-1 or TNF is usually accompanied by severe congestion, and histamine is a potent stimulator of post capillary venule permeability, the histamine produced by the induction of HDC might be expected to be involved in the development of the hepatic congestion.

In this study, therefore, the effects of GalN on the induction of ODC and HDC in the liver by LPS, IL-1 and TNF were examined. Since LPS, IL-1 and TNF induce ODC and HDC in the spleen, also, the effects of GalN on the spleen were compared to those on the liver.

Methods

Determination of putrescine and histamine and assay of ornithine and histidine decarboxylase activities

The mice were killed by decapitation at indicated time intervals after injections of test samples, the liver and spleen rapidly removed and kept in dry ice until assayed.

Putrescine and histamine levels were determined as described previously (Endo, 1983b). Briefly, they were extracted from the tissues with 0.4 M $HClO_4$, separated on a small phosphorylated cellulose column and the amounts present determined fluorometrically after reaction with fluorescamine or *o*-phthalaldehyde.

HDC and ODC activities were assayed by a previously described method (Endo, 1983b), which was modified both in order to remove any histamine and putrescine in the tissues and to simplify the method. The liver (0.2 g) or a single whole spleen was placed in a teflon tube with phosphorylated cellulose powder (50 mg) and 2 ml of ice-cold 0.02 M sodium phosphate buffer (pH 6.2) containing pyridoxal 5'-phosphate (20 μM) and dithiothreitol (200 μM) and homogenized with an Ultra Turrax homogenizer (Janke & Kunkel Co., Germany). The supernatant of the homogenate obtained after centrifugation (10,000 *g* for 15 min at 4°C) was used as the enzyme solution. The histamine and putrescine present in the tissues were bound to the phosphorylated cellulose and removed almost completely from the enzyme solution.

The procedures for simultaneous assay of HDC and ODC activities were the same as described previously (Endo, 1983b). Briefly, the enzyme solution was incubated at 37°C for 3 h with both histidine and ornithine. After terminating the enzyme reaction by adding $HClO_4$ (final concentration 0.33 M), histamine and putrescine formed in the reaction mixture were recovered separately by chromatography on a small phosphorylated cellulose column and quantitated fluorometrically as described above. The small amounts of histamine or putrescine in the non-incubated reaction mixture (i.e., zero-activity; $HClO_4$ was added without incubation) were subtracted from the amounts of these amines in the incubated samples. Since the enzyme reaction progressed linearly for at least 3 h, the activities of HDC and ODC were expressed as nmol of histamine or putrescine formed during 1 h by the enzyme contained in 1 g of the tissue (nmol h^{-1} g^{-1}).

Estimation of the degree of hepatic congestion

When the livers were removed for the assay of ODC and HDC activities, the degree of hepatic congestion was scored by macroscopic observation, i.e., (1) no congestion; (2) slight (congestion in up to 20% of the liver); (3) medium (congestion in 20 to 50% of the liver); (4) severe (congestion in more than 50% of the liver), and the mean ± s.d. of the score was calculated. The incidence of hepatic congestion is also shown together with the mean scores of hepatic congestion.

Materials and animals

Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 prepared by Boivin's method was obtained from Difco Laboratories (Detroit, MI, U.S.A.). Recombinant human interleukin-1α (IL-1α) and recombinant human tumour necrosis factor α (TNFα) were provided by Dainippon Pharmaceutical Co., Osaka, Japan. DL-α-Difluoromethylornithine (DFMO) and DL-α-monofluoromethylhistidine (FMH) were gifts from Dr Wilkins of Merrell Dow Research Institute (Strasburg, Cedex, France) and Dr Kollonitsch of Merck Sharp & Dohme Research Laboratories (Rahway, NJ, U.S.A.), respectively. Uridine and putrescine dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Female BALB/cA and C57BL/6N mice (6 weeks old) were obtained from the Mouse Center at our University. Reagents were dissolved in sterile saline and injected intraperitoneally into mice (0.05–0.1 ml/10 g body weight).

Statistical differences

Statistical differences between two means of data were evaluated by Student's unpaired *t* test and *P* values less than 0.05 were considered to be significant.

Results

Lethality induced by the combination of a lipopolysaccharide with D-galactosamine

The sensitivity to LPS varies among strains of mice. Therefore, the lethal effect of the combination of LPS with GalN was tested in two strains of mice, C57BL/6N and BALB/cA, raised in our University (Table 1). GalN by itself, even at a dose of 1500 mg kg^{-1}, caused no deaths and no obvious ill effects when mice were observed for up to 3 days. Most mice survived for 20 h after the injection of LPS alone at a dose of 10 mg kg^{-1}. However, the combination of smaller amounts of GalN and LPS markedly enhanced lethality. Sudden death occurred within 5–10 h without symptoms until shortly before death, as noted by Galanos *et al.* (1979), and severe congestion in the liver was observed in all the mice that died.

Kinetics of the induction of histidine and ornithine decarboxylases and the effects of D-galactosamine

In normal mice both ODC and HDC activities are very low in the liver (below 1 and 0.2 $nmol^{-1}$ g^{-1}, respectively), and a

Table 1 Lethality of the combination of lipopolysaccharide (LPS) with D-galactosamine (GalN)

GalN (mg kg^{-1})	*LPS* (μg kg^{-1})	*Lethality* C57BL/6N	BALB/cA
1500	0	0/5	0/5
0	10000	0/5	1/5
0	1000	0/5	0/5
800	100	4/4	4/4
800	10	4/4	4/4
800	1	4/4	0/4
800	0.1	0/4	–
800	10	4/4	4/4
600	10	4/4	3/5
400	10	4/4	–
200	10	0/4	–

LPS was injected into mice immediately after the injection of GalN. The lethality values are the sum total of deaths occurring by 20 h. The dashes indicate that no observations were made with these drug combinations.

sham injection with saline does not enhance these activities. However, an injection of LPS induced marked increases in ODC and HDC activities in the liver of both C57BL/6N and BALB/cA mice (Figure 1). GalN suppressed the induction of ODC in the liver of both strains of mice, but the HDC activity in the liver of C57BL/6N mice was not suppressed at all. In the liver of BALB/cA mice given a larger amount of GalN (800 mg kg^{-1}), the induction of HDC activity was enhanced. Such an enhancement of HDC activity in the liver was also observed in C57BL/6N mice when injected with large amounts of GalN (Figure 2).

The ODC and HDC activities in the spleen of normal mice are below 5 and 1 nmol h^{-1} g^{-1}, respectively. These activities were also markedly enhanced by LPS, but not by sham injection. However, GalN did not cause any significant effect on the induction of ODC or HDC activities by LPS in the spleen of either C57BL/6N or BALB/cA mice in this experiment (data not shown).

The scores (and incidence) of hepatic congestion in C57BL/6N mice in this experiment were 1 ± 0 (0/12) within 3.5 h and 1.8 ± 1.0 (2/4) and 3.5 ± 0.6 (4/4) at 4.5 h and 5.5 h, respectively. The scores (and incidence) or hepatic congestion in BALB/cA mice were 1 ± 0 (0/12) within 6 h and 3.5 ± 0.6 (4/4) at 7.5 h. These results indicate that the suppression of ODC induction occurs before the development of hepatic congestion.

In the following experiments C57BL/6N mice were used, because hepatic congestion occurred in this strain more rapidly than in BALB/cA mice. In most of the following experiments, a combination of 50 μg kg^{-1} LPS and 600 mg kg^{-1} GalN was used. All the mice treated with this combination died within 7 h (Figure 6).

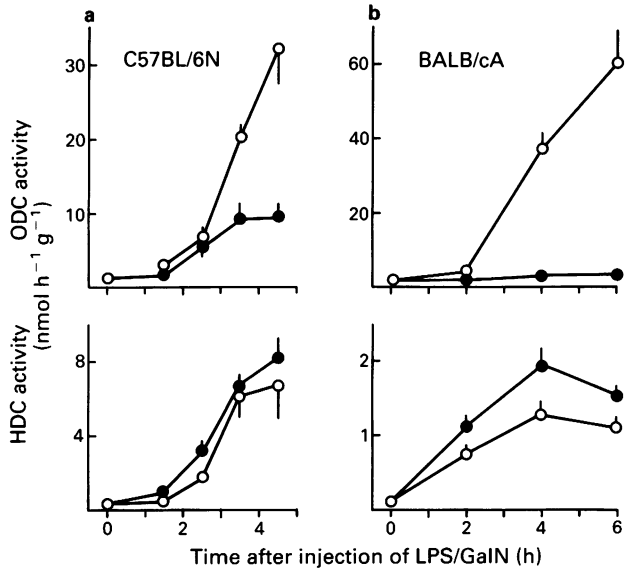

Figure 1 Kinetics of the induction of ornithine decarboxylase (ODC) and histidine decarboxylase (HDC) in the liver by lipopolysaccharide (LPS) and the effect of D-galactosamine (GalN). Mice were killed at the time intervals indicated after the injection of LPS alone (○) or a mixture of LPS and GalN (●). (a) In C57BL/6N mice: the doses of LPS and GalN were 50 μg kg^{-1} and 600 mg kg^{-1}, respectively. (b) In BALB/cA mice: the doses of LPS and GalN were 10 μg kg^{-1} and 800 mg kg^{-1}, respectively. Each value is the mean (± s.d., vertical lines) from 4 mice.

Dose- and time-dependent effects of D-galactosamine

GalN, administered to C57BL/6N mice 1 h before the injection of LPS, suppressed the ODC induction by LPS in the liver in a dose-dependent manner (Figure 2). However, GalN was not effective in suppressing HDC induction in the liver and, at higher doses, enhanced HDC activity, although GalN itself did not induce HDC activity at these doses. GalN opposed ODC and HDC inductions in the spleen only at the highest dose used. The scores (and incidence) of hepatic congestion in this experiment were 1.3 ± 0.5 (1/4) at 400 mg kg^{-1} of GalN and 2.5 ± 1.3 (3/4) at 600 mg kg^{-1}.

As shown in Figure 3, the suppressant effect of GalN on the ODC induction was marked when GalN was administered 1 h before LPS injection or simultaneously with LPS, and the scores (and incidence) of hepatic congestion under these conditions were 2.8 ± 1.0 (4/4) and 1.8 ± 1.0 (2/4), respectively. On the other hand, when GalN was injected 1 or 2 h after LPS injection, its effect was not significant and hepatic congestion did not occur. In this experiment, GalN showed no significant effect on the induction of HDC in the liver nor on the induction of ODC and HDC in the spleen. When GalN (600 mg kg^{-1}) was injected into 5 mice 1 h after LPS injection (50 μg kg^{-1}), all the mice survived for 20 h and no hepatic congestion was observed.

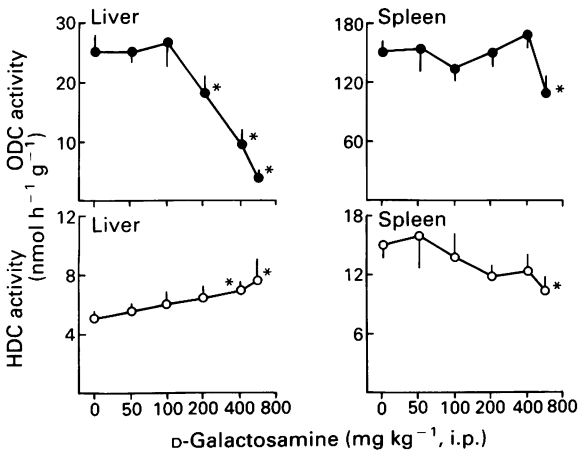

Figure 2 The effects of D-galactosamine (GalN) on the induction of ornithine decarboxylase (ODC) and histidine decarboxylase (HDC) in the liver and spleen. GalN (50–800 mg kg^{-1}) was injected into C57BL/6N mice 1 h before injection of lipopolysaccharide (LPS) (50 μg kg^{-1}). The mice were killed 4.5 h after LPS injection. Each value is the mean (± s.d., vertical lines) from 4 mice.
*$P<0.05$ vs control (LPS alone).

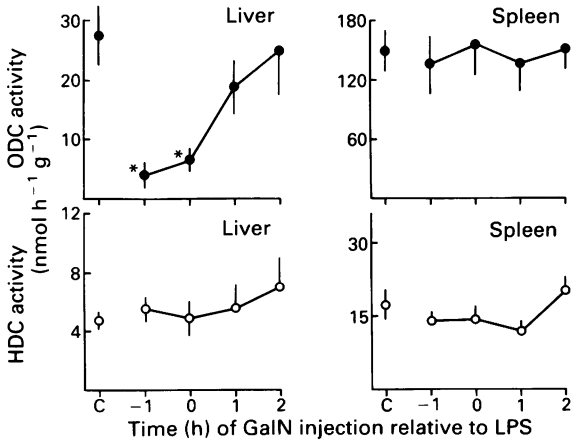

Figure 3 Effects of the injection time of D-galactosamine (GalN) on the induction of ornithine decarboxylase (ODC) and histidine decarboxylase (HDC) by lipopolysaccharide (LPS). GalN (600 mg kg^{-1}) was injected into C57BL/6N mice 1 h before, simultaneously with, or 1 or 2 h after the injection of LPS (50 μg kg^{-1}), and the mice were killed 4.5 h after LPS injection. C: LPS alone (control). Each value is the mean (± s.d., vertical lines) from 4 mice.
*$P<0.05$ vs control.

Effects of uridine on the action of D-galactosamine

Uridine can reverse the depletion of uridine nucleotides by GalN (Decker *et al.*, 1974). As shown in Figure 4, uridine, administered to mice 0.5 h after simultaneous injection of GalN and LPS, diminished the GalN-induced suppression of ODC induction. Moreover, uridine prevented the development of hepatic congestion, i.e., the scores (and incidence) of hepatic congestion in uridine-treated and non-treated mice were 1 ± 0 (0/5) and 2.2 ± 1.3 (3/5), respectively. In another experiment under these conditions, uridine completely abrogated the lethality caused by LPS plus GalN (0/8, 3 days observation). In contrast to its effects on ODC induction in the liver, uridine caused a small but statistically significant suppression of the induction of HDC by LPS in the presence of GalN (Figure 4).

Effects of lipopolysaccharide-pretreatment on the action of D-galactosamine

In order to examine whether prior stimulation of ODC induction would prevent the hepatic congestion and death induced by GalN plus LPS, the effect of LPS pretreatment was tested. In mice that had not received LPS-treatment before injection of GalN plus LPS, the induction of ODC was suppressed (Figure 5) and the score (and incidence) of hepatic congestion were 2.6 ± 1.3 (4/5). However, in mice pretreated with LPS, full ODC activity was induced, and there was no hepatic congestion. This LPS-pretreatment had no clear effect on the HDC induction. In another confirmatory experiment, the LPS-pretreatment again completely abrogated the lethality induced by GalN plus LPS (0/8, 3 days observation).

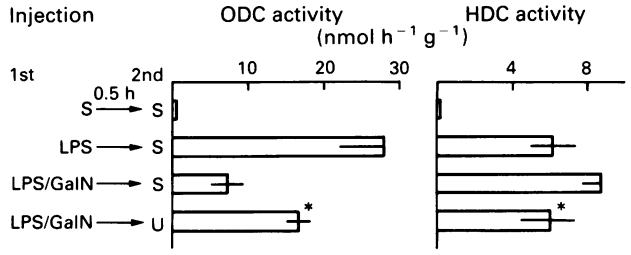

Figure 4 Effects of uridine on the induction of ornithine decarboxylase (ODC) and histidine decarboxylase (HDC) in the liver by D-galactosamine (GalN) plus lipopolysaccharide (LPS). Uridine (U) (500 mg kg^{-1}) or saline (S) was injected into C57BL/6N mice 0.5 h after simultaneous injection of LPS (50 μg kg^{-1}) and GalN (600 mg kg^{-1}). The mice were killed 4.5 h after the second injection. Each value is the mean (± s.d., horizontal lines) from 5 mice. *$P < 0.05$ vs the group injected with saline after LPS plus GalN.

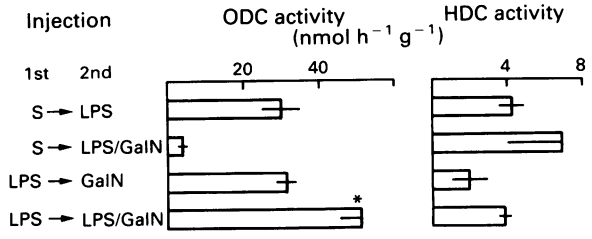

Figure 5 Effects of a prior injection of lipopolysaccharide (LPS) on the induction of ornithine decarboxylase (ODC) and histidine decarboxylase (HDC) in the liver by D-galactosamine (GalN) plus LPS. Saline (S) or LPS (50 μg kg^{-1}) was injected into C57BL/6N mice 2 h before a second injection of LPS (50 μg kg^{-1}), GalN (600 mg kg^{-1}) or their mixture. The mice were killed 5 h after the second injection. Each value is the mean (± s.d. horizontal lines) from 5 mice. *$P < 0.05$ vs the group injected with GalN after LPS.

Effects of putrescine, histamine and inhibitors of ornithine and histidine decarboxylases

The induction of ODC and HDC by LPS resulted in an increase in the levels of putrescine and histamine in the liver (Table 2). GalN inhibited the increase in putrescine but not the increase in histamine.

An injection of putrescine, the product of ODC, into mice given both LPS and GalN prolonged the survival time (Figure 6). In another confirmatory experiment, the development of congestion was also delayed by putrescine, i.e., the scores (and incidence) of hepatic congestion at 5 h after the injection of LPS plus GalN were 1 ± 0 (0/5) in mice given putrescine and 3.0 ± 1.2 (4/5) in mice given saline.

DFMO (Pösö & Pegg, 1982) and FMH (Kollonitsch *et al.*, 1978) are potent irreversible inhibitors of ODC and HDC, respectively. As shown in Table 2, these inhibitors almost completely prevented the increase in putrescine and histamine caused by LPS. However, the administration of DFMO to mice given LPS under these conditions did not produce hepatic congestion and death. In addition, FMH at this dose was ineffective in preventing or in delaying hepatic congestion and death induced by GalN plus LPS, nor did it enhance the protective effect of putrescine (data not shown).

Table 2 Effects of inhibitors of ornithine and histidine decarboxylases on the increase in putrescine and histamine in the liver caused by lipopolysaccharide (LPS)

Treatment	*Amines in the liver* (nmol g^{-1})	
	Putrescine	*Histamine*
Saline + saline	37 ± 4	0.82 ± 0.10
LPS + saline	87 ± 2*	1.69 ± 0.13*
LPS + GalN	47 ± 5	2.35 ± 0.35*
LPS + DFMO	39 ± 2	2.00 ± 0.32*
LPS + FMH	86 ± 6*	0.86 ± 0.06

The mice (C57BL/6N) were killed 5 h after injection of LPS (50 μg kg^{-1}). D-Galactosamine (GalN) (600 mg kg^{-1}) was injected simultaneously with LPS. α-Difluoromethyl ornithine (DFMO) (100 mg kg^{-1}), an inhibitor of ornithine decarboxylase or α-monofluoromethyl histidine (FMH) (50 mg kg^{-1}), an inhibitor of histidine decarboxylase, were injected 1 h after LPS injection. Each value is the mean ± s.d. of 5 or 8 mice.
*$P < 0.05$ vs control.

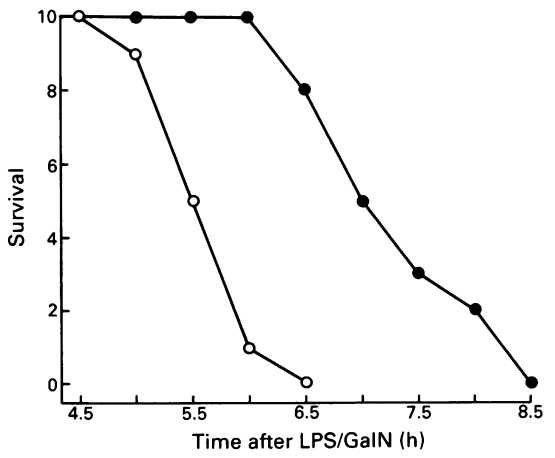

Figure 6 The effect of putrescine on the lethal effect of lipopolysaccharide (LPS) plus D-galactosamine (GalN). Saline (○) or putrescine dihydrochloride (500 mg kg^{-1}) (●) was injected twice into each group of C57BL/6N mice (10 mice/group) at 1.5 h and 4.0 h after simultaneous injection of LPS (50 μg kg^{-1}) and GalN (600 mg kg^{-1}).

Effects of D-galactosamine on the actions of interleukin-1 and tumour necrosis factor

GalN also suppressed the induction of ODC activity in the liver by IL-1α and TNFα (Table 3). Again, neither ODC activity in the spleen nor HDC activity in the liver and spleen were suppressed by GalN. In this experiment, there was no hepatic congestion in the mice given IL-1α plus GalN and the score (and incidence) of hepatic congestion in the mice given TNFα plus GalN was 1.6 ± 0.9 (2/5).

GalN combined with TNFα produced hepatic congestion within 7 h (Table 4). On the other hand, in spite of a higher HDC induction by IL-1α (Table 3), IL-1α in combination with GalN did not produce hepatic congestion (Table 4). In combination with both TNFα and IL-1α, GalN resulted in a high lethality and a high score of hepatic congestion, indicating a synergistic effect between TNFα and IL-1α.

Putrescine, even when administered orally, prolonged the survival time of the mice given GalN in combination with both IL-1α and TNFα (Figure 7). Two of seven mice injected i.p. with putrescine survived in this experiment.

Discussion

LPS, IL-1 and TNF share various biological activities (Dinarello, 1989). As to their effects on the liver, these compounds stimulate the production of various factors involved in the blood coagulation system, acute phase proteins in inflammation and cytokines such as IL-6. They also increase body temperature. They suppress hepatic gluconeogenesis and production of albumin. In addition, we have shown that they induce the enzymes catalyzing the production of polyamines and histamine in the liver. On the other hand, GalN rapidly depletes UTP in the liver, resulting in suppressed RNA synthesis and consequently, may suppress the synthesis of various proteins (Decker & Keppler, 1974). Therefore, the sensitization to LPS or hepatic failure induced by GalN may be due to combined effects on several, but not one single, biochemical event. Wallach *et al.*, (1988) have suggested that the deleterious effects of IL-1, TNF or LPS may be modulated by antagonistic mechanisms; mechanisms

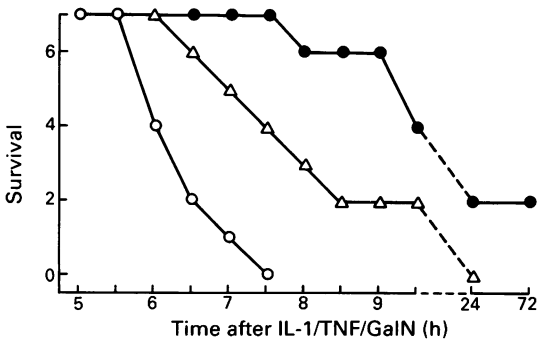

Figure 7 The effect of putrescine on the survival time of mice given D-galactosamine (GalN) in combination with both interleukin-1α (IL-1α) and tumour necrosis factor α (TNFα). Saline (i.p.) (○) or putrescine dihydrochloride (500 mg kg^{-1}) was injected i.p. (●) or given orally (△) twice into each group of C57BL/6N mice (7 mice/group at 2 h and 5 h after injection of GalN (600 mg kg^{-1}) in combination with both IL-1α (20 μg kg^{-1}) and TNFα (20 μg kg^{-1}).

Table 3 Effects of D-galactosamine (GalN) on the induction of ornithine decarboxylase (ODC) and histidine decarboxylase (HDC) by interleukin-1α (IL-1α) or tumour necrosis factor α (TNFα)

Treatment	*ODC activity* (nmol h^{-1} g^{-1})		*HDC activity* (nmol h^{-1} g^{-1})	
	Liver	*Spleen*	*Liver*	*Spleen*
Saline	3 ± 2	3 ± 2	0.2 ± 0.1	1.4 ± 0.7
IL-1α	42 ± 7	109 ± 27	3.0 ± 0.4	9.5 ± 0.5
IL-1α + GalN	13 ± 3*	130 ± 10	3.7 ± 0.4	10.2 ± 0.3
TNFα	30 ± 3	41 ± 2	1.4 ± 0.1	6.4 ± 0.6
TNFα + GalN	14 ± 2*	65 ± 20	1.5 ± 0.2	4.9 ± 0.9

The mice (C57BL/6N) were killed 4.5 h after the injection of IL-1α (100 μg kg^{-1}), TNFα (100 μg kg^{-1}), or a combination of a cytokine with GalN (600 mg kg^{-1}). Each value is the mean ± s.d. of 5 mice.
*$P < 0.05$ vs each cytokine alone.

Table 4 Effects of the combination of interleukin-1α (IL-1α) or tumour necrosis factor α (TNFα) with D-galactosamine (GalN) on hepatic congestion and lethality

Cytokines	(μg kg^{-1})	*GalN* (mg kg^{-1})	*Hepatic congestion*		*Lethality*
IL-1α	500	0	1 ± 0	(0/5)*	0/5
TNFα	500	0	1 ± 0	(0/5)	0/5
IL-1α + TNFα	100 each	0	1 ± 0	(0/5)	0/5
IL-1α	50	600	1 ± 0	(0/5)	0/5
	100	600	1 ± 0	(0/5)	0/5
TNFα	50	600	1.8 ± 1.5	(2/5)	0/5
	100	600	2.4 ± 0.8	(4/5)	0/5
IL-1α + TNFα	10 each	600	3.0 ± 1.2	(4/5)	2/5
IL-1α + TNFα	20 each	600	3.8 ± 0.4	(5/5)	4/5

IL-1α, TNFα or a mixture of the cytokines with or without GalN were injected into C57BL/6N mice. The lethality occurring within 7 h was observed and the scores (and *incidence) of hepatic congestion at death or 7 h after the injection were calculated.

which are suppressed by GalN, but which, in the absence of GalN, are augmented by IL-1 or TNF. However, it is not clear what kinds of deleterious effects or antagonistic mechanisms are involved in the sensitization.

GalN suppressed the induction of ODC in the liver but not in the spleen, and depletion of UTP has been reported to occur predominantly in the liver (Decker & Keppler, 1974). The suppression of ODC induction preceded the development of hepatic congestion. Both the suppression of ODC induction and the development of hepatic congestion were diminished by uridine, the precursor of UTP. Putrescine, the product of ODC, prolonged survival time and delayed the development of hepatic congestion. These results suggest that suppression of ODC induction is necessary to produce the sensitization to LPS by GalN. However, the suppression of ODC induction is not sufficient, because the combination of LPS with an ODC inhibitor did not induce hepatic congestion. Therefore, the induction of ODC seems to be one of the antagonistic mechanisms postulated by Wallach *et al.*, (1988).

It has been reported that the induction of ODC is essential in hepatic regeneration (Luk, 1986) and that putrescine may be involved in the healing or protection from hepatitis induced in rats by GalN alone (Daikuhara *et al.*, 1979; Nishiguchi *et al.*, 1990). Our results indicate that putrescine is also effective in delaying the GalN-induced sensitization to LPS, IL-1 and TNF. However, the detailed role of putresine or the induction of ODC in the antagonistic effects on sensitization is not clear.

In contrast to ODC, the induction of HDC by LPS, IL-1α or TNFα, was not suppressed by GalN. At higher doses, HDC induction was rather augmented by GalN. Histamine is known to be a potent mediator increasing post capillary venule permeability. Therefore, the induction of HDC activity was expected to be one of the deleterious effects described above. However, despite complete inhibition of the LPS-induced increase in histamine by FMH, an inhibitor of HDC, this agent neither prevented the hepatic congestion nor enhanced the protective effect of putrescine. Although IL-1α was more potent than TNFα in inducing HDC (Endo, 1989 and Table 3), its combination with GalN did not produce hepatic congestion (Table 4). Therefore, against our expectations, the present study indicates that induction of HDC is not involved in GalN-induced hepatic congestion or in the sensitization to LPS, TNFα or IL-1α, suggesting that HDC induction is not a deleterious event. Recently, we observed that the injection of LPS, IL-α or TNFα into mice causes the accumulation of platelets, which contain large amounts of 5-hydroxytryptamine, predominantly in the liver (Endo & Nakamura, 1992). The relevance of this phenomenon to the GalN-induced sensitization is now being examined.

The mechanism by which LPS-pretreatment prevented the GalN-induced sensitization to LPS can be explained as follows: there was a time lag of about 2 h before the induction of ODC by LPS (Figure 1), and GalN was ineffective in suppressing the induction of ODC when it was injected 2 h after the LPS injection (Figure 3). These results suggest that although the synthesis of ODC molecules is still low by 2 h after stimulation with LPS, the synthesis of ODC mRNA has been completed in this period, resulting in a full induction of ODC, and this ODC induction contributes to preventing the subsequent sensitization to LPS.

Since the induction of both ODC and HDC by LPS or cytokines is suppressed by cycloheximide, an inhibitor of protein synthesis (Endo, 1983c; 1984; Endo *et al.*, 1985), their induction may depend on *de novo* synthesis of proteins or the ODC and HDC molecules themselves. However, GalN, in spite of suppressing ODC induction in the liver, did not suppress the induction of HDC and, at high doses, enhanced HDC activity in this organ. Since GalN suppresses protein synthesis predominantly in the hepatocytes (Decker & Keppler, 1974), these results suggest that the induction of HDC in the liver may occur in cells other than hepatocytes. We have also shown that the induction of HDC by LPS *in vivo* is enhanced by actinomycin D which inhibits RNA synthesis by intercalating into DNA (Endo, 1983c). The cell types and mechanisms involved in the induction of HDC are of interest. We have evidence that actinomycin D also suppresses ODC induction by LPS preferentially in the liver and produces hepatic congestion (unpublished data).

The cytocidal effects of TNF and IL-1 on tumour cells *in vitro* are known to be markedly potentiated by inhibitors of RNA or protein synthesis or by viral infection (Aderka *et al.*, 1985). The similarity between the cytocidal effects of these cytokines *in vitro* and their lethal effects *in vivo* has been described by Wallach *et al.* (1988). We have demonstrated that the cytocidal effects of IL-1 and TNF on some tumour cells *in vitro* may involve the suppression of ODC induction (Endo *et al.*, 1988). Here we present evidence that the suppression of ODC induction may also be involved in the lethal effects of IL-1 and TNF *in vivo* in the presence of GalN. Finally, the stimulation of IL-1 and/or TNF production by endotoxins (or bacterial infection) in the course of viral infection or during treatment with inhibitors of RNA or protein synthesis might be considered to be an important cause of hepatitis.

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The effect of aliphatic alcohols on the transient potassium current in hippocampal neurones

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1 The transient potassium current was recorded in single hippocampal CA_1 neurones from the rat by use of the whole-cell patch clamp technique. The effects on this current of a homologous series of aliphatic alcohols, ranging from butanol to octanol, were investigated.

2 The predominant effect of octanol (and the other alcohols) was to cause an increase in the initial rate of decay of the transient potassium current together with a slight decrease in the rate of decay of later phases of the current, such that the current decay became markedly non-monotonic. The alcohols also caused a decrease in peak current amplitude which could not be accounted for soley by the change in current decay kinetics.

3 The effect of the alcohols was concentration-dependent and readily reversible. Increasing chain length increased the potency of each alcohol by about 3 fold for each methylene group added. Other than a difference in potency, there appeared to be little difference in the action of aliphatic alcohols of different chain length on the transient current.

4 The alcohols did not appreciably change the voltage-dependence of steady state inactivation or activation of the transient potassium current.

5 The rate of inactivation of the transient current in these cells was only weakly voltage-dependent. This weak voltage-dependence was not changed by the presence of aliphatic alcohols, neither was the effect of the alcohols themselves voltage-dependent.

6 The potencies of each of the aliphatic alcohols were well correlated with their respective membrane/buffer partition coefficients, a finding which implies a hydrophobic locus of action.

Keywords: Transient potassium current; anaesthetics; alcohols; neurone

Introduction

Although aliphatic alcohols are not used clinically as anaesthetics, they nevertheless share many properties with what may be called 'true' anaesthetics and they have been used in many systems to examine the way in which anaesthetic-like molecules change the properties of ionic currents (e.g. Armstrong & Binstock, 1964; Gage *et al.*, 1975; Haydon & Urban, 1983; Wachtel, 1984). They can be a useful tool in investigations of anaesthetic action since they provide a homologous series of molecules whose physico-chemical properties are well documented and vary predictably with chain length (Seeman, 1972). Although the aliphatic alcohols have been shown to affect such sodium channel properties as inactivation (e.g. Oxford & Swenson, 1979), the concentrations of alcohols and other anaesthetics which substantially affect sodium channel gating are generally quite high. Hence it appears unlikely that interference with the action potential generating mechanism is involved in the induction of anaesthesia.

Like sodium currents, transient potassium currents appear to be widely distributed among both neuronal (e.g. Linas, 1988) and non-neuronal tissues (e.g Pelzer & Trautwein, 1987), and it has been suggested that they play a major role in the control of such cell properties as repetitive firing (Connor & Stevens, 1971) and action potential duration (Nakajima *et al.*, 1986). Therefore, if anaesthetics and similar molecules exert an action on the transient potassium current, one would expect that profound alterations of cell behaviour would result, possibly accounting for the anaesthetic action of these agents. In addition, information on the way in which these agents alter the transient current may elucidate the mechanisms which control the properties of the current itself.

The data presented here show that aliphatic alcohols affect the transient potassium current in hippocampal neurones by increasing the rate of decay of the current and causing a reduction in peak current amplitude. In addition, the potency of a series of alcohols in affecting the transient current correlates well with their membrane/buffer partition coefficients, implying that these agents exert their effects by interaction with a hydrophobic environment. How these findings fit with contemporary ideas on the mechanism of inactivation of the transient potassium channel is discussed.

Methods

Cell preparation and solutions

Whole cell patch-clamp recordings were made from isolated neurones obtained from the CA_1 region of hippocampi from newborn rats (less than 12 h post-natal). Primary cultures were made by mechanical dissociation of the cells, which were then grown on coverslips coated with poly-L-lysine. The culture medium used was Minimum Essential Medium (Commonwealth Serum Laboratories) to which was added foetal calf serum (10%), glucose (6%), $NaHCO_3$ (1.5%), penicillin/streptomycin (2%) and fungizone (Flow Laboratories) (1%). Cells were kept in primary culture for 4 to 6 days before use. For experimentation, cells were transferred on a coverslip to a bath and superfused with solution of the following composition (mM): NaCl 150, KCl 3, $CaCl_2$ 2, HEPES 10, pH adjusted to 7.3 ± 0.05 with NaOH. Patch clamp electrodes were made from thick-walled borosilicate glass (Clarke Electromedical) and typically had resistances of 6 to 8 megohm when filled with intracellular solution of composition (mM): KCl 150, NaCl 4, $CaCl_2$ 0.5, EGTA 5, pH adjusted to 7.3 ± 0.05 with KOH. Experiments were done at room temperature (25 ± 2°C). Drug application was made with a rapid switching perfusion system, in which the perfusate entered the bath in the immediate vicinity of the cell. The solution bathing the cell could be changed in 1 to 2 s by this method.

Electrophysiology

Whole cell voltage clamp was performed with a List EPC 7 amplifier, with command potentials being generated by a PDP 11 microcomputer, which also digitized and stored the currents generated. Analysis of the records was performed on an IBM-compatible microcomputer with software specifically written for the purpose. Ohmic leak currents were nullified as well as possible 'on line' with an analogue subtraction circuit, any remaining leakage current being digitally subtracted at the data analysis stage. Transient currents arising from the capacitance of the pipette or cell membrane were cancelled by the circuitry employed in the List EPC7. Liquid junction potentials were not corrected for, and would be of the order of 4 to 5 mV (internal solution negative) (see Fenwick *et al.*, 1982). The 'G-series' control on the amplifier was set to minimize the capacitative transient but otherwise series resistance compensation was not generally used; the voltage error from this source would be of the order of 5% for the largest currents measured. The effect of series resistance on cell membrane charging time, even for the worst case estimate, would not be large enough to affect the currents described here.

Results

Cells selected on the basis of their appearance under phase contrast optics typically had membrane potentials more negative than −45 mV and, in current-clamp mode, responded to current injection with action potentials having an overshoot to about +10 mV. Input resistance of the cells was typically greater then 500 megohm. Decay of voltage transients in current-clamp mode was well fitted by a single exponential, indicating that the cells were electrotonically compact.

In voltage clamp mode the cells responded to depolarizing voltage steps from a holding potential of −50 mV with an outward, non-inactivating current (I_K). However, if the voltage command pulse was preceded by a hyperpolarizing pre-pulse to −120 mV, a large, rapidly inactivating outward current was evoked in addition. This transient current could be blocked by 4-aminopyridine (4-AP) at concentrations above 1 mM, and could not be evoked when internal KCl was replaced by CsCl. The transient current will be called I_A, due to its similarity to a current described in molluscan neurones by Connors & Stevens (1971). It was not possible to isolate I_A pharmacologically, since block of I_K by tetraethylammonium (TEA) was incomplete except at concentrations above 10 mM, a concentration which also blocked I_A by around 20%. It was also considered likely that there may be an interaction between the alcohols and pharmacological agents used to differentiate the currents, perhaps due to a change in the potency of the blocker in the presence of the alcohols. For these reasons I_K and I_A were isolated electrophysiologically, as described in Figure 1. The current evoked by a depolarization to +80 mV from a holding potential of −50 mV (at which potential I_A is entirely inactivated), was subtracted from the current evoked by the same depolarization following a pre-pulse to −120 mV. This procedure effectively produces a record of I_A alone, provided that one ensures that I_A is entirely inactivated at the holding potential used.

Calcium-dependent transient potassium currents have been described in guinea-pig hippocampal cells (Numann *et al.*, 1987). However, in the experiments described here, the addition of Cd^{2+} (200 μM) to the extracellular solution made no appreciable difference to the results (in 3 cells tested under both conditions); hence, there appears to be no calcium-dependent component to the currents examined in these experiments, possibly due to inter-species differences.

The effect of octanol on both I_K and I_A is illustrated in Figure 1. At the concentrations used throughout these experiments octanol caused a very small decrease in the magnitude

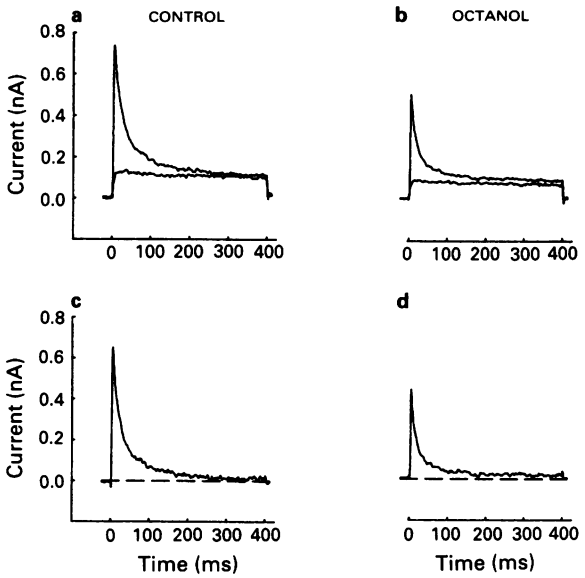

Figure 1 The effect of octanol on I_A and I_K. (a,b) Outward currents were elicited by a voltage step to +80 mV from a holding potential of −50 mV (lower current traces) or following a pre-pulse to −120 mV for 200 ms (current trace with large transient component). Panel (a) shows the currents recorded in control solutions; (b) shows currents recorded with 0.35 mM octanol added. (c,d) Show the result of subtracting the trace recorded with no pre-pulse from that recorded with a pre-pulse, to yield the transient component, or I_A.

of I_K, with no discernible effect on its kinetics. I_A was dramatically changed, however, showing a reduced peak height and increased initial rate of decay. In addition, there appeared to be a late, slowly inactivating component of the current in the presence of octanol, which was not discernible in the control currents.

Figure 2 illustrates the concentration-dependence of the effect of octanol. Each increment in concentration from zero to 1.26 mM caused a decline in the magnitude of the peak outward current. Upon cessation of application of octanol, the peak height recovered to near control values (data record truncated in the figure). These concentrations of octanol (and of the other alcohols described later) are comparable to those used by Haydon & Urban (1986) in a study of anesthetics on the potassium channels in the squid giant axon.

One explanation of the slowly inactivating component of the current revealed in the presence of octanol in Figure 1 could be that there is a contribution to the current from I_D, a slowly inactivating voltage-dependent potassium current described in these cells by Storm (1988), and that this is somehow enhanced by octanol. However, the decay of control records of I_A in these experiments was monotonic, a finding which suggests that I_D was not present. More persuasively, repetition of the experiments in the presence of 50 μM 4-AP, a concentration which has been shown to block I_D (Storm, 1988), did not alter the effects of the alcohols (tested in 5 cells, data not shown). This lack of I_D in the experiments described here may be due to the late post-natal development of I_D in neurones (Surmeier *et al.*, 1991), since previous reports of I_D have been in cells from adult animals (Storm, 1988).

Figure 3 shows the transient current in one cell (obtained by the subtraction procedure illustrated in Figure 1) plotted semi-logarithmically (Figure 3a) and linearly (Figure 3c) against time, each record fitted with a single exponential having a time constant of 58 ms. However, the current decay in the presence of octanol (0.3 mM) became markedly biphasic, with time constants of 37 ms for the initial decay (fitted between 0 and 40 ms) and 120 ms for the decay beyond 150 ms. Figure 3b and d show the current recorded

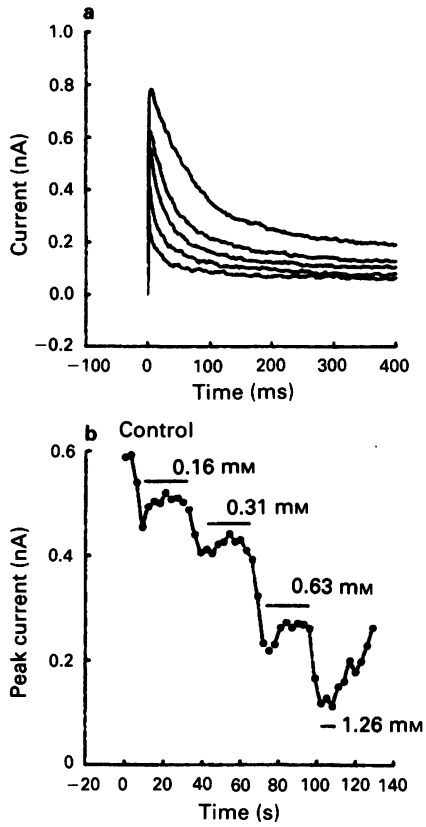

Figure 2 The effect of increasing concentrations of octanol on the transient potassium current. Currents were elicited by a voltage step to +80 mV from a pre-pulse of −140 mV (duration 200 ms). (a) The largest current record is the control; progressively smaller currents show the effect of adding increasing concentrations of octanol to the bathing solution (0.16, 0.31, 0.63 and 1.26 mM). After a period of washout, the size and character of the current returned to near the control trace (data not shown for clarity). (b) Cumulative dose-response curve to octanol. Peak current amplitude of the transient currents is shown plotted against time, the concentration of octanol in the bathing solution being increased in steps as in the left panel. A short period of washout, starting at 110 s, is shown at the end of the trace.

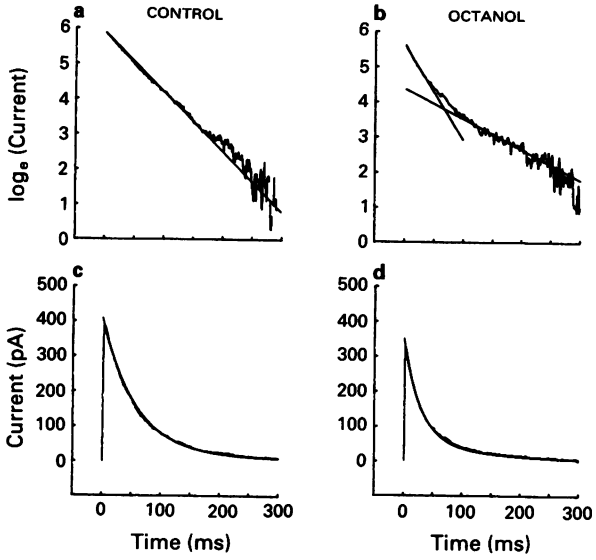

Figure 3 Octanol makes the current decay bi-phasic. Transient currents were elicited by a voltage step to +80 mV from a pre-pulse potential of −140 mV. Records for control current and current recorded in the presence of octanol (0.33 mM) are plotted semi-logarithmically against time in (a) and (b) and linearly in (c) and (d). Control currents were typically mono-exponential, as shown in (a), in which a single regression line with a slope equivalent to a time constant of 58 ms has been fitted to the record. The effect of octanol was to cause a marked increae in the rate of decay of the early phase of the current ($\tau = 35 \pm 4$ ms) along with a decrease in the rate of decay of the latter part ($\tau = 120 \pm 10$ ms). Correlation coefficients of the regressions were 0.99 for the initial decay (fitted between 0 and 40 ms) and between 0.8 and 0.9 for the late decay (fitted between 150 and 250 ms). The regression lines have been extrapolated for clarity. The currents shown are averages of 5 records for each trace. Records from the same cell as in Figure 2.

in the presence of octanol plotted logarithmically or linearly and fitted with two exponentials. This effect of octanol was consistently seen, although it was somewhat variable (in 6 cells control τ was 54 ± 6.5 (mean ± s.e.) which changed to 36 ± 4.7 for the initial decay and 117 ± 16 for the late decay). The increase in the initial rate of decay of the current in octanol was not sufficient to account entirely for the decrease in peak current amplitude, since extrapolation of the fits to the currents to 'zero time' (shown in Figure 3) did not result in a 'corrected' peak current in octanol of the same magnitude as control. (This procedure should correct for the effect of the increased rate of decay during the finite time necessary for the rising phase of the current.) Hence it appears that octanol may exert two independent effects, one to increase the initial rate of current decay, and one to reduce peak current amplitude.

This reduction in the peak current amplitude was not due to a change in the voltage-dependence of either the activation or steady state inactivation of the current (a change which has been shown to be induced in these cells by, for instance, $GABA_B$ agonists (Saint *et al.*, 1990)), as shown in Figure 4, which shows data typical of that recorded in 5 cells. Concentrations of octanol which caused considerable changes in maximum conductance (G_{max}) did not cause a large shift in the voltage-dependence of either activation or inactivation. A small shift to the right of both curves may have occurred

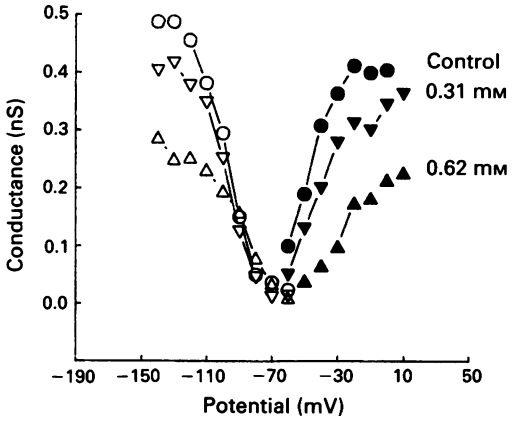

Figure 4 Steady state inactivation and activation curves for the transient current. Peak current amplitude for transient current records was converted to conductance by use of the equation $g = I/(V - V_k)$, where V = membrane potential (see below), V_k = reversal potential for the current (calculated as −100 mV for the potassium concentrations used and a pNa/pK of 0.04). Conductance is shown plotted against potential, where 'potential' represents either the potential of a pre-pulse lasting 200 ms immediately preceding the test pulse to +40 mV (inactivation curves, open symbols), or the potential of the test pulse following a pre-pulse to −140 mV (activation curves, closed symbols). The application of 0.31 mM or 0.62 mM octanol, while causing a substantial decrease in maximum conductance, did not substantially shift the voltage-dependence of either the inactivation or activation of the current. (○,●) Control; (▽,▼) 0.31 mM; (△,▲) 0.62 mM. Filled symbols, activation data; open symbols, inactivation data.

with the higher concentration of octanol used, but this could not account for the change in peak current with the voltage protocol used. Hence the reduction in current amplitude, since it appears not to be due to a change in current kinetics, must be due to the reduction in G_{max}.

A possible explanation of the effect of octanol in causing this decrease in the peak current magnitude and a concurrent increase in the rate of decay could simply be that, for some reason, smaller I_A currents have a faster rate of decay. In other words, the increase in rate of decay could be incidental to the decrease in current magnitude caused by octanol. This possibility was discounted by varying peak current amplitude by changing the degree of inactivation of the transient channels (by varying the potential of the pre-pulse). Little effect on the rate of decay was seen, even when the peak current amplitude was greatly attenuated, as shown in Figure 5a, indicating that transient currents decay at the same rate irrespective of their size. The same procedure carried out in the presence of octanol showed that octanol, although causing an overall depression of peak current, did not alter this lack of correlation between peak height and τ (Figure 5a, filled circles). However, when the same type of analysis was done for currents in which the peak height was varied by exposure to increasing concentrations of octanol, there was a strong correlation between τ and peak current, at least for reductions in current amplitude down to 1.5 nA (Figure 5b). Below this current amplitude (i.e. at higher concentrations of octanol) the relationship between τ and current amplitude seemed to degenerate, implying that the reduction in peak current and the reduction in τ are the product of different processes, and that the effect of octanol in reducing τ saturates before the effect of reducing current amplitude.

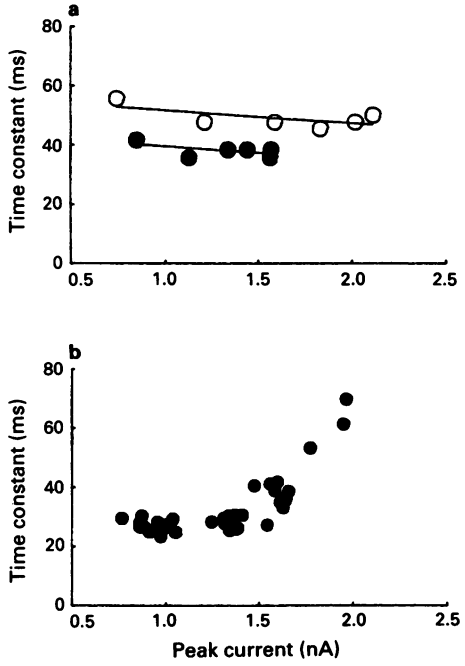

Figure 5 Lack of correlation between peak current and τ for control currents and the effect of octanol. (a) Peak current amplitude was varied by changing the degree of inactivation current (by changing the potential of the pre-pulse over the range -140 to -90 mV). For each current record the peak height and the time constant of decay of the initial 40 ms of the current was measured. Over a range of peak current amplitudes from 0.6 nA to 2.3 nA there was very little change in τ (○). The effect of octanol (0.6 mM) was to reduce peak current amplitude and decrease τ, but there was no change in the lack of correlation between them (●). Lines show regression lines for the data. (b) When the same analysis as above was made for currents in which the peak current amplitude was reduced by increasing the concentration of octanol there was a strong correlation between peak height and τ, at least for reductions of peak height from 2 nA down to 1.25 nA. At smaller peak currents (i.e. for higher concentrations of octanol) the effect to reduce τ appeared to saturate.

The decay of I_A in these cells shows only a weak voltage-dependence (Numan *et al.*, 1987), as shown in Figure 6. Currents were elicited by voltage steps of varying size, from a fixed, hyperpolarizing potential. The time constant of decay of the initial phase (first 40 ms) of each current is shown plotted against the membrane potential at which it was elicited. In the cell illustrated, a weak voltage-dependence of the rate of decay was seen; currents at more depolarized potentials decayed somewhat more slowly. Exposure of the cell to octanol increased the rate of decay of the currents but did not alter this weak voltage-dependence. This result also indicates that the effect of octanol itself is not voltage-dependent, since the decrease in τ produced by octanol was the same at all voltages.

The effects of other aliphatic alcohols, down to a chain length of 4 (butanol), appeared to be qualitatively the same as the effects of octanol. Concentrations of octanol or butanol which caused a similar reduction in peak transient currents also caused a similar increase in the rate of current decay: i.e. the alcohols of different chain length did not produce discernibly different effects, other than a difference in potency. This is similar to the results of Armstrong & Binstock (1964), who also reported that chain length made little difference, other than in potency, to the action of alcohols on squid giant axon. However, it should be noted that Treistman & Wilson (1987) reported that the effects of alkanols on I_A in *Aplysia* did vary qualitatively with chain length, particularly with ethanol.

Aliphatic alcohols with carbon chains of 4, 5, 6 and 7, carbons, as well as octanol, were assessed for their potency in causing a reduction of peak current. Figure 7a shows that, on a logarithmic plot of peak current against concentration, each of the alcohols reduced the peak current height in a parallel manner (i.e. a doubling of the concentration of any one of the alcohols reduces peak height by a similar increment). There was a strong correlation between chain length and potency for the alcohols, the ED_{50} values being (in mM): C8 = 0.8, C7 = 3.5, C6 = 8.8, C5 = 26, C4 = 49. Hence the potency of the alcohols increased by about 3 fold for an increase in chain length of 1 carbon atom. A comparable result is obtained if one uses the reduction in τ as an index of potency, instead of reduction in peak current. These concentrations, and the change in potency with chain length, are similar to those reported for the effects of hexanol and octanol on I_A in *Helix* neurones (Winpenny *et al.*, 1990). A similar relationship between alcohol potency and chain length has also been described for effects upon potassium channels in the squid giant axon (Haydon & Urban, 1986).

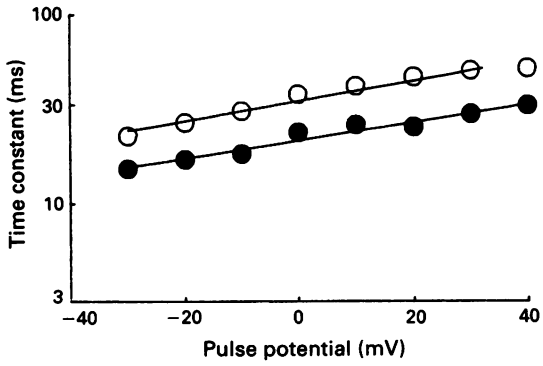

Figure 6 Voltage-dependence of τ. Currents were evoked by a test pulse of varying potential following a fixed pre-pulse of -140 mV for 200 ms. The rate of decay of the current showed a slight voltage-dependence over the range of potentials from -30 mV to 40 mV (○). The addition of octanol (0.6 mM) caused an increase in the initial rate of current decay (decrease in τ), but the voltage-dependence of τ was not affected (●). Note that τ is plotted logarithmically.

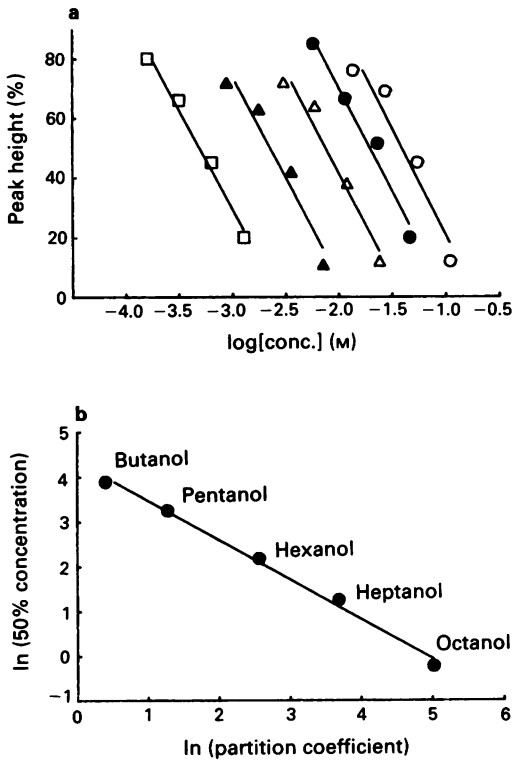

Figure 7 Potency of other aliphatic alcohols. (a) The effect of increasing concentrations of each of the alcohols on the peak height of the transient current. Each data point is the mean of measurements in at least 4 cells. Lines are regression lines through the points, and points show data for octanol (C = 8) (□), C = 7 (▲), C = 6 (△), C = 5 (●) and C = 4 (○). The standard error of the measurements of normalized peak height are of the order ± 5% in all cases. (b) Correlation of the effect of the alipatic alcohols with their membrane buffer partition coefficients. The potency of each of the alcohols from butanol to octanol (expressed as the concentration which produces 50% inhibition of the transient current) is shown plotted against the membrane/buffer partition coefficient (from Seeman, 1972). Both axes are logarithmic. The line through the points is a regression line.

Discussion

The results presented here show that the main effects of aliphatic alcohols on the transient potassium current are to cause a reduction in peak current and an increase in the initial rate of current decay, such that the current decay becomes markedly bi-phasic. There are two possible explanations for the increase in the rate of decay of the current: an increase in the rate of inactivation of the channels, or a 'channel blocking' action of the alcohols. The simplest way in which the behaviour of the transient potassium channel can be depicted is as follows:

$$\text{Closed} \underset{\text{Depolarization}}{\rightleftharpoons} \text{Open} \underset{K_2}{\overset{K_1}{\rightleftharpoons}} \text{Inactivated}$$

In such a scheme the action of the alcohols would be to increase the rate of inactivation (K_1). A simple change in the rate constants of such a scheme by the alcohols is, however, incompatible with the data, since manipulation of the rate constants in such a model cannot yield a biphasic decay. This sort of model can be made to produce a bi-phasic decay with the addition of multiple inactivated (or non-conducting) states of the channel, and a differential effect of the alcohols upon the various rate constants. Such an action of the alcohols is possible, but seems somewhat implausible. It should perhaps be noted that if the alcohols act by increasing the rate of inactivation then they do so without changing the voltage-dependence of steady state inactivation (see Figure 4), a result which also seems implausible.

An alternative explanation is that the alcohols act as channel blockers, i.e. an interaction of the alcohol molecule with the open channel to reduce (or abolish) its conductance, in the same way as the block of nicotinic channels produced by procaine (Adams, 1976). i.e.:

$$\text{Closed} \underset{\text{Depolarization}}{\rightleftharpoons} \text{Open} \rightleftharpoons \text{Inactivated}$$
$$\text{Open} \underset{K_{-2}}{\overset{K_2}{\rightleftharpoons}} \text{Blocked}$$

In this scheme the faster initial rate of decay of the current in the presence of the alcohols would be due to increased rate of conversion of the channels to a non-conducting form by interaction of the open channel with the alcohol molecule, the slower rate of decay at later times representing the channels becoming able to conduct again upon dissociation of the alcohol molecule (K_{-2}), before finally inactivating.

The lack of voltage-dependence of the effect of the alcohols (illustrated in Figure 6) would then not be surprising, since one would not expect that uncharged molecules such as the alcohols would display a voltage-dependence in their interaction with the open channel.

However, if the only effect of the alcohols was a 'channel blocking' action, the reduction of the peak height of the current should be entirely accounted for by blockade of the channels as they are activated. As shown in Figure 2, extrapolation of the 'channel blocked' current in the presence of octanol does not yield an intercept of the same magnitude as control currents, indicating that some mechanism other than a channel block is responsible for at least some of the reduction in the peak current amplitude. Other evidence for a dual effect of octanol is provided by the data in Figure 5b, where the correlation between peak height and τ appears to break down at higher concentrations of octanol. This suggests two distinct effects of the alcohols with two sites in the channel protein, one which changes the kinetics of current decay, and one which affects ion conduction. A similar sort of 'dual action' of anaesthetics on potassium channels has been suggested by Haydon & Urban (1986), and 'one site' or 'two site' models of anaesthetic action have been discussed by Forman & Miller (1989) in the context of the nicotinic channel.

A further consideration to be taken into account in assessing whether a channel blocking mode of action of the alcohols can satisfactorily account for the data is the finding that the potency of the alcohols ranging from butanol to octanol correlates well with their respective membrane/buffer partition coefficients (from Seeman, 1972), as shown in Figure 7b. Such a correlation between potency and partition coefficient means, in effect, that the concentration of each alcohol is approximately the same in the membrane lipid phase when the current is reduced by the same amount, although the 'aqueous' concentration is very different. Such a finding implies a hydrophobic locus of action of the alcohols, either with a hydrophobic region of the channel protein, or the lipid environment around it. The same sort of relationship has been described for the effect of alcohols upon the decay of miniature endplate currents by Gage *et al.* (1975), and these authors advanced the same explanation. Similar results have also been shown for alcohol effects on squid giant axon, for both sodium and potassium channels (Haydon & Urban, 1983; 1986). Although one would imagine that a channel blocking agent should be active in the aqueous phase, Murrell *et al.* (1991) recently suggested that the action of n-alcohols in blocking the nicotinic channel was due to interaction at a hydrophobic site. Hence, the data presented here are not inconsistent with a channel blocking model for the action of the alcohols on the transient potassium current.

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Erratum

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Y.J. Liu, N.P. Shankley, N.J. Welsh & J.W. Black. Evidence that the apparent complexity of receptor antagonism by angiotensin II analogues is due to a reversible and syntopic action.

In the above paper, a number of errors occurred in the published article, most particularly, equations (5), (6) and (11) contained inaccuracies as printed. The items concerned are shown in their correct form below.

Evidence that the apparent complexity of receptor antagonism by angiotensin II analogues is due to a reversible and syntopic action

[1]Y.J. Liu, N.P. Shankley, N.J. Welsh & J.W. Black

Methods

Isolated smooth muscle preparations

Rabbit and guinea-pig aorta ring preparations Aortic ring segments (approximately 3 mm long) from rabbit (male, New Zealand White, approximately 2.5 kg) and guinea-pig (male, Dunkin Hartley, 350 to 450 g) aortae were prepared following Stollak & Furchgott (1983). Briefly, descending thoracic aortae were excised, cleaned of extraneous tissues, and the endothelium was removed. Rings were suspended between 2 stainless steel wires in 20 ml organ baths containing Krebs-Henseleit (K-H) solution of the following composition (in mM): Na^+ 143, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128, HPO_4^{2-} 1.0, SO_4^{2-} 1.2, HCO_3^- 25, D-glucose 10, constantly gassed with 95% O_2 and 5% CO_2, and maintained at 37°C. Tension was continuously recorded with isometric transducers, following the application of 1 g and 3 g pre-loads in the guinea-pig and rabbit preparations, respectively.

Figure 4 Analysis of the relationship between peptide ligand ([Sar[1],Leu[8]]AII) concentration and the depression of the angiotensin II (AII) E/[A] curve maxima, (α). (a) AII E/[A] curves ($n = 6$) obtained in the absence (●) and presence (nM) of [Sar[1],Leu[8]]AII, 0.3 (○), 0.5 (■), 1 (□), 3 (▲) and 10 (△). (b) The change in α has been expressed with reference to the control curve value; α_B is α in the presence of the peptide ligand. The curve shown superimposed on the experimental data was obtained by logistic curve-fitting (see text for details).

Figure 6 Simulation showing the effect of concurrent affinity and efficacy reduction in the explanatory model (equations (10) and (11)) for both (a) high and (b) low efficacy values. Parameters were set as follows: $E_M = 100$, $pK_A = pK_B = 6$, $\beta = 0.9$, $n = 1$. (a) $\tau = 1000$ (b) $\tau = 0.1$. (c) Schild plots corresponding to this simulated data shown in panels (a, ●....●) and (b, ■—■).

$$E = \frac{E_M[A]^n\tau^n}{(K_A + [A])^n + [A]^n\tau^n} \qquad (5)$$

$$E = \frac{E_M[A]^n\tau^n}{(K_A(1 + [B]/K_B) + [A])^n + [A]^n\tau^n} \qquad (6)$$

$$E = \frac{E_M\tau'^n[A]^n}{(K_A(1 + [B]/K_B) + [A])^n + [A]^n\tau'^n} \qquad (11)$$

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