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The selective NK_3 receptor agonist senktide excites a subpopulation of dopamine-sensitive neurones in the rat substantia nigra pars compacta *in vitro*

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Intra- and extracellular recordings were made from substantia nigra zona compacta neurones from an *in vitro* rat brain slice preparation. A subpopulation of dopamine-sensitive neurones were encountered which were potently excited by bath application of the NK_3 receptor agonist, senktide. On these senktide-sensitive neurones, NK_1 and NK_2 receptor agonists were inactive. The excitatory action of senktide supports a role for tachykinins as putative neurotransmitters in the basal ganglia.

Keywords: Basal ganglia; substantia nigra; dopamine; tachykinin; senktide; neurokinin

Introduction There is some evidence for the existence of a striatonigral pathway which utilizes substance P (SP) as a neurotransmitter (Dray, 1980). The highest concentrations of SP-like immunoreactivity (SPLI) occur in the substantia nigra (Ljungdahl *et al.*, 1978) and it has been shown that injection of SP into the substantia nigra results in increases in dopamine cell activity as measured by changes in dopamine release and turnover (Cheramy *et al.*, 1978). In this study we have made use of tachykinin receptor-selective agonists to study the action of SP on substantia nigra compacta (SNC) neurones which respond to dopamine. These have allowed us partially to characterize the receptor type involved in the tachykinin response in the substantia nigra, pars compacta.

Methods Experiments were performed on brain slices (300 μm thick) from 150 g male Wistar rats and extracellular recordings were made by conventional techniques (Keegan *et al.*, 1990). Drugs were applied by bath perfusion and removal was achieved simply by returning to the control drug-free solution. There was a 'dead time' of 1 min due to the perfusion process. Extracellular electrodes were filled with aCSF and

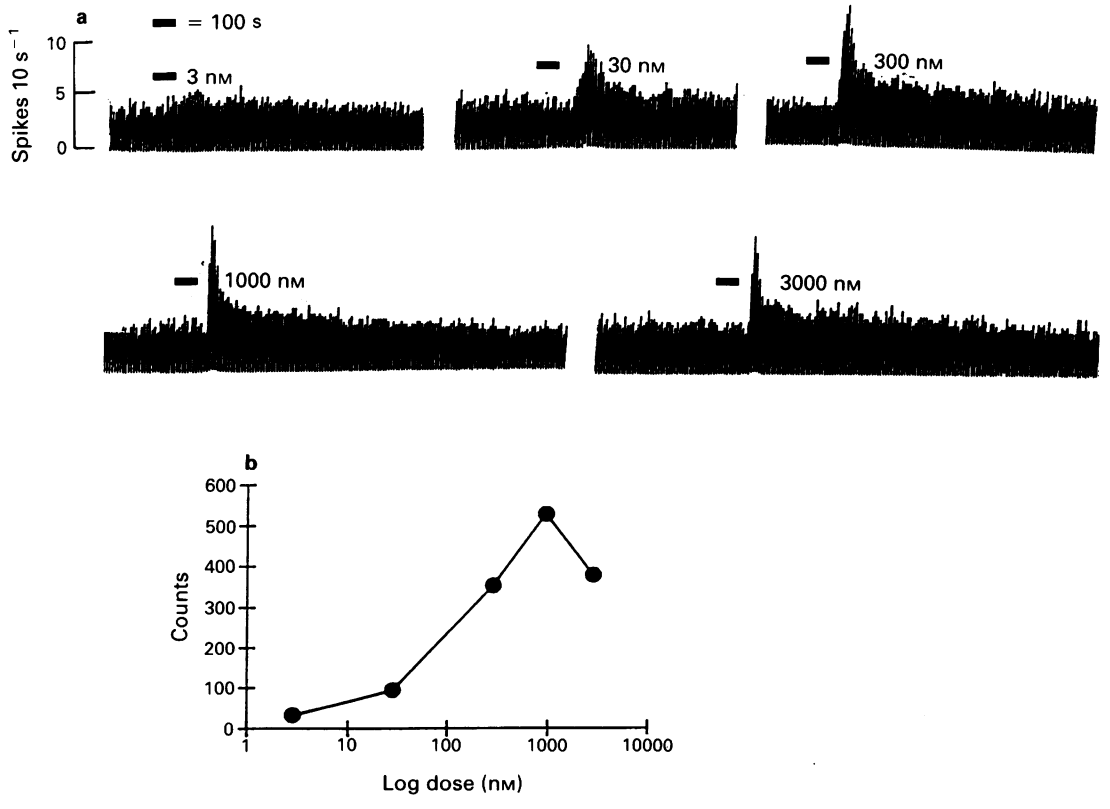


Figure 1 Ratemeter histogram from a substantia nigra neurone showing that the excitatory actions of senktide are concentration-dependent and long lasting. (a) The ratemeter recordings were taken from the same neurone, the responses plotted on the graph (b) were obtained by using the total number of action potentials occurring over a 20 min period and subtracting the number of action potentials occurring over a 20 min control period.

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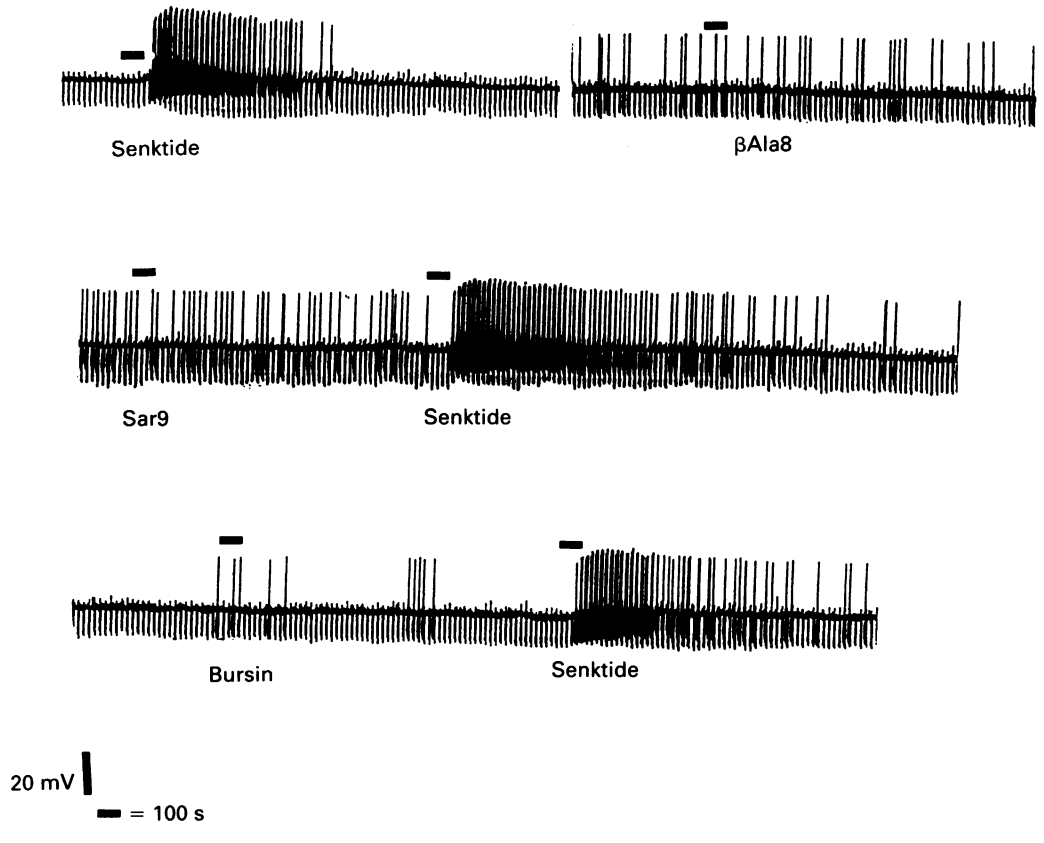


Figure 2 Intracellular recording from a substantia nigra neurone showing that the excitatory response to senktide (500 nM) was caused by a depolarization of the membrane potential without a large change in membrane conductance and the lack of effect of the NK_1 receptor agonist [Sar^9,$Met(O_2)^{11}$]SP(1-11) (1 μM) and the NK_2 receptor agonist [βAla^8]NKA(4–10) (1 μM) on the same neurone. The downward deflections of membrane potential were caused by the injection of constant current (0.2 Hz, 200 ms, 0.5 nA) through the recording electrode. The resting potential of the cell was −51 mV.

had resistances of 5–14 MΩ. For intracellular recordings, electrodes were filled with 1 M potassium acetate and had d.c. resistances of 70–110 MΩ. Neurones were considered to be dopaminergic if they had a characteristic waveform, slow firing rate (~5 Hz) and inhibitory response to dopamine (Pinnock, 1985). β-Ala^8 was obtained from Novabiochem. All other drugs were obtained from Peninsula laboratories.

Results The selective NK_3 receptor agonist senktide (succinyl-[Asp^6, $MePhe^8$]SP(6-11)) excited 24 of 31 dopaminergic neurones in the substantia nigra pars compacta in a concentration-dependent manner. The effective concentration-range was between 3–3000 nM. The mean EC_{50} for senktide was 41.2 ± 9 nM (n = 5). Senktide responses were prolonged and required 10–30 min before recovery (Figure 1). At concentrations greater than 1 μM, there was a marked degree of desensitization. The selective NK_1 receptor agonist, [Sar^9,$Met(O_2)^{11}$]SP(1-11) and the selective NK_2 receptor agonist, [βAla^8]NKA(4-10) were inactive on senktide-sensitive neurones (n = 6; Figure 2) within the concentration-range used (500–3000 nM). In contrast to the potent actions of senktide, SP was only weakly active on senktide-sensitive neurones with responses observed only when concentrations greater than 1 μM were applied (n = 3). The peptide, bursin, was inactive on all three senktide-sensitive neurones on which it was tested. Most senktide-sensitive neurones were encountered in coronal brain sections of the SNC at the level of the interpeduncular nucleus (18 of 24) whilst none were encountered as rostral as the level of the medial mamilliary nucleus.

Discussion Autoradiographic studies of the distribution of tachykinin receptors reveal a surprisingly poor relationship between the distribution of tachykinin receptors and SPLI although a good correlation between [^{3}H]-SP binding sites and inositol phospholipid hydrolysis has been reported (Mantyh *et al.*, 1984). Thus whilst the substantia nigra contains the highest levels of SPLI in the brain (Ljungdhal *et al.*, 1978) there is a sparse distribution of SP binding sites. In agreement with this observation, previous electrophysiological studies *in vivo* have demonstrated a weak excitatory action of SP on identified dopamine neurones of the SNC (Pinnock and Dray 1982). Furthermore, preliminary experiments *in vitro* suggest a differential sensitivity between the SNC and SNR neurones to substance P (Pinnock, 1983). One explanation for the relatively low potency of SP on SNC neurones may be that neurokinin B (NKB) acts as the endogenous ligand for the tachykinin receptor in the substantia nigra. Indeed this tachykinin appears to be the preferred agonist for the NK_3 receptor. Furthermore, the presence of NKB in the substantia nigra has been demonstrated by radioimmunochemical techniques (Kanazawa *et al.*, 1984). In conclusion this study demonstrates that a subpopulation of dopamine-sensitive neurones in the substantia nigra are sensitive to the selective NK_3 receptor agonist, senktide, but the lack of NK_3-selective antagonists prevents definitive characterization of the tachykinin receptor involved. Recent autoradiographic studies with [^{3}H]-senktide have also revealed NK_3 binding sites in the SNC (Dam *et al.*, 1990). The absence of responses to the NK_1 receptor agonist [Sar^9,$Met(O_2)^{11}$]SP(1-11) and the NK_2 receptor agonist [βAla^8]NKA(4-10) suggests that NK_1 and NK_2 receptors are not present on dopamine neurones in the SNC. These results support a role for tachykinins as putative neurotransmitters in the basal ganglia.

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5-Methylurapidil may discriminate between α_1-adrenoceptors with a high affinity for WB4101 in rat lung

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The α_1-adrenoceptors of rat lung with a high affinity for [^{3}H]-prazosin were subdivided into two populations (high and low affinity sites) by WB4101 and 5-methylurapidil but the proportions were different between both drugs. After pretreatment with chlorethylclonidine, WB4101 recognized only high affinity sites, while 5-methylurapidil still detected high and low affinity sites. These results indicate that α_1-adrenoceptors with a high affinity for WB4101 are not homogeneous in the rat lung, suggesting the possible existence of a new α_1-adrenoceptor subtype in addition to α_{1A} and α_{1B} subtypes.

Keywords: α_1-Adrenoceptor subtype; 5-methylurapidil; WB4101; rat lung

Introduction According to recent subclassification of α_1-adrenoceptors, high affinity sites for [^{3}H]-prazosin or [^{125}I]-BE2254 are subdivided into two subclasses (α_{1A} and α_{1B}) (Morrow & Creese, 1986; Minneman, 1988; Hanft & Gross, 1989; Oshita *et al.*, 1991). The α_{1A} subtype shows high affinity for WB4101, benoxathian and 5-methylurapidil, and is relatively insensitive to an alkylating agent chlorethylclonidine (CEC), while the α_{1B} subtype has low affinity for the antagonists mentioned above and is potentially inactivated by CEC. However, recent molecular biological studies have demonstrated the presence of an additional α_1-adrenoceptor subtype (α_{1C}) with high affinity for prazosin (Schwinn *et al.*, 1990; Lomasney *et al.*, 1991). The present study in the rat lung shows that 5-methylurapidil displaces a different proportion of [^{3}H]-prazosin binding from that seen with WB4101 suggesting a possible existence of three distinct binding sites with high affinity for prazosin.

Methods The lungs of male of Wistar rats (250–450 g) were used in the present studies. After isolation of the large bronchi, the lungs were minced and homogenized in 20 volumes of ice cold 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 30 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 15 min at 37°C, and centrifuged again as described above. The final pellets were resuspended in the assay buffer. In some studies the membrane preparations were incubated with 10 μM CEC for 30 min at 37°C and then washed once more with assay buffer before the binding experiments. Incubation with [^{3}H]-prazosin (76.6 Ci mmol^{-1}, NEN, Boston) was carried out in duplicate in a final volume of 1 ml at 30°C. After 45 min the membrane was filtered through Whatman GF/C filters by using a Brandel M30 cell harvester and the radioactivity retained on filters was determined by liquid scintillation counting. Prazosin (10 μM) was used to define non-specific binding. All values were expressed as mean ± s.e.mean. Other experimental conditions and data analysis methods were the same as those described previously (Oshita *et al.*, 1991).

Results *Saturation experiments:* The specific binding of [^{3}H]-prazosin (10–5000 pM) was concentration-dependent and saturated at 1000 pM. Scatchard plots of the binding data resulted in a straight line, suggesting a single class of binding sites ($pK_D = 9.83 \pm 0.16$, $B_{max} = 324 \pm 64$ fmol mg^{-1} protein). In the membranes pretreated with 10 μM CEC also, [^{3}H]-prazosin bound to a single site with an affinity similar to that of CEC-untreated membranes but the number of binding sites was reduced (46% reduction in B_{max}).

Displacement experiments: Unlabelled prazosin monophasically displaced the specific binding of 200 pM [^{3}H]-prazosin to CEC-untreated and -pretreated membranes, resulting in an affinity similar to the pK_D in the saturation experiments (Table 1). On the other hand, WB4101, benoxathian and 5-methylurapidil showed shallow displacement curves in CEC-untreated membranes. Computerized analysis revealed that the antagonists subdivided the binding sites into two populations; the proportion (approximately 65%) of WB4101 – or

Table 1 Inhibition of [^{3}H]-prazosin binding to α_1-adrenoceptors of rat lung by α_1-antagonists

Antagonist	n	*Slope factor*	$pK_{I\,high}$	$pK_{I\,low}$	% *high*
CEC-untreated					
Prazosin	3	0.86	9.47 ± 0.21	—	100
WB4101	3	0.52	9.82 ± 0.24	8.25 ± 0.38	67.6 ± 8.9
Benoxathian	3	0.58	9.61 ± 0.30	8.04 ± 0.16	63.0 ± 7.9
5-Methylurapidil	3	0.55	9.13 ± 0.11	7.25 ± 0.06	43.0 ± 3.2
CEC-pretreated					
Prazosin	3	0.92	9.21 ± 0.12	—	100
WB4101	3	0.96	9.32 ± 0.27	—	100
Benoxathian	3	0.98	9.89 ± 0.25	—	100
5-Methylurapidil	3	0.57	9.38 ± 0.35	7.07 ± 0.28	63.5 ± 3.4

Displacement curves were individually analysed by LIGAND programme. A two site fit was accepted only if it was significantly better than a one site fit.
Data shown are means ± s.e.mean. *n*: number of experiments.
$pK_{I\,high}$ and $pK_{I\,low}$: negative log of the equilibrium dissociation constants (−log M) at high or low affinity sites for antagonists tested.
% high: population binding at the high affinity site compared to the total specific binding sites.
CEC-pretreated: the rat lung membranes were pretreated with 10 μM chlorethylclonidine (CEC) and washed.

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benoxathian – high affinity sites was significantly higher than that (48%) for 5-methylurapidil ($P < 0.05$). In the membranes pretreated with 10 μM CEC, WB4101 and benoxathian recognized only the high affinity site. However, 5-methylurapidil still discriminated two distinct affinity sites, although the proportion of high affinity sites increased to 64% in CEC-pretreated membranes (Table 1).

Discussion In the rat lung, α_1-adrenoceptors were detected as a single population with high affinity for prazosin. However, displacement experiments with WB4101, benoxathian and 5-methylurapidil revealed that the sites consist of at least two distinct populations. According to the α_{1A}, α_{1B} subclassification (Minneman, 1988; Hanft & Gross, 1989), both the populations might simply be considered to correspond to α_{1A} and α_{1B} subtypes, respectively. In fact, the α_{1A}, α_{1B} discriminating drugs (WB4101 and benoxathian) clearly distinguished the high and low affinity sites in a similar way and the low affinity sites were completely inactivated by CEC. However, the proportion of the high affinity sites for WB4101 and benoxathian was approximately 20% higher than that for 5-methylurapidil in CEC-untreated membranes, and 5-methylurapidil still detected two distinct affinity sites in CEC-pretreated membranes. These results strongly suggest that the high affinity sites for WB4101 and benoxathian may not be recognized by 5-methylurapidil homogeneously. The existence of three distinct α_1-adrenoceptor subtypes (α_{1A}, α_{1B} and α_{1C}) with a high affinity for prazosin has been demonstrated by molecular biological approach; the α_{1A} and α_{1C} subtypes show a high affinity for WB4101 and are less sensitive to CEC, as compared with the α_{1B} subtype (Lomasney *et al.*, 1991). The difference (20%) between the populations of high affinity sites for WB4101 and 5-methylurapidil observed in the present study may reflect the existence of an additional subtype (α_{1C} or unknown subtype). Therefore, the present results suggest that prazosin-high affinity sites of the rat lung consist of presumably three different α_1-adrenoceptor subtypes and that 5-methylurapidil may be a useful probe for the subdivision of WB4101-high affinity sites.

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The antagonism of benzodiazepine withdrawal effects by the selective cholecystokinin$_B$ receptor antagonist CI-988

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The ability of a selective cholecystokinin$_B$ (CCK_B) receptor antagonist, CI-988, to block benzodiazepine withdrawal effects was examined in mice. The discontinuation of twice daily administration of diazepam (1 mg kg^{-1}, i.p.) induced withdrawal anxiogenesis and a proconvulsant effect. In contrast, no such effects were seen following withdrawal from similar administration of CI-988. However, CI-988 dose-dependently (0.001–1.0 mg kg^{-1}, s.c.) antagonized both the anxiogenesis and the proconvulsant effect following diazepam-withdrawal.

Keywords: Diazepam; withdrawal effects; anxiogenesis; proconvulsant; antagonism

Introduction Benzodiazepines have been widely used for the treatment of anxiety disorders. Although benzodiazepines are effective anxiolytics, they do exhibit certain characteristics and actions that are highly undesirable (e.g. sedation, interaction with CNS depressants, dependence). Several novel non-benzodiazepine compounds have been synthesized as potential anxiolytics which would be devoid of side effects. One such compound is the dipeptoid CI-988, which is a highly selective and potent cholecystokinin$_B$ (CCK_B) receptor antagonist (Hughes *et al.*, 1990). It has been reported to produce potent anxiolytic-like actions in a number of animal models predictive of anxiety (Hughes *et al.*, 1990; Singh *et al.*, 1991a). Further studies have shown that CI-988 is orally active and does not produce sedation or potentiate the action of CNS depressants (Singh *et al.*, 1991b). The present studies were carried out to examine whether or not CI-988 would block benzodiazepine withdrawal effects.

Methods *Animals* Male TO mice (20–25 g) were obtained from Bantin and Kingman (Hull, U.K.). They were housed in groups of 10 under a 12 h light/dark cycle (lights on at 07 h 00 min) with food and water *ad libitum*. All behavioural tests were carried out between 10 h 00 min and 17 h 00 min.

Mouse light/dark box The activity of mice in the light/dark box was measured as previously described (Singh *et al.*, 1991b), except that the animals were housed under bright light for an hour before testing to obtain a high base line for measuring anxiogenic-like response. The time spent in the illuminated side was measured during the 5 min test period.

Anticonvulsant studies Pentylenetetrazol (PTZ; 10 mg ml^{-1}, 0.224 ml min^{-1}) was infused i.v. via a tail vein of restrained mice. The latency to clonic seizure was noted. This convulsion represented the end-point of the test and mice were killed immediately after it had occurred. The threshold dose of the convulsant required to elicit this seizure was calculated for each mouse.

Treatment schedules Groups of mice were injected i.p. twice daily (09 h 30 min and 16 h 30 min) for 7 days with 1 mg kg^{-1} of either diazepam or CI-988. This dose is 10 and 100 times higher than the respective minimum effective dose of diazepam and CI-988 in the mouse light/dark anxiolytic test (Singh *et al.*, 1991b). At 1 mg kg^{-1} the duration of action of the two compounds is about 24 h as measured in the mouse light/dark box (data not shown). The control animals were injected twice daily with the vehicle alone for 7 days. On the 8th day animals were dosed only in the morning and were tested at various times in the light/dark box and immediately afterwards each mouse was infused with PTZ. All experiments were carried out by an observer unaware of the drug treatments.

Drugs The following drugs were used: diazepam and pentylenetetrazol were obtained from Sigma Chemical Co. CI-988 [R-(R*, R*)]-4-[[2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo[3.3.1.1.^{3,7}]dec - 2 - yloxy)carbonyl]amino]propyl] amino]-1-phenylethyl]amino]-4-oxobutanoic acid compound with 1-deoxy-1-(methylamino)-D-glucotil (1:1) was synthesized at Parke-Davis Neuroscience Research Centre. Diazepam was suspended in 1.0% w/v carboxymethylcellulose (CMC) with the aid of ultrasonification. CI-988 and PTZ were dissolved in 0.9% w/v NaCl. Drugs were administered in a volume of 10 ml kg^{-1} for mice.

Results *Effect of withdrawal from chronic administration of diazepam and CI-988 in the mouse.* Withdrawal from 7 days of diazepam administration decreased the time spent by mice in the illuminated side of the light/dark box in a time related fashion (Figure 1). This withdrawal anxiogenic-like response peaked at 16–24 h into withdrawal and thereafter it slowly decreased reaching control level within 48 h (Figure 1). In addition to the anxiogenesis, a significant decrease in the convulsive dose of PTZ required to induce a clonic seizure was also seen in mice withdrawn from diazepam for 24 h. In contrast, no such withdrawal effects were present in animals pretreated with CI-988 during the 5 days of withdrawal (Figure 1).

Effect of CI-988 on diazepam-induced withdrawal effects in the mouse The effect of CI-988 on diazepam withdrawal behaviours was examined 24 h after the last administration of diazepam (i.e. at the peak withdrawal effect). CI-988 administered 40 min before test, antagonized dose-dependently (0.001–1.0 mg kg^{-1}, s.c.) both the diazepam-induced withdrawal anxiogenesis and the proconvulsant effect (Figure 2).

Discussion Previous animal studies have suggested that changes in anxiety level and seizure threshold are reliable measures of benzodiazepine withdrawal (Emmett-Oglesby *et al.*, 1990; Moreau *et al.*, 1990). This is supported by clinical studies showing similar changes during discontinuation of chronic treatment with benzodiazepines (Marks, 1978). The present data show that in the mouse, discontinuation of diazepam administration for 7 days produced withdrawal anxiogenic-like response and a proconvulsant action in the PTZ infusion model. In contrast, no such withdrawal effects were seen in CI-988 treated animals.

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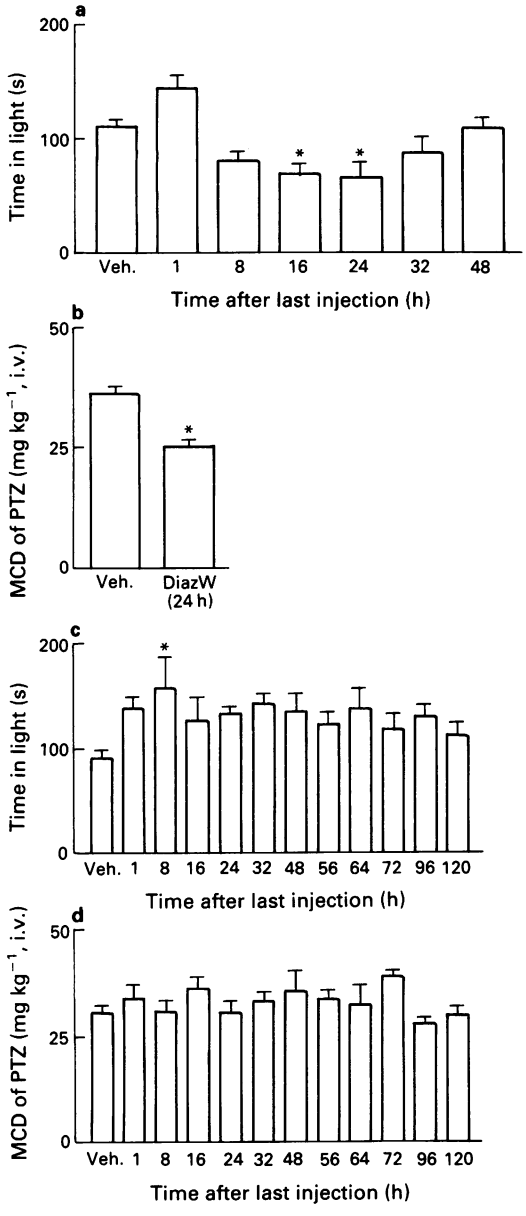


Figure 1 Lack of withdrawal effects following discontinuation of chronic administration of CI-988. Diazepam (a,b), CI-988 (c,d) (both, 1 mg kg^{-1}, i.p.), or vehicle was administered twice daily for 7 days. At various times after the last injection, the mice were tested in the light/dark box and immediately afterwards were infused with pentylenetetrazol (PTZ). Results are shown as the mean time spent in the illuminated side during the 5 min observation period and the mean convulsive dose (MCD) of PTZ (vertical bars represent ± s.e.mean; 10 animals per group). Significant differences from vehicle (Veh.) are shown by asterisks (* $P < 0.05$), determined by Dunnett's t test after significant ANOVA. DiazW (24 h) = 24 h Diazepam withdrawal group.

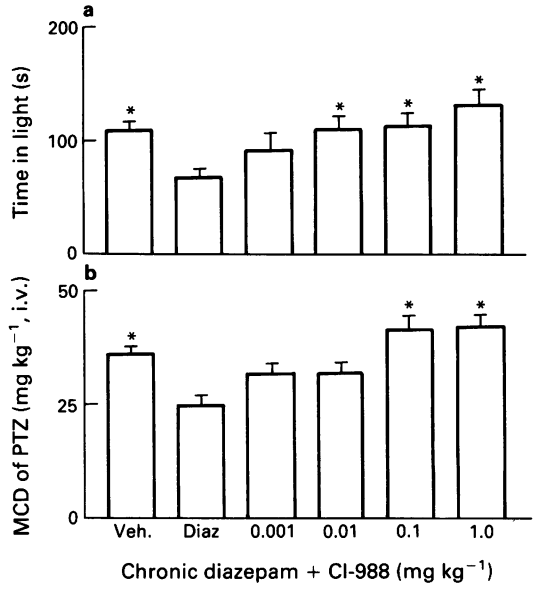


Figure 2 The antagonism of diazepam-induced withdrawal anxiogenic-like (a) and proconvulsant (b) effects by CI-988. Diazepam (Diaz, 1 mg kg^{-1}, i.p.) or vehicle (Veh.) was administered twice daily for 7 days. At 24 h after the last injection mice were tested in the light/dark box and immediately afterwards were infused with pentylenetetrazol (PTZ). CI-988 was administered (s.c.) 40 min before test. Results are shown as the mean time spent in the illuminated side during the 5 min observation period and the mean convulsive dose (MCD) of PTZ (vertical bars represent ± s.e.mean; 10 animals per group). Significant differences from diazepam are shown by asterisks (* $P < 0.05$) ANOVA.

The antagonism of diazepam-induced withdrawal anxiety by CI-988 is consistent with its ability to antagonize benzodiazepine receptor-mediated anxiogenic-like behaviour in animals (Singh *et al.*, 1991b). However, the exact mechanism by which CI-988 antagonizes the diazepam-induced withdrawal proconvulsant effect is unclear. It is not due to a general anticonvulsant activity because CI-988 does not antagonize PTZ-induced seizures (Singh *et al.*, 1991b). It has been reported that chronic benzodiazepine administration induces changes in brain CCK neurotransmission (Bouthillier & De Montigny, 1988; Harro *et al.*, 1990). The possible antagonism of these changes by a CCK receptor antagonist may offer some explanation for the ability of CI-988 to block benzodiazepine-induced withdrawal effects.

Buspirone and ipsapirone are effective anxiolytics which are thought to act via the 5-HT$_{1A}$ receptor (Goa & Ward, 1986). However, in animal studies unlike CI-988 they are unable to antagonize benzodiazepine withdrawal effects and in the clinic benzodiazepine-experienced patients respond poorly to buspirone (Schweizer *et al.*, 1986). Therefore, it is predicted that CI-988 may have an improved anxiolytic profile compared with the currently available treatments and it may also be useful in the treatment of benzodiazepine dependence.

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The role of nitric oxide in endothelial cell damage and its inhibition by glucocorticoids

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Incubation of vascular endothelial cells with *S.typhosa* endotoxin and interferon-γ caused a time- and concentration-dependent reduction in the viability of the cells. This cytotoxic effect was inhibited in a concentration-dependent manner by N^G-monomethyl-L-arginine, an inhibitor of nitric oxide (NO) synthesis, and by the glucocorticoids dexamethasone and hydrocortisone, two inhibitors of the induction of NO synthase. These findings indicate that in these cells the cytotoxic effect of endotoxin is mediated by the NO synthesized by an inducible NO synthase. This induction of NO synthase in vascular endothelial cells may represent a mechanism of local endothelial damage during endotoxin shock and other immunologically based conditions.

Keywords: Endothelium; nitric oxide synthase; cytotoxicity; glucocorticoids; endotoxin

Introduction Vascular endothelial cells synthesize nitric oxide (NO) from L-arginine by the action of a Ca^{2+}-dependent constitutive NO synthase as a transduction mechanism for the activation of the soluble guanylate cyclase in vascular smooth muscle. The consequent rise in guanosine 3′:5′-cyclic monophosphate (cyclic GMP) causes vascular relaxation which contributes to the regulation of blood pressure in animals and man (for review see Moncada *et al.*, 1991).

Nitric oxide also plays a role in the cytotoxic activity of macrophages (for review see Hibbs *et al.*, 1990). These cells synthesize NO by a Ca^{2+}-independent enzyme that is induced following activation by endotoxin (LPS) and/or cytokines (Marletta *et al.*, 1988). Induction of this enzyme also occurs in other cells, including vascular endothelium (Radomski *et al.*, 1990). The vascular endothelial cells are the only cells so far known to possess both the constitutive and inducible NO synthases. The functional consequences of the formation of NO by the inducible NO synthase have not been clearly established, but it is likely that, besides cytotoxicity for microorganisms and tumour cells, it plays a role in pathological vasodilatation and tissue damage (for review see Moncada *et al.*, 1991). The expression, but not the activity, of the inducible NO synthase is inhibited by glucocorticoids such as dexamethasone and hydrocortisone. This action is distinct from that of arginine analogues such as N^G-monomethyl-L-arginine (L-NMMA) which are specific inhibitors of both the constitutive and inducible NO synthases.

Using these two types of inhibitors we have now examined whether endothelial cells are damaged by the NO synthesized as a consequence of the expression of the inducible NO synthase.

Methods Vascular endothelial cells were isolated as described previously (Gryglewski *et al.*, 1986) by collagenase digestion from porcine aortae obtained from a local abattoir. The cells were cultured at 37°C for 48 h in Dulbecco's modification of Eagle's medium (400 μM L-arginine) containing 20% foetal calf serum, penicillin (100 u ml^{-1}) and streptomycin (100 μg ml^{-1}). The cultures were then screened microscopically for microbial contamination, before being subcultured until confluent (72 h) in 6-well plates as described above (approximately 1×10^6 cells per well).

When confluent, the cells were incubated for the appropriate time (generally 24 h) with LPS and/or interferon-γ (IFN-γ) and, where indicated, with test compounds. After incubation, the culture supernatant was removed and replaced with 0.5% trypan blue in phosphate buffered saline and incubation continued for a further 10 min at 37°C. The number of dead cells was then determined, by light microscopy at ×200 magnification, by counting those cells that failed to exclude the dye in each of three representative fields in each of three replicate wells. Statistical significance was determined by Student's paired t test and $P < 0.05$ taken as significant.

Results Incubation of vascular endothelial cells with LPS (10 μg ml^{-1}) or IFN-γ (150 u ml^{-1}) for 24 h caused concentration-dependent cytotoxicity, as measured by exclusion of trypan blue. LPS alone was significantly more effective than IFN-γ (Figure 1a). The maximum effect observed with LPS (100 μg ml^{-1}) was 137.4 ± 9.1 as compared with 52.0 ± 1.9 dead cells per field ($n = 4$ for each) for IFN-γ (1500 u ml^{-1}). These represented 22.9 ± 1.9 and 8.7 ± 0.3% of the total number of cells in each field respectively. Concomitant incubation of the cells with threshold concentrations of these agents for 24 h did not lead to a synergistic interaction; LPS 0.1 μg ml^{-1}, IFN-γ, 15 u ml^{-1} and LPS 0.1 μg ml^{-1} with IFN-γ 15 u ml^{-1} caused 21.8 ± 6.8, 8.8 ± 1.6 and 32.1 ± 4.9 dead cells per field respectively, $n = 3$ for each.

Incubation of the cells with LPS (10 μg ml^{-1}) and IFN-γ (150 u ml^{-1}) caused time-dependent cytotoxicity which was significant at 6 h and reached a maximum at 24 h (Figure 1b). This level was not significantly increased by incubation of the cells for a further 24 h either in the same medium (Figure 1b) or in fresh medium containing LPS (10 μg ml^{-1}) and IFN-γ (150 u ml^{-1}; $n = 3$).

Cytotoxicity in arginine-containing (400 μM) medium was inhibited in a concentration-dependent manner by L-NMMA (Figure 2), but not by D-NMMA (100 μM) or by 10 μM haemoglobin ($n = 3$ for each). Hydrocortisone and dexamethasone also caused concentration-dependent inhibition of cytotoxicity under these conditions (Figure 2). The cytotoxic effect of incubation with LPS (10 μg ml^{-1}) and IFN-γ (150 u ml^{-1}) for 24 h was reduced by 40.8 ± 5% when the cells were cultured in L-arginine-free medium. In contrast, indomethacin (5 μM) significantly potentiated the cytotoxic effect of these concentrations of LPS and IFN-γ from 68.0 ± 6.6 to 129.5 ± 7.9 dead cells/field.

Discussion LPS and IFN-γ, which induce the expression of a Ca^{2+}-independent NO synthase in vascular endothelial cells (Radomski *et al.*, 1990), caused a time-dependent decrease in viability, which became apparent after a lag period of 6 h and was inhibited by L-NMMA but not D-NMMA. These findings indicate that this cytotoxicity is mediated by NO synthesized by an inducible enzyme. A similar effect associated with the induction of NO synthesis has recently been described in

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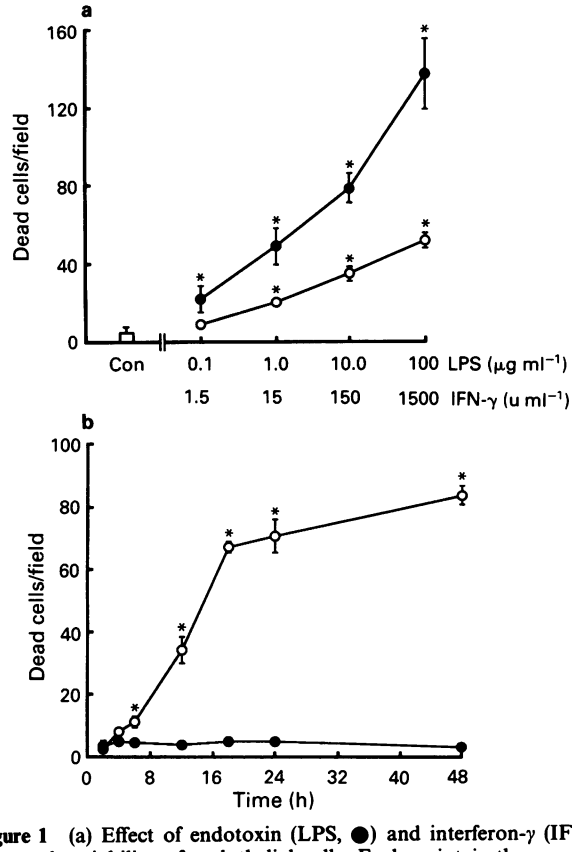


Figure 1 (a) Effect of endotoxin (LPS, ●) and interferon-γ (IFN-γ, ○) on the viability of endothelial cells. Each point is the mean of three or four separate experiments; s.e.mean shown by vertical bars. Con = medium alone. (b) Time-dependent development of cytotoxicity (○) during incubation of endothelial cells with LPS ($10\,\mu g\,ml^{-1}$) and IFN-γ ($150\,u\,ml^{-1}$); (●) medium alone. * $P < 0.05$ compared to corresponding time control.

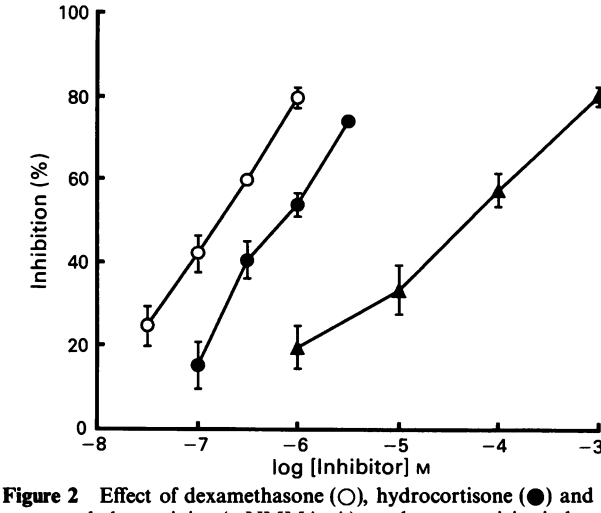


Figure 2 Effect of dexamethasone (○), hydrocortisone (●) and N^G-monomethyl-L-arginine (L-NMMA, ▲) on the cytotoxicity induced in endothelial cells by endotoxin (LPS, $10\,\mu g\,ml^{-1}$) and interferon-γ (IFN-γ, $150\,u\,ml^{-1}$). Each point is the mean of four separate experiments; vertical bars show s.e.mean.

EMT-6 cells (O'Connor & Moncada, 1991). Interestingly, the cytotoxic effect of NO was not affected by haemoglobin, suggesting that the NO synthesized exerts its cytotoxic effect directly without being released into the extracellular space.

The development of this NO-mediated cytotoxicity was also inhibited by the glucocorticoids dexamethasone and hydrocortisone, which prevent the induction of NO synthase in several tissues *in vitro* and *in vivo* (see Moncada *et al.*, 1991). In addition, the cytotoxic effect of LPS and IFN-γ was attenuated in the absence of L-arginine. These findings provide further evidence for an NO-dependent cytotoxicity in endothelial cells. Since cytotoxicity was only partially reduced in the absence of L-arginine, it is likely that endothelial cells contain sufficient L-arginine to synthesize substantial amounts of NO or that they are able to synthesize L-arginine from other cellular components (Hecker *et al.*, 1990).

The cytotoxic effect of LPS and IFN-γ reached a maximum at 24 h and affected less than 25% of the cells. There was no additional increase in cytotoxicity following a further 24 h incubation in fresh medium containing these agents. The reason for this limited cytotoxicity requires further investigation.

The cytotoxic effect of LPS and IFN-γ was significantly enhanced by concomitant incubation with indomethacin. The mechanism by which eicosanoid synthesis affects NO-mediated cytotoxicity is not known but it may either be due to a cytoprotective effect of prostacyclin (Blackwell *et al.*, 1982) or to a down-regulation of the induction of NO synthase. Interestingly cyclo-oxygenase has recently been reported to be induced in endothelial cells by LPS and this induction is inhibited by glucocorticoids (Masferrer *et al.*, 1990). This suggests that during immunological activation these cells are able to release cytoprotective and cytotoxic substances, the balance of which may determine the final outcome in terms of host tissue viability.

Our finding that endothelial cells are damaged as a consequence of the induction of NO synthase provides an explanation for the local endothelial damage that can occur in immunologically-based conditions. That the vascular endothelium releases NO constitutively for physiological purposes and also expresses NO synthesis as part of the host defence mechanism exemplifies the diverse roles of NO as a biologically active molecule.

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Non-competitive inhibition of $GABA_A$ responses by a new class of quinolones and non-steroidal anti-inflammatories in dissociated frog sensory neurones

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1 The interaction of a new class of quinolone antimicrobials (new quinolones) and non-steroidal anti-inflammatory agents (NSAIDs) with the $GABA_A$ receptor-Cl^- channel complex was investigated in frog sensory neurones by use of the internal perfusion and 'concentration clamp' techniques.

2 The new quinolones and the NSAIDs (both 10^{-6}–10^{-5} M) had little effect on the GABA-induced chloride current (I_{Cl}) when applied separately. At a concentration of 10^{-4} M the new quinolones, and to a lesser degree the NSAIDs, produced some suppression of the GABA response.

3 The co-administration of new quinolones and some NSAIDs (10^{-6}–10^{-14} M) resulted in a marked suppression of the GABA response. The size of this inhibition was dependent on the concentration of either the new quinolone or the NSAID tested. The inhibitory potency of new quinolones in combination with 4-biphenylacetic acid (BPAA) was in rank order norfloxacin (NFLX) $\gg$ enoxacin (ENX) > ciprofloxancin (CPFX) $\gg$ ofloxacin (OFLX), and that of NSAIDs in combination with ENX was BPAA $\gg$ indomethacin = ketoprofen > naproxen > ibuprofen > pranoprofen. Diclofenac, piroxicam and acetaminophen did not affect GABA responses in the presence of ENX.

4 In the presence of ENX or BPAA, there was a small shift to the right of the concentration-response curve for GABA without any effect on the maximum response. However, the co-administration of these drugs suppressed the maximum of the GABA concentration-response curve, indicating a non-competitive inhibition, for which no voltage-dependency was observed.

5 Simultaneous administration of ENX and BPAA also suppressed pentobarbitone (PB)-gated I_{Cl}. On the other hand, both PB and phenobarbitone reversed the inhibition of GABA-induced I_{Cl} by co-administration of ENX and BPAA.

6 The effect on $GABA_A$ responses of co-administration of new quinolones and NSAIDs was not via an interaction with benzodiazepine receptors coupled to the $GABA_A$ receptor, since this effect was not reversed by Ro15-1788 or diazepam.

7 It is concluded that the co-administration of new quinolones and some of the NSAIDs inhibit GABAergic transmission, and could result in convulsions.

Keywords: Frog dorsal root ganglia; γ-aminobutyric acid (GABA); new quinolones; non-steroidal anti-inflammatory drugs; $GABA_A$ receptor

Introduction

Quinolone antimicrobials can be divided into at least two types, old and new. The new quinolones are extremely useful in the oral treatment of urinary, biliary, intestinal and pulmonary tract infections because of the following advantages: a wide antimicrobial spectrum (including Gram positive and negative), few serious adverse reactions, low mutagenecity, resistance to metabolism and a large distribution volume (Ball, 1989; Bergeron, 1989; Nicolle, 1989; Toma, 1989; Vellend, 1989). Recently, however, serious convulsions have been reported to occur in patients co-treated with new quinolone antimicrobials and with fenbufen, a non-steroidal anti-inflammatory drug (NSAID). Hence, the Japanese government has warned against the joint use of these drugs (Ministry of Health and Welfare, Japan, 1986). Since there is no effective clinical therapy against the convulsions induced by co-administration of these drugs, it is important to clarify the mechanism underlying these convulsions.

New quinolones inhibit the specific binding of either [^{3}H]-γ-aminobutyric acid ([^{3}H]-GABA) or [^{3}H]-muscimol to synaptic plasma membranes from rat brain, in a concentration-dependent manner (Hori *et al.*, 1985; Segev *et al.*, 1988; Tsuji *et al.*, 1988a). New quinolones also reverse the inhibitory effect of GABA on the binding of [^{35}S]-butylbicyclophosphorothionate (TBPS) to rat brain membranes (Squires & Saederup, 1984). Moreover, the GABA-induced current in rat dorsal root ganglia (DRG) neurones is inhibited by co-administration of ciprofloxacin (CPFX), a new quinolone, and 4-biphenylacetic acid (BPAA), a metabolite of fenbufen (Halliwell *et al.*, 1991). However, both barbiturates and benzodiazepines do not inhibit the convulsions induced by new quinolones and fenbufen (Murayama *et al.*, 1987). Therefore, the mechanism underlying quinolone-induced convulsions remains to be elucidated.

For the last eight years we have carried out physiological and pharmacological studies on the response mediated by $GABA_A$ receptors using single neurones dissociated from frog DRG and the 'concentration-clamp' technique. This experimental system has been found to be useful for evaluating the effects of drugs acting on the $GABA_A$ receptor-Cl^- channel complex (Hattori *et al.*, 1984; 1986; Akaike *et al.*, 1985; 1986; 1987a,b,c; Inoue *et al.*, 1986). The aim of the present study was to examine whether new quinolones in the presence of NSAIDs modulate the $GABA_A$ receptor-Cl^- channel complex in frog sensory neurones.

Methods

The experimental methods were similar to those described previously (Hattori *et al.*, 1984; Ishizuka *et al.*, 1984; Akaike

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et al., 1986). In brief, DRG of bull-frog (*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 with 95% O_2 and 5% CO_2. Thereafter, single neurones were mechanically dissociated from the digested ganglia with fine pins under a binocular microscope. Dissociated neurones were maintained in a solution which contained equal parts of Eagle's minimum essential medium (Nissui, Tokyo) and normal Ringer solution at a room temperature of 22 to 25°C. Dissociated neuronal cell bodies were perfused internally and externally with respective test solutions for recording the Cl^- current (I_{Cl}) by a suction-pipette technique (Ishizuka *et al.*, 1984). The external and internal perfusion solution were composed as follows; external solution (in mM): Tris-Cl 83, CsCl 2, $MgCl_2$ 5, TEA-Cl 25, glucose 5, HEPES 10. Internal solution: CsCl 95, Cs-aspartate 10, TEA-Cl 25, $CaCl_2$ 0.1, HEPES 10, EGTA 2.5. The inclusion of EGTA at this concentration results in a calculated free Ca^{2+} concentration of 10^{-8} M. The pHs of external and internal solutions were adjusted to 7.4 and 7.2 with Tris-OH, respectively. Neurones were voltage clamped at a holding membrane potential (V_H) of −20 mV with a single electrode voltage-clamp system (Ishizuka *et al.*, 1984). Drugs were applied by 'concentration-clamp' technique (see Figure 1 of Akaike *et al.*, 1986).

Enoxacin (ENX), ofloxacin (OFLX), acetaminophen, ibuprofen, ketoprofen, pranoprofen, diclofenac, piroxicam, indomethacin, naproxen and Ro15-1788 were at first dissolved in dimethyl sulphoxide (DMSO) at 5×10^{-2} M, and then the drugs were diluted with external solution. The final concentration of DMSO never exceeded 0.2%. Norfloxacin (NFLX) and ciprofloxacin (CPFX) were directly dissolved in external solution. BPAA was first dissolved in ethanol at 10^{-1} M, and then diluted with external solution. The final concentration of ethanol never exceeded 0.1%. These maximum concentrations of DMSO and ethanol did not affect GABA and pentobarbitone-induced responses. New quinolones, NSAIDs and diazepam were produced by Yoshitomi Pharmaceutical Industries Co., Ltd. Pentobarbitone and phenobarbitone were obtained from Tokyo-kasei, and Ro 15-1788 was provided by Dr Scherschlicht (F. Hoffman—La Roche & Co., Ltd., Basel, Switzerland).

Results

The GABA-induced current was rapidly activated and then subsequently desensitized towards a steady state level during continuous application. The desensitization became more rapid when the concentration of GABA was increased. The threshold concentration, K_a (apparent association constant) and Hill coefficient of the GABA concentration-response curve were 10^{-6} M, 1.3×10^{-5} M and 1.8, respectively (Figure 5a). Hence, 10^{-5} M GABA was used as a test response in these experiments. The reversal potential of the GABA-induced current (E_{GABA}) was $+6 \pm 0.5$ mV (mean ± s.e.mean). The value was close to the Cl^- equilibrium potential (E_{Cl}) of +4 mV calculated from the Nernst equation, using the external and internal Cl^- concentration (Figure 6). The results clearly show that there is adequate internal perfusion and purity of the Cl^- current (I_{Cl}).

Inhibitory interaction of new quinolones and anti-inflammatory drugs on $GABA_A$ receptor-Cl^- channel complex

Pretreatment of DRG neurones with new quinolones and NSAIDs for approximately 5 s was sufficient to produce a steady-state inhibition of the GABA response. In the following experiments, therefore, the preparations were pretreated with new quinolones and NSAIDs for 10 to 20 s. Figure 1a shows the effects of new quinolones (10^{-6}–10^{-4} M) on the peak component of I_{Cl} induced by 10^{-5} M GABA. New-quinolones only inhibited the GABA-induced I_{Cl} at a concentration of 10^{-4} M. The rank order for the inhibitory potency of new quinolones was norfloxacin (NFLX) ≫ ciprofloxacin (CPFX) > enoxacin (ENX); ofloxacin (OFLX) was inactive. On the other hand, NSAIDs (10^{-6}–10^{-4} M) had little inhibitory action on GABA responses even at a concentration of 10^{-4} M where the most potent compounds, indomethacin and BPAA, produced less than 25% inhibition (Figure 1b).

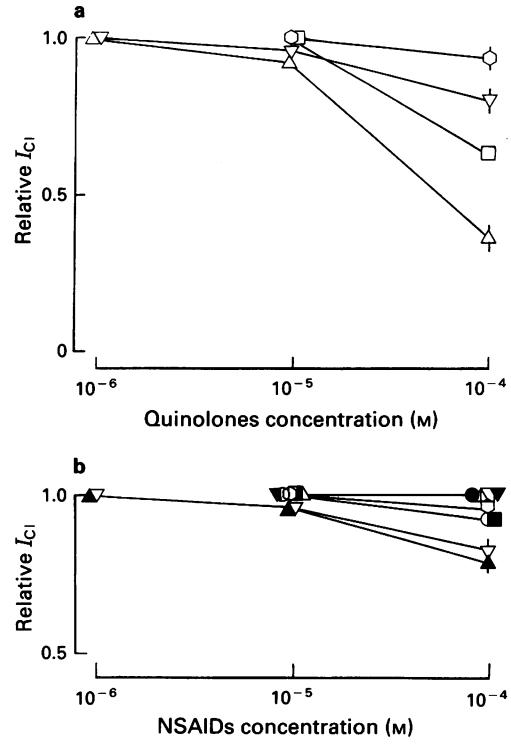


Figure 1 Effects of new quinolone antimicrobials and non-steroidal anti-inflammatories (NSAIDs) on 10^{-5} M GABA-induced I_{Cl}. V_H was −20 mV. (a) Effects of quinolones on the GABA response. Y-axis indicates the peak current amplitude after adding one of drugs, measured relative to the control. Data are mean values obtained in 4 experiments and bars showing ± s.e.mean. Norfloxacin (NFLX) (△) had substantial inhibitory effect on GABA response at 10^{-4} M, but ofloxacin (OFLX) (○) had almost no effect. (▽) Enoxacin; (□) ciprofloxacin (CPFX). (b) Effects of NSAIDs (△, diclofenac-Na; ▲, indomethacin; ▽, BPAA; ▼, ibuprofen; ○, ketoprofen; ●, acetaminophen; □, pranoprofen; ■, (+)-naproxen; ⬡, piroxicam) on the GABA response.

It has been reported that convulsions occur when patients are co-treated with ENX and fenbufen. The inhibition of [^{3}H]-GABA binding to plasma membrane from rat brain by new quinolones is potentiated by addition of fenbufen (Hori *et al.*, 1987; Tsuji *et al.*, 1988b). Therefore, we investigated the effect of the simultaneous administration of one new quinolone (ENX) and the fenbufen metabolite BPAA on the 10^{-5} M GABA-induced I_{Cl}. As shown in Figure 2, the GABA response was not affected by adding ENX (10^{-6}–10^{-5} M) or BPAA (10^{-5} M), a concentration similar to the plasma concentration in patients treated with fenbufen (Chiccarelli *et al.*, 1980). However, after pretreatment with both ENX and BPAA for 10 s, repeated administration of the two drugs and GABA dramatically suppressed the GABA-induced I_{Cl}. The inhibitory effect was completely reversible. The results are summarized in Figure 3a. The inhibitory potency was in the rank order NFLX > ENX > CPFX, the concentrations causing 50% inhibition (IC_{50}) being 2.2×10^{-7} M, 8.8×10^{-7} M and 2.2×10^{-6} M, respectively. OFLX had little inhibitory effect on the GABA response in the presence of 10^{-5} M BPAA.

To determine whether the inhibitory effect of new quinolones applied in combination with BPAA on GABA-induced

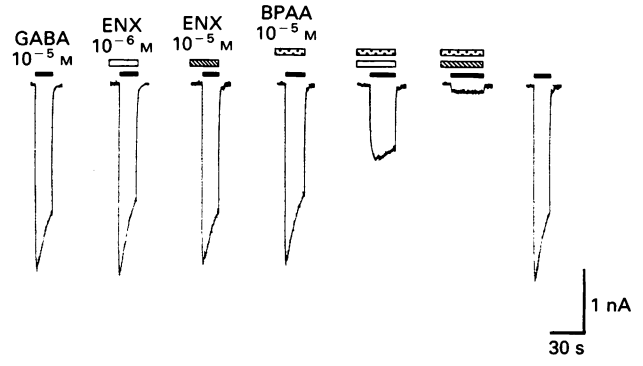


Figure 2 Inhibition of GABA-induced I_{Cl} by co-administration of enoxacin (ENX) and 4-biphenylacetic acid (BPAA) and the lack of inhibition when applied separately. V_H was −20 mV. Drugs were continuously applied for the period shown by the bars above each response. All data were obtained from the same cell.

I_{Cl} is specific to BPAA, the effects of various NSAIDs on the GABA response were examined in the presence of 10^{-5} M ENX. The results are summarized in Figure 3b. BPAA was the most potent inhibitor of the GABA response, followed by indomethacin and naproxen which were about 30 times weaker than BPAA. The inhibitory potency was in the rank order (with IC_{50} values in parentheses): BPAA (8×10^{-7} M) ≫ indomethacin (2.1×10^{-5} M) > ketoprofen (3.5×10^{-5} M) > naproxen (> 10^{-4} M) > ibuprofen (> 10^{-4} M) > pranoprofen (> 10^{-4} M). Diclofenac, piroxicam and acetaminophen had little inhibitory effect on the GABA response. The dose-effect curve for the inhibition of the GABA response by ENX in the presence of BPAA was shifted to the left with increasing BPAA concentrations (Figure 4).

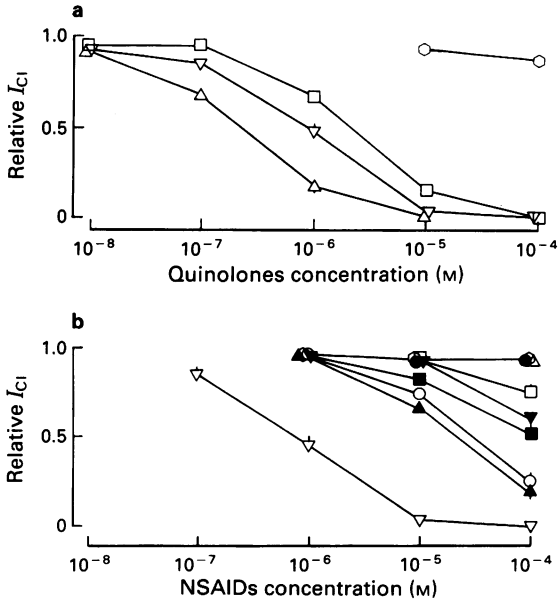


Figure 3 Effects of co-administration of new quinolones and NSAIDs on 10^{-5} M GABA-induced I_{Cl}. V_H was −20 mV. (a) The augmentation of quinolone-induced inhibition of the GABA response in the presence of 10^{-5} M 4-biphenylacetic acid (BPAA). Each point is the average of 4 neurones. NFLX (△), ENX (▽) and CPFX (□) showed marked inhibition at the therapeutic dosages. ○, OFLX. (b) The inhibition of 10^{-5} M GABA responses by the application of 10^{-5} M ENX and various NSAIDs (△, diclofenac-Na; ▲, indomethacin; ▽, BPAA; ▼, ibuprofen; ○, ketoprofen; ●, acetaminophen; □, pranoprofen; ■, (+)-naproxen; ⬡, piroxicam). BPAA in combination with ENX showed the most potent inhibition of the GABA response. Each point is the average of 4 neurones. Abbreviations as in Figure 1.

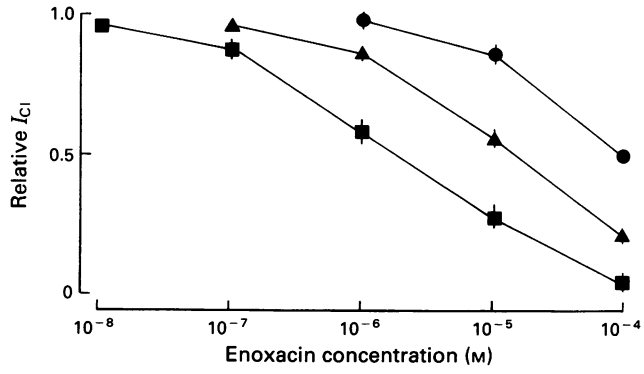


Figure 4 Parallel shifts of the GABA inhibition curves of enoxacin in the presence of 4-biphenylacetic acid (●, 10^{-6} M; ▲, 10^{-5} M, ■, 10^{-4} M). Each point is the average result from 4 neurones.

Mechanism of GABA inhibition produced by co-administration of new quinolones and NSAIDs

The effects of 10^{-4} M ENX and 10^{-6} M BPAA on the concentration-response relationship for GABA were investigated. In the presence of either ENX or BPAA, there was a small parallel shift to the right of the GABA concentration-response curve without any effect on the maximum response (Figure 5a). However, the simultaneous administration of ENX and BPAA caused both an increase in the threshold response concentration and a suppression of the maximum response (Figure 5b), indicating a non-competitive inhibition. The values of half-maximum concentration (EC_{50}) estimated from the concentration-response curves were 1.3×10^{-5} M for control, and 2.5×10^{-5} and 5×10^{-5} M after exposure to

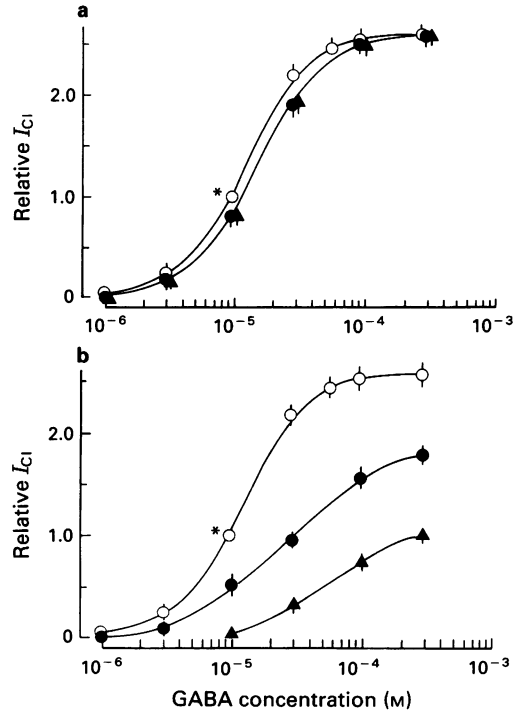


Figure 5 Concentration-response relationships for GABA-induced I_{Cl} in the absence and presence of enoxacin (ENX) and 4-biphenylacetic acid (BPAA). All responses were normalized to the peak current (*) induced by 10^{-5} M GABA alone. (a) Parallel shift of the GABA concentration-response curve in the presence of either 10^{-4} M ENX (●) or 10^{-4} M BPAA (▲); (○) control. (b) Concentration-response curves of GABA response in the presence of both ENX (●, 10^{-6} M; ▲, 10^{-5} M) and 10^{-5} M BPAA; (○) control. Each point gives mean from 4 neurones; s.e.mean shown by vertical lines.

10^{-6} and 10^{-5} M ENX both in the presence of 10^{-5} M BPAA, respectively. When current amplitudes were normalized to the peak amplitude induced by 10^{-5} M GABA alone, the maximum response changed from 2.6 for control to 1.8 and 1.0 after exposure to 10^{-6} and 10^{-5} M ENX respectively, both in the presence of BPAA.

From the results shown in Figure 5 it was expected that the inhibitory action of the new quinolones in combination with BPAA might show a voltage-dependency in the current-voltage (*I-V*) relationship of the GABA response. Thus, the inhibitory action of ENX in the presence of BPAA was examined in neurones held at various V_Hs. The *I-V* relationships for the GABA response obtained in the presence and in the absence of ENX and BPAA showed that the GABA-induced peak I_{Cl} was depressed by about 60% at every V_H (Figure 6). The result indicates that the blockade of the GABA response with ENX and BPAA was voltage-independent. In addition, co-administration of ENX and BPAA did not shift E_{GABA}.

Interaction of enoxacin and 4-biphenylacetic acid on the barbiturate and benzodiazepine receptors

Biochemical, neurochemical and electrophysiological studies have revealed that the $GABA_A$ receptor-Cl^- channel complex is coupled with two drug binding sites, the barbiturate and the benzodiazepine receptors (Olsen & Leeb-Lundberg, 1981), and that barbiturates and benzodiazepine derivatives potentiate the $GABA_A$ receptor-induced I_{Cl} acting via their respective receptors (MacDonald & Barker, 1978; Placheta & Karobath, 1980; Alger & Nicoll, 1982; Higashi & Nishi, 1982; Chan *et al.*, 1983; Akaike *et al.*, 1985; Hattori *et al.*, 1986). In addition, pentobarbitone (PB) itself activates the Cl^- current at concentrations above 3×10^{-5} M via the barbiturate receptor (Barker & Ransom, 1978; Nicoll & Wojtowicz, 1980; Akaike *et al.*, 1987b).

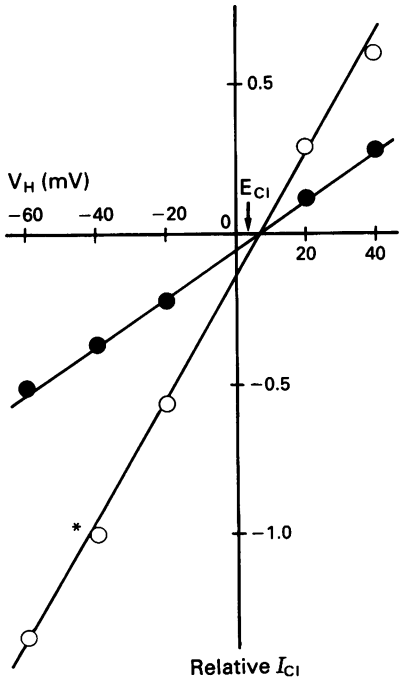


Figure 6 Effect of simultaneous application of enoxacin (ENX) and 4-biphenylacetic acid (BPAA) on current-voltage (*I-V*) relationship of the GABA response. All points were normalized to the peak response (*) induced by 10^{-5} M GABA alone at a V_H of −40 mV. (○) Control; (●) in the presence of 10^{-5} M ENX and 10^{-5} M BPAA. Similar results were obtained from another two neurones. V_H, holding potential. E_{Cl}, Cl^- equilibrium potential.

Figure 7a shows the concentration-response relationship for PB in which the blockade of the Cl^- channel by high concentrations of PB is responsible for the bell-shaped concentration-response curve (Akaike *et al.*, 1987b). In the presence of 10^{-5} M BPAA and 10^{-6} M ENX the PB-induced response was reversibly inhibited, with a suppression of the maximum of the concentration-response curve. When current amplitudes were normalized to the peak amplitude induced by 10^{-5} M GABA, maximum responses were 1.02 for PB control concentration-response curve and 0.86 after adding 10^{-5} M ENX in the presence of 10^{-5} M BPAA. On the other hand, the addition of barbiturates (PB and phenobarbitone) reversed the suppression of the GABA concentration-response curve by co-administration of ENX and BPAA (Figure 7b). In the presence of both ENX and BPAA, the maximum response and K_a for the GABA concentration-response curve were 1.0 and 5.2×10^{-5} M. After additional exposure to either 3×10^{-4} M phenobarbitone or 10^{-4} M PB these values were 1.43 and 4.2×10^{-5} M, and 2.03 and 2.5×10^{-5} M, respectively.

As shown in Figure 8a, a benzodiazepine antagonist, Ro15-1788(10^{-6} M), did not reverse the inhibition of the GABA response produced by simultaneous administration of ENX (10^{-6} M) and BPAA (10^{-5} M), indicating that ENX in the presence of BPAA does not act on the benzodiazepine receptor. Moreover, the addition of diazepam (3×10^{-7} to 3×10^{-6} M) did not prevent the inhibition of the GABA response induced by ENX and BPAA (Figure 8b).

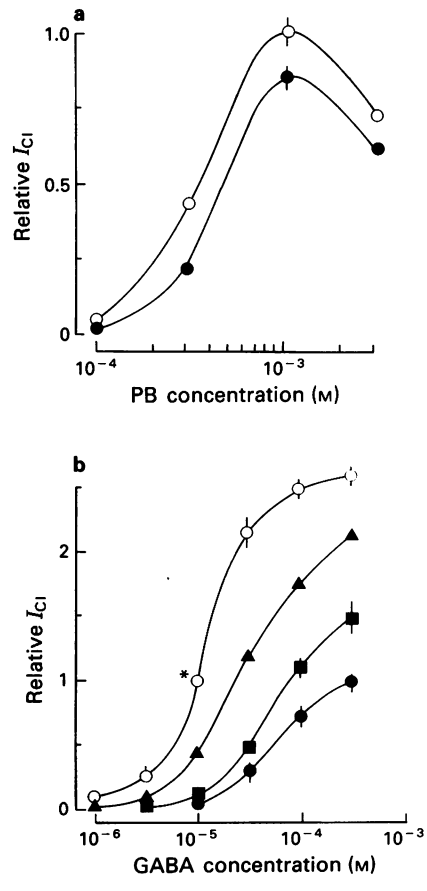


Figure 7 (a) Concentration-response curves for pentobarbitone (PB)-induced I_{Cl} in the presence (●) and absence (○) of 10^{-6} M enoxacin (ENX) and 10^{-5} M 4-biphenylacetic acid (BPAA). V_H was −20 mV. All points were normalized to the peak current induced by 10^{-5} M GABA. Data were obtained from 4 cells. (b) Effect of barbiturate on the inhibition of the GABA-induced I_{Cl} by ENX in combination with BPAA. V_H was −20 mV. All responses were normalized to the peak response (*) induced by 10^{-5} M GABA alone. Both 10^{-4} M PB (▲) and 3×10^{-4} M phenobarbitone (■) reversed the inhibition of GABA response by 10^{-5} M ENX in combination with 10^{-5} M BPAA (●); (○) control. Each point presents the average value from 4 neurones.

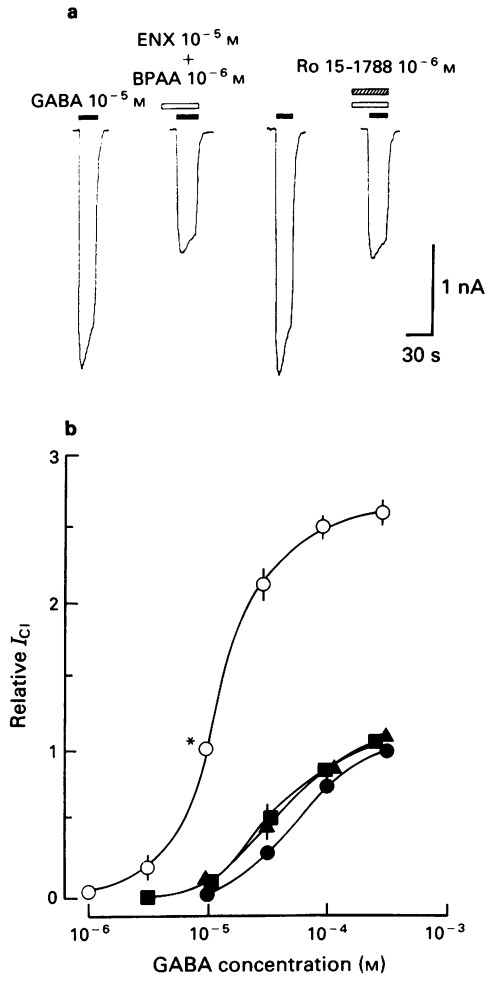


Figure 8 The lack of effect of a benzodiazepine agonist and antagonist on the GABA response suppressed by enoxacin (ENX) and 4-biphenylacetic acid (BPAA). V_H was −20mV. (a) Effects of a benzodiazepine antagonist (Ro15-1788). Similar results were obtained in another 2 neurones. (b) Diazepam (■, 3×10^{-7}; ▲, 3×10^{-6} M) did not affect the inhibition of the GABA response by 10^{-5} M ENX and 10^{-5} M BPAA (●); (○) control. All responses were normalized to the peak response (*) induced by 10^{-5} M GABA alone. Data are mean value obtained in 4 experiments. The vertical lines are s.e.mean.

Discussion

The results from this study indicate that when either the new quinolones or the NSAIDs are applied alone, they produce only a moderate or little effect on $GABA_A$ responses. However, the co-application of new quinolones with NSAIDs results in a pronounced enhancement of inhibitory potency compared with their combined individual effects. The rank order of potency for the new quinolones in the presence of the NSAID, BPAA, is consistent with that observed for the inhibitory effect of the new quinolones in the presence of the NSAID, fenbufen, on [^{3}H]-GABA binding (Tsuji *et al.*, 1988b). The inhibitory effect of ENX and BPAA on GABA-induced I_{Cl} is not antagonized by added the benzodiazepine antagonist, Ro15-1788 (10^{-6} M). Since this concentration of Ro15-1788 is almost maximally effective in blocking benzodiazepine receptors (Sigel & Baur, 1988; Yakushiji *et al.*, 1989), our results suggest that the new quinolones in combination with BPAA do not act as inverse agonists at the benzodiazepine receptor. This observation is consistent with binding experiments where the specific binding of [^{3}H]-diazepam to the $GABA_A$ receptor is inhibited by extremely high concentrations of ENX (10^{-4} to 10^{-3} M) beyond the normal therapeutic plasma levels (Yamamoto *et al.*, 1988; Dodd *et al.*, 1989). The lack of a specific antagonist for the barbiturate site on the $GABA_A$ receptor-complex limits further conclusions as to the site of action of new quinolones and NSAIDs.

As the chemical structure of new quinolones is similar to that of theophylline and caffeine, there is a possibility that new quinolones induce convulsions by the blockade of adenosine-mediated inhibitory neurotransmission. Indeed, new quinolones inhibit the specific binding of adenosine receptor agonists. However the inhibitory effects of new quinolones on adenosine ligand binding are generally observed at a level higher than the normal therapeutic plasma concentrations (Dodd *et al.*, 1989). Thus, the adverse CNS reactions of quinolone antimicrobials are likely to be due to a predominant interaction with the $GABA_A$ rather than the adenosine receptors.

Clinically, patients with no evidence of epilepsy or other underlying cerebral dysfunction have experienced clonic or acute convulsions when given normal therapeutic doses of the three new quinolones, NFLX, ENX or CPFX administered simultaneously with fenbufen. As a result, the Ministry of Health and Welfare, Japan (1989) has required doctors to proceed with caution when prescribing fenbufen or ketoprofen during new quinolone therapy. The results in our study indicate that fenbufen (or its metabolite) could augment the neurotoxic potency of the new quinolones via a pharmacological interaction with the $GABA_A$ receptor. Bicuculline, a specific antagonist of $GABA_A$ receptor and a convulsant, inhibits the GABA response in dissociated frog DRG neurone with an IC_{50} of 2×10^{-6} M (Yakushiji *et al.*, 1987). Using a concentration of BPAA similar to that found in the serum of patients treated with fenbufen, the IC_{50} values of new quinolones are 2.2×10^{-7} M for NFLX, 8.8×10^{-7} M for ENX, and 2.2×10^{-6} M for CPFX, indicating that the blockade of the GABA response by simultaneous administration of BPAA and NFLX, ENX or CPFX is greater than or equivalent to that produced by bicuculline. The observed reversal by barbiturates of the inhibition of $GABA_A$ responses by the co-administration of new quinolones and NSAIDs suggests that the barbiturates may prove useful clinically in protecting against the convulsive effect of joint new quinolone/NSAID therapy, if mediated via the $GABA_A$ receptor-complex. However, when intracellular ATP is reduced, $GABA_A$ receptor gated I_{Cl} shows a run-down (Chen *et al.*, 1990; Shirasaki *et al.*, 1992). Therefore, the protective effects of barbiturates on convulsion induced by new quinolones and NSAIDs may be limited to immediately after the convulsion. In addition, the present experiments suggest that this experimental approach of measuring GABA-induced I_{Cl} in frog DRG neurones is useful for evaluating the convulsant action of co-administered new quinolones and NSAIDs.

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Effect of murine recombinant interleukin-5 on the cell population in guinea-pig airways

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1 An intratracheal injection of murine recombinant interleukin 5 (mrIL-5, 2–15 μg/0.25 ml/animal) induced a dose-dependent increase in the number of macrophages, eosinophils, neutrophils and epithelial cells in the bronchoalveolar lavage fluid (BALF) of guinea-pigs 24 h after administration. Bovine serum albumin (15 μg/0.25 ml/animal), used as a reference material, did not cause any change of this type.

2 The intratracheal administration of mrIL-5 at a dose of 15 μg showed a tendency to increase the number of these pulmonary inflammatory cells and epithelial cells in the BALF at 12 h with a significant increase observed at 24 h.

3 Prednisolone (20 mg kg^{-1}, i.p.) inhibited the mrIL-5-induced increase in macrophages, eosinophils, neutrophils and epithelial cells. Ketotifen (2 mg kg^{-1}, i.p.) reduced the mrIL-5-induced increase in the eosinophil, neutrophil and epithelial cell populations. The simultaneous injection of 2% disodium cromoglycate (DSCG) into the trachea prevented the mrIL-5-induced increase in the number of airway epithelial cells, without affecting changes in the other inflammatory leukocytes.

4 These results suggest that mrIL-5 is a potent inducer of lung inflammation, in terms of increased inflammatory leukocytes and epithelial cells in guinea-pig BALF. Prednisolone, DSCG and ketotifen are effective against mrIL-5-induced pulmonary inflammation, especially the desquamation of bronchial epithelial cells.

Keywords: Interleukin-5 (IL-5); airway cell populations; anti-asthmatic drugs; airway inflammation

Introduction

Airway inflammation is an important component in the pathogenesis of bronchial asthma (Barnes, 1989; Dolovich *et al.*, 1989). The incidence of inflammatory cells, such as neutrophils and eosinophils, increases in the bronchoalveolar lavage fluid (BALF) in the late phase airway response of man and animals (Barnes, 1989; Frew & Kay, 1990). Chemotactic factors, such as platelet activating factor (PAF), leukotriene B_4 (LTB_4) and complement fragments, which are thought to contribute to these events, lead to the induction of nonspecific airway hyperreactivity (Barnes, 1989). However, airway epithelial cells also play an important part in bronchial asthma, because the desquamation of these cells enhances stimulated contraction of the trachea in man and animals (Vanhoutte, 1989). Moreover, the number of airway epithelial cells present in the BALF of guinea-pigs with airway hyperreactivity induced by repeated antigen provocation, is more than in normal animals (Motojima *et al.*, 1989). Creola bodies, which are agglomerations of bronchial epithelial cells, are found in the BALF of asthma patients (Frigas & Gleich, 1986). Recently, interleukin 5 (IL-5), secreted from T lymphocytes, has been identified as a T cell-replacing factor (Takatsu *et al.*, 1980) or B cell growth factor II (Swain, 1985). This cytokine also acts as an eosinophil activating factor (Clutterbuck & Sanderson, 1988; Lopez *et al.*, 1988) and is thought to be closely related to the control of immune responses and inflammatory reactions (Frew & Kay, 1990; Szelenyi, 1991). However, the role of IL-5 in pulmonary inflammation has not yet been fully elucidated. In this study, we have found that intratracheal administration of mrIL-5 induces an increase in the number of inflammatory leukocytes and epithelial cells in guinea-pig BALF. We also investigated the effect of various drugs, used in the treatment of human bronchial asthma, on this bronchopulmonary inflammation.

Methods

Male Hartley guinea-pigs weighing 300–400 g (Nihon SLC, Japan) were used.

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Administration of mrIL-5

Guinea-pigs were anaesthetized by an initial injection of pentobarbitone sodium (20 mg kg^{-1}, i.p.) followed, where necessary (in about 80 to 95% of the animals), by additional doses of the drug (in increments of 10 mg kg^{-1} up to a cumulative maximum of 70 mg kg^{-1}, i.p.) until the animal could no longer experience pain as evidenced by a cry or shudder. After the animals were sedated and restrained, the trachea was then surgically exposed and 0.25 ml sterile mrIL-5, bovine serum albumin (BSA) or 0.9% NaCl solution was injected into the trachea with a 26-G syringe. The wound was closed with sterile stitches. The lungs were lavaged 5, 12 and 24 h after the mrIL-5 challenge. In order to study the dose-dependency and the effect of drugs, the number of each cell type in the BALF was determind 24 h after mrIL-5 treatment. In the preliminary study, inhalation of human recombinant interleukin 5 (hrIL-5) (50 μg over 30 min) and an intraperitoneal injection of mrIL-5 (20 μg/kg/day for two days) did not cause a significant change in the number and population of cells in the BALF after 24 h (unpublished observations). The aerosol (spray volume 1 ml min^{-1}, mass particle diameter distribution 2.0–6.0 μm) was generated by an TUR-3200 ultrasonic nebulizer (Nihon Koden, Japan). We thus tried to investigate the effect of locally injected mrIL-5 on pulmonary inflammation in guinea-pigs.

Broncheolar lavage fluid study

Guinea-pigs were killed by the intraperitoneal injection of sodium pentobarbitone (150 mg kg^{-1}). The trachea was cannulated and the airway lumen was washed with two aliquots (5 ml) of saline containing 0.1% BSA warmed to 37°C. Typically, more than 80% of the fluid was recovered in an individual animal. In the control group, the mean percentage of recovery of fluid was 85 ± 0.1% and this was unaffected by treatment with the test drugs. Lavaged fluid from each animal was pooled in a plastic tube cooled in ice and centrifuged (150 *g*) at 4°C for 10 min. The cell pellet was resuspended in 2 ml saline containing 0.1% BSA. Fifty μl of cell suspension was added to 450 μl Turk's solution (1:2 dilution with saline) to count the total leukocyte numbers under microscopy

(× 500). In order to count differentiated cell types, the cell suspension was then diluted with saline to give a final concentration to 5×10^5 cells ml^{-1}, and then centrifuged at 400 r.p.m. for 4 min at room temperature in a Cytospin II (Shandon Southern Instruments, Sewickley, PA, U.S.A.). Thereafter the cytocentrifuged preparations were stained with May-Gruenwald and Giemsa dye solution. A total of approximately 500 differentiated leukocytes (macrophages, eosinophils, neutrophils and lymphocytes) were counted and the proportion of each cell type was calculated as a percentage of the total. The number of each leukocyte was calculated from the total leukocytes in the BALF and the percentage of the individual cell type in the smear. Simultaneously, the number of epithelial cells in the smear was also counted. Results are expressed as the total number of cells recovered in the BALF.

Drug administration

Prednisolone (20 mg kg^{-1}, i.p.) was adminstered 3 h, and ketotifen (2 mg kg^{-1}, i.p.) was administered 30 min before mrIL-5 treatment. DSCG was administered into the trachea mixed with the mr-IL5 solution.

Materials

Murine recombinant interleukin-5 (mrIL-5) and human recombinant interleukin 5 (hrIL-5) were synthesized at the Suntory Institute for Biochemical Research (Osaka, Japan). Ketotifen was provided by Sandoz Ltd. (Basel, Switzerland). DSCG (disodium cromoglycate) was also a gift from Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Prednisolone acetate (Takeda Chemical Ind. Ltd., Osaka, Japan), BSA (type IV, Sigma Chemical Co., St. Louis, U.S.A.), May-Gruenwald stain and Giemsa stain (Merk Co. Inc., N.J., U.S.A.), Turk solution (Wako Pure Chemicals, Japan) and sodium pentobarbitone (Abbot Lab, Chicago, U.S.A.) were purchased commercially. mrIL-5, DSCG and BSA were dissolved in 0.9% NaCl. Prednisolone acetate was suspended in saline containing 0.5% CMC-Na.

Statistical analysis

All data were represented as mean ± s.e.mean and analysed by Dunnet's multiple range test.

Results

mrIL-5-induced leukocyte infiltration in bronchoalveolar lavage fluid

Twenty-four hours after intratracheal injection of mrIL-5 (2-15 μg/animal), the number of macrophages, eosinophils and neutrophils in the BALF had increased in a dose-dependent manner (Table 1). mrIL-5 at a dose of 5 μg tended to increase, but not significantly, the macrophage and neutrophil level in the BALF. When 15 μg mrIL-5 was administered, a significant increase in the number of macrophages, eosinophils and neutrophils in the BALF was observed, but no significant change in the number of lymphocytes occurred. In contrast, the same dose of BSA did not affect the number of macrophages, eosinophils, neutrophils and lymphocytes (Table 1). In the time-course experiments to measure cell increase in the BALF induced by mrIL-5 (15 μg), the number of macrophages, eosinophils, neutrophils and lymphocytes increased after 5 and 12 h, but these effects were not significant (Table 2).

Effect of mrIL-5 on pulmonary epithelial cells

The intratracheal injection of mrIL-5 (2 and 5 μg) exhibited a tendency to increase the number of epithelial cells in the BALF after 24 h, but the effect was not significant (Figure 1a). However, a significant increase in BALF epithelial cells was observed at 24 h following intratracheal administration of 15 μg mrIL-5. In contrast, BSA at a dose of 15 μg did not increase the epithelial cell number (Figure 1a). In the time course experiments, intratracheal administration of 15 μg mrIL-5 tended to increase the number of epithelial cells in the BALF 12 h after treatment, although no change had occurred at 5 h (Figure 1b).

Table 1 Dose-response for mrIL-5-induced changes in bronchial leukocyte populations in guinea-pigs

	Leukocyte populations (× 10^6)				
Dose	*Total*	*Macro*	*Eosin*	*Neut*	*Lymph*
Saline	6.0 ± 1.3	4.6 ± 0.9	0.5 ± 0.2	0.5 ± 0.1	0.3 ± 0.1
BSA 15 μg	6.3 ± 0.3	4.9 ± 0.2	0.5 ± 0.1	0.7 ± 0.2	0.2 ± 0.02
mrIL-5 2 μg	8.7 ± 1.7	6.5 ± 1.4	0.6 ± 0.2	1.3 ± 0.5	0.4 ± 0.03
5 μg	12.5 ± 2.4	8.1 ± 1.2	0.9 ± 0.1	3.3 ± 1.2	0.3 ± 0.1
15 μg	17.7 ± 4.0*	8.7 ± 1.2*	1.9 ± 0.5*	6.4 ± 2.0*	0.8 ± 0.2

Total = total leukocytes; Macro = macrophages; Neut = neutrophils; Eosin = eosinophils; Lymph = lymphocytes. Results are expressed as the mean ± s.e.mean of the numbers (× 10^6 cells) of total leukocytes and each leukocyte type recovered from BALF of 5–8 animals 24 h after the intratracheal injection of saline, bovine serum albumin (BSA) or mrIL-5.
* $P < 0.05$, significantly different from saline response.

Table 2 Time-course for changes in bronchial leukocyte populations after mrIL-5 challenge in guinea-pigs

	Leukocyte populations (× 10^6)				
Time (h)	*Total*	*Macro*	*Eosin*	*Neut*	*Lymph*
0	6.0 ± 0.4	5.1 ± 0.3	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.02
5	9.3 ± 1.1	6.5 ± 1.1	1.1 ± 0.3	1.5 ± 0.5	0.2 ± 0.05
12	13.8 ± 4.8	8.0 ± 1.6	1.3 ± 0.6	4.0 ± 2.4	0.5 ± 0.2
24	17.7 ± 4.0*	8.7 ± 1.2*	1.9 ± 0.5*	6.4 ± 2.0*	0.8 ± 0.2

Total = total leukocytes; Macro = macrophages; Neut = neutrophils; Eosin = eosinophils; Lymph = lymphocytes. Results are expressed as the mean ± s.e.mean of the numbers (× 10^6 cells) of total leukocytes and each leukocyte type recovered from BALF of 6–8 animals after the intratracheal injection of mrIL-5 (15 μg/animal).
* $P < 0.05$, significantly different from zero time response.

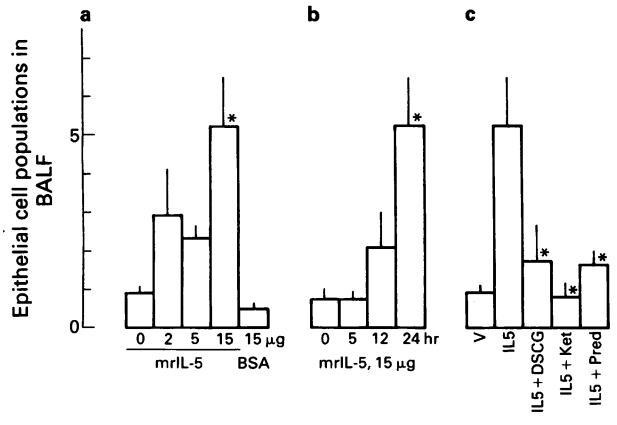


Figure 1 mrIL-5-induced increase in the number of pulmonary epithelial cells in guinea-pigs: (a) indicates the dose-response relationship for the changes in bronchial epithelial cell numbers 24 h after the intratracheal injection of mrIL-5 and BSA; (b) shows the time-course for the changes in bronchial epithelial cell numbers induced by the intratracheally administered mrIL-5 (15 μg/body); (c) illustrates the effect of anti-asthmatic drugs on the intratracheally administered mrIL-5 (15 μg/body)-induced increase in bronchial epithelial cell numbers 24 h after challenge. V (drug vehicle) = 0.9% NaCl (saline); IL5 = mrIL-5; Ket = ketotifen; Pred = prednisolone (c). Results are expressed as the mean number of cells ($\times 10^5$) of 6–8 animals; vertical lines show s.e.mean. * $P < 0.05$, significantly different from the zero dose response (a), the zero time response (b), and the mrIL-5, no drug, response (c).

Effects of anti-asthmatic drugs on bronchoalveolar lavage fluid cellular content

As shown in Table 3 and Figure 1c, prednisolone (20 mg kg^{-1}, i.p.) inhibited the increase in macrophages, eosinophils, neutrophils and epithelial cells induced by intratracheal injection of 15 μg mrIL-5. Ketotifen (2 mg kg^{-1}, i.p.) reduced the mrIL-5-induced increase in eosinophils, neutrophils and epithelial cells. DSCG (2%), when administered at the same time into the trachea, prevented the mrIL-5-induced increase in pulmonary epithelial cells, without affecting the number of inflammatory leukocytes.

Discussion

IL-5 was first reported as a T cell-replacing factor which differentiates B cells into antibody producing cells (Takatsu *et al.*, 1980) and as the B cell growth factor II (Swain, 1985). It is now known that IL-5 promotes the proliferation and differentiation of eosinophils (Clutterbuck & Sanderson, 1988; Lopez *et al.*, 1988), the production of cytotoxic T cells (Takatsu *et al.*, 1987), the expression of the IL-2 receptor on activated B cells (Takatsu *et al.*, 1987), and the production of IgA (Yokota *et al.*, 1987). These observations suggest that this cytokine is closely related to the control of immune responses and inflammatory reactions. Here we have examined mrIL-5-induced increase of inflammatory leukocytes and epithelial cells in the BALF of guinea-pigs.

Table 3 Effect of anti-asthmatic drugs on mrIL-5-induced increase in the numbers of macrophages, eosinophils and neutrophils in bronchoalveolar lavage fluid (BALF) of guinea-pigs

	Leukocyte populations ($\times 10^6$)		
Treatment	*Macro*	*Eosin*	*Neut*
Saline, no drug	4.6 ± 0.9	0.5 ± 0.2	0.5 ± 0.1
mrIL-5, no drug	8.7 ± 1.2	1.9 ± 0.5	6.4 ± 2.0
DSCG	8.9 ± 1.8	2.4 ± 1.1	4.3 ± 1.6
Ketotifen	7.9 ± 1.7	0.8 ± 0.3*	1.1 ± 0.5*
Prednisolone	5.0 ± 1.1*	0.6 ± 0.3*	1.0 ± 0.6*

Macro = macrophages; Neut = neutrophils; Eosin = eosinophils. Results are expressed as the mean ± s.e.mean of the numbers ($\times 10^6$ cells) of macrophages, eosinophils and neutrophils recovered from BALF of 6–8 animals, 24 h after the intratracheal injection of mrIL-5 (15 μg/animal).
* $P < 0.05$, significantly different from 'mrIL-5, no drug' response.

In the present study, the injection of mrIL-5 into the trachea of guinea-pigs induced a dose-dependent accumulation of macrophages, eosinophils, neutrophils and lung epithelial cells in the BALF after 24 h. However, in our preliminary study, neither the inhalation of hrIL-5 nor the intraperitoneal injection of mrIL-5 caused any change in guinea-pig BALF cell counts and populations after 24 h. In addition, intratracheally administered BSA did not cause any change. These results indicate that IL-5 formed in the airway lumen may be important in the induction of airway inflammation.

Airway inflammation is an important pathogenic symptom in asthma patients. A number of animal models of airway inflammation have been described. Antigen challenge elicits the pulmonary recruitment of eosinophils and neutrophils in guinea-pigs, the eosinophilia, but not the neutrophilia, being inhibited by a LTB_4 antagonist (Richards *et al.*, 1989) and a PAF antagonist (Coyle *et al.*, 1988). Endotoxin-induced eosinophilia and neutrophilia are reduced by a PAF antagonist (Rylander *et al.*, 1988), and the inhalation of PAF produces a selective accumulation of eosinophils in the airways of guinea-pigs (Coyle *et al.*, 1988; Sanjar *et al.*, 1990a). Granulocyte macrophage colony stimulating factor (GM-CSF), IL-3 and tumour necrosis factor (TNF) also stimulate pulmonary eosinophil accumulation in guinea-pigs (Kings *et al.*, 1990). Furthermore, GM-CSF enhances the PAF-induced pulmonary eosinophilic response, whereas IL-3 suppresses it (Sanjar *et al.*, 1990c). This effect of IL-3 may be explained by an induced blood neutrophilia, since infected animals with high blood or bronchial neutrophils do not exhibit an eosinophilia in response to PAF (Sanjar *et al.*, 1990c). In our study, intratracheally administered mrIL-5 clearly induced the accumulation of macrophages, eosinophils and neutrophils in the BALF. These observations demonstrate that IL-5 shares a common effect with PAF, LTB_4, GM-CSF, IL-3 and TNF.

The glucocorticoid, prednisolone, reduced the mrIL-5-induced increase in the macrophage, eosinophil and neutrophil populations of BALF in guinea-pigs. Ketotifen inhibited the increase in eosinophils and neutrophils. DSCG tended partially to inhibit the pulmonary neutrophilia, without affecting pulmonary macrophage and eosinophil accumulation. Others have studied the effects of anti-asthmatic drugs on pulmonary eosinophilia and neutrophilia with similar results. Thus, DSCG was found to inhibit antigen-induced BALF neutrophilia in guinea-pigs (Hutson *et al.*, 1988), while ketotifen and dexamethasone prevented respiratory antigen-induced eosinophilia and neutrophilia (Sanjar *et al.*, 1990b) and PAF-induced eosinophilia (Sanjar *et al.*, 1990a). On the other hand, the previous finding that DSCG inhibits PAF-induced eosinophilia (Sanjar *et al.*, 1990a) contradicts our results. However, Hutson *et al.* (1988) reported that DSCG given after antigen challenge modifies eosinophilia but not when given beforehand. Whether the inhibitory effects of these anti-asthmatic drugs on BALF inflammatory leukocyte increases are based on inhibition of cell chemotaxis, cell proliferation, cell differentiation or other mechanisms remains to be determined.

Airway epithelial cells play an important role in modulating bronchial smooth muscle tone. The airway epithelium is capable of releasing relaxant factors and epithelial destruction leads to airway hyperreactivity (Barnes, 1989; Vanhoutte, 1989). It has been observed that there are more respiratory epithelial cells in the BALF of guinea-pigs with airways rendered hyperreactive by antigen challenge than in normal animals (Motojima *et al.*, 1989). It has also been reported that clumps of airway epithelial cells, called Creola bodies (Naylor, 1985), are found in the sputum of asthmatics (Frigas & Gleich,

1986). In addition, epithelial desquamation is related to increased bronchial hyperresponsiveness following antigen challenge in asthma patients (Kushima *et al.*, 1990). In our experiments, mrIL-5 induced an increase in the number of epithelial cells in the BALF of guinea-pigs. Prednisolone attenuated the mrIL-5-induced respiratory epithelial desquamation, as shown by a smaller increase in the number of macrophages, eosinophils and neutrophils. Ketotifen inhibited epithelial cell desquamation and prevented the pulmonary eosinophilia and neutrophilia induced by mrIL-5. These results suggest that the inhibitory effect of prednisolone and ketotifen on mrIL-5-induced epithelial cell desquamation may be related to that inhibition of the increase in BALF leukocytes, especially that of eosinophils and neutrophils. However, it is interesting that DSCG suppressed mrIL-5-induced epithelial cell desquamation without significantly affecting pulmonary leukocyte accumulation. At present, the mechanism by which DSCG inhibits the desquamation of epithelial cells is unclear and further studies are required e.g. investigation of the blockade by DSCG of direct epithelial damage by IL-5; epithelial damage by IL-5-induced active oxygen species and toxic proteins from macrophages, neutrophils and eosinophils; epithelial damage by IL5-induced cytotoxic T cell activation, or a combination of these.

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Indirect inhibitory effect of succinylcholine on acetylcholine-activated channel activities and its modulation by external Ca^{2+} in mouse skeletal muscles

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1 The effect of extracellular calcium on single acetylcholine (ACh)-activated channel activities when desensitizing concentrations of succinylcholine (SuCh) were applied to the surrounding endplate membrane was investigated by the cell-attached patch-clamp technique at endplates of single skeletal muscle (flexor digitorum brevis) fibres of adult mice.

2 Bath-applied SuCh (0.1–3 μM, in 2.5 mM Ca^{2+}) increased in a concentration-dependent manner the mean open time of ACh-activated channel currents recorded at membrane potentials which cancelled the SuCh-induced depolarizations.

3 In the presence of 0.5 and 2.5 mM external Ca^{2+}, SuCh (3 μM) applied outside the patch pipette prolonged the mean open time of ACh-activated channel currents in a time-dependent manner (by 45% and 52%, respectively), and simultaneously significantly decreased the single channel conductance (by 14% and 10%, respectively). These SuCh-induced effects did not occur in a nominally Ca^{2+}-free extracellular medium.

4 Under the same conditions, SuCh (3 μM) augmented the time-dependent decline in the opening frequency of ACh-activated channel currents obtained in nominally Ca^{2+}-free medium.

5 These results suggest that external calcium ions act to modulate nicotinic ACh receptor channel activity, and accelerate desensitization of the receptor.

Keywords: Calcium; succinylcholine; desensitization; nicotinic acetylcholine receptor; single channel recording

Introduction

The mechanisms by which nicotinic cholinergic responses are desensitized by the prolonged application of agonists are not yet well understood. The process of desensitization by the continuous application of acetylcholine (ACh) at the motor endplate has been demonstrated electrophysiologically to involve: (1) a gradual decay of depolarization (Miledi, 1980; Chesnut, 1983), (2) a frequency-dependent depression of responses to iontophoretically applied ACh (Katz & Thesleff, 1957; Anwyl & Narahashi, 1980; Magazanik *et al.*, 1982) and (3) a time-dependent decline in the opening frequency of ACh-activated channel currents (Sakmann *et al.*, 1980; Hopfield *et al.*, 1988; Dionne, 1989).

Succinylcholine (SuCh), like other depolarizing neuromuscular blockers, acts to block the postsynaptic response in part through desensitization of the nicotinic ACh receptor (Durant & Katz, 1982). The rate of onset of desensitization can be accelerated by increasing the intracellular or extracellular free calcium levels (Miledi, 1980; Fiekers *et al.*, 1980; Magazanik & Vyskočil, 1982; Chesnut, 1983). Depolarizing neuromuscular blockers affect the mobilization of intracellular Ca^{2+} (Kimura *et al.*, 1990), and extracellular calcium modulates nicotinic ACh receptor-channel activities in single skeletal muscle fibres (Kimura *et al.*, 1991).

The aim of the present study was to elucidate the effect of extracellular calcium on the activity of single ACh-activated channels in the presence of SuCh at endplates of flexor digitorum brevis (FDB) muscle fibres in adult mice by use of the cell-attached patch-clamp technique.

Methods

Adult male ddY mice (32–38 g) were used.

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Intracellular recordings

Diaphragm muscles were isolated, and maintained in Krebs-Henseleit solution (KHS) saturated with 95% O_2 and 5% CO_2, at 35–37°C. The composition of the solution was as follows (mM): NaCl 137, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 15 and glucose 10. ACh was applied by iontophoresis (rectangular current pulses; 1 nA, 5 s duration) from a microelectrode (100–200 MΩ) filled with 2 M ACh. To avoid leakage of ACh, a few nA of braking current was applied.

Single channel recordings

The FDB muscles were isolated from the hind feet. Dissection and enzyme treatment were carried out in modified KHS (KCl reduced from 5 to 2.5 mM) which did not contain tetrodotoxin and was saturated with 95% O_2 and 5% CO_2. The muscles were incubated at 37°C for 75 min in KHS containing 0.2% collagenase, transferred to KHS containing 0.05% trypsin but no $CaCl_2$ for 15 min, rinsed in KHS, and then triturated gently to obtain single fibres. Experiments were performed 2 h after enzyme treatment.

Single channel currents were recorded from the endplate of each muscle fibre in KHS at 24–26°C in the cell-attached patch configuration (Hamill *et al.*, 1981) with a patch-clamp amplifier (Axopatch-1D; Axon Instruments, CA, U.S.A.). Currents were low-pass filtered at 2 kHz and stored in a PCM data recorder (RP-880; NF Electric Instruments, Japan). The sampling frequency was 56.88 kHz. The data were analysed with a histogram analyser (QC-111J; Nihon Kohden, Japan). Data replayed from a PCM data recorder were sampled at 100 μs per point and stored on a memory oscilloscope (VC-11, Nihon Kohden, Japan). The traces of channel currents shown were drawn by a pen-recorder from the stored data. The threshold for detecting opening and closing transitions was set at a current amplitude of 2 pA, which corresponded to a value of 35–47% of the mean channel current amplitude in both the control and test recordings. Only data from one channel in

the patch were adopted. The mean open time and mean current amplitude were determined at the resting membrane potential in a non-depolarized state, or at various membrane potentials with various pipette potentials to cancel the degree of depolarization, from 400–1600 events in each membrane patch. The opening frequency (the number of events per second) was determined simultaneously. Channel conductance was obtained from the slope of the current-voltage relationship, using the ACh reversal potential of 0 mV determined from the measurement of ACh-activated channel currents at various pipette potentials in the cell-attached patch configuration. ACh (1 μM)-activated channel currents were recorded continuously at an endplate for 20 min immediately after patch-seal formation. In these experiments, SuCh (3 μM) was applied in the bath after a control recording period of 3 min. At the end of the experiments, the fibres were impaled with the patch pipette in order to measure directly the intracellular membrane potentials. The membrane potential during the patch recordings was calculated as the sum of the pipette potential and the membrane potential measured directly at the end of the recordings.

Patch pipettes were prepared from capillary glass tubing (Pyrex, U.S.A.) which was pulled to a tip diameter of less than 1 μm. The resistance of pipettes filled with KHS containing 1 μM ACh was 15–20 MΩ. When small-tipped patch-pipettes were used, multi-level channel openings were not observed frequently at the resting membrane potential.

Statistics

The paired or unpaired t test and Student's range test (the analysis of variance with a subsequent multiple comparisons test) or one-way analysis of variance (ANOVA) were used, and $P < 0.05$ or $P < 0.01$ were accepted as significant.

Drugs

Acetylcholine chloride (Dai-ichi, Japan), succinylcholine chloride (Nacalai tesque, Japan), collagenase (for cell dispersion; Wako Pure Chemical Industries, Japan) and trypsin (Type III; Sigma, St. Louis, MO, U.S.A.) were used.

Results

The iontophoretic application of a prolonged pulse of ACh evoked endplate potentials which gradually declined with the desensitization of the nicotinic ACh receptors. After the application of 0.2 μM SuCh for 30 min, the peak of the ACh potential was reduced and the ACh potential decayed more rapidly than without SuCh (Figure 1).

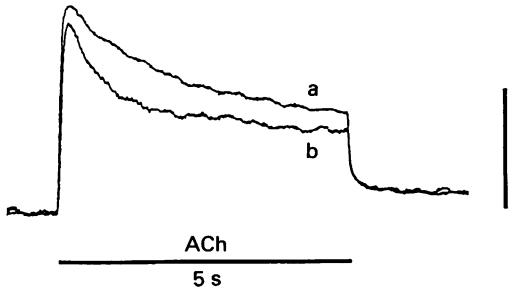


Figure 1 The effect of succinylcholine (SuCh, 0.2 μM) on the decay phase of depolarization produced by a prolonged acetylcholine (ACh) pulse at an endplate in mouse diaphragm muscle. Depolarizations generated by iontophoretically-applied ACh were recorded from the same fibre before (a) and 30 min after the application of SuCh (b). The horizontal bar represents the duration of the ACh pulse. The vertical calibration bar represents 7 mV in (a) and 5 mV in (b).

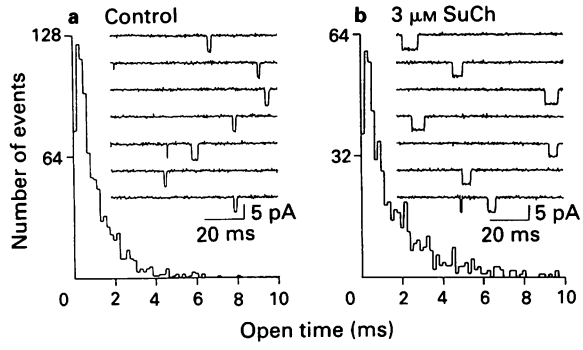


Figure 2 Frequency distribution histograms for the open time and typical data of acetylcholine (ACh, 1 μM)-activated channel currents recorded from the endplates of single FDB muscle fibres from mice at a resting membrane potential (−73 mV, which was a value directly measured at the end of the recording) before (a) and at a membrane potential (−75 mV, which was a sum of a membrane potential of −35 mV directly measured at the end of the recording and a pipette potential of 40 mV hyperpolarization during a patch recording) 10 to 15 min after bath application of 3 μM succinylcholine (SuCh) (b). (a) The mean open time and opening frequency without SuCh were 1.13 ms (n = 895) and 6.86 Hz respectively; (b) the mean open time and opening frequency with SuCh were 1.81 ms (n = 568) and 5.11 Hz respectively. The records are analysed quantitatively in Table 1.

ACh (1 μM)-activated single channel currents in a FDB muscle fibre were recorded 10 to 15 min after the bath application of SuCh (0.1–3 μM). Bath-applied SuCh at concentrations greater than 0.1 μM depolarized the membrane in a concentration-dependent manner. The SuCh-induced depolarization was then cancelled with a pipette potential of 10 mV hyperpolarization at 0.3 μM SuCh and 40 mV hyperpolarization at 1 and 3 μM SuCh, and the open time was measured. SuCh prolonged the mean open time of ACh currents in a concentration-dependent manner, but did not significantly alter opening frequency (Figure 2 and Table 1).

ACh (1 μM)-activated single channel currents were recorded continuously at an endplate for 20 min immediately after patch-seal formation, with a patch pipette containing various concentrations of Ca^{2+} (nominally free, 0.5 and 2.5 mM). With 2.5 mM Ca^{2+} in the patch pipette, the opening frequency of ACh-activated channel currents was decreased time-dependently from 7.3 ± 0.8 Hz (mean ± s.e.mean, n = 6) at 0–3 min after the seal, to 4.0 ± 0.2 Hz at 10–15 min, and simultaneously the mean open time was significantly increased from 1.21 ± 0.07 ms to 1.39 ± 0.08 ms (n = 6, $P < 0.01$; paired t test). Nominally Ca^{2+}-free solution and 0.5 mM Ca^{2+} in the patch pipette also decreased the opening frequency time-dependently from 6.6 ± 0.9 Hz to 4.4 ± 0.6 Hz (n = 6) and from 8.4 ± 1.0 Hz to 4.8 ± 0.5 Hz (n = 6), respectively. Ca^{2+}, 0.5 mM also significantly prolonged time-dependently, the mean open time from 0.92 ± 0.05 ms to 1.07 ± 0.03 ms (n = 6, $P < 0.05$; paired t test). Nominally Ca^{2+}-free medium in the patch pipette did not change the mean open time measured 10–15 min after a control recording, but the values for the mean open time were significantly lower than those recorded in 0.5 and 2.5 mM Ca^{2+} (Table 2).

ACh (1 μM)-activated single channel currents were recorded continuously at an endplate in the presence of various concentrations of Ca^{2+} (nominally 0, 0.5 and 2.5 mM) in the extracellular medium. SuCh (3 μM) was applied in the bath after a control recording for 3 min. SuCh induced a maintained depolarization which was independent of the Ca^{2+} concentration (Table 2). In 2.5 mM $[Ca^{2+}]_o$, SuCh prolonged the mean open time by 52% 10–15 min after its bath application (Figure 3a) and in 0.5 mM $[Ca^{2+}]_o$ SuCh prolonged the mean open time by 45%. However, in nominally Ca^{2+}-free medium, SuCh failed to prolong the mean open time 10–15 min after its bath

Table 1 Effects of bath-applied succinylcholine (SuCh) on acetylcholine (ACh)-activated channel activities and resting membrane potentials

SuCh (μM)	n	*Membrane potential* (mV) (pipette potential, mV)	*Mean open time* (ms)	*Opening frequency* (Hz)
0	(7)	−73.3 ± 0.9 (0)	1.17 ± 0.04	6.52 ± 0.79
0.1	(6)	−73.0 ± 0.6 (0)	1.19 ± 0.04	6.44 ± 0.82
0.3	(6)	−61.8 ± 2.3** (10)	1.40 ± 0.05**	5.44 ± 1.07
1	(7)	−35.6 ± 1.3** (40)	1.48 ± 0.02**	4.91 ± 0.44
3	(8)	−32.6 ± 1.5** (40)	1.95 ± 0.06**	4.77 ± 0.51

ACh (1 μM)-activated channel currents were recorded 10 to 15 min after the bath application of SuCh. The mean open time and opening frequency were estimated at membrane potentials with various pipette potentials (hyperpolarizing pulse in parentheses) to cancel the SuCh-induced depolarization. Significant differences (** $P < 0.01$) were detected by an unpaired t test between the values with and without SuCh. The values are means ± s.e.mean (n = number of cells).

Table 2 Effects of succinylcholine (SuCh) and calcium applied to the non-patched membrane on single acetylcholine (ACh)-activated channel currents

SuCh (μM)	$CaCl_2$ (mM)	*Membrane potential* (mV) (pipette potential, mV)	*Conductance* (pS) 10–15 min		*Mean open time* (ms) Pre	*Mean open time* (ms) 10–15 min		*Opening frequency* 10–15 min/Pre (%)	
0	0	−74.5 ± 1.4 (0)	75.0 ± 0.9	NS	0.66 ± 0.02	0.69 ± 0.04	NS	69.2 ± 5.9	
0	0.5	−75.3 ± 1.4 (0)	71.9 ± 1.0	**	0.92 ± 0.05	1.07 ± 0.03*	*	57.8 ± 5.0	
0	2.5	−73.5 ± 0.7 (0)	65.9 ± 1.0		1.21 ± 0.07	1.39 ± 0.08**		58.4 ± 7.0	
3	0	−33.8 ± 0.8 (40)	73.4 ± 1.4		0.69 ± 0.03	0.68 ± 0.04	**	68.8 ± 7.3	†
3	0.5	−33.5 ± 2.2 (40)	61.9 ± 1.2	**	0.87 ± 0.04	1.26 ± 0.07**		44.5 ± 4.7	†
3	2.5	−33.9 ± 0.7 (40)	59.2 ± 0.7		1.22 ± 0.05	1.86 ± 0.08**		44.9 ± 5.3	

ACh (1 μM)-activated channel currents were recorded continuously at an endplate, with a patch pipette containing various concentrations of $CaCl_2$ (0, 0.5 and 2.5 mM). A solution of SuCh (3 μM) containing the same concentration of $CaCl_2$ as in the pipette was applied to the non-patched membrane after a control recording period for 3 min (Pre); 10–15 min after the application of 0 or 3 μM SuCh, the mean open time, the opening frequency and the mean current amplitude were estimated at membrane potentials with various pipette potentials (hyperpolarizing pulse in parentheses) to cancel the SuCh-induced depolarization. Conductance was determined as described in Methods. Significant differences: * $P < 0.05$ and ** $P < 0.01$ were detected by a paired t test between the values at Pre and 10–15 min; * $P < 0.05$ and ** $P < 0.01$ by an unpaired t test between the values with and without SuCh, and † $P < 0.05$ by a Student's range test. NS: not significant. The values are means ± s.e.mean of 6 cells.

application (Figure 3b). In 0.5 and 2.5 mM $[Ca^{2+}]_o$ SuCh augmented the decrease of opening frequency, and this was accompanied by a prolongation of the mean open time and by a decrease of channel conductance. These effects were not produced by SuCh in nominally Ca^{2+}-free extracellular medium.

Discussion

This paper describes the effect of extracellular calcium on ACh-activated channel activities in the presence of SuCh in skeletal muscle fibres of adult mice. At concentrations higher than 0.2 μM, SuCh accelerated nicotinic ACh receptor desensitization, and concentration-dependently prolonged the mean open time at a membrane potential which cancelled the SuCh-induced depolarization. This effect may reflect an increased affinity of the receptor for ACh during the process of desensitization (Burgermeister *et al.*, 1977; Heidmann *et al.*, 1983). In single channel recordings, the desensitization of nicotinic ACh receptors induced by ACh is manifested as a time-dependent decline in the opening frequency of ACh-activated channel currents (Hopfield *et al.*, 1988; Dionne, 1989) or as the occurrence of 'bursts' of single channel currents observed at irregular intervals (Sakmann *et al.*, 1980; Colquhoun & Ogden, 1988). We showed that the opening frequency of ACh-activated channel currents is reduced independently of the

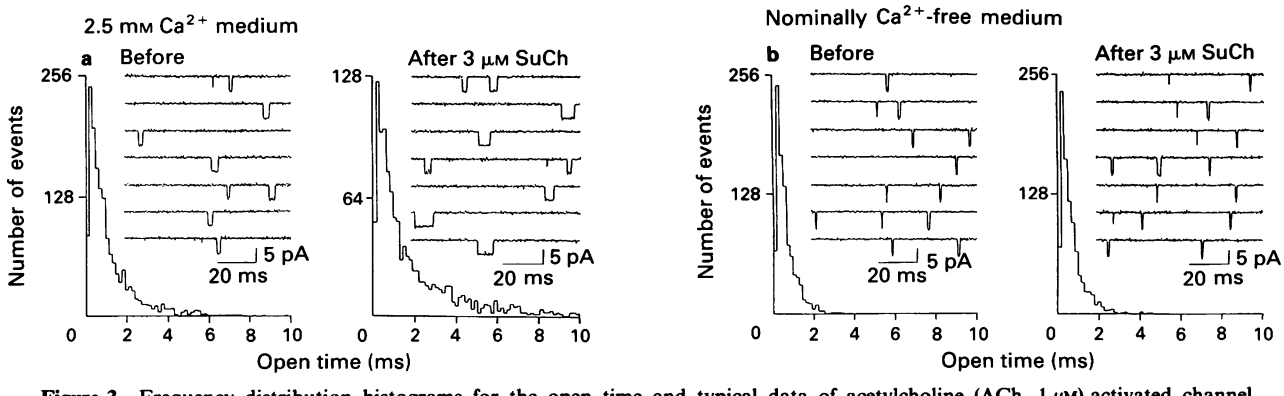


Figure 3 Frequency distribution histograms for the open time and typical data of acetylcholine (ACh, 1 μM)-activated channel currents recorded from the endplates of single muscle fibres at a resting membrane potential before (left) and at a membrane potential of −73 mV (a) and −74 mV (b) 10 to 15 min after (right) the application of 3 μM succinylcholine (SuCh) to the non-patched membrane in 2.5 mM Ca^{2+} (a) and nominally Ca^{2+}-free (b) medium. The mean open time and opening frequency were 1.17 ms (n = 1599) and 8.88 Hz respectively without SuCh and 1.86 ms (n = 1172) and 3.91 Hz respectively with SuCh in (a), and 0.67 ms (n = 982) and 8.18 Hz respectively without SuCh and 0.70 ms (n = 938) and 5.21 Hz respectively with SuCh in (b). The records are analysed quantitatively in Table 2.

presence of external calcium, and that the mean open time is prolonged by increasing the extracellular calcium concentration. These results indicate that calcium is certainly not necessary for the desensitization of nicotinic ACh receptors by ACh alone, and that Ca^{2+} influx induced by nicotinic ACh receptor-activation (Miledi *et al.*, 1980) may prolong the mean open time.

Desensitizing concentrations of SuCh applied to the membrane outside the patch pipette augmented the time-dependent prolongation of the mean open time, accelerated the time-dependent decline of the opening frequency, and decreased the conductance of single ACh-activated channel currents. All these effects were dependent on the extracellular calcium concentration. Prolongation of the mean open time of the ACh-activated channel has been observed during hyperpolarization (Anderson & Stevens, 1973; Aracava *et al.*, 1984; Greenberg *et al.*, 1985). The effects of SuCh are not associated with SuCh-induced depolarization, because SuCh induced a constant depolarization which was independent of changes in the extracellular calcium concentration. These results indicate that the indirect effects of SuCh on the nicotinic ACh receptor channel are triggered by the SuCh-induced Ca^{2+} influx (Kimura *et al.*, 1990). In a previous paper, we showed that increasing the calcium concentrations in a patch pipette reduces both the conductance and the opening frequency of ACh-activated channels, and prolongs their mean open time (Kimura *et al.*, 1991). In the presence of desensitizing concentrations of SuCh, channel activities may be modulated by external calcium ions with the consequence that SuCh amplifies the time-dependent changes in the ACh-activated channel activities.

However, our data are at variance with those of Eusebi *et al.* (1987) in chick myotubes, where the decreases in both channel opening frequencies and conductance induced by ACh applied to the membrane outside the patch pipette also occurred when the non-patched membrane was exposed to nominally Ca^{2+}-free extracellular medium.

In conclusion, the modulating effects of SuCh on ACh-activated channel activities probably resulted from an increase of intracellular Ca^{2+} during nicotinic ACh receptor-activation, because these effects were not produced when calcium was not present in the extracellular medium. These results suggest that extracellular calcium ions modulate nicotinic ACh receptor channel activity, and accelerate desensitization of the receptor.

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Pharmacological characterization of RP 62203, a novel 5-hydroxytryptamine 5-HT_2 receptor antagonist

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1 RP 62203 (2-[3-(4-(4-fluorophenyl)-piperazinyl)propyl]naphto[1,8-cd]isothiazole-1,1-dioxide) is a novel naphtosultam derivative which shows very high affinity for 5-HT_2 receptors in the rat cerebral cortex (K_i = 50.0 pM).

2 RP 62203 is relatively selective for this sub-type of 5-hydroxytryptamine (5-HT) receptor, having lower affinity for the 5-HT_{1A} receptor and very low affinity for the 5-HT_3 receptor. RP 62203 displayed low to moderate affinity for α_1-adrenoceptors, dopamine D_2 receptors and histamine H_1 receptors.

3 *In vivo* binding experiments demonstrated that oral administration of low doses of RP 62203 led to a long-lasting (>6 h) occupation of cortical 5-HT_2 receptors (ID_{50} = 0.39 mg kg^{-1}).

4 In cortical slices from the neonatal rat, RP 62203 potently inhibited inositol phosphate formation evoked by 5-HT, with an IC_{50} of 7.76 nM.

5 The activity of neurones in the raphé and their responses to microiontophoretically applied 5-HT were studied with extracellular recording electrodes in the anaesthetized rat. RP 62203 potently and dose-dependently blocked excitations evoked by 5-HT when administered at doses of 0.5–4.0 mg kg^{-1}, i.p. In contrast, neither 5-HT-evoked depressions nor glutamate-evoked excitations of raphé neuronal firing were blocked by RP 62203 at doses as high as 8.0 mg kg^{-1}, i.p.

6 Head twitches induced by 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) could be abolished by low doses of RP 62203 in mice (ED_{50} = 0.44 mg kg^{-1}, p.o.) and in rats (ED_{50} = 1.54 p.o.). Similar results were obtained with mescaline and 5-hydroxytryptophan (5-HTP).

7 The potency of RP 62203 was compared with that of three other 5-HT_2 receptor antagonists, ritanserin, ICI 169,369 and ICI 170,809. In all models, RP 62203 showed similar activity to ritanserin, whilst either ICI 169,369 or ICI 170,809 was several fold less active.

8 It is concluded that RP 62203 is a potent and selective antagonist at 5-HT_2 receptors in the rodent central nervous system.

Keywords: 5-Hydroxytryptamine; 5-HT_2 receptor antagonists; RP 62203; binding; inositol phosphate; raphé; neuronal activity; head-twitch

Introduction

5-Hydroxytryptamine (5-HT) is a widely-distributed neurotransmitter in the mammalian central nervous system, where it is thought to play a role in the neural circuits involved in pain perception, sleep, mood, anxiety, appetite and sexual behaviour (see Willis, 1984 for review). Understanding of the physiological role of 5-HT has, however, been hampered by the multiplicity of receptor subtypes and the complexity of their pharmacology (Bradley *et al.*, 1986), discrepancies between binding studies and functional tests (Leysen, 1984), and by species and tissue differences (Hoyer *et al.*, 1986a,b; Hoyer & Middlemiss, 1989; Mylecharane, 1989). The best-characterized of these receptor subtypes is the 5-HT_2 receptor, due to the availability of reasonably selective agonists and antagonists (Shannon *et al.*, 1984; Leysen *et al.*, 1985; Blackburn *et al.*, 1988a).

From electrophysiological studies in the rat, both *in vivo* and *in vitro*, it has been shown that 5-HT excites neurones in the cortex (Davies *et al.*, 1987), the nucleus accumbens (North & Uchimura, 1989), the hippocampus (Andrade & Nicoll, 1987) and the brainstem (Davies *et al.*, 1988) through a 5-HT_2 receptor. The neuronal excitation which follows 5-HT_2 receptor activation results from the closure of an inwardly rectifying K^+ conductance which induces a slow depolarization. It has been suggested that 5-HT_2 receptors could be linked to protein kinase C through inositol phospholipid hydrolysis (North & Uchimura, 1989), although in rat facial motoneurones Aghajanian (1990) has reported that this response is mediated by G proteins but not protein kinase C. This 5-HT-evoked firing is readily antagonised by ketanserin, which agrees well with biochemical data from rat brain (Leysen, 1984.

The raphé nuclei of the mammalian brainstem are the origin of the major 5-hydroxytryptaminergic projections throughout the brain and spinal cord and the rate of raphé neuronal firing thus exerts considerable control over many regions in the central nervous system. Compounds which interact with raphé receptors are therefore of significant clinical interest.

In behavioural models of 5-HT-mediated functions, however, the involvement of 5-HT_2 receptors is less clear. Perhaps the best evidence for a behavioural response mediated by 5-HT_2 receptors has been found in the case of 5-HT agonist-induced head-twitches (Green *et al.*, 1983). These can be observed after administration of the 5-HT precursor, 5-hydroxytryptophan (5-HTP), and the 5-HT_2 receptor agonists 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) and mescaline (Corne & Pickering, 1967; Peroutka *et al.*, 1981; Heaton *et al.*, 1988). These responses can be blocked by 5-HT_2 receptor antagonists, such as ritanserin (Goodwin & Green, 1985).

This report describes the properties of a novel 5-HT_2 receptor antagonist belonging to the naphthosultam family, RP 62203 (Figure 1), which shows high affinity and selectivity for 5-HT_2 receptors in rodent brain. The activity of RP 62203 has been compared to those of three reference compounds, ritanserin (Leysen *et al.*, 1985), ICI 169,369 (Blackburn *et al.*,

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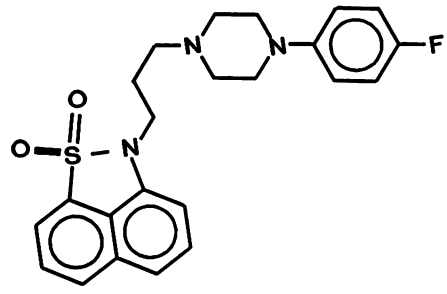


Figure 1 Structure of RP 62203 (2-(3-(4-(4-fluorophenyl)-piperazinyl)propyl)naphto(1,8-c,d)isothiazole-1,1-dioxide).

1988a; 1990) and ICI 170,809 (Blackburn *et al.*, 1988b; Frenken & Kauman, 1989). The properties of RP 62203 have been studied in radioligand binding assays and three functional tests of 5-HT_2 receptor activity, inositol phosphate formation, neuronal firing in the raphé nuclei and 5-HT agonist-induced head-twitches. Preliminary reports of some of these data have been published (Doble *et al.*, 1990; Piot *et al.*, 1990; Dupuy *et al.*, 1991).

Methods

Binding assays

The affinity of the different compounds for 5-HT_2 receptors was assessed by use of the [^{3}H]-ketanserin binding assay described by Leysen *et al.* (1982). Male Sprague-Dawley rats (Charles River France) were killed by decapitation and their brains rapidly removed on ice. The cerebral cortices were dissected out and homogenized in 10 volumes of ice-cold Tris-HCl buffer (50 mM; pH 7.6). The homogenate was centrifuged for 10 min at 50,000 *g* at 4°C and the supernatant discarded. The pellet was washed by resuspension in seven volumes of the same buffer and recentrifugation, the final pellet was then resuspended in five volumes of Tris-HCl buffer, distributed in 1 ml aliquots in plastic vials (Nunc) and frozen at −80°C until required. Protein content was measured according to the BCA method (Smith *et al.*, 1985) with bovine serum albumin as the standard.

Aliquots (1 ml) of cortical membranes at a protein concentration of 0.15 mg ml^{-1} in Tris-HCl buffer were incubated for 15 min at 37°C with the compound of interest or with 10^{-6} M methysergide (to define the non-specific binding) in the presence of [^{3}H]-ketanserin (final concentration 0.4 nM). The binding interaction was terminated by filtration across Whatman GF/B glass fibre filters using a Skatron cell harvester, and was followed by two washes with 2.5 ml of ice-cold buffer. The radioactivity retained on the filters was determined by liquid scintillometry in 4.5 ml of Ready-Gel scintillant (Beckman).

The specific binding was defined as that displaceable by 10^{-6} M methysergide. The percentage of the specific binding obtained with each concentration of the test compound was calculated, and the IC_{50} (concentration that inhibits 50% of the specific binding) determined by non-linear regression analysis using an iterative curve-fitting procedure to a simple Langmuir isotherm performed on an IBM-PC computer (McPherson, 1985).

The other binding assays were carried out on frozen crude membrane preparations from rat brain (or guinea-pig brain, in the case of histamine H_1, cholecystokinin, neurotensin and opiate receptors). The protocols were taken from published methods: α_1-adrenoceptors (Greengrass & Bremner, 1979), α_2-adrenoceptors (Rouot & Snyder, 1979), benzodiazepine binding sites (Speth *et al.*, 1978), bradykinin receptors (Manning *et al.*, 1986), CCK_A and CCK_B cholecystokinin receptors (Saito *et al.*, 1981), dopamine D_2 receptors (Urwyler & Coward, 1987), histamine H_1 receptors (Laduron *et al.*, 1982), 5-HT_{1A} receptors (Hall *et al.*, 1985), 5-HT_{1B} receptors (Hoyer *et al.*, 1985), 5-HT_3 receptors (Kilpatrick *et al.*, 1987), 5-HT uptake sites (Hebert *et al.*, 1985), N-methyl-D-aspartate receptors (Vignon *et al.*, 1983), muscarinic receptors (Yamamura & Snyder, 1974), neuropeptide Y receptors (Unden *et al.*, 1984), neurotensin receptors (Schotte *et al.*, 1986), opiate receptors (Kosterlitz *et al.*, 1981), somatostatin receptors (Tran *et al.*, 1985), tachykinin NK_1 receptors (Lee *et al.*, 1983), vasoactive intestinal peptide receptors (Robberecht *et al.*, 1978), calcium channels (Galizzi *et al.*, 1984) and sodium channels (Pauwels *et al.*, 1986).

In vivo *binding experiments*

In the rat, [^{3}H]-spiperone can be used to determine the occupation *in vitro* of 5-HT_2 receptors in the frontal cortex and dopamine D_2 receptors in the striatum (Leysen *et al.*, 1985). Similarly, in the mouse brain, [^{3}H]-methylspiperone has been proposed as a useful ligand for the study of D_2 and 5-HT_2 receptors *in vivo* (Frost *et al.*, 1987).

Male rats (170–190 g, Charles River France) were used for all experiments. Each animal received an injection of [^{3}H]-spiperone (0.185 MBq in 100 µl, approximately 0.5 µg kg^{-1}) into the left saphenous vein. Animals were pretreated orally or subcutaneously 60 min before administration of [^{3}H]-spiperone, and killed a further 60 min later. The cerebellum, striatum and cerebral cortex were dissected out, and homogenized in 1 ml H_2O (frontal cortex and striatum) or 2.5 ml H_2O (cerebellum). The radioactivity was determined by counting the entire homogenate (frontal cortex and striatum) or a 1 ml aliquot (cerebellum) in 10 ml of HP Beckman scintillant.

Male mice (23 g, Iffa-Credo France) were injected with [^{3}H]-methylspiperone (2.22 or 3.03 MBq ml^{-1}, New England Nuclear) at a dose of 0.5 µg kg^{-1} into the dorsal tail vein. Animals were pretreated subcutaneously (RP 62203) or intraperitoneally (ritanserin) 30 min before the injection of [^{3}H]-methylspiperone, and killed 60 min later. The cerebellum, hippocampus and cerebral cortex were dissected out, weighed, solubilized in 2 ml Soluene, and the radioactivity estimated by liquid scintillometry in 10 ml Instagel.

The non-specific binding was attributed to the radioactivity accumulated in the cerebellum, and this 'blank' subtracted from the values obtained in the striatum and frontal cortex to yield the 'specific' binding. The doses which inhibited specific [^{3}H]-spiperone binding by 50% *in vivo* (ID_{50}) were determined by linear regression analysis of the log dose-response curves.

Inositol phosphate formation

5-HT-stimulated inositol phosphate formation was measured in cortical slices prepared from neonatal rat brain by a modification of the method of Claustre *et al.* (1988). Neonatal rats of either sex (8–10 days, Sprague-Dawley, Charles River France) were killed by decapitation and their cortices removed. These were cut into slices (350 µm bidimensionally) using a McIlwain tissue chopper and preincubated for 1 h in Krebs-HCO_3 buffer, composition as follows (mM): NaCl 144, KCl 5, $MgSO_4$ 1.8, NaH_2PO_4 1.8, $CaCl_2$ 2, $NaHCO_3$ 10 and glucose 20.2, equilibrated with O_2/CO_2 (95%/5%) at 37°C before use. The slices were then resuspended in 10 ml of fresh buffer containing [^{3}H]-*myo*-inositol (4.8×10^{-7} M; 3.7×10^6 Bq) for a further 60 min, then rinsed and allowed to settle. Aliquots of the slices (50 µl) were distributed into assay tubes containing pargyline (10 µM), LiCl (10^{-2} M) and the antagonist to be studied in a total volume of 250 µl. Fifteen minutes later, 5-HT (10^{-5} M) was added, and the incubation continued for a further 60 min before isolation of the inositol phosphates. All incubations were performed at 37°C. Quadruplicate determinations were performed for each treatment condition.

At the end of the incubation period, chloroform/methanol (1:2 v:v; 0.9 ml) was added to lyse the cells and liberate the

inositol phosphates formed. A further 0.3 ml of chloroform and of water were added to separate the organic and aqueous phases. An aliquot (0.75 ml) of the aqueous phase was removed and applied to an anion exchange column (0.25 ml Dowex). The inositol phosphates were eluted by serial elution with increasing concentrations of formate as described by Berridge *et al.* (1982) and modified by Brown *et al.* (1984). Free inositol was eluted with distilled water (10 ml), glycerophosphoinositides with sodium formate (60 mM)/sodium tetraborate (5 mM) (10 ml) and the inositol phosphates with ammonium formate (1.0 M)/formic acid (0.1 M) (1 ml). Preliminary experiments showed that 95% of the radioactivity in the last fraction co-eluted with inositol monophosphate. The radioactivity in the inositol phosphate fraction was determined by liquid scintillometry in 4 ml of Ready-Gel scintillant (Beckman).

Inositol formation stimulated by histamine (10^{-5} M) was studied using a similar protocol in slices of guinea-pig cerebellum, and by noradrenaline (10^{-5} M) in primary cultures of mouse astrocytes using an identical procedure to that described for tachykinins by Torrens *et al.* (1989).

Electrophysiological recordings in the raphé nuclei

All experiments were carried out on 250–350 g male Sprague-Dawley rats (Charles River France). The animals were anaesthetized with chloral hydrate (400 mg kg^{-1}, i.p.) and subsequent doses of anaesthetic were administered when necessary to maintain a stable level of anaesthesia throughout the experiment. An intraperitoneal cannula was inserted and the animals were then placed in a stereotaxic frame and their rectal temperature was maintained at 36–37°C. The skin overlying the skull was incised and reflected and a small hole was drilled in the skull overlying the region of study. The smallest possible piece of dura was removed to allow insertion of the recording electrodes into the brain. For stereotaxic coordinates, the atlas of Paxinos & Watson (1982) was used. The coordinates for the raphé were (mm): dorsal raphé nucleus (DRN) A-P Bregma −7.8, Lateral O, Vertical 6.0–7.0 and median raphé nucleus (MRN) A-P Bregma −7.3, Lateral O, Vertical 8.0–9.0.

Extracellular recordings of action potentials were made with the central barrel (containing 3 M NaCl) of seven barrelled glass microelectrodes with a tip diameter of 5–7 μm. The remaining barrels were filled with combinations of the following drugs and solutions: 200 mM NaCl (current balancing), sodium-L-glutamate (0.2 M, pH 8), γ-aminobutyric acid (GABA) (0.2 M, pH 3.5), 5-hydroxytryptamine creatinine sulphate (0.05 M, pH 4). All solutions were freshly prepared immediately before the experiment. One electrode barrel contained pontamine sky blue (PSB, 2% in 0.5 M sodium acetate) for histological verification of the recording site at the end of the experiment.

Extracellular recordings of single unit activity were amplified, filtered and displayed by conventional methods. For microiontophoretic application of drugs, automatic current balancing was routinely used and a small retaining current was applied to each drug barrel to prevent unwanted diffusional leakage. On-line analysis of firing rate was carried out with an IBM microcomputer and interface and recordings were also stored on magnetic tape for further analysis off-line.

Measurement of 5-hydroxytryptamine agonist-induced head-twitches

Rats (male, 180–250 g, Sprague-Dawley, Charles River France or Wistar, Iffa-Credo France) and mice (male or female, 19–25 g, CD_1 COBS, Charles River France) were used. Animals were given free access to food and water and maintained on a 12 h light/dark cycle at an ambient temperature of 22 ± 2°C. To observe head-twitches, the animals were placed in individual perspex chambers without bedding material (12.5 × 13.5 × 11.5 cm for mice and 18 × 22 × 32 cm for rats). The head-twitch response consisted of a rapid movement of the head with little or no involvement of the trunk.

5-Hydroxytryptophan-induced head-twitches in mice Mice were injected with carbidopa (6 mg kg^{-1}, s.c.) and 15 min later with DL-5-HTP (180 mg kg^{-1}, i.p.). The presence or absence of head-twitches was recorded during a 2 min period, 15 min after 5-HTP administration (Corne *et al.*, 1963).

DOI-induced head-twitches in mice and rats Animals were injected with DOI (0.63 mg kg^{-1}, i.p.) and head-twitches recorded between the fifth and fifteenth minute after treatment in mice and during the 20 min following treatment in rats. Animals showing less than three head-twitches were considered to be protected (Heaton *et al.*, 1988).

Mescaline-induced head-twitches in mice and rats Mice were injected with mescaline (50 mg kg^{-1}, i.p.) and head-twitches recorded as described above (see also Corne & Pickering, 1967). In rats, head-twitches were counted during the 15 min following mescaline administration (20 mg kg^{-1}, i.v.; Niemegeers, 1983).

In mice, antagonists (6 or 8 mice per dose) were administered 60 (s.c.) or 90 (p.o.) min before the agonist. In rats, antagonists (6 rats per dose) were administered 60 min (s.c. or p.o.) before the agonist. The results are expressed as the ED_{50}, defined as the dose which protected 50% of the animals from head-twitches.

Measurement of amphetamine-induced stereotypies

Antagonists were administered orally or subcutaneously to groups of five rats (male, 160–200 g, Sprague-Dawley, Charles River France) 30 min before treatment with a single dose of amphetamine sulphate (12.5 mg kg^{-1}, i.p.). The rats were placed in individual cages (14 × 19 × 19 cm) and scored for stereotypies 60 and 90 min following amphetamine treatment, with a visual rating scale of 0–3. The results are expressed as the ED_{50}, defined as the dose which produced a 50% decrease in the mean stereotypy score compared to animals treated with amphetamine alone.

Measurement of tetrabenezine-induced ptosis

Groups of six mice (male or female, 16–26 g, CD_1 COBS, Charles River France) were treated orally with antagonists 1 h before administration of tetrabenazine (50 mg kg^{-1}, p.o.). The appearance of the palpebral fissure was evaluated 30 and 60 min after tetrabenazine treatment; a visual rating scale of 0 (fully closed) to 2 (fully open) was used. The results are expressed as the ED_{50}, defined as the dose which produced a 50% decrease in the mean ptosis score compared to animals treated with tetrabenazine alone.

Protection against noradrenaline-induced lethality

Antagonists were administered subcutaneously to groups of eight mice (male or female, 10–26 g, CD_1 COBS, Charles River France) 1 h before intravenous injection of noradrenaline (0.5 mg kg^{-1}). The number of mice surviving was counted after 15 min and 24 h. The results are expressed as the ED_{50}, defined as the dose which protected 50% of the animals against noradrenaline-induced lethality.

Drugs

RP 62203 (2-[3-(4-(4-fluorophenyl)-piperazinyl)propyl]-naphto[1,8-cd]isothiazole-1,1-dioxide), mescaline, amphetamine sulphate, prazosine, tetrabenazine and mepyramine were synthesized by the Chemistry Department at Rhône-Poulenc Rorer, CRVA, Vitry-sur-Seine, France.

ICI 169,369 (2-(2-dimethylaminoethylthio)-3-phenylquinoline hydrochloride) and ICI 170,809 (2-(2-dimethylamino-2-

Table 1 Affinity of RP 62203 and other 5-HT_2 antagonists for 5-HT_2 receptors in rat frontal cortex

Antagonist	IC_{50} (nM)
RP 62203	0.12 ± 0.03
Ritanserin	0.24 ± 0.03
Spiperone	0.27 ± 0.10
Ketanserin	0.76 ± 0.14
ICI 170,809	0.77 ± 0.20
Pipamperone	1.13 ± 0.13
Methysergide	2.00 ± 0.32
Carpipramine	3.00 ± 0.20
Mianserin	3.67 ± 0.45
ICI 169,369	61.3 ± 17.1

The concentrations at which the compounds displaced 50% of the specific binding (IC_{50}) were obtained from seven-point displacement curves using [^{3}H]-ketanserin as the radiolabel. Data are expressed as the mean ± s.e.mean of three to four independent determinations of the IC_{50} values.

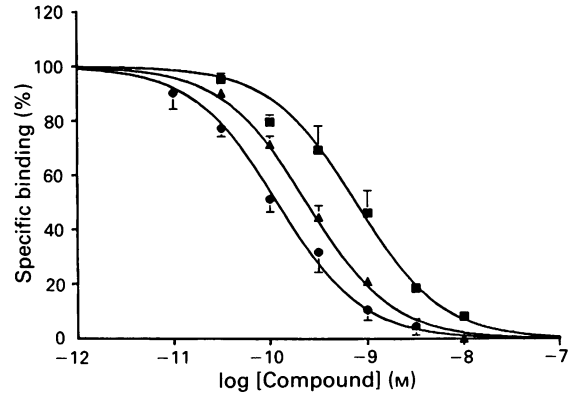


Figure 2 Displacement by RP 62203 (●), ritanserin (▲) and ICI 170,809 (■) of [^{3}H]-ketanserin binding to 5-HT_2 receptors in rat cortical membranes. Each point represents the mean of three independent determinations; s.e.mean shown by vertical bars. The curves represent the best-fit solutions to Langmuir isotherms generated by computer-assisted non-linear regression analysis using the IC_{50} values in Table 1. The Hill coefficients calculated by regression analysis of the corresponding Hill transformations were 0.93 (RP 62203), 1.10 (ritanserin) and 0.91 (ICI 170,809).

methylpropylthio)-3-phenylquinoline hydrochloride) were kindly supplied by Dr G.R. Birchall, I.C.I. Pharmaceuticals, Macclesfield.

Radioligands were purchased from Amersham, unless otherwise stated. Other compounds used were obtained from the following commercial sources: ritanserin, Janssen Life Sciences Products; noradrenaline bitartrate, sodium-L-glutamate, γ-aminobutyric acid (GABA), 5-hydroxytryptamine creatinine sulphate and 5-hydroxytryptophan, Sigma; 8-OH-DPAT (8-hydroxy-N,N-dipropyl-2-aminotetralin) and DOI (1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane), Research Biochemicals Incorporated.

Results

In vitro *binding studies*

RP 62203 potently inhibited the binding of [^{3}H]-ketanserin to 5-HT_2 receptors on rat frontocortical membranes with an $IC_{50} = 1.2 \times 10^{-10}$ M (Figure 2). Of all the compounds tested in this binding assay, RP 62203 is as yet the most potent detected (Table 1), being twice as active as ritanserin ($IC_{50} = 2.4 \times 10^{-10}$ M vs [^{3}H]-ketanserin) and six times more active than ICI 170,809 ($IC_{50} = 7.7 \times 10^{-10}$ M). ICI 169,369 was considerably less potent ($IC_{50} = 6.1 \times 10^{-8}$ M), in agreement with published data (Blackburn *et al.*, 1988a).

RP 62203 was less active at 5-HT_{1A} binding sites in the rat hippocampus, labelled with [^{3}H]-8-OH-DPAT ($IC_{50} = 7.4 \times 10^{-8}$ M) and displayed very low affinity ($IC_{50} > 10^{-6}$ M) for 5-HT_{1B} receptors in rat cortex labelled with [^{125}I]-iodocyanopindolol and for 5-HT_3 receptors in rat entorhinal cortex labelled with [^{3}H]-GR 65630 (Table 2). Neither ritanserin nor ICI 170,809 was active ($IC_{50} > 10^{-6}$ M) in these binding assays.

With respect to other monoamine receptors, RP 62203 displaced the binding of [^{3}H]-mepyramine to histamine H_1 receptors in the guinea-pig cerebellum with an affinity of 1.31×10^{-8} M. The affinities of ritanserin and ICI 170,809 in this assay were 2.24×10^{-8} M and 6.78×10^{-7} M respectively (Table 2). At dopamine D_2 receptors in the rat striatum labelled with [^{3}H]-spiperone, RP 62203 ($IC_{50} = 3.94 \times 10^{-7}$ M) and ICI 170,809 ($IC_{50} = 7.79 \times 10^{-7}$ M) displayed only very low affinity, whilst ritanserin ($IC_{50} = 3.5 \times 10^{-8}$ M) was much more active (Table 2). RP 62203 was most active in binding to α_1-adrenoceptors in the rat cortex labelled with [^{3}H]-prazosin ($IC_{50} = 4.33 \times 10^{-9}$ M). The affinities of ritanserin and ICI 170,809 at this binding site were 1.33×10^{-8} M and 9.23×10^{-8} M respectively (Table 2).

No displacement by RP 62203 of [^{3}H]-*p*-aminoclonidine binding to α_2-adrenoceptors or of [^{3}H]-dihydroalprenolol to β-adrenoceptors was observed in the rat cortex at a concentration of 10^{-6} M. The binding of [^{3}H]-quinuclidinyl benzoate ([^{3}H]-QNB) to muscarinic acetylcholine receptors in the rat cortex was not displaced by RP 62203 at a concentration of

Table 2 Receptor binding profile of RP 62203

Receptor	*Tissue source*	*Non-specific* (M)	*RP 62203*	IC_{50} (M) *ritanserin*	*ICI 170,809*
5-HT_2	Rat frontal cortex	Methysergide (10^{-6})	$1.2 \pm 0.3 \times 10^{-10}$	$2.4 \pm 0.3 \times 10^{-10}$	$7.7 \pm 2.0 \times 10^{-10}$
5-HT_{1A}	Rat hippocampus	5-HT (10^{-5})	$7.5 \pm 2.6 \times 10^{-8}$	55.5% at 10^{-6}*	NA 10^{-6}
5-HT_{1B}	Rat cortex	5-HT (10^{-5})	22.5% at 10^{-6}*	32.7% at 10^{-6}*	ND
5-HT_3	Rat entorhinal cortex	Ondansetron (10^{-6})	$6.6 \pm 2.0 \times 10^{-6}$	$2.5 \pm 1.1 \times 10^{-6}$	ND
α_1-Adrenoceptor	Rat cortex	Phentolamine (10^{-6})	$4.3 \pm 0.7 \times 10^{-9}$	$1.3 \pm 0.1 \times 10^{-8}$	$9.2 \pm 0.5 \times 10^{-8}$
α_2-Adrenoceptor	Rat cortex	Phentolamine (10^{-6})	NA 10^{-6}	$8.6 \pm 0.2 \times 10^{-8}$	ND
Dopamine D_2	Rat striatum	Sulpiride (10^{-5})	$3.9 \pm 0.6 \times 10^{-7}$	$3.5 \pm 0.4 \times 10^{-8}$	$7.8 \pm 0.8 \times 10^{-7}$
Histamine H_1	Guinea-pig cerebellum	Promethazine (10^{-5})	$5.0 \pm 0.6 \times 10^{-8}$	$4.2 \pm 0.2 \times 10^{-9}$	$4.8 \pm 0.2 \times 10^{-7}$
Muscarinic	Rat cortex	Atropine (10^{-6})	NA 10^{-6}	NA 10^{-6}	$1.1 \pm 0.4 \times 10^{-7}$

Data are expressed as the mean ± s.e.mean of three to four independent determinations of the IC_{50} values. NA: no displacement observed at the highest concentration tested (10^{-6} M). ND: not determined. * Percentage displacement seen at the indicated concentration. In addition, RP 62203 (10^{-6} M) did not displace radioligand binding to the following sites: benzodiazepine binding site ([^{3}H]-flunitrazepam), bradykinin receptors ([^{3}H]-bradykinin), CCK_A and CCK_B cholecystokinin receptors ([^{3}H]-pCCK$_8$), 5-HT uptake sites ([^{3}H]-paroxetine), N-methyl-D-aspartate receptors ([^{3}H]-TCP), neuropeptide Y receptors ([^{3}H]-neuropeptide Y), neurotensin receptors ([^{3}H]-neurotensin), opiate receptors ([^{3}H]-ethylketocyclazocine), somatostatin receptors ([^{125}I]-SRIF), tachykinin NK_1 receptors ([^{3}H]-substance P), vasoactive intestinal peptide receptors ([^{125}I]-VIP), calcium channels ([^{3}H]-desmethoxyverapamil) and sodium channels ([^{3}H]-batrachotoxin).

Table 3 Occupation of 5-HT$_2$ receptors *in vivo*

Antagonist	*Species*	ID_{50} (mg kg^{-1}) *p.o.*	*s.c.*	*i.p.*
RP 62203	Rat	0.39 ± 0.10	0.07 ± 0.04	ND
	Mouse	ND	0.06 ± 0.02	ND
Ritanserin	Rat	3.28 ± 0.90	0.58 ± 0.18	ND
	Mouse	ND	ND	0.10 ± 0.04

The ability of RP 62203 and ritanserin to inhibit the *in vivo* binding of [^{3}H]-spiperone (rats) or [^{3}H]-methylspiperone (mice) was evaluated as described in the Methods. ID_{50} values (with standard errors) were determined from five-point dose-response curves (0.02–20 mg kg^{-1}) obtained on groups of five animals per dose. ND: not determined.

10^{-6} M. RP 62203 was inactive in radioligand binding assays for a number of other neurotransmitter receptors.

The affinity of ICI 169,369 for receptors other than the 5-HT$_2$ receptor was not assessed, since this information is already published (Blackburn *et al.*, 1988a).

In vivo *binding studies*

In rats, RP 62203 was a potent inhibitor of specific [^{3}H]-spiperone binding in the frontal cortex (Table 3). When administered orally, the ID_{50} value for RP 62203 was 0.39 mg kg^{-1}, compared to 3.28 mg kg^{-1} for ritanserin. The activity of RP 62203 was also observed after subcutaneous administration (ID_{50} = 0.07 mg kg^{-1}). It is noteworthy that in neither case did RP 62203 modify the binding of [^{3}H]-spiperone to D_2 receptors in the striatum: binding in animals injected with RP 62203 (20 mg kg^{-1}) corresponded to 93.5% (p.o.) and 96.7% (s.c.) of the binding seen in vehicle-injected controls.

Occupation of 5-HT$_2$ receptors by RP 62203 was long-lasting, substantial inhibition of [^{3}H]-spiperone binding still being observed 6 h following treatment (ID_{50} = 0.78 mg kg^{-1}, p.o. and 0.33 mg kg^{-1}, s.c.).

Similar findings were obtained in mice, where RP 62203 and ritanserin also potently inhibited specific [^{3}H]-methylspiperone binding in the frontal cortex, yielding ID_{50} values of 0.06 mg kg^{-1}, s.c. and 0.102 mg kg^{-1}, i.p. respectively. Again, RP 62203 did not modify the binding of [^{3}H]-methylspiperone to D_2 dopamine receptors in the striatum (binding in animals injected with 2.5 mg kg^{-1} s.c. corresponded to 103% of that seen in vehicle-injected controls).

Effects on 5-hydroxytryptamine-stimulated inositol phosphate formation

Incubation of neonatal rat cortical slices with 5-HT (10^{-5} M) led to a two fold increase in the accumulation of inositol phosphate. This effect was also seen with the selective 5-HT$_2$ receptor agonist, DOI, but not with the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT (data not shown).

RP 62203 potently antagonized the stimulatory effect of 5-HT, with an IC_{50} of 7.76 ± 2.09 × 10^{-9} M. A similar activity

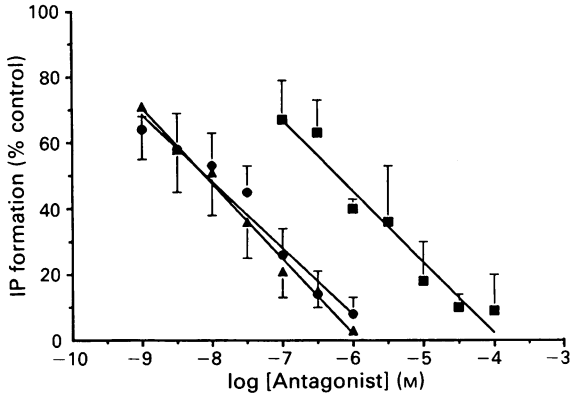


Figure 3 Antagonism by RP 62203 (●), ritanserin (▲) and ICI 170,809 (■) of 5-hydroxytryptamine (5-HT)-stimulated inositol phosphate (IP) formation in slices of neonatal rat cortex. The data are presented as a percentage of the maximal 5-HT-evoked response (214 ± 9% of basal IP formation) and represent the mean of three (ICI 170,809) or four (RP 62203 and ritanserin) independent determinations; s.e.mean shown by vertical bars.

was observed for ritanserin (IC_{50} = 7.58 ± 1.32 × 10^{-9} M), whilst ICI 170,809 was much less active (IC_{50} = 5.89 ± 1.55 × 10^{-7} M) (Figure 3). RP 62203 (10^{-6} M) also antagonised inositol phosphate formation stimulated by 10^{-4} M DOI (66 ± 3% inhibition, n = 3).

RP 62203 seemed to be a selective antagonist of the 5-HT$_2$ receptor-mediated response (Table 4), since it had little effect on either basal or carbachol-stimulated inositol phosphate (IP) accumulation. In the guinea-pig cerebellum, IP formation stimulated by histamine (10^{-4} M) was only weakly inhibited by RP 62203 (35% inhibition at 10^{-6} M). High concentrations (IC_{50} = 1.48 ± 0.13 × 10^{-6} M) of RP 62203 were needed to antagonize noradrenaline-stimulated IP formation in primary cultures of mouse astrocytes, compared to the reference α_1-receptor antagonist, prazosin (IC_{50} = 6.20 ± 1.33 × 10^{-8} M).

Electrophysiological recordings in the raphé nuclei

Spontaneously active neurones in the raphé nuclei typically fired short duration action potentials at frequencies of 5–20 Hz. Cells giving reproducible responses to microiontophoretically applied 5-HT (50–100 nA for 30–60 s) were used for the antagonist studies. Changes in activity following drug administration were expressed as a percentage of the control firing rate of the cell. Histological examination of the location of the PSB dye spot confirmed correct electrode placement in all studies.

The effects of RP 62203 were studied on 29 cells affected by 5-HT. RP 62203 was administered intraperitoneally in physiological saline after dissolving the compound in 1% Tween vehicle. Injection time was 1 ml min^{-1} and in a volume of not more than 1 ml kg^{-1} at pH 6. In 3 control injections, no

Table 4 Effect of RP 62203 on monoamine-stimulated inositol phosphate formation

Agonist		*Tissue*	IC_{50} (M)
None		Rat cortex	9.5 ± 2.9%*
5-Hydroxytryptamine	10^{-5} M	Rat cortex	7.8 ± 2.1 × 10^{-8}
Carbachol	10^{-4} M	Rat cortex	24.4 ± 11.1%*
Histamine	10^{-4} M	Guinea-pig cerebellum	39.1 ± 16.1%*
Noradrenaline	10^{-5} M	Mouse astrocytes	1.5 ± 0.2 × 10^{-6}

The indicated tissues and agonists were used as described in the Methods. Data are expressed as the mean ± s.e.mean of four independent determinations.

* Percentage displacement seen at a concentration of 10^{-6} M.

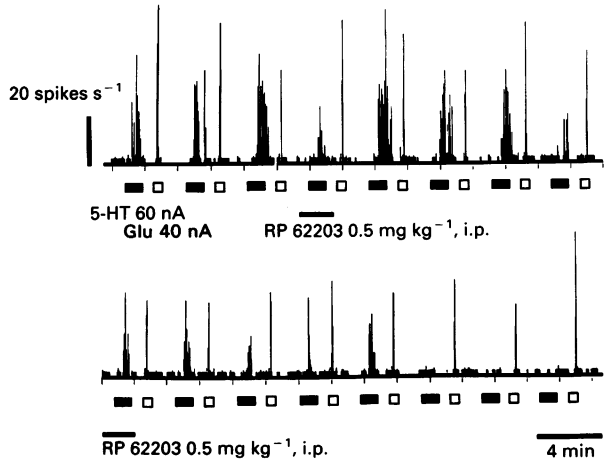


Figure 4 Antagonism of 5-HT_2 receptor-mediated responses in raphé neurones. Excitatory responses of a median raphé neurone to microiontophoretically applied 5-hydroxytryptamine (5-HT, 60 nA, 40 s) and glutamate (Glu; 40 nA, 30 s). RP 62203 was administered as a cumulative dose of 1.0 mg kg^{-1}, i.p. at the times indicated by the horizontal bars. The diminution of the neuronal responses to 5-HT is clear, whereas the responses to the control agonist, glutamate, were not antagonized by RP 62203.

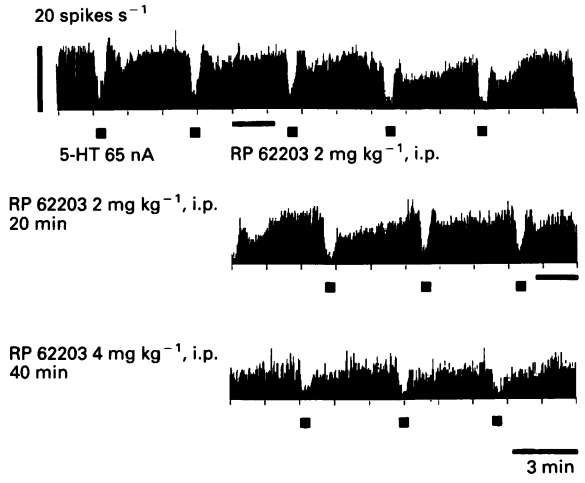


Figure 5 The lack of effect of RP 62203 at neuronal 5-HT_1-like receptors. Microiontophoretically applied 5-hydroxytryptamine (5-HT, 65 nA, 50 s) evoked depressions of dorsal raphé neuronal firing which were not blocked by a cumulative dose of RP 62203 (6.0 mg kg^{-1}, i.p.) administered at the times indicated by the horizontal bars.

effects of vehicle alone were seen on the rate of firing of recorded neurones.

In all studies, RP 62203 depressed and eventually blocked excitatory responses to microiontophoretically applied 5-HT in a dose-dependent manner at doses of 0.1–4.0 mg kg^{-1}, i.p. ($n = 21$, e.g. Figure 4). At a dose of 0.5 mg kg^{-1}, i.p., RP 62203 reduced 5-HT responses by 57.5 ± 6.6%, and at 1.0 mg kg^{-1}, i.p., the responses were blocked completely. The effects of RP 62203 were long lasting and recovery from the effects of RP 62203 was not observed within a period up to 2 h after administration of the compound. In 5 studies, glutamate (20–40 nA, 10–20 s) was cycled with 5-HT as the control agonist to examine the specificity of the antagonist. RP 62203 was administered at doses of 0.5–2.0 mg kg^{-1}, i.p. The excitatory responses to 5-HT were potently depressed and eventually blocked at these doses, whereas responses to glutamate remained. In 5 studies, gutamate was cycled alone on cells that responded to 5-HT. RP 62203 at higher doses of 6.0 mg kg^{-1}, i.p. did not affect the responses to glutamate.

Eight neurones were studied in which 5-HT depressed their activity. This effect has been correlated with the activation of a 5-HT_1-like receptor. RP 62203 had no effect at this receptor when injected at doses from 2.0 to 8.0 mg kg^{-1}, i.p. (e.g. Figure 5). In 3 of the studies, 5-HT and GABA (10–20 nA for 5–10 s) were cycled together. Inhibitory responses to these two transmitters were not blocked by RP 62203 at doses as high as 8.0 mg kg^{-1} i.p. However, it was observed that at this high dose, RP 62203 decreased the firing rate of the recorded neurone.

Ritanserin ($n = 5$) and ICI 169,369 ($n = 4$) also depressed 5-HT-evoked excitations. Ritanserin had similar activity to RP 62203, depressing responses to 5-HT by 71.8 ± 6% at 0.5 mg kg^{-1}, i.p. whereas ICI 169,369 was less potent, depressing 5-HT-evoked firing by 41.3 ± 6% at 2.0 mg kg^{-1}, i.p. (Figure 6). With neither antagonist was a recovery from the effects seen.

In some studies in which microiontophoretically applied glutamate was cycled with 5-HT as the control agonist, an increase in the amplitude of the glutamate response accompanied the antagonism of the excitatory response to 5-HT. An illustration of this effect is shown in Figure 4.

All three compounds were found to depress action potential amplitude by approximately 5% of control amplitudes in at least some studies. Since the amplitude was carefully monitored throughout counting, on no occasion did the decrease in spike amplitude introduce an error into the recording of the correct frequency of firing.

Arterial blood pressure was not measured during these studies. Administration of RP 62203 at the doses studied had no visible effect on respiration and resulted in no change of the recorded temperature of the animal. These observations were supported by data obtained from experiments carried out in rats in which administration of RP 62203 at doses of 30 mg kg^{-1}, p.o. did not have any effect on arterial blood pressure (S. Mondot, personal communication).

Effects on 5-hydroxytryptamine agonist-induced head-twitches

The effects of RP 62203 on 5-HT agonist-induced head-twitches are presented in Table 5. RP 62203 effectively abolished 5-HTP-induced head-twitches in the mouse after oral and subcutaneous administration ($ED_{50} = 1.27$ mg kg^{-1} and 0.17 mg kg^{-1} respectively). The values obtained for ritanserin were 0.17 mg kg^{-1}, p.o. and 0.15 mg kg^{-1}, s.c.

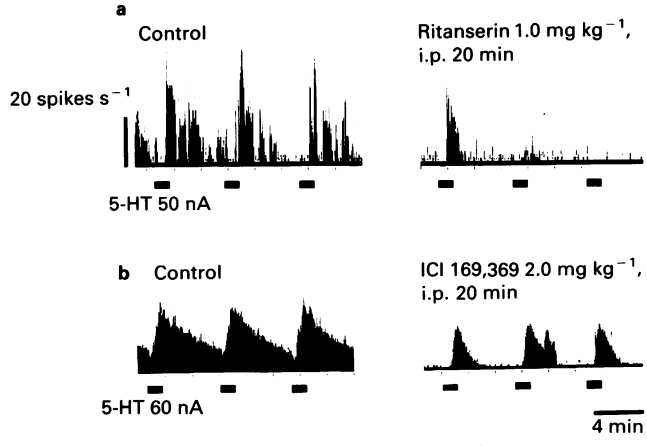


Figure 6 The effect of (a) ritanserin (1.0 mg kg^{-1}, i.p.) and (b) ICI 169,369 (2.0 mg kg^{-1}, i.p.) on excitatory responses to microiontophoretically applied 5-hydroxytryptamine (5-HT). Antagonists were administered after three control applications of 5-HT.

Table 5 Antagonism by RP 62203, ritanserin and ICI 170,809 of head-twitches in rats and mice

		ED_{50} (mg kg^{-1})				
		5-HTP	*Mescaline*		*DOI*	
Antagonist	*Route*	mice	mice	rats	mice	rats
RP 62203	p.o.	1.27 (0.55–2.95)	0.43 (0.08–2.28)	0.99 (0.63–1.55)	0.44 (0.24–0.78)	1.54 (0.85–2.78)
	s.c.	0.17 (0.09–0.34)	0.16 (0.08–0.34)	0.65 (0.38–1.12)	0.04 (0.03–0.07)	1.82 (0.90–3.65)
Ritanserin	p.o.	0.17 (0.08–0.28)	0.09 (0.04–0.23)	2.55 (1.22–5.33)	0.08 (0.05–0.13)	2.59 (1.52–4.40)
	s.c.	0.15 (0.08–0.28)	0.29 (0.08–1.07)	0.38 (0.24–0.62)	0.05 (0.04–0.08)	1.54 (0.76–3.12)
ICI 170,809	p.o.	ND	ND	>20	1.78 (1.27–2.40)	>20

The data represent the ED_{50} values (5–95% confidence limits) calculated from four-point dose-response curves, obtained in groups of six to eight animals per dose. ND: not determined.
5-HTP: 5-hydroxytryptophan; DOI: 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane.

In the case of mescaline-induced head-twitches, protection was observed with RP 62203 in both mice (ED_{50} = 0.43 mg kg^{-1}, p.o. and 0.17 mg kg^{-1}, s.c.) and rats (ED_{50} = 0.99 mg kg^{-1}, p.o. and 0.65 mg kg^{-1}, s.c.). The duration of this effect was examined in rats: no decrease in protection was observed after 6 h, and effects could still be seen 16 h after administration of RP 62203 (Table 6).

When head-twitches were evoked by the more selective 5-HT$_2$ receptor agonist DOI, these were blocked by RP 62203 in both mice (ED_{50} = 0.44 mg kg^{-1}, p.o. and 0.04 mg kg^{-1}, s.c.) and rats (ED_{50} = 0.05 mg kg^{-1}, p.o. and 1.82 mg kg^{-1}, s.c.) at doses similar to those for ritanserin, whereas ICI 170,809 (ED_{50} = 1.78 mg kg^{-1}, p.o. in mice) was less active (Figure 7). This latter compound was considerably less potent in rats.

Effects on dopamine- and noradrenaline-mediated responses

RP 62203 did not affect amphetamine-induced stereotypies in rats up to the highest dose tested (93.4% of controls at 40 mg kg^{-1}, p.o.), suggesting that this compound does not interact with dopamine receptor function *in vivo*. Furthermore, in two tests of adrenergic function in mice, tetrabenazine-induced ptosis (83.4% of controls at 40 mg kg^{-1}, p.o.) and noradrenaline-induced lethality (ED_{50} > 40 mg kg^{-1}, s.c.), RP 62203 was inactive.

Discussion

These studies reveal the extremely potent 5-HT$_2$ receptor antagonist properties of RP 62203 both *in vitro* and *in vivo*. In radioligand binding studies, RP 62203 showed the highest affinity for 5-HT$_2$ receptors labelled with [^{3}H]-ketanserin of all compounds tested (K_i = 50 pM). Similarly, *in vivo* binding studies demonstrated occupation of central 5-HT$_2$ receptors following administration of low doses of RP 62203 (< 1.0 mg kg^{-1}, p.o.), suggesting good penetration of RP 62203 into the brain following oral administration. Moreover, receptor occupation could still be observed 6 h after treatment.

In functional tests of 5-HT$_2$ receptor activity, RP 62203 showed potent 5-HT receptor antagonist properties. For example, antagonism of 5-HT-stimulated inositol phosphate formation was observed in the nanomolar range (IC_{50} = 7.76 nM). RP 62203 was equiactive with ritanserin, but considerably more potent than ICI 170,809.

Similarly, 5-HT$_2$ receptor antagonist activity could be demonstrated *in vivo*: in the raphé nuclei, the electrophysiological data clearly illustrate that RP 62203 can block central effects of 5-HT that are mediated by 5-HT$_2$ receptors. The absence of activity at other receptors mediating neuronal excitation, such as the excitatory amino acid receptors studied in these experiments, confirms the ability of the compound to discriminate between different excitatory receptors on the same neurone. This demonstrates that RP 62203 does not block 5-HT-evoked firing by a general depressant effect on neuronal activity. This observation is supported by other results obtained in the cortex, where similar doses of RP 62203 can block postsynaptic 5-HT$_2$ receptors, but not dopamine receptors (Godbout *et al.*, 1992). The two reference compounds, ritanserin and ICI 160,369 also blocked 5-HT$_2$ receptors in the raphé. As seen with RP 62203, a slight decrease

Table 6 Antagonism by RP 62203 and ritanserin of mescaline-evoked head-twitches in rats: duration of action

	Time	ED_{50} (mg kg^{-1})	
Antagonist	(h)	p.o.	s.c.
RP 62203	1	0.99 (0.63–1.55)	0.65 (0.38–1.12)
	6	2.38 (1.08–5.25)	0.45 (0.22–0.89)
	16	14.2 (10.7–18.8)	5.91 (8.06–11.4)
Ritanserin	1	2.55 (1.22–5.33)	0.38 (0.24–0.62)
	6	1.54 (0.74–3.20)	0.55 (0.34–0.93)
	16	18.4 (9.17–37.0)	>5

The data represent the ED_{50} values (5–95% confidence limits) calculated from four-point dose-response curves, obtained on groups of six rats per dose.

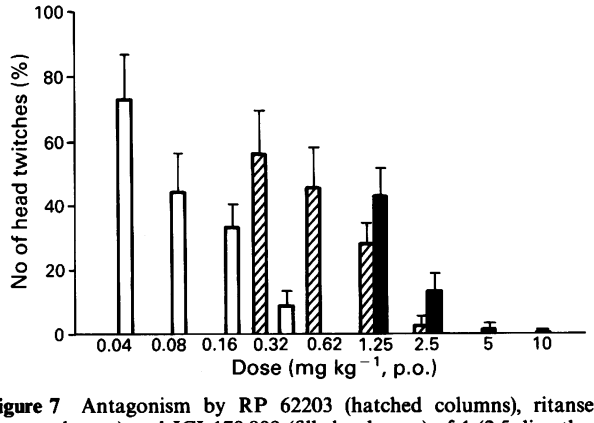


Figure 7 Antagonism by RP 62203 (hatched columns), ritanserin (open columns) and ICI 170,809 (filled columns) of 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI)-evoked head-twitches in mice. The data are presented as a percentage of the number of head-twitches observed in control animals (6.29 ± 0.44) and represent the values (mean with s.e.mean shown by vertical bars) obtained in groups of eight mice.

(<5%) in action potential amplitude was seen in some cells after administration of the antagonists; this was not, however, seen in all cases and was not correlated with the dose administered. A possible explanation of this effect could be a local anaesthetic action of the compounds, although this cannot be concluded with certainty. In addition, with high doses of RP 62203 a variable degree of depression of the spontaneous activity of recorded neurones was seen in both regions of study. Since this effect was seen in a heterogeneous population of cells and the pharmacological basis of the effect was not explored, the mechanism underlying this effect cannot at present be proposed. It will be interesting to see if this effect is retained *in vitro*.

Electrophysiological evidence for ritanserin potency has already been published (Uego *et al.*, 1989) and the doses used by these authors (0.5–2.0 mg kg^{-1}, i.v.) agree well with our findings. The effects of ICI 169,369 on neuronal firing are however, reported here for the first time. This compound also displayed 5-HT$_2$ receptor antagonist activity in the raphé, although at higher doses. The relative potencies of the three compounds were ritanserin > RP 62203 > ICI 169,369.

A behavioural consequence of central 5-HT$_2$ receptor activation is the head-twitch syndrome induced by 5-HT$_2$ receptor agonists (Goodwin & Green, 1985). In such tests, RP 62203 antagonized head-twitches induced by 5-HTP, mescaline and DOI in both rats and mice at doses comparable to those active in the *in vivo* binding studies. Again, RP 62203 showed comparable activity to ritanserin in rats, whilst being somewhat less potent following oral administration in mice; ICI 170,809 was once again less active. The effects of RP 62203 are long-lasting, no loss of activity being observed 6 h after administration. These results are in agreement with the data obtained in *in vivo* binding studies.

A major drawback with currently available 5-HT$_2$ receptor antagonists is their lack of selectivity for this receptor subtype (Fozard, 1987; Hoyer, 1988; Canton *et al.*, 1990). In this respect, RP 62203 is relatively selective for 5-HT$_2$ receptors compared to other 5-HT receptor sub-types. In binding studies, its affinity for 5-HT$_{1A}$ receptors, although not negligible (IC_{50} = 75 nM), is more than five hundred times lower than that at 5-HT$_2$ receptors. This selectivity was also confirmed in electrophysiological experiments *in vivo*. In the dorsal raphé nuclei, where 5-HT depresses neuronal activity by activation of a 5-HT$_{1A}$ receptor (Lum & Piercey, 1988), RP 62203 did not block 5-HT-evoked depressions at doses as high as 8.0 mg kg^{-1}, i.p.

The activity of RP 62203 at 5-HT$_{1C}$ receptors in the choroid plexus has been evaluated by [^{3}H]-mesulergine autoradiography (IC_{50} = 35 nM, compared to 0.1 nM for cortical 5-HT$_2$ receptors in the same slices); these data will be published elsewhere. It should be noted that neither ritanserin nor ICI 169,369 (Blackburn *et al.*, 1990) discriminate between 5-HT$_2$ and 5-HT$_{1C}$ receptors. Like other known 5-HT$_2$ antagonists (Hoyer *et al.*, 1985), RP 62203 had very low (IC_{50} > 10^{-6} M) affinity for 5-HT$_{1B}$ receptors in rat cerebral cortex.

Binding studies revealed that RP 62203 displayed significant activity at α_1-adrenoceptors (IC_{50} = 4.3 nM); this selectivity ratio of 40 compared to 5-HT$_2$ receptors is of the same order as that for ritanserin. Antagonism of noradrenaline-stimulated IP formation was seen at concentrations 150 times higher than those needed to antagonize 5-HT-stimulated IP formation. However, the consequences of this affinity *in vivo* seem to be minimal, since neither noradrenaline-induced lethality nor tetrabenazine-induced ptosis were affected. Furthermore, autoradiographic studies performed *ex vivo* have failed to demonstrate occupation of α_1-adrenoceptors at the highest dose tested (10 mg kg^{-1}, i.p.) (Malgouris *et al.*, 1990). Overall, the selectivity profile of RP 62203 in binding studies was superior to that of ritanserin, but this compound was less selective than ICI 170,809 (Blackburn *et al.*, 1988b) and ICI 169,369 (Blackburn *et al.*, 1988a).

Since very little is known about the role of particular 5-hydroxytryptaminergic pathways in pathological states, nor about the receptor sub-types activated at individual synapses, the high *in vivo* potency and selectivity of RP 62203 would make it an ideal tool with which to explore such mechanisms.

In the clinic, selective 5-HT$_2$ receptor antagonists have been proposed to have therapeutic potential in the treatment of dysthymic disorders; indeed, ritanserin has been studied in certain forms of anxiety and depression, as well as in sleep disorders (Idzikowsky *et al.*, 1986; Paiva *et al.*, 1988), and, in experimental animals, has been shown to modify sleep cycles (Dugovic & Wauquier, 1987) and to be active in certain models of anxiety (Critchley & Handley, 1987). The superior selectivity of RP 62203 compared to ritanserin makes it a promising candidate for the treatment of these disorders.

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Changes in adrenoceptors and monoamine metabolism in neonatal and adult rat brain after postnatal exposure to the antihypertensive labetalol

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1 The purpose of the present study was to investigate the acute (single injection), direct (chronic treatment) and the long-lasting effects after exposure to the α_1/β-adrenoceptor antagonist labetalol during rat brain development on adrenoceptors and monoamine metabolism.

2 In 10-day-old rat pups, subcutaneously administered labetalol ($10\,mg\,kg^{-1}$) passed the blood-brain barrier, reaching a level of $2.1\,\mu g\,g^{-1}$ tissue in the brain 90 min after injection.

3 Chronic labetalol treatment ($10\,mg\,kg^{-1}$, s.c., twice daily) during the first 10 days of life significantly increased α_1-adrenoceptor binding in the hypothalamus (+39%), but not in the occipital cortex.

4 This chronic postnatal labetalol treatment did not result in long-lasting changes in α_1- and β-receptors measured on day 60.

5 A single labetalol injection ($10\,mg\,kg^{-1}$, s.c.) on postnatal day 10 significantly increased noradrenaline (NA) metabolism in all brain regions tested (+25 to 105%), but had no effects on 5-hydroxytryptamine (5-HT) or dopamine metabolism.

6 Chronic labetalol treatment between postnatal (PN) days 1 and 10 also increased NA metabolism on PN 10 (3-methoxy-4-hydroxyphenylglycol (MHPG)/NA, +20 to 100%), suggesting that tolerance to the acute effect of labetalol did not occur. A slight increase in 5-HT metabolism (20%) was induced by the chronic labetalol treatment in the hippocampus and meso-limbic system.

7 In general, long-lasting effects on NA metabolism could not be detected on day 60, more than one month after the treatment. However, 5-HT metabolism was significantly increased in all four brain regions measured (+20 to 70%).

8 We conclude that chronic labetalol exposure during early postnatal rat brain development does not cause long-lasting changes in β-receptor number or NA metabolism, but appears to be critical for the rate of 5-HT metabolism in later life.

Keywords: Labetalol; propranolol; β-receptor subtypes; α_1-receptor; monoamines; noradrenaline metabolism; brain development

Introduction

Labetalol is widely used for management of essential hypertension or hypertension associated with toxaemia in pregnancy (Michael, 1979; Goa *et al.*, 1989; Sibai *et al.*, 1990). The antihypertensive action of labetalol is considered to be due to its dual α- and β-adrenoceptor antagonism (Brittain & Levy, 1976; Aggerbeck *et al.*, 1978). Labetalol has been regarded as safe for the foetus during pregnancy since treatment does not significantly interfere with foetal heart rate or blood flow (Woods & Malan, 1983; Jouppila *et al.*, 1986; MacPherson *et al.*, 1986). However, labetalol has been demonstrated to cross the placenta (Michael, 1979; Lardoux *et al.*, 1983; Haraldsson & Geven, 1989) and, as is pointed out in the present study, can also pass the blood-brain barrier and subsequently interact with the maturing noradrenergic system.

In the human foetal central nervous system, noradrenaline (NA) can be detected at 12 weeks of gestation (Nobin & Björklund, 1973), while β-adrenoceptor binding is first demonstrated between 15 and 21 weeks of gestation (Sogos *et al.*, 1989). Therefore, the second half of human pregnancy might be a sensitive period for the induction of neuroteratological effects by therapy with drugs acting on adrenoceptors. The overall stage of development of the monoamine neurone system in the 3- to 5-month-old foetuses can roughly be compared to that of the rat in the first week after birth (Nobin & Björklund, 1973; Olson *et al.*, 1973), because the rat brain noradrenergic system matures primarily after birth (Coyle, 1977; Berger & Verney, 1984). The α_1- and β-receptor densities in the newborn rat are only about 20% and 10%, respectively, of the adult value and increase to adult densities within three weeks (Morris *et al.*, 1980; Bruinink & Lichtensteiger, 1984; Erdtsieck-Ernste *et al.*, 1991b). The regional NA levels develop more gradually and approach their adult levels between 5–10 weeks after birth (Kohno *et al.*, 1982).

Literature on animal studies indicates that perinatal drug exposure could have a continuum of effects on the foetus or newborn from long-lasting behavioural or functional alterations to mortality, depending on the dose and the time of exposure (Vorhees, 1986). In rats, blockade or activation of noradrenaline receptors during development has been shown to induce long-lasting changes in the density of receptors and in transmitter release and metabolism (Del Rio *et al.*, 1988; Miller & Friedhoff, 1988; Boer *et al.*, 1990; Erdtsieck-Ernste *et al.*, 1991a; Feenstra *et al.*, 1991). Accordingly, the present study addressed the question as to whether such neuroteratological changes in noradrenergic transmission could be induced by labetalol exposure during brain development.

Therefore, we investigated (1) whether a single injection with labetalol in 10-day-old rat pups affected NA metabolism, (2) whether labetalol treatment between postnatal days (PN) 1 and 10 resulted in adaptational changes in adrenoceptor number and NA metabolism, and (3) whether labetalol treatment resulted in changes in the number of the adult adrenoceptors and in monoamine metabolism. The treatment period of PN 1–10 was also chosen on the basis of previous studies in

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our laboratory. Chronic propranolol treatment during several stages of brain development has shown that rat pups are especially sensitive to the induction of long-lasting changes in NA metabolism in the first ten days after birth (Erdtsieck-Ernste *et al.*, 1991a and unpublished observations).

Methods

Acute and chronic drug treatment

Pregnant Wistar rats, mated in our animal house, were kept under controlled conditions with free access to food and water. The pups were born on the 21st or 22nd day of pregnancy and the day of birth was defined as PN 0. In each experiment we used 4–6 litters consisting of 6–8 male pups randomly assigned to the nursing mothers just after birth. The pups were weighed daily.

In the first experiment, sets of two pups (PN 10) of each litter received either a single subcutaneous injection of $10\,mg\,kg^{-1}$ labetalol HCl or $15\,mg\,kg^{-1}$ (±)-propranolol HCl, both dissolved in physiological saline, or received the vehicle only. The pups were decapitated 90 min after injection to determine brain labetalol concentrations and acute effects on monoamine metabolism. The labetalol dosage of $10\,mg\,kg^{-1}$ was chosen because in adult rats this dose completely antagonized the positive chronotropic response (increase in heart rate) to the β-agonist isoprenaline, whereas the vasopressor response (increase in diastolic blood pressure) to the α-adrenoceptor agonist, phenylephrine was antagonized by 70% (Brittain & Levy, 1976).

In two other experiments, 3–4 rat pups per litter were injected subcutaneously twice daily (8 h 00 min and 17 h 00 min) with $10\,mg\,kg^{-1}$ labetalol HCl from PN 1 to PN 10 or treated as saline controls. The rat pups of the first chronic treatment group (four litters) were used to study the direct effects. Labetalol concentration and monoamine metabolism were determined 90 min after the last morning injection on PN 10, and α_1-receptors 20 h after the last evening injection on PN 9. Rats of the second chronic treatment group survived after the labetalol treatment and were killed on PN 60 in order to establish long-term effects.

After decapitation, several brain regions (frontal cortex, hippocampus, medulla pons, occipital cortex, hypothalamus and mesolimbic system, i.e., septal area, olfactory tubercle and nucleus accumbens) were quickly dissected and frozen on dry ice (Feenstra *et al.*, 1990). Tissue samples were always stored at −80°C until they were used in the further assay procedures.

Labetalol determination

Brain labetalol concentration was measured in the occipital cortex by means of high performance liquid chromatography (h.p.l.c.) with ultraviolet detection (u.v.) detection (Botterblom & Feenstra, unpublished). Tissue samples (approx. 65 mg) were homogenized in 40 volumes 0.4 M perchloric acid (PCA), to which propranolol ($60\,ng\,ml^{-1}$ PCA) was added as an internal standard. The homogenate was centrifuged for 15 min at 3000 *g*. In a polypropylene-capped tube, 1.0 ml of the supernatant was mixed with 10 µl 10 M sodium hydroxide, 350 µl carbonate buffer (1.07 M $NaHCO_3$, 0.23 M K_2CO_3; pH = 9.0) and 8 ml diethylether. The samples were shaken for 45 min and centrifuged for 8 min at 2000 *g*. The organic phase was transferred to a polypropylene tube containing 200 µl 0.2 M sulphuric acid. Labetalol and propranolol were then back-extracted by the same shaking and centrifugation steps. The organic phase was discarded and the tube was placed in a water bath (60 min, 45°C) to evaporate the remaining diethylether.

For chromatographic separation an aliquot of 50 µl extract was injected on a Supelco LC-18-DB column (250 mm × 4.6 mm; with guard column) and eluted at $1.0\,ml\,min^{-1}$ by acetonitrile/0.05 M NaH_2PO_4/triethylamine (35:65:0.1 v/v) adjusted to pH = 3.0 with H_3PO_4. Chromatographic analysis was performed at 40°C and monitored by means of a Hewlett Packard 1090 u.v. detector at 230 nm.

α_1-Adrenoceptor binding assay

Binding of [^{3}H]-prazosine ([^{3}H]-Praz; NEN, $25\,Ci\,mmol^{-1}$) to α_1-adrenoceptors was assayed according to the method of Dausse *et al.* (1982) with minor modifications. In brief, brain areas (hypothalamus and occipital cortex) were homogenized by means of a glass/Teflon homogenizer (10 times 1500 r.p.m.) in 20–30 volumes of ice-cold 50 mM Tris-HCl (pH = 7.4 at 4°C), containing 1.0 mM $MgCl_2$, 0.25 M sucrose and 0.05% ascorbic acid. The homogenate was centrifuged for 20 min at 49,000 *g* (4°C). The pellet was resuspended by gentle homogenization (8 times 400 r.p.m.) in 50 mM Tris-HCl (pH = 7.4 at 30°C), containing 10 mM $MgCl_2$ and 0.05% ascorbic acid, and diluted to $25\,mg\,ml^{-1}$ original tissue. Incubations containing 100 µl of the membrane suspension and [^{3}H]-Praz (0.5 nM) in a final volume of 350 µl were carried out in triplicate for 35 min at 30°C. Non-saturable binding was defined in the presence of 10 µM phentolamine. Incubation was stopped by adding 2 ml washing buffer (50 mM Tris-HCl, 10 mM $MgCl_2$ and 0.05% ascorbic acid, pH = 7.4 at 4°C) followed by rapid automatic filtration (Skatron Cell Harvester) through glass fibre filters (Schleicher & Schuell 3362) that had been presoaked for 2 h in 0.2% bovine serum albumin and subsequently dried. The filters were washed with 6 ml buffer and the radioactivity remaining on the filter was counted in a liquid scintillation counter (Tri-carb 2200CA, 'Ultima Gold' liquid scintillation cocktail) at about 65% counting efficiency.

β-Adrenoceptor binding assay

β-Receptor binding was measured with [^{125}I]-cyanopindolol ([^{125}I]-Cyp; NEN, $2200\,Ci\,mmol^{-1}$) binding assay as described previously (Erdtsieck-Ernste *et al.*, 1991b). In short, PN 60 brain tissue samples (meso-limbic system and hippocampus) were homogenized and centrifuged at 500 *g* for 5 min. The supernatant was diluted to a concentration of 3 mg original wet tissue per ml. An aliquot of 100 µl of this crude membrane suspension was incubated for 30 min at 37°C with [^{125}I]-Cyp (approx. 90 pM) in a final volume of 350 µl buffer (50 mM Tris-HCl, 145 mM NaCl, 5 mM $MgCl_2$; pH = 7.4 at 37°C). All incubations were carried out in the presence 2 µM of 5-HT for specific measurements of the total β-adrenoceptor binding sites, and the monoamine-oxidase inhibitor, pargyline (10 µM) in order to prevent metabolism of 5-HT. The non-saturable binding was determined with 5 µM timolol. Specific β_1-receptor binding is defined as total [^{125}I]-Cyp binding minus binding obtained in the presence 86 nM of the β_1-selective antagonist ICI-89406 (Engel *et al.*, 1981). The specific β_2-binding was determined as the [^{125}I]-Cyp binding in the presence of ICI-89406 minus the non-saturable binding. Incubation was stopped by rapid automatic filtration (Skatron Cell Harvester) through a glass fibre filter (Schleicher & Schuell 3362). The filters were counted in a Packard gamma spectrometer (Cobra 5005) at 82% counting efficiency. The binding assays were performed so that bound ligand was less than 10% of the added ligand and specific binding was a linear function of the protein concentration.

Protein content determination

The protein concentration was determined in an aliquot of the membrane homogenate used in the receptor binding assays by the method of Lowry *et al.* (1951), with bovine serum albumin used as a standard.

Monoamine determination

Monoamines and metabolites were determined in several brain areas (frontal cortex, hippocampus, meso-limbic system,

medulla-pons) as described by Erdtsieck-Ernste *et al.* (1991a). In this procedure, tissue samples were homogenized and the monoamines and metabolites were separated by means of small G10 columns in three fractions, which contained, respectively: (i) free 3-methoxy-4-hydroxyphenylglycol (MHPG); (ii) noradrenaline (NA) and dopamine; (iii) 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine (5-HT), 5-hydroxyindolacetic acid (5-HIAA) and homovanillic acid (HVA).

Samples (250 μl) of each fraction were injected on a reversed phase h.p.l.c. column with amperometric detection.

Statistical analysis

In order to estimate the effects of chronic labetalol treatment on noradrenaline receptors, we have used single point determinations in the individual tissue samples of each rat (receptor binding at one radioligand concentration). Additionally, the equilibrium dissociation constant (K_d) and the total number of binding sites (B_{max}) in a single saturation experiment (6–200 pM [^{125}I]-Cyp or 0.03–1.0 nM [^{3}H]-Praz; 8–10 points) with a pooled homogenate were calculated by the LIGAND computer programme (Munson & Rodbard, 1980).

Significant differences between the chronic saline- and the labetalol-treated group in the body and brain weight, receptor binding, monoamines and their metabolites were determined by Student's unpaired t test (two-tailed). After the acute treatment, in which both propranolol and labetalol were compared with the saline-treated group, the Dunnett's unpaired t-test was used. A P value <0.05 was considered to be significant.

Drugs

The following drugs were kindly donated: ICI 89406 by Imperial Chemical Industries, Macclesfield U.K., and phentolamine by Ciba-Geigy, Arnhem, The Netherlands. Labetalol and (±)-propranolol were purchased from Sigma, [^{3}H]-Praz and (−)-[^{125}I]-Cyp from New England Nuclear Corp. (Boston, MA, U.S.A.). Other reagents were commercially obtained.

Results

Body and brain weights

Chronic postnatal exposure to labetalol significantly reduced the body weight on the last three days of the treatment (−6%, $n = 32$). However, within a week after stopping the treatment the body weights had caught up to those of controls. The labetalol treatment had no significant detectable effects on adult body and brain weights (data not shown).

Labetalol concentrations

Ninety minutes after a single injection of labetalol on PN 10, occipital cortex tissue contained $2.13 \pm 0.18\,\mu g\,g^{-1}$ of the drug (average ± s.e.mean, $n = 8$). Repeated treatment from PN 1–10 resulted in a 56% higher labetalol concentration ($3.32 \pm 0.16\,\mu g\,g^{-1}$) as compared with the single injection.

Direct and long-term effects on α_1- and β-receptors

At PN 10, 20 h after the last evening injection of labetalol, the α_1-receptor binding at 500 pM [^{3}H]-Praz was significantly increased (38%) in the hypothalamus, but unchanged in the occipital cortex compared with the saline-treated animals (Table 1).

In the rats surviving up to PN 60 after postnatal labetalol treatment, no significant treatment-related changes were found on α_1-receptor number of the hypothalamus and occipital cortex in single point assays (Table 1) and in saturation experiments with pooled homogenates (data not shown). Neither could any significant long-lasting changes be demonstrated in β-receptor number or β_1/β_2-receptor ratio in the meso-limbic system and hippocampus, as measured both in single point assays (Table 2) and saturation experiments (data not shown).

Table 1 Direct and long-lasting effects of labetalol exposure from postnatal day (PN) 1 to PN 10 on α_1-adrenoceptors in hypothalamus and occipital cortex (occ. cortex)

		[^{3}H]-prazosin binding	
		PN 10	PN 60
Hypothalamus	Control	31.0 ± 1.4 (5)	23.3 ± 1.9 (8)
	Labetalol	43.0 ± 0.6 (6)*	25.2 ± 1.9 (8)
Occ. cortex	Control	13.5 ± 0.4 (5)	31.8 ± 1.3 (8)
	Labetalol	14.4 ± 0.5 (6)	33.0 ± 1.4 (7)

[^{3}H]-prazosin ([^{3}H]-Praz) binding (fmol mg^{-1} protein) was determined in single point determinations at approx. 500 pM [^{3}H]-Praz in the homogenates of 5–8 individual rats (numbers given in parentheses), except for the α-binding of the hypothalamus on PN 60, which was determined at 200 pM. Values given are means ± s.e.mean. * $P < 0.05$ is significantly different (Student's t-test) from the saline-treated rats.

Acute effects of labetalol and propranolol on PN 10 monoamine metabolism

Ninety minutes after a single injection on PN 10, both labetalol and propranolol significantly decreased the NA concentration in the frontal cortex to, respectively, 68% and 86% of the control value (Table 3). Propranolol had no acute effects on the NA levels on the other brain regions. Labetalol, however, significantly decreased NA in the hippocampus (−23%) and medulla pons (−19%) as well. The MHPG concentrations and the MHPG/NA ratios were significantly elevated (MHPG +25 to 45% and MHPG/NA +25%–105%) in all brain areas measured after both treatments, except for the medulla pons after acute propranolol administration. No significant changes in the dopaminergic (dopamine, DOPAC, HVA, DOPAC/dopamine, HVA/dopamine) or 5-hydroxytryptaminergic (5-HT, 5-HIAA, 5-HIAA/5-HT) transmitter systems were measurable (data not shown).

Direct and long-term effects of chronic labetalol on monoamine metabolism

Monoamine and metabolite concentrations were determined 90 min after the last morning labetalol injection on PN 10 (Table 4). NA levels were significantly decreased in the frontal cortex (−36%) and hippocampus (−21%), whereas the levels

Table 2 Absence of long-lasting effects of labetalol exposure from postnatal day (PN) 1 to 10 on β-adrenoceptors in hippocampus and meso-limbic system

		PN 60: [^{125}*I*]*-Cyp binding*		
		β-total	β_1-	β_2-
Limbic system	Control	24.7 ± 1.0	16.0 ± 1.1	8.6 ± 0.3
	Labetalol	26.0 ± 0.8	17.3 ± 0.6	8.7 ± 0.3
Hippocampus	Control	17.0 ± 0.4	12.9 ± 0.2	4.0 ± 0.2
	Labetalol	16.6 ± 1.0	12.2 ± 0.8	4.4 ± 0.2

[^{125}I]-Cyp binding (fmol mg^{-1} protein) was determined in separate homogenates from 8 adult rats in a single point assay at approx. 90 pM. Values given are means ± s.e.mean. β-Binding in the labetalol group was not significantly different (Student's t test) from control in both brain areas.

Table 3 Acute effects on the noradrenergic system of a single injection with labetalol or propranolol in postnatal day (PN) 10 rats

	Saline	*Labetalol*	%	*Propranolol*	(%)
Noradrenaline					
Frontal cortex	79 ± 3	53 ± 3	68**	68 ± 2	86**
Meso-limbic system	254 ± 10	229 ± 16	90	254 ± 19	97
Hippocampus	101 ± 5	78 ± 7	77*	101 ± 12	100
Medulla pons	443 ± 20	360 ± 20	81*	453 ± 15	102
MHPG					
Frontal cortex	38.8 ± 2.3	53.8 ± 2.7	140**	54.0 ± 2.1	140**
Meso-limbic system	24.2 ± 1.5	31.6 ± 2.2	131*	35.1 ± 1.7	145**
Hippocampus	20.3 ± 1.1	29.5 ± 1.5	145**	26.2 ± 1.6	129*
Medula pons	28.3 ± 1.6	38.2 ± 2.7	135**	35.7 ± 3.9	126
MHPG/NA					
Frontal cortex	0.496 ± 0.45	1.02 ± 0.05	206**	0.799 ± 0.027	161**
Meso-limbic system	0.097 ± 0.007	0.143 ± 0.14	148*	0.148 ± 0.022	153**
Hippocampus	0.202 ± 0.013	0.406 ± 0.47	201**	0.275 ± 0.011	136*
Medulla pons	0.065 ± 0.005	0.110 ± 0.013	169**	0.080 ± 0.010	123

Noradrenaline (NA) and 3-methoxy-4-hydroxyphenylglycol (MHPG) concentrations and NA metabolism (MHPG/NA) on PN 10, 90 min after a single subcutaneous injection with saline, labetalol (10 mg kg^{-1}) or propranolol (15 mg kg^{-1}). All values are means ± s.e.mean (n = 7–8). The data are expressed in ng g^{-1} tissue. The drug effect in percentage (%) compared with the saline-treated rats (100%) is given. Statistically significant differences (Dunnett's t test) are indicated: * $P < 0.05$; ** $P < 0.01$.

in the meso-limbic system and medulla pons were not affected. The labetalol treatment increased the concentration of free MHPG in the frontal cortex, meso-limbic system and medulla pons to 120–140% of the control value. The MHPG/NA ratio was therefore also greatly increased (+20% to 100%) depending on the brain region (Figure 1). The direct effects of chronic labetalol were not restricted to the noradrenergic system. The measurements of 5-HT and 5-HIAA revealed slight, but significant alterations (5-HT: −10% in meso-limbic system and medulla pons, 5-HIAA: +19% in hippocampus). 5-HT metabolism, measured as the 5-HIAA/5-HT ratio, was increased in the hippocampus and meso-limbic system (+20%). Dopamine was significantly decreased in the frontal cortex (−28%) and the medulla pons (−31%). DOPAC and DOPAC/dopamine were significantly elevated in the medulla pons only (Table 4 and Figure 1).

Most of the changes seen in NA and its metabolism directly after the chronic labetalol treatment on PN 10 had disappeared in the 60-day-old rats (Table 5 and Figure 1), except that the NA concentration in the hippocampus was still significantly reduced (−13%). In contrast, changes seen in 5-HT and its metabolism directly after the treatment were even more apparent in the 60-day-old rats. The 5-HT concentration in the hippocampus was significantly diminished (−32%), whereas the 5-HIAA levels in that area and in the meso-limbic system were increased +15% and +17%, respectively. In all four brain areas studied the 5-HIAA/5-HT ratio was significantly increased (+20% to 70%, Figure 1). No long-lasting changes were seen in dopamine and its metabolites.

Acute versus chronic effects on NA, MHPG and MHPG/NA

If we compare the effects on the noradrenergic system of acute administration of labetalol and chronic PN 1–10 treatment generally no differences in the NA and MHPG could be detected in all four brain areas (Table 3 and 4), except for the MHPG of hippocampus, which was significantly increased after acute labetalol, but not after chronic treatment (acute = 145 ± 7% of control value and chronic = 118 ± 5% of control) and the NA in the medulla pons, which was only significantly decreased after acute labetalol (acute = 81% and chronic = 89%).

Table 4 Direct effects of chronic labetalol treatment on postnatal day (PN) 10 monoamines and metabolites in the brain

	Noradrenaline			*MHPG*		
Brain region	Control	Labetalol	%	Control	Labetalol	%
Frontal cortex	104 ± 4	67 ± 3	64**	22.8 ± 1.9	30.0 ± 0.9	132**
Meso-limbic system	207 ± 9	213 ± 7	103	28.1 ± 0.7	34.6 ± 1.9	123**
Hippocampus	102 ± 4	81 ± 7	79*	19.7 ± 1.8	23.3 ± 1.1	118
Medulla pons	430 ± 23	383 ± 25	89	28.1 ± 1.3	38.6 ± 2.3	138**
	5-Hydroxytryptamine			*5-HIAA*		
	Control	Labetalol	%	Control	Labetalol	%
Frontal cortex	167 ± 4	144 ± 12	86	160 ± 7	169 ± 7	106
Meso-limbic system	297 ± 11	269 ± 5	91*	356 ± 16	389 ± 13	109
Hippocampus	156 ± 7	154 ± 5	99	253 ± 13	302 ± 13	119*
Medulla pons	551 ± 19	495 ± 10	90*	1389 ± 49	1369 ± 73	99
	Dopamine			*DOPAC*		
	Control	Labetalol	%	Control	Labetalol	%
Frontal cortex	9.1 ± 0.4	6.5 ± 0.9	72*	17.8 ± 1.3	16.5 ± 0.8	93
Meso-limbic system	802 ± 67	810 ± 31	101	152 ± 9	140 ± 5	92
Medulla pons	22.2 ± 0.9	15.3 ± 0.8	69**	32.4 ± 2.3	40.5 ± 1.9	125*

Monoamine and metabolite concentrations (ng g^{-1} tissue) in several brain regions, 90 min after the last injection (PN 10) with saline or labetalol (10 mg kg^{-1}). All values are means ± s.e.mean (n = 7–8). The labetalol effects in percentage (%) compared with the saline treated rats (100%) is given. Statistically significant differences (Student's t test) are indicated: * $P < 0.05$; ** $P < 0.01$.
MHPG: 3-methoxy-4-hydroxyphenylglycol; 5-HIAA: 5-hydroxy-indoleacetic acid; DOPAC: 3,4-dihydroxyphenylacetic acid.

Table 5 Long-term effects of chronic labetalol treatment on postnatal day (PN) 60 monoamines and metabolites in the brain

	Noradrenaline			*MHPG*		
Brain region	Control	Labetalol	%	Control	Labetalol	%
Frontal cortex	342 ± 15	330 ± 18	96	16.4 ± 1.4	16.2 ± 1.3	99
Meso-limbic system	602 ± 32	555 ± 35	92	18.3 ± 1.1	19.7 ± 1.7	108
Hippocampus	418 ± 14	364 ± 12	87*	10.9 ± 0.5	10.4 ± 1.1	95
Medulla pons	693 ± 34	694 ± 26	100	25.9 ± 1.4	29.7 ± 2.5	115
	5-Hydroxytryptamine			*5-HIAA*		
	Control	Labetalol	%	Control	Labetalol	%
Frontal cortex	440 ± 22	410 ± 19	94	173 ± 13	195 ± 15	113
Meso-limbic system	1046 ± 65	897 ± 43	86	527 ± 24	619 ± 16	117**
Hippocampus	351 ± 19	238 ± 17	68**	248 ± 10	285 ± 8	115*
Medulla pons	767 ± 46	726 ± 44	95	445 ± 25	499 ± 19	112
	Dopamine			*DOPAC*		
	Control	Labetalol	%	Control	Labetalol	%
Frontal cortex	29.5 ± 2.1	33.5 ± 2.1	114	17.2 ± 1.9	17.7 ± 2.5	103
Meso-limbic system	3596 ± 360	3252 ± 155	90	654 ± 49	676 ± 31	103
Medulla pons	47.8 ± 2.5	47.5 ± 1.5	100	20.7 ± 1.6	21.8 ± 1.5	105

Monoamine and metabolite concentrations ($ng\,g^{-1}$ tissue) in several brain regions of adult rats, postnatally treated with saline or labetalol (PN 1–10, $10\,mg\,kg^{-1}$ 2× daily). All values are means ± s.e.mean (n = 7–8). The labetalol effect in percentage (%) compared with the saline-treated rats (100%) is given. Statistically signicant differences (Student's t test) are indicated: * $P < 0.05$, ** $P < 0.01$. Abbreviations as in Table 4.

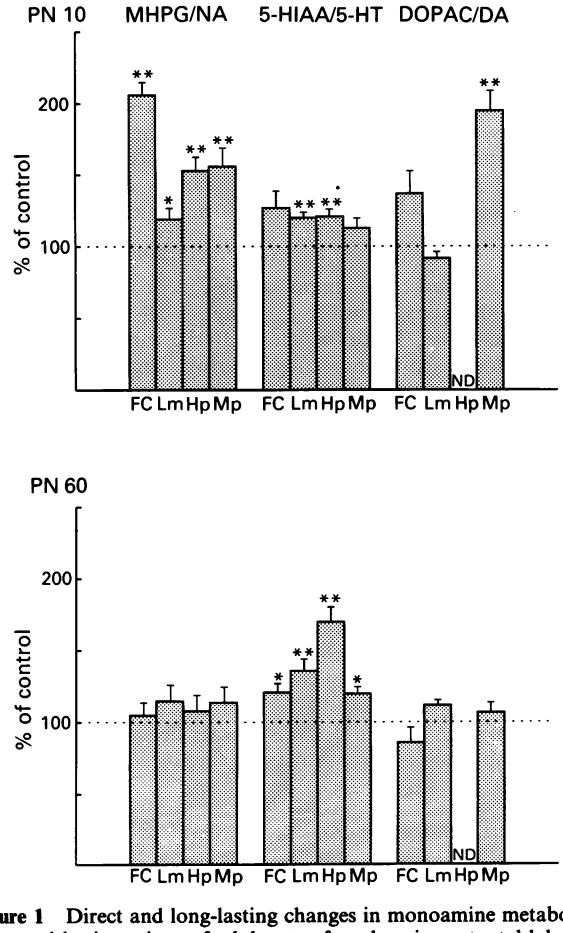


Figure 1 Direct and long-lasting changes in monoamine metabolism in several brain regions of adult rats after chronic postnatal labetalol treatment. Labetalol-induced changes are shown as percentage (%) of the saline treated group. Abbreviations: PN, postnatal day; FC, frontal cortex; Lm, meso-limbic system; Hp, hippocampus; Mp, medulla pons. MHPG, 3-methoxy-4-hydroxyphenylglycol; NA, noradrenaline; 5-HIAA, 5-hydroxy-indoleacetic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; DA, dopamine. Columns are means with s.e.mean shown by vertical bars (n = 7–8, four different litters). Significant changes (Student's t test) are indicated: * $P < 0.05$; ** $P < 0.01$. ND = not determined.

Discussion and conclusions

Up-regulation of α_1-adrenoceptors

The α_1- and β-adrenoceptors are present very early in rat brain development (Morris *et al.*, 1980; Erdtsieck-Ernste *et al.*, 1991b). Evidence is becoming available that one of the characteristics of adult adrenoceptors, i.e. regulation by prolonged presence of agonists or antagonists, is also present in early life. Directly after the postnatal labetalol treatment we found an up-regulation of the α_1-sites in the hypothalamus, but not in the occipital cortex. In adult rats denervation by means of N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP-4) or 6-hydroxydopamine (6-OHDA) as well as chronic α_1-blockade by prazosin has repeatedly been found to increase the α_1-receptor density (Swann *et al.*, 1981; Menkes *et al.*, 1983; Johnson *et al.*, 1987). Moreover, Johnson *et al.* (1987) have reported that the induction of a compensatory α_1-receptor increase is regionally specific. The explanation of our results could, therefore, be either that α_1-receptor regulation mechanisms are not yet functioning in the occipital cortex or that the occipital α_1-receptor number is not regulated at all by noradrenergic activity. With respect to the β-adrenoceptors, it has been reported by others that the postnatal β-adrenoceptor number can be regulated. Depletion of NA by administration of 6-OHDA to newborn rats induces an increase in β-receptor number measurable from PN 6 (Harden *et al.*, 1977), whereas *in vitro* observations have additionally demonstrated a down-regulation of β-binding in cortical slices of PN 7 rats after incubation with the β-agonist, isoprenaline (Nomura *et al.*, 1983). Moreover, results from our laboratory have shown that the β-adrenoceptor number in the rat brain has significantly increased directly after a chronic prenatal β-blockade with propranolol (unpublished results). In the present study, we have not measured the direct effects of labetalol on the central β-adrenoceptors. Therefore, more studies have to be performed to establish a possible β-receptor up-regulation.

The changes in α_1-binding demonstrate that during early postnatal development chronic blockade can result in adaptive changes of the adrenoceptor number. In several clinical studies it has been reported that maternal labetalol administration also results in adrenoceptor blockade in the foetus or newborn, as evidenced by physiological effects (Woods & Malan, 1983; MacPherson *et al.*, 1986; Haraldsson & Geven,

1989). However, as far as we know, drug withdrawal effects as a measure for possible adaptive receptor changes have never been described.

Absence of long-lasting effect on adrenoceptors

It has often been demonstrated that chronic receptor blockade or activation during vulnerable periods of brain development can have profound consequences for the setting of the receptor number in adulthood (Del Rio *et al.*, 1988; Saleh & Kostrzewa, 1988; Miller & Friedhoff, 1988). However, in our experiments the rats exposed postnatally to labetalol showed no differences in adult α_1-, β_1- or β_2-receptor binding as compared with controls. The outcome of the β-receptor binding corroborates our previous conclusion after postnatal propranolol treatment (Erdtsieck-Ernste *et al.*, 1991a), that β-receptor blockade during early postnatal life does not lead to long-lasting changes in adult β-receptor number.

Mechanisms behind the acutely increased noradrenaline metabolism

The present study shows that a single dose of labetalol given on PN 10 is able to increase the level of MHPG, which is a good measure of NA activity. It is unclear how labetalol has generated this effect. Autoregulation of NA activity proceeds mainly through α_2-adrenoceptors, but labetalol has very little affinity for these receptors (McPherson & Summers, 1982). As a combined α_1/β-antagonist, labetalol could have increased NA activity by blockade of the α_1- and β-receptors, because other α_1- and β-antagonists, e.g. prazosin and propranolol, have been reported to increase MHPG as well (Wiesel, 1976; Fuller *et al.*, 1978; Swann *et al.*, 1981; Keeton & Hall, 1986) although the relation between the receptor blockade and the increase in MHPG is unclear. However, labetalol has also been demonstrated to block the *in vitro* reuptake of NA, and its **R,R**-stereoisomer dilevalol was reported to facilitate NA release by activation of presynaptic β_2-receptors. (Farmer *et al.*, 1972; Brittain & Levy, 1976; Misu & Kubo, 1986; Pauwels *et al.*, 1988; Howes *et al.*, 1990). *In vivo*, both these effects would result in an increased extracellular concentration of NA. Indeed, preliminary results with '*in vivo* dialysis' experiments in the adult rat cortex (Van Veldhuizen *et al.*, 1990) have revealed that extracellular NA is elevated by locally applied labetalol at concentrations which can be compared with the labetalol present in the brain after a subcutaneous injection on PN 10 (Erdtsieck-Ernste, unpublished observations). However, whether NA reuptake blockade alone could explain the increase in MHPG levels remains unclear: whereas chronic treatment with the uptake blocker imipramine increased MHPG, acute treatment actually decreased central MHPG levels (Sedlock & Edwards, 1985).

Indirect involvement of peripheral effects of labetalol, such as hypotension, cannot be ruled out, since in adult rats, hypotension induced by non-adrenergic drugs or by blood volume depletion has been reported to activate the NA neurones in the locus coeruleus leading to at least regionally increased NA metabolism (Wijnen *et al.*, 1978; Elam *et al.*, 1985; Kubo *et al.*, 1988). Our data do not indicate whether hypotension was induced in our rat pups and whether this could have affected our results.

Adaptive and long-lasting changes on monoamine metabolism

If we compare the labetalol effects after single and after repeated treatment, in general effects on NA and MHPG levels in the various brain areas appeared to be the same, suggesting that tolerance has not developed. In adulthood the NA concentration in the hippocampus was still slightly (13%), but significantly, decreased. However, we think that the relevance of this finding remains questionable until other studies have confirmed this result. All the other direct effects on NA and its metabolism had disappeared in the adult rats. This is interesting, because in our previous study, propranolol treatment from PN 1 to PN 10 induced a lasting increase in NA metabolism (Erdtsieck-Ernste *et al.*, 1991a). However, besides the increased MHPG levels, an increase of NA content was induced after chronic propranolol treatment in some of the brain areas, whereas after chronic labetalol NA levels were unchanged or decreased. Therefore, different mechanisms may have established the direct effects and may be responsible for the presence or absence of long-lasting effects on NA metabolism (see also Boer *et al.*, 1990).

The major difference in the outcome of the chronic versus the single injection treatment with labetalol is that after the chronic treatment an increase in medulla pons dopamine content and a more widespread increase in 5-HT metabolism were additionally induced. A comparable postnatal treatment with propranolol also increased medulla pons dopamine and 5-HT metabolism (Erdtsieck-Ernste *et al.*, 1991a). Because neurotransmitter systems are strongly interacting, these changes can be induced indirectly by the effects on the noradrenergic system. However, an alternative explanation for the effects on 5-HT metabolism may be that the concentration of labetalol in the brain was high enough to occupy the 5-HT_{1a}-receptor sites (Pauwels *et al.*, 1988) and to block the inhibitory effects of endogenous 5-HT on raphe cell firing (Peroutka, 1988).

In the 60-day-old rats, more than one month after the cessation of the treatment, 5-HT metabolism was significantly increased in all brain regions studied. It is unclear what molecular or cellular mechanisms underlie this effect, but apparently the labetalol treatment had interfered with the maturation of the 5-HT system. Since 5-HT is known to be involved in several types of behaviour, additional studies are now planned to study possible behavioural consequences of this increased 5-HT metabolism. If such behavioural changes can be found, these studies could provide possible directions for clinical studies on long-lasting changes after the use of labetalol to control blood pressure during human pregnancy.

Rat brain labetalol levels in relation to human therapy

Labetalol is given to pregnant women in doses ranging from 400 to 2400 mg day^{-1} orally (Michael, 1979; Lardoux *et al.*, 1983; Sibai *et al.*, 1990), which is in the same range as the labetalol dose used in this study. During pregnancy, maternal and foetal labetalol plasma concentrations are correlated, resulting in foetal cord: maternal vein ratios varying from 0.2 to more than 1 (Lardoux *et al.*, 1983; Morselli *et al.*, 1989; Rogers *et al.*, 1990). Therefore, therapeutic labetalol concentrations may be reached in the foetus as well and may interact with the monoaminergic neurones and adrenoceptors which appear during the second and third trimester of human pregnancy (Nobin & Bjorklund, 1973; Olson *et al.*, 1973; Sogos *et al.*, 1989). In our 10-day-old rats a dose of 10 mg kg^{-1}, s.c., yielded brain concentrations of 2.1 $\mu g\,g^{-1}$ after 90 min. As far as we know, brain labetalol levels in man have never been reported but two studies have provided data of brain propranolol levels (Myers *et al.*, 1975; Neil-Dwyer *et al.*, 1981) and indicate that our labetalol levels in rats are comparable with brain propranolol concentrations reported in man (1–7 $\mu g\,g^{-1}$).

Concluding remarks

As far as we know, this is the first study concerning the direct and long-lasting effects of postnatal labetalol exposure on central monoaminergic systems. In discussing our results we have had to make comparisons with effects described for adult rats. It must, however, be kept in mind that during development, labetalol might have pharmacological effects other than those reported for adult rats because of different kinetics or metabolism and because neuronal organisation in the foetus might differ considerably from that in adulthood.

The present study clearly demonstrates that chronic exposure of rats to labetalol during the first 10 days of life (1) increased NA and 5-HT metabolism in the brain and led to

adaptational changes in α_1-receptor binding, and (2) did not cause long-lasting changes in central α_1- and β-receptor binding or in NA metabolism, but (3) affected the rate of 5-HT metabolism in later life. Although these findings do not directly question the reported safety of maternal labetalol treatment for the foetus or neonate, they should stimulate further basic and clinical research on the long-lasting effects of chronic labetalol exposure.

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Characterization of vascular neuropeptide Y receptors

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1 In the present study we compared neuropeptide Y (NPY) and NPY-related analogues for their ability to activate or bind to vascular NPY receptors in four experimental set-ups. Previous results have suggested the existence of different receptor subtypes, Y_1 receptors requiring full-length NPY (1–36) or [Pro^{34}]-NPY, and Y_2 receptors recognizing also N-terminally truncated forms of NPY but not [Pro^{34}]-NPY.

2 NPY 1–36 and [Pro^{34}]-NPY dose-dependently increased arterial pressure in the anaesthetized rat with a similar magnitude and potency. NPY 2–36 was much less potent than NPY 1–36. NPY 4–36 and NPY 11–36 were inactive even at a dose as high as 10 nmol kg^{-1}.

3 NPY 1–36, [Pro^{34}]-NPY, NPY 2–36 and NPY 5–36 concentration-dependently increased the coronary resistance in the Langendorff preparation of the rat. NPY 1–36 and [Pro^{34}]-NPY were equipotent, while NPY 2–36 and NPY 5–36 were about 7 and 20 times less potent. At 0.3 μM, NPY 11–36, NPY 20–36 and NPY 22–36 induced a slight contraction while NPY 23–36 was inactive.

4 NPY 1–36, [Pro^{34}]-NPY, NPY 2–36, NPY 4–36, NPY 5–36 and NPY 11–36 evoked concentration-dependent contractions in the isolated inferior caval vein of the rat and guinea-pig. [Pro^{34}]-NPY was more potent than NPY 1–36. NPY 2–36 was equipotent with NPY 1–36, while NPY 4–36, NPY 5–36 and NPY 11–36 were approximately 30 times less potent.

5 [Pro^{34}]-NPY was equipotent with NPY 1–36 in displacing the ^{125}I-labelled gut hormone peptide ([^{125}I]-PYY) from rat aortic smooth muscle cells, while NPY 2–36 and shorter forms of NPY were much less potent or inactive.

6 In caval vein smooth muscle cells of the rat, the displacement pattern was more complex than in aortic smooth muscle cells, in that both [Pro^{34}]-NPY and NPY 13–36 effectively displaced the radioligand, albeit none of them completely.

7 In conclusion, the NPY-evoked pressor response in the whole rat and coronary vessels seems to be mediated by vascular Y_1 receptors and the binding characteristics of the NPY-related peptides in the aortic smooth muscle cells correspond to a population of such receptors. In the caval vein, the profile of the bioactivity and the binding affinity of the NPY-related peptides suggest a mixed population of Y_1/Y_2 receptors.

Keywords: Hypertension; vasoconstriction; coronary resistance; radioligand binding; neuropeptide Y (NPY); Y_1-receptors; Y_2-receptors

Introduction

Neuropeptide Y (NPY) has a wide distribution in the peripheral and central nervous system. It seems to be co-localized with noradrenaline (NA) in cardiovascular sympathetic fibres (Lundberg *et al.*, 1982b; Ekblad *et al.*, 1984; Uddman *et al.*, 1985; Sundler *et al.*, 1986). The cardiovascular effects of NPY have been analyzed in some detail during the last few years. Intravenous injection of NPY raises the arterial pressure (AP) (Lundberg *et al.*, 1982b; Grundemar *et al.*, 1990) but this apparently simple response is the net result of a complex mode of action. Briefly, NPY may exert three effects at sympathetic neuroeffector junctions: (1) a direct postjunctional response (vasoconstriction); (2) a postjunctional potentiation of the response to vasoconstrictors, such as NA; and (3) a prejunctional suppression of stimulated NA release (Lundberg & Stjärne, 1984; Håkanson *et al.*, 1986; Wahlestedt *et al.*, 1986; 1990b). There is much evidence to suggest that NPY acts on two receptor subtypes, tentatively referred to as Y_1 and Y_2 (for reviews see Wahlestedt *et al.*, 1990a; Schwartz *et al.*, 1990). The Y_1-type receptor seems to be located postjunctionally and is able to evoke vasoconstriction, while the Y_2-type receptor seems to be predominantly prejunctional, suppressing the stimulated release of NA. The Y_2-type receptor recognizes not only the parent molecule but also truncated C-terminal fragments of NPY (Wahlestedt *et al.*, 1986). In fact, on the latter receptor NPY 13–36 is only slightly less potent than NPY 1–36; a progressive N-terminal shortening of the molecule results in a gradual loss of potency (Grundemar & Håkanson, 1990). The Y_1-type receptor seems to require the whole NPY molecule in order to become fully activated since C-terminal fragments are inactive or much less potent (Rioux *et al.*, 1986; Wahlestedt *et al.*, 1986). [Pro^{34}]-NPY is a recently developed analogue that acts as a selective Y_1-type receptor agonist (Krstenansky *et al.*, 1990; Dumont *et al.*, 1990). The gut hormone peptide YY (PYY) displays 70% homology with NPY and is about equipotent with NPY on either receptor subtype (Lundberg *et al.*, 1982a; Wahlestedt *et al.*, 1986; Grundemar & Håkanson, 1990). Another homologous peptide hormone, pancreatic polypeptide (PP), has virtually no affinity for either receptor subtype (Schwartz *et al.*, 1990). Supporting evidence for the Y_1/Y_2 receptor subtype concept has come from binding studies of neuroblastoma cell lines that seem to express either Y_1 or Y_2 receptors (Sheikh *et al.*, 1989; Schwartz *et al.*, 1990).

In the present study we compared NPY 1–36, C-terminal fragments of NPY and the Y_1-type receptor selective analogue [Pro^{34}]-NPY for their ability to evoke vasoconstriction or bind to vascular NPY receptors. In four different experimental set-ups we measured: (1) AP in the anaesthetized rat, (2) coronary resistance in the Langendorff preparation of the rat heart, (3) contractile effects in guinea-pig isolated inferior

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caval vein, and (4) binding of NPY 1–36 and NPY-related peptides to cultured rat aortic and caval vein smooth muscle cells.

Methods

Arterial blood pressure

Male Sprague-Dawley rats (200–250 g) were anaesthetized with intraperitoneal administration of 0.4–0.5 ml ketamine (50 mg ml^{-1}) (Ketalar, Parke-Davis) and 0.2–0.25 ml xylazine (20 mg ml^{-1}) (Rompun vet, Bayer). The left jugular vein and right femoral artery were cannulated with polyethylene catheters for i.v. administration of the agents and for continuous recording of systemic blood pressure via a Statham P23 pressure transducer to a Grass model 7 polygraph. The histamine H_1-receptor antagonist, mepyramine, was injected (10 mg kg^{-1} i.v.) in order to prevent the effects of NPY-evoked histamine release (Grundemar *et al.*, 1990) and experimentation was started (usually 10 min later) when the AP had stabilized at 100–120 mmHg. The agents were injected slowly (approx. 20 s) in a volume of 100 μl (room temperature), followed by washing the catheter with 100 μl saline. Dose-response curves were constructed from experiments in which single doses of each peptide were administered to each rat.

Perfused Langendorff

Male Sprague-Dawley rats (250–350 g) were killed, the chest was opened, the heart and lungs were excised and placed in Krebs solution containing 10 units ml^{-1} heparin (room temperature). The lungs, connective tissue and large vessels were removed from the heart to expose the aortic arch which was cannulated retrogradely to allow perfusion of the coronary circulation by the Langendorff technique (Vleeming *et al.*, 1989). Perfusion was performed at a constant pressure of 80 cm water. The perfusion fluid consisted of Krebs solution of the following composition (in mM): NaCl 100, KCl 4.0, $MgSO_4$ 1.5, $CaCl_2$ 2.0, $NaHCO_3$ 20.0, NaH_2PO_4 1.5, Na-acetate 20.0 and D-glucose 10.0; maintained at 33°C, pH 7.4 and equilibrated with 95% O_2 and 5% CO_2. Coronary flow was measured by determination of the volume of perfusate through the isolated heart per unit time. The results are presented as coronary resistance, i.e. the inverted value of the coronary flow. Before addition of peptides the coronary flow was about 3 ml min^{-1}. Peptides were administered by changing the Krebs solution to one containing Krebs plus the appropriate concentration of each peptide. Each preparation was exposed to only 1 or 2 concentrations of each peptide (at least 20 min interval).

Isolated vessels

Sprague-Dawley rats (200–250 g) and guinea-pigs (Dunklin-Hartley, 350–450 g) were killed by a blow on the head and exsanguinated. The proximal part of the inferior caval vein was removed close to the right auricle. The vessel was divided into three circular segments (2 mm wide) which were transferred to a thermostated bath (37°C), containing Krebs solution of the following composition (in mM): NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15.0, NaH_2PO_4 1.2 and glucose 6.0. The solution was bubbled continuously with 95% O_2 and 5% CO_2, giving a pH of 7.4. The vascular segments were mounted on two L-shaped stainless steel wires (0.1 mm ø), one of which was connected to a force-displacement transducer (Grass Instrument FT 03C) for continuous tension recording on a Grass polygraph (Högestätt *et al.*, 1983). The resting tension was gradually adjusted to 2 mN. The contractile capacity was examined by adding an isotonic 60 mM potassium Krebs solution (NaCl replaced by KCl); these contractions served as an internal control. After wash-out of the potassium-enriched solution, increasing doses of the different peptides were added to each bath in a cumlative manner (at least 3 min interval). Each segment was exposed to one peptide only. Contractile responses were expressed in relation to the potassium-evoked contraction.

Cultured rat aortic and caval vein smooth muscle cells

The aorta and the inferior caval vein were taken out from male Sprague-Dawley rats under sterile conditions and primary cultures of smooth muscle cells were prepared essentially as described by Meyer-Lehnert & Schrier (1989). Thus, the vessels were collagenase-treated (2 mg ml^{-1}) in Eagle's minimum essential medium, followed by removal of the adventitia and outer media. The vessels were then cut longitudinally and the intima scraped off. Following mincing and further collagenase treatment (3 h) with continuous stirring, the material was triturated 20 times through 18 and 16 gauge needles. The resulting single-cell suspension was centrifuged (1000 r.p.m.; 5 min) and resuspended in an incubation medium containing 10% foetal calf serum (GIBCO). This was repeated three times. Cells were plated on 6-well plates (35 × 35 mm) for the binding experiments (10^6 smooth muscle cells/well) after 8 passages. In parallel experiments, cell viability was tested (exclusion of trypan blue). Routinely, the cells were inspected with phase-contrast microscopy. The cells were defined as smooth muscle cells if stained for actin and desmin by immunocytochemistry (Chamley-Campbell *et al.*, 1979). Binding of [^{125}I]-PYY (New England Nuclear) was tested as previously described (Walker & Miller, 1988; Wahlestedt *et al.*, 1990a). Briefly, intact smooth muscle cells were washed three times in buffer of the following composition (in mM): NaCl 137, KCl 5.4, KH_2PO_4 0.44, $CaCl_2$ 1.25, $MgSO_4$ 0.81, HEPES 20, 0.1% bacitracin and 0.3% bovine serum albumin. The cells were incubated in 1 ml buffer with [^{125}I]-PYY (100 pmol) for 60 min at 37°C with or without various concentrations of competing non-labelled peptides. Binding reaction was interrupted by washing the cells four times in ice-cold buffer and the cells were then exposed to lysis buffer (3 M acetic acid with 8 M urea and 2%, v/v, Nonidet P-40; 1 + 0.5 ml). The cell membrane fragments were transferred to test tubes and the radioactivity counted. All binding experiments were performed in triplicate. Specific binding was defined in the presence of 1 μM unlabelled NPY 1–36 and was approximately 50 and 65% of total binding in smooth muscle cells from aorta and caval vein, respectively.

NPY and analogues: NPY 1–36 (porcine), NPY 20–36 and NPY 22–36 were obtained from Peninsula, Merseyside, St Helens, UK. NPY 2–36 and NPY 5–36 were purchased from Richelieu Biotech, QC, Canada. [Pro^{34}]-NPY and NPY 4–36 were kind gifts from Prof. T.W. Schwartz, Copenhagen, Denmark. NPY 11–36 and NPY 23–36 were synthesized by solid-phase synthesis and purified to at least 96% by h.p.l.c. (W. Krzeminsky, Ferring AB, Malmö, Sweden and H. Franzén, Dept of Med. Chem, Univ. of Lund, Lund, Sweden, respectively). NPY 13–36 and NPY 18–36 were kind gifts from Dr A. Fournier, Canada. All NPY-related peptides were based on porcine NPY.

Statistics

The pharmacological response to each peptide was characterized by the maximum response (E_{max}) and the pD_2 values (the negative logarithm of the dose or molar concentration of the peptide that elicits 50% of the maximum response). In the binding studies the potency with which each peptide displaced the radiolabelled ligand was expressed as pIC_{50} (the negative logarithm of the molar concentration of the peptide that displaces 50% of the ligand). These parameters were estimated by linear regression analysis of the results in the 10–90% response interval. Statistical analysis of the pD_2 or pIC_{50} values for the different peptides was performed by means of unpaired Student's t test. Data are presented as means ± s.e.means.

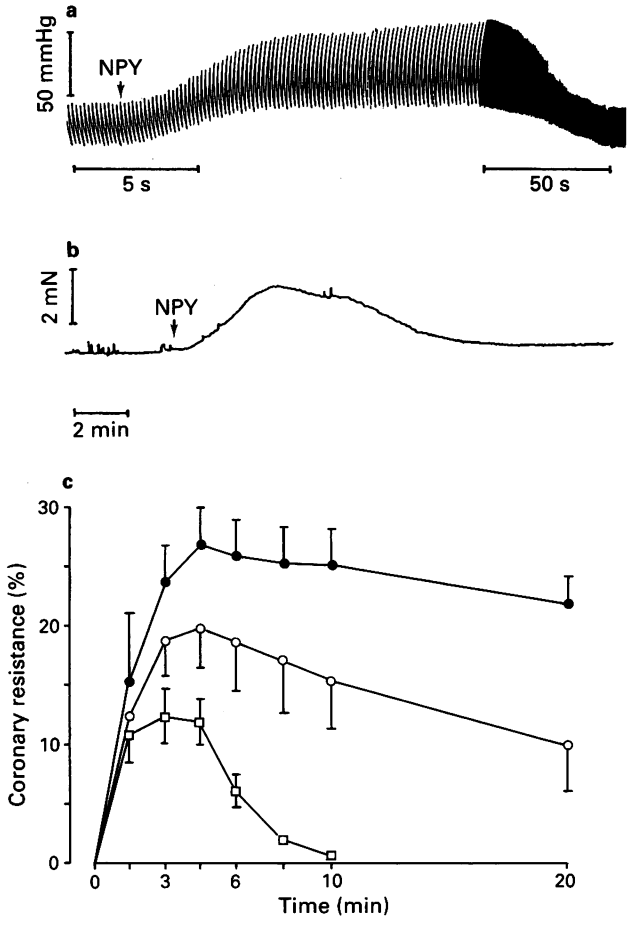


Figure 1 Tracings showing effects on arterial pressure (AP) of neuropeptide Y (NPY, 10 nmol kg^{-1}) injected i.v. into an anaesthetized rat (a), vasoconstriction evoked by NPY (0.1 μM) in the guinea-pig isolated inferior caval vein (b) and time course of the increase in coronary resistance in the perfused rat Langendorff preparation evoked by 5 nmol NPY 1–36 (●), NPY 11–36 (○) or NPY 22–36 (□) (c). See text for further information. Each value is the mean of 4–6 experiments. Vertical bars give s.e.mean.

Results

Pressor effects in the anaesthetized rat

In rats pretreated with the histamine H_1-receptor antagonist, mepyramine, i.v. injection of NPY 1–36 or [Pro^{34}]-NPY (0.1–10 nmol kg^{-1}) raised the AP dose-dependently (Figures 1 and 2). The responses were of a similar magnitude and potency; the pD_2 values were 9.06 ± 0.25 and 9.19 ± 0.38, respectively (expressed as bolus dose in neg log mol kg^{-1}). At 10 nmol kg^{-1} the pressor response to NPY 1–36 lasted for 1–2 min (Figure 1). The heart rate (HR) was not affected. NPY 2–36 was much less potent than NPY 1–36 (Figure 2; Table 1). NPY 4–36 (n = 4) and NPY 11–36 (n = 3) were inactive at 10 nmol kg^{-1}.

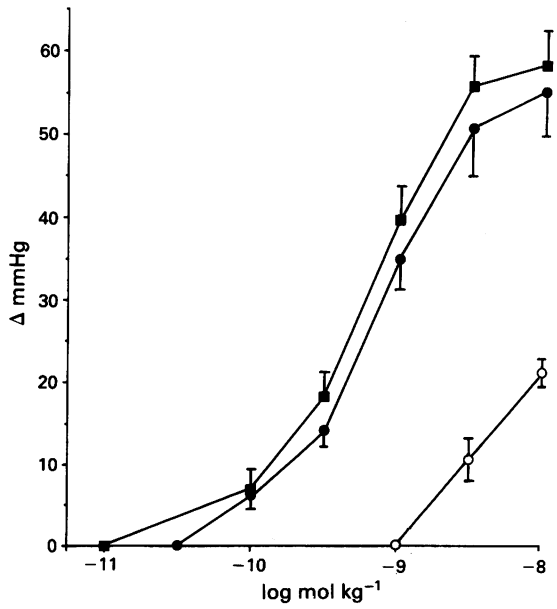


Figure 2 Dose-response curves show the change in arterial pressure (AP) evoked by NPY 1–36 (●), [Pro^{34}]-NPY (■) and NPY 2–36 (○) in the anaesthetized rat. The negative log ED_{50} values for NPY 1–36 and [Pro^{34}]-NPY were 9.06 ± 0.25 and 9.19 ± 0.38, respectively. n = 4–5 for each dose and peptide.

Increased coronary resistance in the perfused Langendorff heart

NPY 1–36, [Pro^{34}]-NPY, NPY 2–36 and NPY 5–36 concentration-dependently increased the coronary resistance (Figure 3). NPY 1–36 and [Pro^{34}]-NPY were approx. equipotent; the pD_2 values were 8.79 ± 0.24 and 8.50 ± 0.27, respectively (expressed as neg log molar concentration). NPY 2–36 and NPY 5–36 were less potent than NPY 1–36 with pD_2 values of 7.97 ± 0.27 ($P < 0.05$) and 7.46 ± 0.23 ($P < 0.05$), respectively (Figure 3; Table 1). NPY 11–36, NPY 20–36 and NPY 22–36 induced a slight increase in coronary resistance at the highest concentration tested (0.3 μM) (Figure 3), while NPY 23–36 was without effect (n = 4). In a series of experiments 5 nmol NPY 1–36, NPY 11–36 or NPY 22–36 were injected into the catheters supplying the coronary vessels with perfused Krebs solution. It was shown that the maximum response was elicited after about 4–5 min and that the duration of the effect was reduced with progressive shortening of the moleculte (Figure 1).

Contractile responses of the isolated inferior caval vein

NPY 1–36 elicited concentration-dependent contractions of the rat and guinea-pig caval vein. However, since the NPY-evoked responses were greater in the guinea-pig caval vein, these vessels were used in the following series of experiments.

NPY 1–36, [Pro^{34}]-NPY, NPY 2–36, NPY 4–36, NPY 5–36 and NPY 11–36 evoked concentration-dependent contractions (Figure 4). The pD_2 and E_{max} values are shown in

Table 1 The rank order, in terms of pharmacological effect on or binding affinity to the vascular neuropeptide Y (NPY) receptor in five experimental set-ups

	Pharmacological effect
Arterial pressure	[Pro^{34}]-NPY = NPY 1–36 ⩾ NPY 2–36; NPY 4–36 (inactive at 10 nmol kg^{-1})
Coronary resistance	[Pro^{34}]-NPY = NPY 1–36 > NPY 2–36 ⩾ NPY 5–36 > NPY 11–36
Vasoconstriction	[Pro^{34}]-NPY > NPY 1–36 = NPY 2–36 ⩾ NPY 4–36 = NPY 5–36 > NPY 11–36
	Binding to vascular smooth muscle cells
Binding to aorta	[Pro^{34}]-NPY = NPY 1–36 ⩾ NPY 2–36 > NPY 11–36 = NPY 13–36 > NPY 18–36
Binding to caval vein	[Pro^{34}]-NPY = NPY 1–36 > NPY 13–36

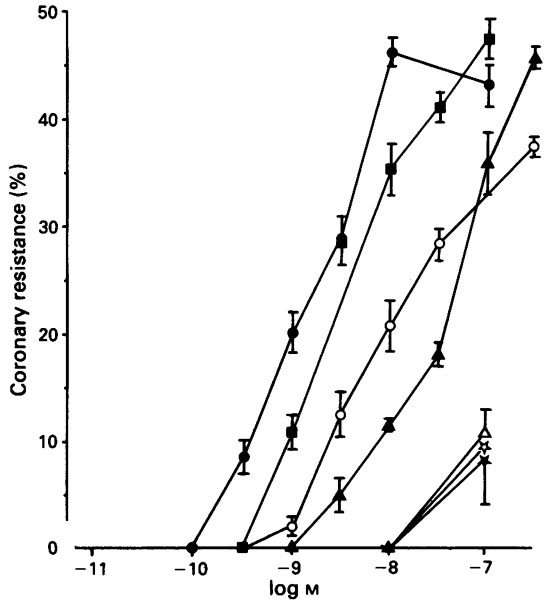


Figure 3 Concentration-response curves showing effects of NPY 1–36 (●), [Pro^{34}]-NPY (■), NPY 2–36 (○), NPY 5–36 (▲), NPY 11–36 (△), NPY 20–36 (★) and NPY 22–36 (☆) on the coronary resistance in the Langendorff perfused heart of the rat. E_{max} for [Pro^{34}]-NPY and NPY 5–36 were assumed to be the same as the E_{max} for NPY 1–36 and NPY 2–36. $n = 4$–6 for each concentration and peptide.

Table 2. [Pro^{34}]-NPY was approximately three times more potent than NPY 1–36 ($P < 0.05$). NPY 2–36 was about equipotent with NPY 1–36, whereas NPY 4–36, NPY 5–36 and NPY 11–36 were about 30 times less potent than NPY 1–36 (Table 1). Since the concentration-response curves for the latter peptides did not reach maximum, the respective pD_2 and E_{max} values could not be calculated. The onset of the vasoconstriction by NPY and related peptides was gradual, reached maximum after several minutes and relaxed spontaneously (Figure 1).

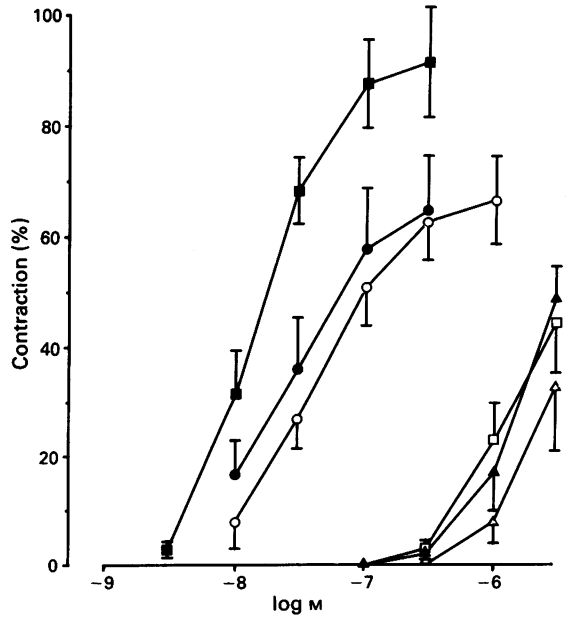


Figure 4 Contractile responses to NPY 1–36 ($n = 9$) (●),[Pro^{34}]-NPY ($n = 8$) (■), NPY 2–36 ($n = 10$) (○), NPY 4–36 ($n = 7$) (□), NPY 5–36 ($n = 5$) (▲) and NPY 11–36 ($n = 4$) (△) in the guinea-pig isolated inferior caval vein. Responses are expressed as a percentage of the 60 mM potassium-induced contraction.

Table 2 Ability of neuropeptide Y (NPY) and various analogues to contract the inferior caval vein of the guinea-pig

	pD_2	E_{max} (%)	n
NPY 1–36	7.5 ± 0.2	65 ± 10	9
[Pro^{34}]-NPY	8.0 ± 0.1*	92 ± 10	8
NPY 2–36	7.3 ± 0.1	67 ± 8	10
NPY 4–36	(+)		7
NPY 5–36	(+)		5
NPY 11–36	(+)		4

Values are means ± s.e.mean.
* Statistically different from NPY 1–36. (+), the peptide induced a contraction at ⩾0.3 μM only. (%), contraction as a percentage of the potassium-evoked contraction. *n*, the number of animals.

Binding to aortic and caval vein smooth muscle cells

NPY binding sites can be labelled by [^{125}I]-PYY (e.g. Walker & Miller, 1988). PYY is known to have qualitatively the same vascular effects as NPY (e.g. Wahlestedt *et al.*, 1990a), therefore [^{125}I]-PYY was used in this study. Displacement curves were generated by co-incubation with unlabelled peptides in increasing concentrations.

Displacement curves for NPY 1–36, [Pro^{34}]-NPY and NPY 13–36 in aortic smooth muscle cells (passage 8) are shown in Figure 5. It can be seen that NPY 13–36 is much less potent (2–3 orders of magnitude) than the other two peptides. The following pIC_{50} values were calculated: NPY 1–36,

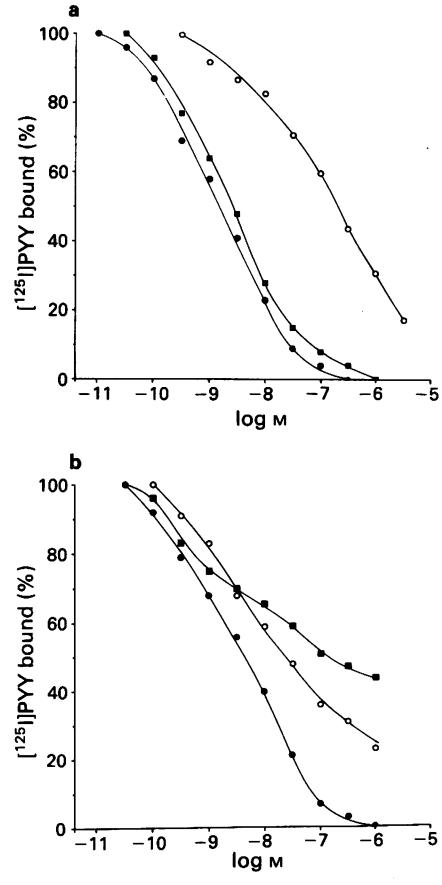


Figure 5 Displacement of [^{125}I]-PYY from (a) rat aortic or (b) inferior caval vein smooth muscle cells with NPY 1–36 (●), [Pro^{34}]-NPY (■) and NPY 13–36 (○). Each experiment was done in triplicate and repeated three times. For clarity only the mean values are indicated.

8.89 ± 0.14; [Pro^{34}]-NPY, 8.66 ± 0.14; NPY 2–36, 7.21 ± 0.11; NPY 11–36, 6.93 ± 0.09; NPY 13–36, 6.72 ± 0.13; NPY 18–36, 6.19 ± 0.20 (n = 3–5 for each peptide) (Table 1). These values differ slightly from those reported by Shen *et al.* (1990), who used cells from passage 2 or 3. Displacement occurred in a monophasic pattern, indicating the presence of a homogeneous population of binding sites displaying the binding characteristics of a Y_1-type receptor (e.g. see Wahlestedt *et al.*, 1990a).

In the caval vein smooth muscle cells, the displacement pattern was more complex, conforming to a mixed rather than single receptor population. As can be seen in Figure 5b, the curves could not be used to calculate useful pIC_{50} values. Both the Y_1-receptor-selective agonist [Pro^{34}]-NPY and the Y_2-receptor selective agonist NPY 13–36, potently, albeit incompletely, displaced [^{125}I]-PYY.

Discussion

A number of authors have shown that NPY is a powerful vasoconstrictor *in vivo* (e.g. Maturi *et al.*, 1989). Thus, i.v. injection of NPY evokes a dose-dependent increase in AP (Lundberg *et al.*, 1982b; Grundemar *et al.*, 1990). High doses of NPY evoke an initial rise followed by a long-lasting fall in AP that can be prevented by pretreatment with histamine H_1-receptor antagonists or with the histamine liberator compound 48/80, indicating that the fall in AP can be attributed to histamine release from mast cells (Grundemar *et al.*, 1990). The depressor response can be reproduced by high doses of different C-terminal NPY fragments (Boublik *et al.*, 1989a,b; Wahlestedt *et al.*, 1990; Shen *et al.*, 1991), and these responses are also abolished by pretreatment with histamine H_1-receptor antagonists (Shen *et al.*, 1991). The NPY-evoked histamine release from mast cells seems not to be mediated by Y_1/Y_2 receptors (Wahlestedt *et al.*, 1990a; Grundemar & Håkanson, 1990). In the present study we sought to determine which NPY analogues are capable of activating the vascular pressor receptors for NPY. It was shown in the mepyramine-pretreated rat that NPY 1–36 and the Y_1 receptor-selective analogue, [Pro^{34}]-NPY, were equally effective in increasing AP, whereas NPY 2–36 was much less potent and NPY 4–36 and NPY 11–36 were inactive. The present results suggest that the hypertensive response to NPY reflects activation of postjunctional Y_1 receptors. The low efficacy of the C-terminal NPY fragments on the AP may in addition reflect a more rapid metabolism compared to the parent molecule. The lack of reflex bradycardia in response to the NPY-evoked pressor effects may be due to the low HR (approx. 250 b.p.m.) in ketamine-xylazine anaesthetized rats compared to conscious or pentobarbitone anaesthetized rats where the HR is about 350–400 b.p.m.

NPY constricts coronary vessels of several species (Allen *et al.*, 1983; Rioux *et al.*, 1986; Macho *et al.*, 1989). In the perfused rat coronary vessels, NPY 1–36, [Pro^{34}]-NPY, NPY 2–36 and NPY 5–36 concentration-dependently increased the coronary resistance. NPY 1–36 and [Pro^{34}]-NPY were roughly equipotent whereas NPY 2–36 and NPY 5–36 were about 7 and 20 times less potent than NPY 1–36, respectively. NPY 11–36, NPY 20–36, NPY 22–36 had a slight effect only at very high concentrations; NPY 23–36 was inactive. The rapid loss of potency induced by progressive N-terminal truncation is in agreement with previous results on guinea-pig coronary vessels (Rioux *et al.*, 1986). Thus, the vasoconstriction evoked by NPY in the rat coronary vessel seems to be mediated predominantly by Y_1 receptors.

In the guinea-pig isolated inferior caval vein [Pro^{34}]-NPY, NPY 1–36, and NPY 2–36 were very effective in inducing a concentration-dependent contraction. [Pro^{34}]-NPY was more potent than NPY 1–36 while NPY 2–36 was equipotent with NPY 1–36. NPY 4–36, NPY 5–36 and NPY 11–36 were much less potent than the parent molecule. Nonetheless, in comparison with the perfused coronary vessels, several C-terminal fragments (longer than 11–36) were relatively more effective in this preparation, suggesting the presence of both Y_1 and Y_2 receptors in the vein.

The curves illustrating the displacement of [^{125}I]-PYY from rat aortic smooth muscle cells by the NPY-related peptides are consistent with a homogeneous population of Y_1 receptors, since [Pro^{34}]-NPY displayed the same affinity as NPY 1–36 on the binding sites and NPY 13–36 had 2–3 orders of magnitude lower affinity. The corresponding displacement curves for the smooth muscle cells from the rat caval vein suggest a mixed population of Y_1/Y_2 receptors since both [Pro^{34}]-NPY and NPY 13–36 displayed a high affinity for the binding sites. For detailed studies of which parts of the ligand are involved in binding, Y_1 and Y_2 receptors can be studied selectively in neuroblastoma cell lines that seem to express either of the two receptor subtypes (Schwartz *et al.*, 1990; Wahlestedt *et al.*, 1990a).

In conclusion, this study has shown the presence of Y_1 receptors in both arteries and veins. The NPY-evoked pressor response in the whole rat and in the coronary vessels seems to reflect the activation of Y_1 receptors, and the characteristics of NPY binding to aortic smooth muscle cells correspond to such receptors. In the caval vein, the contractile effects and the binding characteristics of the NPY-related peptides suggest a mixed population of Y_1 and Y_2 receptors.

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The resistance of some rat cerebral arteries to the vasorelaxant effect of cromakalim and other K^+ channel openers

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1 Cromakalim (0.01–30 μM) and sodium nitroprusside (SNP, 0.01–100 μM) were tested for their ability to relax a number of pre-contracted small arteries (approximate diameter 200–700 μM at 100 mmHg) from the rat, rabbit and guinea-pig.

2 In the rat, SNP (0.01–100 μM) caused near maximal relaxation in all vessels studied including the middle cerebral, anterior cerebellar, basilar, mesenteric and renal arteries. Cromakalim (0.01–30 μM) relaxed pre-contracted mesenteric and renal arteries but was only a weak relaxant of all the rat cerebral arteries with the exception of the basilar artery. Similar experiments using mesenteric and cerebral vessels from the rabbit and guinea-pig showed cromakalim could relax pre-contracted vessels in a concentration-dependent manner.

3 Two other K^+ channel openers, nicorandil and pinacidil, were also tested for their ability to relax rat cerebral arteries. Nicorandil (0.01–100 μM) was ineffective in the rat anterior cerebellar artery at concentrations up to 100 μM. Pinacidil (0.01–100 μM) caused significant vasorelaxation, although high concentrations were required (> 10 μM) and the response was insensitive to the effects of glibenclamide (3 μM).

4 Electrophysiological experiments with the rat anterior cerebellar artery showed that cromakalim (up to 30 μM) failed to influence the resting membrane potential of impaled single smooth muscle cells.

5 The results showed that some rat small cerebral arteries were resistant to the effects of K^+ channel openers including cromakalim, pinacidil and nicorandil. This is peculiar to this vascular tree since the same vessels from other species do not exhibit the same behaviour. Such a phenomenon could result from a lack of K^+ channels opened by cromakalim in rat cerebral arteries.

Keywords: K^+ channels; cromakalim; pinacidil; nicorandil; cerebral arteries; vascular specialization

Introduction

Cromakalim is representative of a new class of smooth muscle relaxants (Hamilton *et al.*, 1986; Edwards & Weston, 1990) which act by opening a type of K^+ channel (termed K_c^+ in this paper) in the cell membrane resulting in membrane hyperpolarization. There is a great deal of interest in the vascular actions of cromakalim, not only does it represent a new class of therapeutic agent, but also its haemodynamic profile appears to differ from other anti-hypertensives, in particular Ca^{2+} antagonists such as nifedipine (Buckingham, 1988; Shoji *et al.*, 1990). That cromakalim and other K_c^+ channel openers may exhibit some organ specificity is an intriguing property. A number of *in vivo* studies have shown cromakalim to dilate selectively some vascular beds compared with others (Buckingham *et al.*, 1986; Hof *et al.*, 1988). In addition cromakalim and other K_c^+ channel openers appear to be more efficacious, *in vivo* at least, in ischaemic tissues (Angersbach & Nicholson, 1988; Maruyama *et al.*, 1989). The mechanisms behind such selectivity are currently unknown.

In some preliminary studies we observed that cromakalim was an unusually poor vasorelaxant in some isolated small cerebral arteries of the rat. The purpose of the present work was to extend these observations to determine the extent of such resistance in a variety of vessels of the same size. We found that such resistance was confined to the rat cerebral circulation and possibly results from a lack of K_c^+ channels in this vascular bed. A preliminary account of this work was presented at the 24th annual meeting of the Australian Society of Clinical and Experimental Pharmacologists (Stork *et al.*, 1990).

Methods

Isolation of small resistance arteries

Wistar Kyoto (WKY) or spontaneously hypertensive (SHR) rats were killed by CO_2 asphyxia. In some experiments WKY rats were also killed by a blow to the head. Guinea-pigs were killed by a blow to the head and rabbits were killed by an overdose of pentobarbitone (60 mg kg^{-1} i.v.). The organ under study was rapidly removed and placed in ice cold Krebs solution (composition in mM: NaCl 119, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, $CaCl_2$ 2.5 and glucose 11) gassed with 5% CO_2 in O_2. Two mm segments were mounted in a small vessel myograph to record isometric tension development as previously described (Angus *et al.*, 1988). Briefly two 40 μm wires were threaded through the lumen of the vessel segment. One wire was attached to a stationary support driven by a micrometer while the other was attached to an isometric force transducer which measured force development. Force development was recorded on a dual flat bed recorder (W&W Scientific Instruments, model 320). Vessels were allowed to equilibrate under zero tension for 30 min at 36°C.

A passive length-tension curve was constructed as previously described (Mulvany & Halpern, 1977). Since some vessels (the rat cerebral vessels) displayed spontaneous myogenic tone, sodium nitroprusside (SNP, 0.1 mM) was included in the bath to cause full relaxation while constructing the curve. The vessel was set at a diameter 0.9 times the diameter of the vessel at 100 mmHg. This point on the passive length-tension curve corresponds to the point of maximum active tension development (Mulvany & Halpern, 1977). Non-linear curve fitting of the passive length-tension curve was achieved using a custom written program for the IBM PC (NORMALIZE, GA McPherson) which uses the Marquart-Levenberg modification of the Gauss-Newton technique (McPherson, 1985). In the text, vessel diameter is expressed as the diameter at an equivalent transmural pressure of 100 mmHg (D_{100}). Upon completion of the passive length-tension curve, vessels were washed and allowed to equilibrate for 30 min before the addition of any drugs. The viability of the tissue was then assessed by adding a K^+ depolarizing solution (in which all NaCl in the Krebs buffer was replaced by an equimolar concentration of KCl) to activate the tissue maximally.

Unless otherwise stated the vasorelaxant activity of cromakalim was assessed in tissues precontracted with a sub-

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maximal concentration of 5-hydroxytryptamine (5-HT; approximately 0.3–1 μM). The rat cerebral vessels were unusual in that they displayed spontaneous myogenic tone. However, in the majority of experiments with these vessels, active tone was induced with a sub-maximal concentration of 5-HT so that a constant level of tone could be generated throughout the duration of the experiment. Vasorelaxant responses of the drugs tested were expressed as a percentage of the maximum level of tone. This was calculated as the difference between tone in the presence of the constrictor and that in the presence of SNP (0.1 mM), which was added at the end of every concentration-effect curve constructed to a vasorelaxant.

Cumulative concentration-effect curves were constructed to cromakalim (0.01–30 μM) and SNP (0.01–100 μM) which was used as the control vasorelaxant. In additional experiments, we also determined the ability of pinacidil and nicorandil (K_c^+ channel openers, 0.01–100 μM) and three other vasorelaxants (acetylcholine 0.01–3 μM, glyceryl-trinitrate 0.01–3 μM and nitric oxide 0.1–100 μM) for their ability to cause vasorelaxant responses in selected tissues. Nitric oxide (NO) solutions were prepared from acidified sodium nitrite as previously described (Cocks & Angus, 1990). The final concentration of NO generated was assumed to be equivalent to the final concentration of acidified sodium nitrite used.

We were also interested in the effect of cromakalim on spontaneous myogenic tone which was displayed by the rat small cerebral arteries. It has been our experience that myogenic behaviour of small resistance arteries is better observed when vascular reactivity is assessed under isobaric/isotonic conditions. Consequently in some experiments the ability of cromakalim to alter vascular tone of the small resistance arteries was assessed isobarically using a newly developed computer based system (ISOTONIC, McPherson, unpublished data). In these experiments the manual micrometer on the small vessel myograph was replaced with a computer controlled motor (Burleigh Inchworm motor controlled by a 6000 series controller, Burleigh, U.S.A.). Wall tension in the small resistance artery was continually monitored using an analogue to digital card (DASH16, Metrabyte, U.S.A.) and transmural pressure calculated. In isobaric mode the computer program could adjust the separation distance of the wires, by the use of the inchworm motor, such that pressure was held constant at a pre-set level. Vascular reactivity was therefore assessed by monitoring changes in vessel diameter.

Electrophysiological experiments

In some experiments the intracellular resting membrane potential was monitored in rat anterior cerebellar vessels mounted in the small vessel myograph. A conventional glass electrode (see McPherson & Angus, 1991 for details) was used to impale a single smooth muscle cell. Cumulative concentrations of cromakalim were added to the bath and membrane potential and tension changes were monitored simultaneously. Since the rat anterior cerebellar artery displayed intrinsic myogenic tone, vasorelaxant responses could be assessed without the addition of a vasoconstrictor agent.

Statistics and data analysis

Statistical comparisons between two groups were made by use of Student's t test. Results in the text are the mean ± s.e.mean for the specified number of experiments. The potency of the vasorelaxants are expressed as the −log concentration of the drug required to produce 50% relaxation (pEC_{50}). Relaxation responses were calculated as a percentage of active tone with 100% implying full relaxation.

Drugs

The following drugs were used: cromakalim (Beecham); pinacidil monohydrate (Leo); nicorandil (Chugai); glibenclamide (Hoechst); sodium nitroprusside (Nipride, Roche); glyceryl-trinitrate (David Bull Laboratories, Australia); acetylcholine bromide (Sigma); U46619 (1,5,5-hydroxy-11a,9a (epoxy-methano) prosta-E2,13-dienoic acid, Upjohn); endothelin-1 (Austpep, Australia).

Cromakalim (10 mM), pinacidil (10 mM), nicorandil (10 mM) and glibenclamide (1 mM) stock solutions were made in 100% methanol. All other drugs were made up on the day of experiment. Dilutions of all drugs were made in distilled water.

Results

Characteristics of the small arteries studied

Figure 1 shows a schematic diagram of the rat cerebral circulation showing the origin of the cerebral vessels studied. These were the basilar, anterior cerebellar and the middle cerebral arteries. In selected studies anatomically similar vessels were also taken from the rabbit and guinea-pig. Several peripheral small arteries were also studied including a mesenteric and renal artery. The diameters of all vessels studied, at an estimated transmural pressure of 100 mmHg, are listed in Table 1. The vessels ranged in size from 200–700 μm depending on the species and origin of the vessel. The viability of each vessel was assessed at the commencement of each experiment by fully activating the preparation using a K^+ depolarizing solution. All vessels developed significant amounts of tension, which were similar when the values were standardized for differences in vessel diameter (Table 1).

Comparison of the vasorelaxant effect of cromakalim in cerebral and peripheral vessels from rat, rabbit and guinea-pig

SNP was an effective vasorelaxant in all SHR and WKY rat cerebral and peripheral vessels studied although its potency did vary to some extent (Figures 2, 3 and 5; Table 2). In the mesenteric artery SNP had a pEC_{50} of approximately 6.6 while in the anterior cerebellar it was approximately 20 fold less potent (5.3). In all cases SNP could reverse nearly all the vasoconstrictor-induced tone (greater than 95% relaxation), except in the WKY small renal vessel where 80% of active tone was reversed at the highest concentration of SNP used.

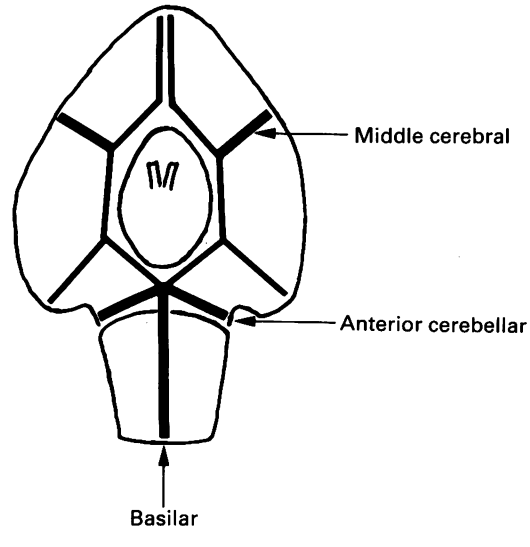


Figure 1 Dorsal view (schematic diagram) of the rat brain showing the basilar, anterior cerebellar and middle cerebral arteries which were used. Anatomically similar vessels were also obtained from the rabbit and guinea-pig.

Table 1 (A) Arterial diameters (μm) at 100 mmHg, of the vessels studied estimated during the normalization procedure and (B) mean active tension (mN mm^{-1}) developed to a K^+ depolarizing solution which was used to assess tissue viability

Animal	*Mesenteric*	*Renal*	*Basilar*	*Anterior cerebellar*	*Middle cerebral*
A			*Diameter* (μm)		
WKY	303 (14)	486 (46)	399 (16)	251 (8)	266 (8)
SHR	298 (11)	—	351 (19)	226 (12)	233 (5)
Rabbit	383 (45)	—	788 (54)	443 (10)	397 (19)
Guinea-pig	398 (16)	—	—	362 (8)	—
B			*Tension* (mN mm^{-1})		
WKY	4.7 (0.6)	5.3 (0.4)	5.9 (0.7)	2.9 (0.5)	2.7 (0.5)
SHR	5.3 (0.4)	—	7.4 (0.6)	2.9 (0.6)	2.7 (0.3)
Rabbit	5.5 (0.9)	—	7.7 (1.0)	4.6 (0.6)	3.8 (0.7)
Guinea-pig	6.3 (0.9)	—	—	4.4 (0.05)	—

Values are the mean (s.e.mean) of 4–10 separate determinations.

Cromakalim was particularly ineffective as a vasorelaxant on rat small cerebral arteries with the exception of the basilar artery. In the WKY and SHR basilar artery, cromakalim was able to produce significant vasorelaxant response in tissues pre-constricted with 5-HT (Figure 2, Table 2). In the WKY vessels approximately 60% and in the SHR 35% of the active tone was reversed. These were not significantly different ($P > 0.05$, unpaired t test). In addition cromakalim was approximately 10 fold more potent in rat mesenteric and renal small arteries (pEC_{50} basilar 5.58 cf 6.42 mesenteric). In the anterior cerebellar and middle cerebral artery from both strains cromakalim was a particularly poor relaxant, with only the highest concentration of cromakalim used (30 μM) causing any marked relaxation (Figure 3). Approximately 20% of active tone could be reversed by cromakalim in these tissues. In the majority of studies using the rat, vessels were obtained from animals killed by CO_2 asphyxia. Cerebral vessels are known to be quite sensitive to CO_2. Consequently we repeated some experiments with rats killed by a blow to the head to determine whether the method of killing the animals affected the response to cromakalim. Figure 4 shows the vasorelaxant responses to cromakalim in anterior cerebral or basilar arteries from these rats in which the tissues were precontracted with either 5-HT or a low concentration of K^+ (20 mM). Again, particularly in the anterior cerebellar artery, cromakalim was a weak relaxant causing only 20–40% maximum relaxation at the highest concentration of cromakalim used (30 mM).

In contrast, a number of peripheral rat vessels were found to be sensitive to cromakalim. Thus in the WKY mesenteric and renal artery and SHR mesenteric artery, cromakalim was an effective vasorelaxant with a pEC_{50} of approximately 6.4 and with an ability to cause greater than 95% relaxation (Figure 5, Table 2).

For comparative purposes the ability of cromakalim to relax pre-contracted cerebral and mesenteric vessels from the rabbit and guinea-pig was also studied. In contrast to the results obtained in the rat, cromakalim was an effective vasorelaxant in rabbit and guinea-pig cerebral arteries (Figures 2, 3 and 5; Table 3). In all cases the pEC_{50} value for cromakalim was approximately 6.4. In addition cromakalim caused greater than 90% relaxation. As in the rat cerebral vessels, SNP was approximately 10 times less potent on the anterior cerebellar artery than on the mesenteric artery in the rabbit (Figure 3, Table 2). However, in the guinea-pig both cromakalim and SNP appeared to be relatively potent vasorelaxants (Figure 3, Table 2).

Responses of the rat anterior cerebellar artery to pinacidil and nicorandil

Nicorandil (0.01–100 μM), like cromakalim, was ineffective as a vasorelaxant in the WKY anterior cerebellar artery (Figure 6). This compound caused only 11% relaxation at a concentration of 100 μM. However, pinacidil (0.01–100 μM) was able to cause significant relaxation (approximately 75% relaxation of

Table 2 pEC_{50} and maximum response values for cromakalim and sodium nitroprusside (SNP) obtained in rat cerebral and peripheral small resistance arteries pre-contracted with 5-hydroxytryptamine (0.3–1 μM)

	Cromakalim		*SNP*	
	pEC_{50}	*%relax.*	*pEC_{50}*	*%relax.*
WKY				
Basilar	5.58 (0.07)	61 (10)	5.86 (0.06)	97 (2)
Ant. cereb.	—*	17 (4)	5.29 (0.07)	95 (4)
Middle cer.	—*	23 (3)	5.52 (0.07)	98 (1)
Mesenteric	6.42 (0.08)	96 (1)	6.56 (0.2)	94 (2)
Renal	6.18 (0.07)	96 (2)	6.21 (0.3)	80 (5)
SHR				
Basilar	5.48 (0.1)	35 (9)	5.74 (0.1)	91 (3)
Ant. cereb.	—*	8 (4)	5.52 (0.2)	95 (3)
Middle cer.	—*	19 (4)	5.58 (0.1)	94 (4)
Mesenteric	6.28 (0.1)	95 (2)	6.98 (0.1)	97 (1)
Renal	NS			

Results are the mean (s.e.mean) of 4 to 12 separate determinations.
pEC_{50} is the $-\log$ (M) of the concentration of drug required to cause 50% relaxation.
% relax. is the percentage reduction in total active tone.
* Curve could not be processed by the computer due to an absence of points, therefore, %relax. is the maximum relaxation observed at the highest concentration of cromakalim used (30 μM). NS, not studied.

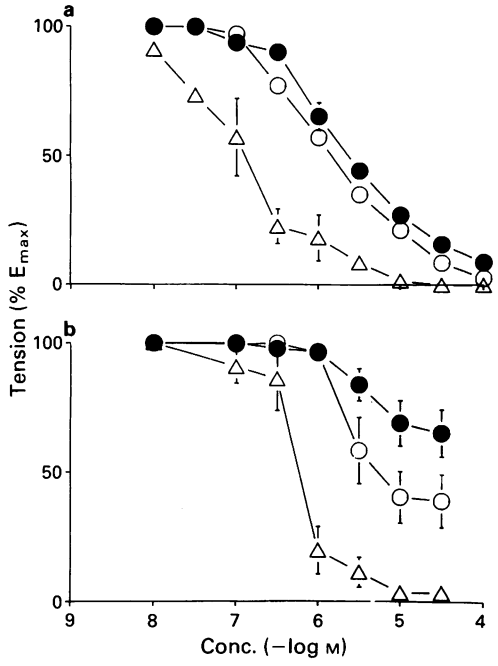


Figure 2 Mean concentration-effect curves for sodium nitroprusside (SNP) (a) and cromakalim (b) in the basilar artery from WKY (○), SHR (●) and rabbit (△). Tissues were precontracted with 5-hydroxytryptamine (0.3–1 μM). Results are expressed as a percentage of the total amount of constrictor tone (%E_{max}) defined by a maximal concentration of SNP (0.1 mM) added at the end of each experiment. Values are the mean from 4 to 12 separate experiments.

active tone) with a pEC_{50} of approximately 4.5 (Figure 6). Additional experiments showed that the response to pinacidil (30 μM) in the presence of glibenclamide (3 μM, 50 ± 6% relaxation) was not significantly different from that in its absence (53 ± 5% relaxation, $n = 3$).

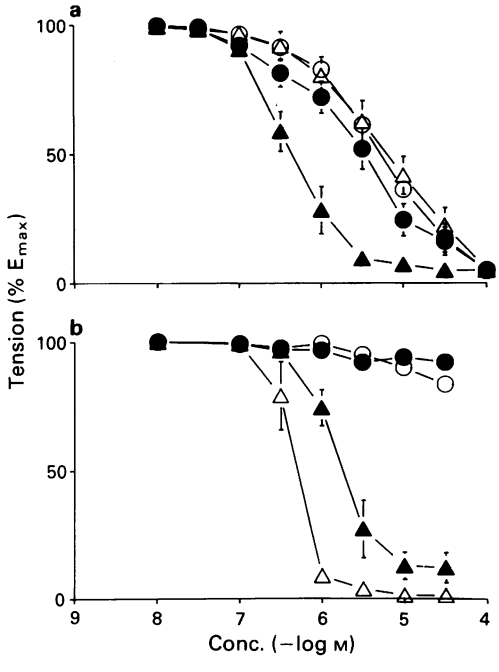


Figure 3 Mean concentration-effect curves for sodium nitroprusside (SNP) (a) and cromakalim (b) in the anterior cerebellar artery from WKY (○), SHR (●), rabbit (△) and guinea-pig (▲). Tissues were precontracted with 5-hydroxytryptamine (rat and rabbit, 0.3–1 μM) or histamine (guinea-pig 1–30 μM). Results are expressed as a percentage of total constrictor tone (%E_{max}) and are the mean from 4 to 12 separate experiments.

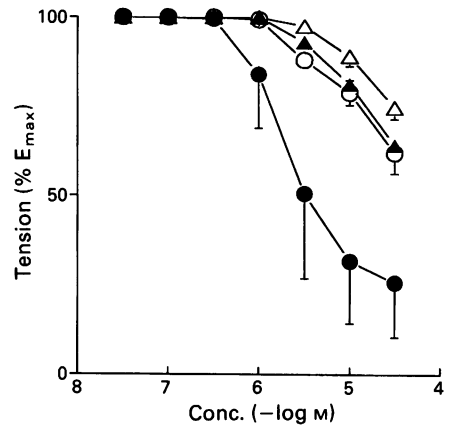


Figure 4 Mean concentration-effect curves for cromakalim in the anterior cerebellar (△, ▲) or the basilar artery (○, ●) from WKY rats killed by a blow to the head. Tissues were precontracted with 5-hydroxytryptamine (solid symbols, 0.3–1 μM) or K^+ (open symbols, 20 mM). Results are expressed as a percentage of total constrictor tone (%E_{max}) defined by sodium nitroprusside (0.1 mM) and are the mean from 4 to 6 separate experiments.

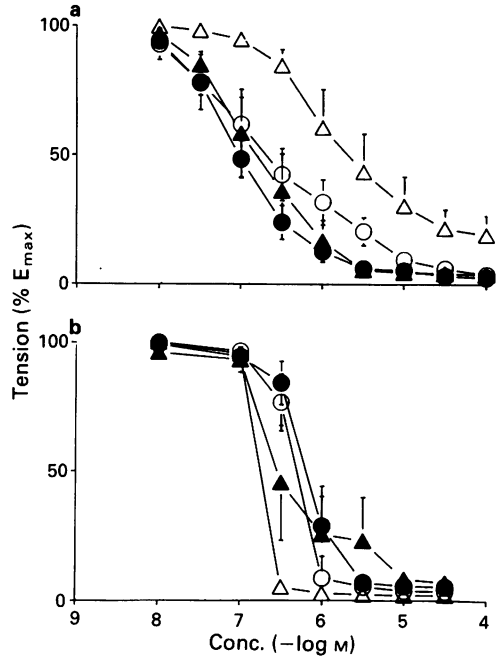


Figure 5 Mean concentration-effect curves for sodium nitroprusside (SNP) (a) and cromakalim (b) in the mesenteric artery from WKY (○), SHR (●), rabbit (△) and guinea-pig (▲). Tissues were precontracted with 5-hydroxytryptamine (rat, 0.3–1 μM) or noradrenaline (rabbit and guinea-pig, 30–100 μM). Results are expressed as a percentage of the total constrictor tone (%E_{max}) and are the mean from 4 to 12 separate experiments.

Electrophysiological characteristics of the rat anterior cerebellar artery

The rat anterior cerebellar artery had a resting membrane potential (Em) of −38 ± 2 mV (7 impalements from 3 arteries) in the absence of any vasoconstrictor agent. The membrane potential was unstable and oscillated with an amplitude of between 2 to 5 mV. Figure 7 shows the results from an experiment examining the effects of cromakalim. Cromakalim caused only a small hyperpolarization (−2 ± 1 mV, $n = 3$) even at concentrations up to 30 μM. In addition there was no effect on the spontaneous tone displayed by these vessels. In contrast SNP (0.1 mM) caused a hyperpolarization (−14 ± 5 mV, $n = 3$) and relaxation (Figure 7) in vessels displaying spontaneous myogenic tone.

Table 3 pEC_{50} and maximum response values for cromakalim and sodium nitroprusside (SNP) obtained in rabbit and guinea-pig cerebral and peripheral small resistance arteries

	Cromakalim		*SNP*	
Vessel	pEC_{50}	*%relax.*	pEC_{50}	*%relax.*
Rabbit				
Basilar	6.39 (0.14)	97 (2)	6.82 (0.3)	100 (0)
Ant. cereb.	6.33 (0.05)	99 (1)	5.26 (0.2)	95 (3)
Middle cer.	6.28 (0.1)	99 (1)	5.76 (0.2)	98 (1)
Mesenteric	6.75 (0.04)	98 (1)	5.83 (0.2)	81 (7)
Guinea-pig				
Ant. cereb.	5.84 (0.06)	88 (6)	6.39 (0.1)	95 (4)
Mesenteric	6.36 (0.3)	93 (2)	6.96 (0.2)	96 (1)

Mesenteric arteries of both species were pre-contracted with noradrenaline (30–100 μM). Rabbit cerebral vessels were pre-contracted with 5-hydroxytryptamine (0.3–1 μM) while the guinea-pig vessel was pre-contracted with histamine (1–30 μM). Results are the mean (s.e.mean) of 4 to 7 separate determinations.
pEC_{50} is the $-\log$ (M) of the concentration of drug required to cause 50% relaxation.
%relax. is the percentage reduction in total active tone.

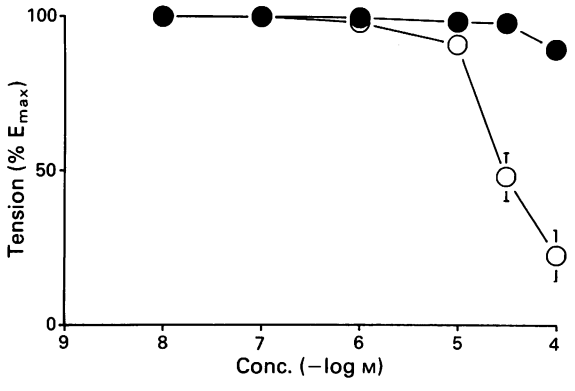


Figure 6 Mean concentration-effect curves obtained for nicorandil (●) and pinacidil (○) in WKY anterior cerebellar artery. Tissues were pre-contracted with 5-hydroxytryptamine (0.3–1 μM). Responses are the mean from six separate experiments expressed as a percentage of total constrictor tone.

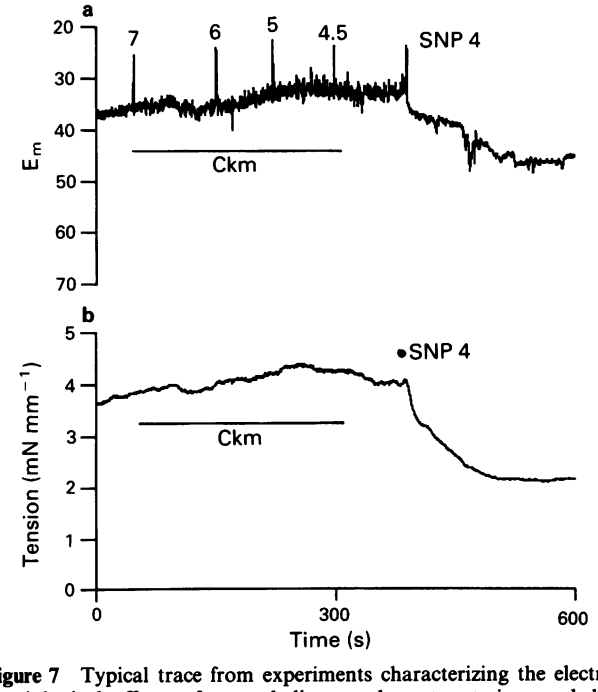


Figure 7 Typical trace from experiments characterizing the electrophysiological effects of cromakalim on the rat anterior cerebellar artery. (a) Shows resting membrane potential (E_m) and the bottom panel the tension trace. Tone in the vessel was present by virtue of spontaneous myogenic tone displayed by this particular vessel. Responses to cromakalim (Ckm) and sodium nitroprusside (SNP) are the $-\log$ (M).

Responses of the rat anterior cerebellar artery to glyceryl trinitrate, nitric oxide and acetylcholine

Glyceryltrinitrate (GTN, 0.01–3 μM), nitric oxide (NO, 0.1–100 μM) and acetylcholine (0.01–3 μM) were tested for their ability to relax rat anterior cerebellar and mesenteric arteries precontracted with a submaximal concentration of 5-HT (0.3–1 μM). In the mesenteric artery all three compounds caused marked relaxant responses with approximate pEC_{50} values of 7.5, 6.8 and 5 for acetylcholine, GTN and NO respectively (Figure 8). Acetylcholine caused near maximal relaxation while GTN caused approximately 70% at the highest concentration used (3 μM). Full concentration-effect curves to NO could not be constructed. However, at the highest concentration used (100 μM) NO cause approximately 50% relaxation (Figure 8).

Relaxation responses to these compounds in the anterior cerebellar artery were dissimilar to those in the mesenteric artery (Figure 8). In the anterior cerebellar artery acetylcholine was a relatively poor vasorelaxant causing only 20%

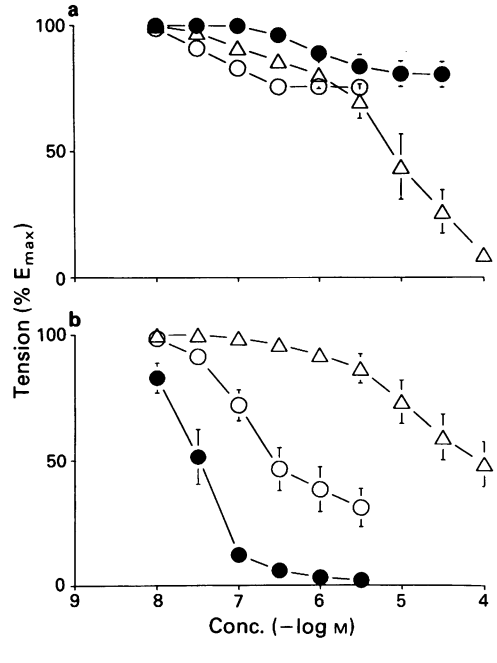


Figure 8 Mean concentration-effect curves obtained for acetylcholine (●), glyceryl trinitrate (○) and nitric oxide (NO, △) in WKY anterior cerebellar artery (a) and mesenteric artery (b) preconstricted with 5-hydroxytryptamine (0.1–30 μM). Responses are the mean of 4–8 separate experiments expressed as a percentage of the total constrictor tone.

relaxation with a pEC_{50} of approximately 6. GTN displayed a similar potency as it did in the mesenteric artery (pEC_{50} = 7.25). However, it caused only 25% reversal of maximum active tone. Conversely, NO was a relatively powerful relaxant causing near maximal relaxation of tissue with a pEC_{50} of 5.25 (Figure 8).

The effect of cromakalim on spontaneously contracted rat anterior cerebellar arteries and arteries pre-contracted with U46619

We also determined whether the inability of rat cerebral vessels to relax to cromakalim depended on the nature of the vasoconstrictor used. In tissues pre-contracted with a submaximal concentration (0.1 μM) of the thromboxane-mimetic U46619, cromakalim, pinacidil and SNP displayed similar vasorelaxant actions to those seen when 5-HT was used to induce tone (Figure 9).

The ability of cromakalim to relax rat anterior cerebellar vessels which displayed spontaneous myogenic tone was also studied. In these studies vascular reactivity was assessed under isobaric conditions where calculated transmural pressure was held constant (100 mmHg) by altering vessel diameter. Vascular reactivity was also assessed isometrically. Figure 10a and b shows the result of an experiment with a single rat anterior cerebral vessel, where cromakalim was unable to affect tone at concentrations up to 30 μM under isobaric and isometric conditions. However, SNP was an effective vasorelaxant in both circumstances.

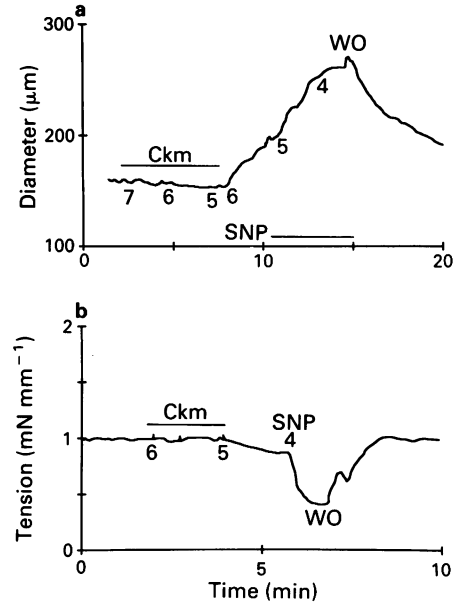


Figure 10 Original trace showing the effect of cromakalim (Ckm) and sodium nitroprusside (SNP) to relax rat anterior cerebellar artery which has spontaneous myogenic activity. (a) Vascular reactivity recorded isobarically (pressure = 100 mmHg) where vessel diameter is monitored. (b) Isometric recording in the same blood vessel. Concentrations are the −log M. WO = washout of drug.

Discussion

The main finding from this work is that the effects of cromakalim on a number of rat cerebral vessels are poor compared with its actions on rat peripheral vessels (mesenteric and renal) and both cerebral and mesenteric vessels from the rabbit and guinea-pig. The resistance of the rat cerebral arteries to cromakalim was not uniform. The basilar artery from both strains of rat studied (SHR and WKY) was responsive to cromakalim, although the concentrations required to cause vasorelaxation were somewhat higher (pEC_{50} = 5.5) than those required to relax a range of vascular (this study) and non-vascular smooth muscle preparations (pEC_{50} = 6–6.5; McPherson & Angus, 1990). However, in the rat anterior cerebellar and middle cerebral arteries, cromakalim was a particularly weak vasorelaxant with less than 30% of 5-HT induced tone reversed by this compound at the highest concentration used. In addition, we recently tested the active isomer of cromakalim (BRL 38227) and found that it also failed to relax rat anterior cerebellar artery (data not shown), indicating that the results with cromakalim did not result from the use of the racemate.

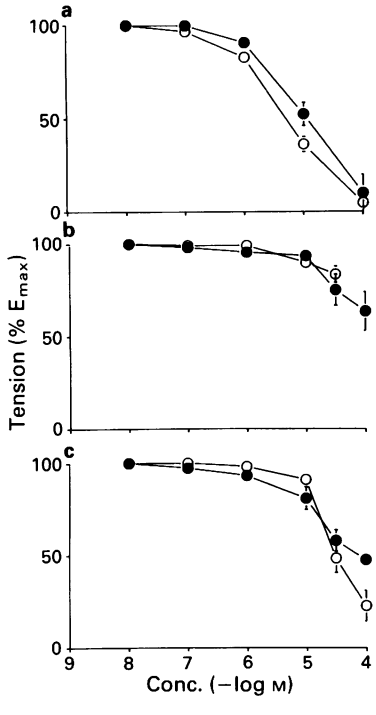


Figure 9 Mean concentration-effect curves constructed to sodium nitroprusside (SNP) (a), cromakalim (b) and pinacidil (c) in the rat anterior cerebellar artery in vessels precontracted with either 5-hydroxytryptamine (0.3–1 μM, ○) or U46619 (0.1 μM, ●). Responses are the mean from 4 to 10 separate experiments and are expressed as a percentage of the total constrictor tone.

There has been some conflicting data recently published over whether K^+ channel openers are, in fact, able to relax rat cerebral vessels. The studies of Wahl and co-workers (Parsons *et al.*, 1991a; Ksoll *et al.*, 1991), described near maximal relaxation responses to cromakalim in 5-HT pre-contracted rat basilar arteries. In their studies the pEC_{50} for cromakalim was approximately 6.35, while in our study the reversal of tone was only partial (40–60%) and the potency was less (pEC_{50} = 5.5). More recently this group (Parsons *et al.*, 1991b) has also shown that pinacidil could relax rat pial arterioles *in situ*. In a separate study, the ability of cromakalim to relax isolated perfused segments of the rat superior cerebellar artery has also been described (Nagao *et al.*, 1991). Collectively these data appear at odds with the results described in our study. However, in agreement with our results, Halpern and co-workers (McCarron *et al.*, 1991) have recently shown in a preliminary study that perfused isolated cerebral arteries of the rat are resistant to the effects of pinacidil and cromakalim. The reasons for these differences are unclear at this time. The fact that the rat basilar (present study; Ksoll *et al.*, 1991) and rat arterioles (Parsons *et al.*, 1991b) respond to K_c^+ channel openers, may indicate that the resistance is only displayed by vessels of a diameter (250 μm) predominantly used in our study.

We also tested two other K^+ channel opening compounds, nicorandil and pinacidil, and found that they were also poor vasorelaxants in the rat cerebral vasculature. There were two

unusual observations made during these studies. First, nicorandil was particularly ineffective despite the fact that previous studies by us and others (see McPherson & Angus, 1989) have shown that nicorandil can produce vasorelaxant responses which do not involve the opening of K_c^+ channels. Consequently our expectation was that this compound would cause a vasorelaxant response. Conversely pinacidil did cause significant vasorelaxant responses which were insensitive to glibenclamide, a compound known to antagonize the K^+ opening induced by pinacidil (see McPherson & Angus, 1990; 1991). It would appear therefore that pinacidil, along with cromakalim and nicorandil, may be able to relax vascular smooth muscle using a number of mechanisms; although their predominant effect appears to result from the opening of K_c^+ channels.

We considered a number of possibilities as to why rat cerebral vessels should display such resistance in our studies. First, the inability of cromakalim to relax rat cerebral vessels may result from the type of vasoconstrictor used to induce tone. This appeared not to be the case since similar results were obtained when either 5-HT, U46619 or low concentrations of K^+ (20 mM) were used. In addition, the rat cerebral vessels also displayed spontaneous myogenic tone which was little affected by cromakalim but was sensitive to SNP.

The fact that rat cerebral vessels display spontaneous myogenic tone may result from the absence of K_c^+ channels in these vessels. We have previously provided evidence that K_c^+ channels may tonically influence the resting membrane potential (by hyperpolarizing it) in selected vascular beds such as the rat small mesenteric artery (McPherson & Angus, 1991). In the case of the rat mesenteric artery it would appear that spontaneously opened K_c^+ channels hyperpolarize the vessel from approximately -50 to -60 mV (McPherson & Angus, 1991). Electrophysiological experiments revealed that the rat anterior cerebellar artery has a low resting membrane potential compared with other tissues (-38 mV anterior cerebellar cf -60 mV mesenteric). It is possible that, in the absence of spontaneously opened K_c^+ channels in this tissue, voltage operated Ca^{2+} channels open to cause the spontaneous tone. The spontaneous myogenic activity displayed by this vessel was abolished by the Ca^{2+} channel antagonist felodipine (30 nM), which is consistent with this hypothesis (data not shown). The finding that cromakalim did not hyperpolarize the rat anterior cerebellar artery would indicate that the K_c^+ is absent rather than being fully closed in this vessel. SNP did cause a hyperpolarization in association with relaxation in the rat anterior cerebellar artery. We have observed that high concentrations of SNP can cause hyperpolarization in vessels obtained from a number of vascular beds. However, the mechanism behind the response is currently unknown.

It is possible that the rat cerebral vessels were less responsive to the vasorelaxants studied by an, as yet, unidentified mechanism. The ability of GTN and acetylcholine to relax rat cerebral vessels was less than that observed using rat mesenteric arteries. In addition, SNP was generally more potent in rat mesenteric and rabbit and guinea-pig small arteries than in rat anterior cerebral arteries; a finding which would support this idea. However, there were exceptions, in particular the rabbit anterior cerebral artery where cromakalim was a more potent relaxant than SNP. Also nitric oxide (NO) was a more powerful vasorelaxant in the rat anterior cerebral artery than in the rat mesenteric artery. Consequently, there does not appear to be a clear correlation between the potency of the vasorelaxants and the ability of cromakalim to elicit a response. The possibility also exists that rat cerebral vessels were in someway damaged upon removal and mounting of the vessel in the small vessel myograph, which had a deleterious effect on responses to the vasorelaxants. We used a number of approaches to kill the animal (CO_2 asphyxia or a blow to the head) and a number of vasoconstrictors to induce tone and showed that these had no effect on the poor response to cromakalim. Despite this, the relatively low and unstable resting membrane potential displayed by the anterior cerebellar artery (-40 mV) is unusual and may be indicative of some form of damage. However, work by Hirst and his colleagues (Edwards *et al.*, 1988) have also shown segments of the rat cerebral vasculature to have a similar unstable resting membrane potential associated with spontaneous myogenic activity. This does not preclude the possibility that the rat cerebral vessels were deleteriously affected in both studies. However, given that the vessels are functionally normal in terms of their response to a number of vasoconstrictors we suspect that this is not the case. This idea is also supported by a number of *in vivo* studies.

That cromakalim may display some regional selectivity in its action is supported by a number of *in vivo* studies. For example, in the anaesthetized rabbit cromakalim preferentially dilates the coronary, gastrointestinal and cerebral blood vessels but to a lesser extent the renal and skeletal muscle vasculature (Hof *et al.*, 1988). In anaesthetized cats cromakalim increases flow in carotid, mesenteric and renal beds but not to the femoral circulation (Buckingham *et al.*, 1986). In a recent study in conscious spontaneously hypertensive rats (Shoji *et al.*, 1990) cromakalim decreased vascular resistance in a number of beds including the heart, stomach, kidney, skeletal muscle and skin. A significant finding from that study was that cromakalim was without effect on cerebral vascular resistance. The results from this study support the idea that the rat cerebral circulation is resistant to the vasorelaxant actions of cromakalim and other potassium channel openers.

In summary, the results from this study show that a number of small arteries from the rat cerebral circulation are resistant to the vasorelaxant effects of cromakalim and other K_c^+ channel openers. The property is specific for these vessels since such resistance is not displayed in other rat vessels or in the same vessels obtained from other species. While there may be a number of reasons for this phenomenon, it is possible that these vessels may be devoid of K_c^+ channels under the conditions in which the experiments were performed.

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Effect of milacemide on extracellular and tissue concentrations of dopamine and 5-hydroxytryptamine in rat frontal cortex

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1 Milacemide is a glycine prodrug which is both an inhibitor and a substrate for monoamine oxidase-type B (MAO-B) and also an inhibitor of MAO-type A (MAO-A). Its effects on dopamine and 5-hydroxytryptamine (5-HT) metabolism in rat frontal cortex tissue and dialysate were evaluated.

2 Dialysate dopamine concentrations increased linearly and dose-dependently after milacemide administration (100, 200, 400 mg kg^{-1}, i.p.), peaking at 1 h. A concomitant dose-dependent decrease in dialysate 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) concentrations was observed but these changes were smaller (27% and 40% respectively) than the change in dopamine (125% after 400 mg kg^{-1} milacemide).

3 Dialysate 5-HT was significantly increased only at 1.5 h after giving milacemide 400 mg kg^{-1}. Dialysate 5-hydroxyindoleacetic acid (5-HIAA) concentration was not affected.

4 Milacemide (400 mg kg^{-1}) at 1.5 h post-administration significantly increased frontal cortex tissue concentrations of dopamine and 5-HT; the percentage increase in dopamine being about four times that of 5-HT. Metabolite concentrations, including 5-HIAA, decreased. Changes in tissue and dialysate dopamine, DOPAC and HVA were approximately proportionate to each other.

5 The results are explicable in terms of an inhibition by milacemide of MAO-A.

Keywords: Dopamine; glycine prodrug; microdialysis; milacemide; monoamine oxidase; 5-hydroxytryptamine

Introduction

Milacemide (2-n-pentylaminoacetamide) is a drug with reported anticonvulsant (van Dorsser *et al.*, 1983; Houtkooper *et al.*, 1986; Roba *et al.*, 1986), antimyoclonic (Truong *et al.*, 1986), mood elevating and cognitive enhancing properties (Saletu *et al.*, 1986; Handelmann *et al.*, 1989). It readily enters the brain, where it is metabolized by monoamine oxidase type B (MAO-B) to glycinamide (de Varebeke *et al.*, 1988; 1989; Semba *et al.*, 1991) and then to the inhibitory neurotransmitter glycine (Cavalier *et al.*, 1983; Christophe *et al.*, 1983). The drug also increases brain γ-aminobutyric acid (GABA; de Varebeke *et al.*, 1983) and blocks convulsions induced by bicuculline (van Dorsser *et al.*, 1983) which suggest a role for GABA in its pharmacological effects. However, according to a recent microionophoretic study, neither glycine nor GABA is involved in the depressant action of milacemide (Godfraind, 1990). Another possibility is that some of its actions may be mediated via dopamine or 5-hydroxytryptamine (5-HT) because it is an inhibitor as well as a substrate of MAO-B and also inhibits monoamine oxidase type A (MAO-A; de Varebeke *et al.*, 1989; Truong *et al.*, 1989b).

Acute treatment with milacemide is reported to increase tissue 5-HT in various rat brain regions including the cortex (Truong *et al.*, 1989a) and to have similar effects when given chronically to rhesus monkey (de Varebeke *et al.*, 1990). These findings are of clinical interest as milacemide opposes p,p'-DDT-induced myoclonus (Truong *et al.*, 1989a) which has been both proposed (Chung Hwang & Van Woert, 1978) and contested (Pratt *et al.*, 1985) as a rat model for human post-anoxic myoclonus. As intraventricular glycine alleviated the model (Truong *et al.*, 1986) and systemic treatment with the 5-HT precursor 5-hydroxytryptophan alleviates both the model (Chang Hwang & Van Woert, 1978) and human post-anoxic myoclonus (Chadwick *et al.*, 1977) it was reasonable to suggest that milacemide might be of use in the human disorder (Truong *et al.*, 1986).

In the present study, the roles of 5-HT and dopamine in the neurochemical effects of milacemide and their possible implications for its behavioural and therapeutic properties were investigated by monitoring the effects of the drug on amine metabolism by means of a dialysis probe implanted in the frontal cortex of freely moving rats.

Methods

Male Sprague-Dawley rats (Charles River, Margate, Kent) weighing 250–300 g with free access to a normal laboratory diet (SDS R and M number 1 expanded, Scientific Dietary Services, Witham, Essex) and water were individually housed under a 12 h light–dark cycle (lights on 06 h 00 min).

Intracerebral microdialysis

Rats were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and dialysis probes implanted in the frontal cortex (3.2 mm anterior and 5.5 mm ventral to bregma, 2.5 mm lateral to the midline) according to Paxinos & Watson (1986). Concentric dialysis probes 4 mm long, 200 μm diameter, were prepared as previously described (Hutson *et al.*, 1985) except that the internal glass capillary tubes were replaced by vitreous silica tubing (SGE, Milton Keynes) and Filtral 12 (Hospal, Rugby) was used for the dialysis membrane. The following day, artificial CSF (composition mM: NaCl 125, KCl 2.5, $MgCl_2$ 1.18 and $CaCl_2$ 1.26) was perfused through the probes at 2 μl min^{-1}. Dialysate samples (60 μl) were collected every 30 min for 3 h. Animals were then injected with milacemide (100, 200, or 400 mg kg^{-1}; Continental Pharma Inc., Belgium) or saline vehicle (0.9% NaCl) intraperitoneally and sampling continued for a further 3.5 h.

The first two dialysate samples were discarded and subsequent samples immediately analysed for dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA),

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5-HT and 5-hydroxyindoleacetic acid (5-HIAA). Fifty microlitres was directly injected and analysed by high performance liquid chromatography (h.p.l.c.) with electrochemical detection. An Altex Ultrasphere 3-μm ODS column (4.6 mm x 7.5 cm: Beckman Ltd, U.K.) was used; mobile phase, 0.1 M phosphate buffer (pH 2.7) containing 18% methanol, 1 mM octanesulphonate and 0.1 mM EDTA. The flow rate was 1.3 ml min^{-1}. An ESA Coulochem electrochemical detector (model 5100A; Severn Analytical Ltd, U.K.) with a dual-electrode analytical cell (model 5100; Severn Analytical Ltd, U.K.) was used with electrode 1 set at 0.0 V, electrode 2 at 0.5 V and the guard cell (model 5020; Severn Analytical Ltd, U.K.) at +0.7 V. Limits of detection for dopamine, DOPAC, HVA, 5-HT and 5-HIAA were 0.30, 0.25, 1.00, 0.30 and 0.25 pmol ml^{-1} respectively.

Frontal cortex monoamine and metabolite concentrations

In a separate experiment, rats were given milacemide (400 mg kg^{-1}, $n = 8$) or saline ($n = 8$) as before, decapitated 1.5 h later and the frontal cortex removed, weighed, and homogenized in 10 volumes of the mobile phase described above. After centrifugation at 16,000 g for 5 min, 5 μl of the supernatant was analysed for dopamine, DOPAC, HVA, 5-HT and 5-HIAA by h.p.l.c. as before.

Statistics

Statistical analysis of the dialysis values was made by summary measures and analysed by one-way ANOVA followed by Dunnett's test. The Mann-Whitney U-test was used to compare tissue values in the different groups.

Results

Dialysates

Figure 1 shows dialysate dopamine, DOPAC and HVA in the frontal cortex after giving saline or milacemide (100, 200, 400 mg kg^{-1}). Results are expressed as percentage changes with respect to baseline. Dopamine values rose linearly and dose-dependently, peaking at 1 h after injection. Values for rats given milacemide (100 and 200 mg kg^{-1}) returned to baseline at 2 and 4 h after injection respectively but animals given 400 mg kg^{-1} of the drug had values that were still significantly ($P < 0.01$) raised at 3.5 h after injection. DOPAC and HVA decreased dose-dependently but these changes were less striking (27% and 40% respectively after 400 mg kg^{-1} milacemide) than the increases in dopamine (125%). Maximum changes of DOPAC were somewhat later (1.5–2.5 h) than those of dopamine (1 h); HVA changes were slightly more delayed (2–3 h).

A significant ($P < 0.05$) increase (25%) of 5-HT was observed only at 1.5 h after giving the highest dose of milacemide (Figure 2; Table 1). 5-HIAA values were unaffected.

Tissue

The effects of milacemide (400 mg kg^{-1}) on dopamine and 5-HT metabolism in frontal cortex tissue are shown in Table 1 together with values for dialysates from rats given the same dose in a separate experiment (see Figures 1 and 2). Tissue and dialysate was taken 1.5 h after drug administration. Milacemide significantly increased dopamine and 5-HT and significantly decreased their metabolites in the tissue, the percentage increase of dopamine being about 4 times that of 5-HT. Percentage changes of dopamine, DOPAC and HVA in dialysate were in general comparable but somewhat smaller than corresponding tissue changes. Ratios of concentrations in tissue to concentrations in dialysates were much larger for 5-HT than for dopamine and very much smaller for the metabolites than for the corresponding amines.

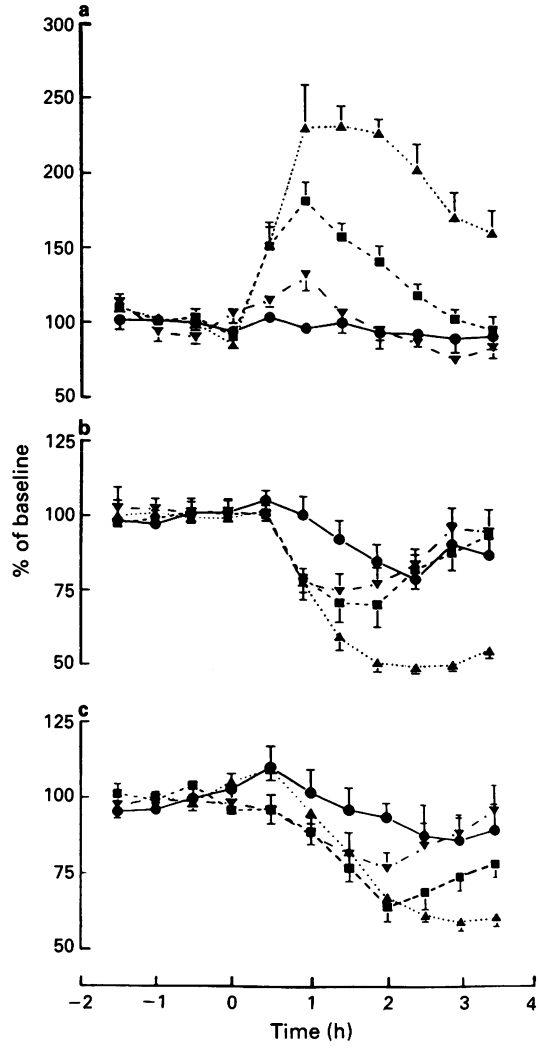


Figure 1 Dialysate dopamine (a), 3,4-dihydroxyphenylacetic acid (DOPAC; b) and homovanillic acid (HVA; c) before and after the administration of milacemide or 0.9% saline (vehicle). Values are mean of 6–7 rats and are expressed as a percentage of baseline; vertical bars show s.e.mean. Basal dialysate values for dopamine, DOPAC and HVA were 2.9 ± 0.2, 170 ± 37 and 160 ± 27 pmol ml^{-1} respectively ($n = 26$); saline (●); milacemide 100 mg kg^{-1} (▼), 200 mg kg^{-1} (■) and 400 mg kg^{-1} (▲).

Discussion

The tissue/dialysate ratios for the amines and their metabolites (Table 1) are worth some comment. The much larger values for amines than for metabolites agree with previous data (Westerink *et al.*, 1987; Adell *et al.*, 1989) but the ratio for cortical dopamine is much lower than that for 5-HT and also much lower than that reported by Westerink *et al.* (1987) for striatal dopamine. This latter discrepancy could reflect both the higher firing rate of cortical than of striatal dopaminergic neurones (Bannon & Roth, 1983) and less efficient dopamine reuptake by the more diffuse cortical dopamine terminals. On the other hand, data on monkeys (Elchisak *et al.*, 1983) suggest that conjugation of cortical dialysate dopamine might influence the ratio in the opposite direction. The tissue/dialysate ratio for dopamine may also have been influenced by the perfusate Ca^{2+} concentration as we used 1.26 mM (which approximates the value in brain interstitial fluid; Hansen, 1985) but Westerink *et al.* (1987) used 3.4 mM which may have somewhat increased dopamine efflux (De Boer *et al.*, 1990). The lower cortical tissue/dialysate ratios for DOPAC and HVA than for 5-HIAA may however be explicable in terms of the removal of the dopamine metabolites from

Table 1 Effect of milacemide (400 mg kg^{-1}, i.p.) on dopamine, 5-hydroxytryptamine (5-HT) and their metabolites in frontal cortex tissue and dialysate

	Vehicle			*Milacemide*		
	Tissue	*Dialysate*	*Tissue/dialysate*	*Tissue*	*Dialysate*	*Tissue/dialysate*
Dopamine	512 ± 44	3.5 ± 0.9	146	1312 ± 355[a] (+156%)	8.1 ± 1.8[b] (+131%)	162
DOPAC	360 ± 36	120 ± 55	3.0	157 ± 23[a] (−56%)	63 ± 24[b] (−48%)	2.5
HVA	451 ± 17	123 ± 26	3.7	323 ± 26[a] (−29%)	98 ± 18[c] (−20%)	3.6
5-HT	2961 ± 171	1.6 ± 0.4	1851	4110 ± 278[a] (+38%)	2.0 ± 0.3[c] (+25%)	2055
5-HIAA	2208 ± 56	348 ± 74	6.3	1573 ± 76[a] (−29%)	352 ± 74 (+1%)	4.5

DOPAC = 3,4-dihydroxyphenylacetic acid; HVA = homovanillic acid; 5-HIAA = 5-hydroxyindoleactic acid. Values (pmol g^{-1} or pmol ml^{-1}) are for rats 1.5 h after injection of saline vehicle (0.9% NaCl) or milacemide and are given as means ± s.e.mean, $n = 6$–8. Dialysis values are not corrected for recovery. Percentage differences from vehicle-treated animals are shown in parentheses. [a] $P < 0.01$ vs vehicle-treated group (Mann-Whitney U test); [b] $P < 0.01$, [c] $P < 0.05$ vs vehicle-treated group (one-way ANOVA followed by Dunnett's test).

cortical dialysates as conjugates (Elchisak *et al.*, 1977; Sarna *et al.*, 1984).

Although in the present study experiments were not undertaken to determine the possible contribution of diffusion of dopamine, 5-HT and metabolites into frontal cortex dialysate from other regions, the recent study of Schwartz *et al.* (1990) suggests that diffusion effects would be minimal. By use of two microdialysis probes, one placed in the medial hypothalamus and the other in the lateral hypothalamus, drug-induced increases in 5-HT at one site were not detected at the other site even though the probes were only 1 mm apart in the same side of the brain (Schwartz *et al.*, 1990).

Rats given milacemide (400 mg kg^{-1}) are reported to show increases of 5-HT in various brain regions including the frontal cortex and a relatively slight rise of striatal dopamine (Truong *et al.*, 1989a); rhesus monkeys given milacemide chronically had substantial increases of 5-HT in various parts of the cortex and of dopamine in the striatum (de Varebeke *et al.*, 1990). These changes were explained in terms of inhibition of MAO-B. In the present investigation, rats given milacemide (400 mg kg^{-1}) had moderate but significant increases and decreases of 5-HT and 5-HIAA respectively in cortical tissue and a small transient increase (significant only at 1.5 h post administration) of 5-HT in cortical dialysates. Changes of dialysate 5-HT concentration are more indicative of changes at receptors than are brain tissue 5-HT changes. Thus, behavioural responses to the 5-HT releaser *p*-chloroamphetamine paralleled cortical dialysate but not tissue 5-HT (Hutson & Curzon, 1989).

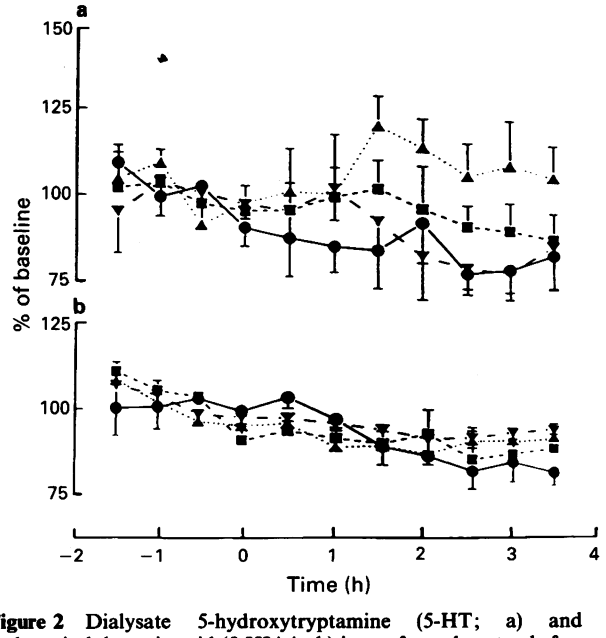


Figure 2 Dialysate 5-hydroxytryptamine (5-HT; a) and 5-hydroxyindoleacetic acid (5-HIAA; b) in rat frontal cortex before and after the administration of milacemide or 0.9% saline (vehicle). Values are mean of 6–7 rats and are expressed as a percentage of baseline; vertical bars show s.e.mean. Basal dialysate values for 5-HT and 5-HIAA were 1.15 ± 0.12 and 233 ± 25 pmol ml^{-1} respectively ($n = 26$): saline (●); milacemide 100 mg kg^{-1} (▼), 200 mg kg^{-1} (■) and 400 mg kg^{-1} (▲).

The lack of an effect of milacemide (400 mg kg^{-1}) on dialysate 5-HT is consistent with the recent brief report (Brown *et al.*, 1991) that although patients with post-anoxic myoclonus respond therapeutically to the 5-HT precursor 5-hydroxytryptophan (Chadwick *et al.*, 1977) similar patients did not respond to milacemide (approx 12–34 mg kg^{-1} day^{-1}). Nevertheless, as lower doses of milacemide than those used in the present study enhanced behavioural effects of 5-HT on rats (Truong *et al.*, 1989b) the drug may have greater effects on 5-HT concentrations at extra-cortical sites. Alternatively, the dopamine changes may conceivably have potentiated responses to 5-HT.

A previous dialysis study (Sleight *et al.*, 1988) has shown that extracellular 5-HT is substantially increased only if both MAO-A and MAO-B are inhibited. Thus, in the present study, the relatively large increases of both cortical tissue and dialysate dopamine in association with decreases of DOPAC and HVA and the absence of significant changes of 5-HT metabolism in rats given milacemide are explicable by inhibition of MAO-A. That dopamine is mainly deaminated by MAO-A is indicated by both brain tissue and dialysate studies (Green *et al.*, 1977; Fowler & Benedetti, 1983; Kato *et al.*, 1986; Waldmeier, 1987; Butcher *et al.*, 1990; Garrett & Soares de Silva, 1990). Figure 4 of Kato *et al.* (1986) is particularly relevant as interpolation reveals that when the specific MAO-A inhibitor clorgyline decreased rat striatal dialysate DOPAC by 44%, the decreases of HVA and 5-HIAA are 28% and 5% respectively; changes which are essentially identical with the effect of milacemide on dialysate amine metabolites in the present study (Table 1). Although de Varebeke *et al.* (1990) emphasize the role of MAO-B in the rise of dopamine in the caudate nucleus of rhesus monkeys given milacemide chronically and show that, under these conditions, MAO-B is inhibited more markedly than MAO-A, evidence is strong (see above) that rat brain DA is largely metabolized by MAO-A though MAO-B is probably involved when MAO-A is inhibited (Green *et al.*, 1977).

De Varebeke *et al.* (1990) propose milacemide as an adjuvant to L-DOPA in the treatment of Parkinson's Disease. This is worthy of further consideration since correction of the cortical dopamine deficiency which occurs in the disease (Scatton *et al.*, 1983) could oppose associated cognitive defects (Brozoski *et al.*, 1979; Simon *et al.*, 1980).

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Blockade by local anaesthetics of the single Ca^{2+}-activated K^+ channel in rat hippocampal neurones

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1 Effects of local anaesthetics on single Ca^{2+}-activated K^+ channels were investigated using the inside-out configuration of the patch-clamp technique in single pyramidal neurones, which were freshly dissociated from rat hippocampus by use of proteolytic enzymes.

2 No significant effect was observed when 2 mM benzocaine was applied on either side of the membrane patch, or when 2 mM lignocaine or QX-314 was applied to the external surface of the membrane.

3 Lignocaine 1 mM, applied to the internal surface, slightly reduced the amplitude of the single K^+ channel current. When applied to the internal surface, QX-314 reduced the amplitude of the K^+ channel current, accompanied by an increase in noise in the open channel current, suggesting a fast flickering block. The blocking effect of QX-314 on the outward current increased with depolarization, suggesting a binding site for the drug at an electrical distance of about 0.5 across the membrane field.

4 The open time histogram showed one exponential component and the closed time histogram showed at least two components. The mean open time of the outward current was increased when the amplitude was reduced by the drugs.

5 The ionized form of the local anaesthetics had a similar action on the Ca^{2+}-activated K^+ channels to that on Na^+ channels, that is, they enter into the channel from the cytoplasmic side to induce open channel block. The blocking kinetics, however, might be so fast that they were beyond the frequency response of our recording apparatus, thus the recorded current amplitude was decreased. In contrast the K^+ channel was not accessible via hydrophobic pathways for the neutral form, which is also known to block the sodium channel.

Keywords: Lignocaine; benzocaine; QX-314; Ca^{2+}-activated K^+ channel; patch clamp; hippocampal neurone

Introduction

Mechanisms of ion channel blockade by local anaesthetics have been extensively studied in sodium (Na^+) channels in various tissues (Strichartz, 1973; Courtney, 1975; Khodorov *et al.*, 1976; Hille, 1977a,b; Hondeghem & Katzung, 1977; Schwarz *et al.*, 1977; Cahalan, 1978; Starmer, 1987; Strichartz & Ritchie, 1987). It is thought that an electrically neutral form of anaesthetic molecules applied in the external solution partition into the membrane. A proportion of these molecules directly affects the Na^+ channel, and others diffuse into the cytoplasm, where their cationic form blocks the channel from the internal mouth of the channel (Frazier *et al.*, 1970; Strichartz, 1976; Hille, 1977a,b). However, detailed studies on the effect of local anaesthetics on potassium (K^+) channels are still lacking at the single channel level, with the exception of work on the epithelial K^+ channels (Richards & Dawson, 1986). In the case of general anaesthetics, it has been demonstrated that they hyperpolarize the hippocampal pyramidal cells by increasing potassium conductance (Nicoll & Madison, 1982; see also Franks & Lieb, 1988). In this study, we describe the actions of local anaesthetics on single calcium (Ca^{2+})-activated K^+ channels.

The Ca^{2+}-activated K^+ channels, having a large conductance of around 250 pS at symmetrical 150 mM K^+ concentrations ($[K^+]$), are widely distributed at high densities in a variety of cells (Marty, 1981; Latorre & Miller, 1983). In neurones, the influx of Ca^{2+} through Ca^{2+} channels and/or release of Ca^{2+} from the endoplasmic reticulum increase the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) during the action potential, which in turn may activate the Ca^{2+}-activated K^+ channels. Therefore, the Ca^{2+}-activated K^+ channel may provide an outward current for repolarization and after-hyperpolarization (Kuba, 1980; Adams *et al.*, 1982; McDermott & Weight, 1982; Kuba *et al.*, 1983; Petersen & Maruyama, 1984; Storm, 1987; Tanaka & Kuba, 1987; for review see, Latorre *et al.*, 1989). Yoshida *et al.* (1991) recently demonstrated openings of the 235 pS K^+ channel immediately after the spontaneous action potential in the hippocampal cell, prepared in the same way as in the present study. It might be speculated that seizure following an overdose of a local anaesthetic is related to a possible blocking action of the drug on K^+ channels in the central nervous system (Usubiaga *et al.*, 1966; de Jong *et al.*, 1969; Warnick *et al.*, 1971). The present study demonstrates that ionized forms of the local anaesthetics block the Ca^{2+}-activated K^+ channels from the cytoplasmic side, in essentially the same manner as for Na^+ channels. Benzocaine (a neutral anaesthetic at physiological pH) failed to block the K^+ channel.

Methods

Single hippocampal pyramidal cells were isolated from the brain of Wistar rats (7–10 days old) of either sex by a technique similar to that developed by Kaneda *et al.* (1988). Briefly, the brain was rapidly dissected out and sliced at thicknesses of 400–600 μm, which were then treated for 20 min at 37°C with collagenase (0.04%, Sigma, type I) and actinase (0.056%, Kaken Chemical Co., Japan) dissolved in the control external solution. The enzyme was washed out with external solution containing 20% foetal calf serum. The treated brain pieces of hippocampal tissue were stored in the external solution. The neurones were mechanically isolated just before use.

A drop of the cell suspension was added to the external solution in the recording chamber. After the cells settled down on the glass bottom, perfusion of the recording chamber was started. Single channel currents were recorded in the inside-out configuration (Hamill *et al.*, 1981) by use of a patch clamp amplifier (EPC-7, List, Darmstadt, F.R.G.). The glass electrode was usually filled with a 150 mM K^+ pipette solution,

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and had a tip resistance of 5–10 MΩ. The local anaesthetics were applied in the internal solution by use of the rapid application technique (Kakei & Ashcroft, 1987; Akaike *et al.*, 1986). All experiments were performed at room temperature (20–25°C). Statistical values are given in mean ± s.d. (*n*).

The single channel current was replayed and fed to the computer via a Bessel-type low pass filter (28 dB/octave) with a cut-off frequency of 2.5 kHz. The sampling frequency of the a.d. converter was 5 kHz. In some preliminary experiments, a current noise induced by applying local anaesthetics was examined on the oscilloscope using a higher cut-off frequency (10 kHz), but we failed to see clear improvement of the frequency resolution.

The composition of the control external solution was (in mM): NaCl 150.0, KCl 5.0, $MgCl_2$ 1.0, $CaCl_2$ 1.0, glucose 10.0, N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid (HEPES) 10.0 and titrated to pH 7.4 with Tris base (Tris(hydroxymethyl)aminomethane). The composition of the standard internal solution used for the inside-out patch recordings was as follows (in mM): KCl 150.0, HEPES 5.0, ethyleneglycol-bis(β-aminoethylether)N,N'-tetraacetic acid (EGTA) 1.0 (pH 7.2 with KOH). The concentration of free Ca^{2+} ($[Ca^{2+}]$) was adjusted by adding an appropriate amount of $CaCl_2$ according to Fabiato & Fabiato (1979) and Tsien & Rink (1980). The pipette solution contained (in mM): KCl 150.0, $CaCl_2$ 1.0, and HEPES 5.0 (pH = 7.4 with KOH).

Drugs

The drugs used were purchased from the following companies: collagenase (Sigma type 1, U.S.A.), actinase (Kaken Chemical Co., Japan), foetal calf serum (Dainippon Chemical Co., Japan). The local anaesthetics used were lignocaine (lidocaine; pK_a = 7.9, Sigma, U.S.A.), QX-314 (N-ethyl derivative of lignocaine) predominantly in the cationic form (Ishizu Chemical Co., Japan), and uncharged benzocaine (pK_a = 2.6, Tokyokasei, Japan).

Results

Effects of lignocaine and benzocaine applied to the internal surface

After a gigaohm seal had been made on a hippocampal cell, the patch electrode was drawn back to excise the patch membrane in the control external solution. Under this inside-out patch condition, the activity of Ca^{2+}-activated K^+ channels was observed in more than 90% of the membrane patches. Representative records of the K^+ current activated by 12.6 μM $[Ca^{2+}]_i$ in symmetrical 150 mM K^+ solutions are illustrated in Figure 1. The channel activity increased with increasing depolarization, and at +20 and at +40 mV the channel showed sustained bursting activity. The channel closing events of relatively long durations observed at negative potentials became less frequent with depolarization, leaving the repetitive brief closing events in the burst. This K^+ channel had an ohmic conductance of about 235 pS. In 18 experiments, the average conductance was 234 ± 10 pS (n = 18). Our study on the control characteristics (Yoshida *et al.*, 1991) also indicated that blocking of the channel by tetraethylammonium (TEA) and Ba^{2+}, as well as the channel gating kinetics produced by divalent cations are consistent with those of the Ca^{2+}-activated K^+ channel (BK channel) described in other tissues (Latorre *et al.*, 1989).

After the control current had been recorded, 1 mM lignocaine or benzocaine was applied to the internal surface of the membrane. The gating kinetics of the channel were not markedly changed by these local anaesthetics. However, the amplitude of outward channel current was clearly decreased by 1 mM lignocaine. Benzocaine failed to affect the channel current.

The amplitude histograms were constructed from records obtained at +20 mV as shown in Figure 2. The open channel current showed a symmetrical distribution, and the very brief closing events caused a small continuous distribution between the full open level and the closed level in every experimental

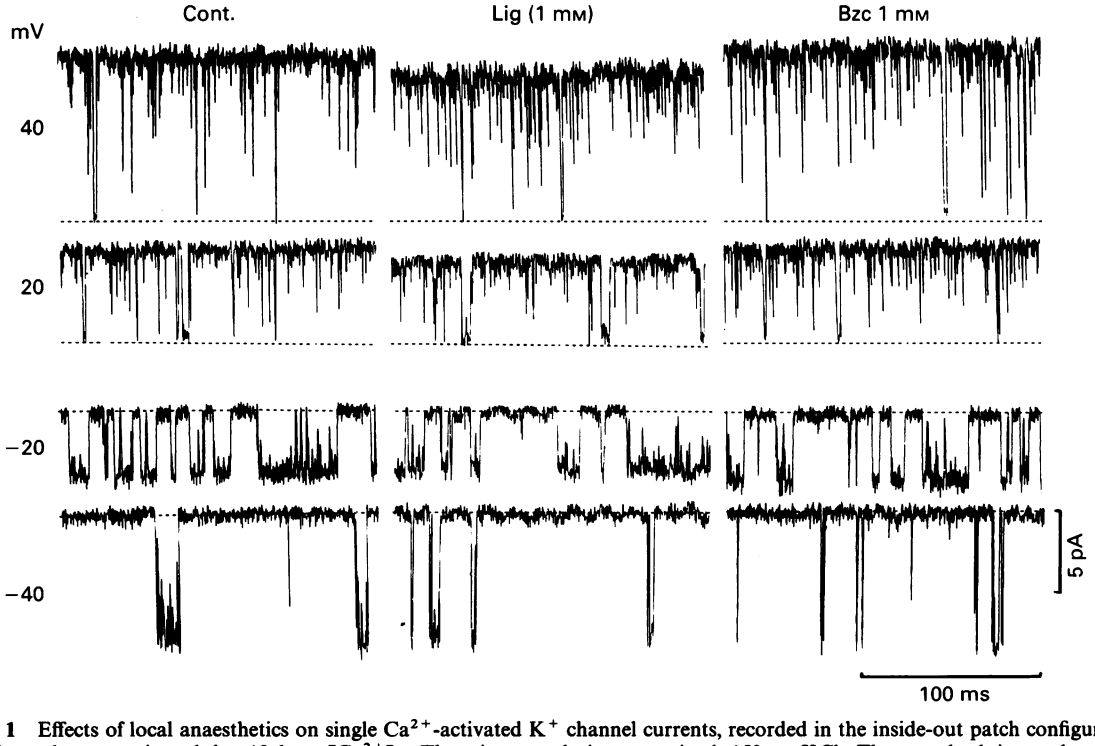


Figure 1 Effects of local anaesthetics on single Ca^{2+}-activated K^+ channel currents, recorded in the inside-out patch configuration. The channel was activated by 12.6 μM $[Ca^{2+}]_i$. The pipette solution contained 150 mM KCl. The standard internal solution (150 mM K^+) containing 1 mM lignocaine (Lig) or benzocaine (Bzc) was perfused after recording the control current (Cont). The membrane potentials are given at the left in mV. The upward deflections represent outward current, and the dashed lines indicate closed channel current level. All records were sampled at 5 kHz through a low pass filter of 2.5 kHz cut-off frequency. All data were taken from the same patch.

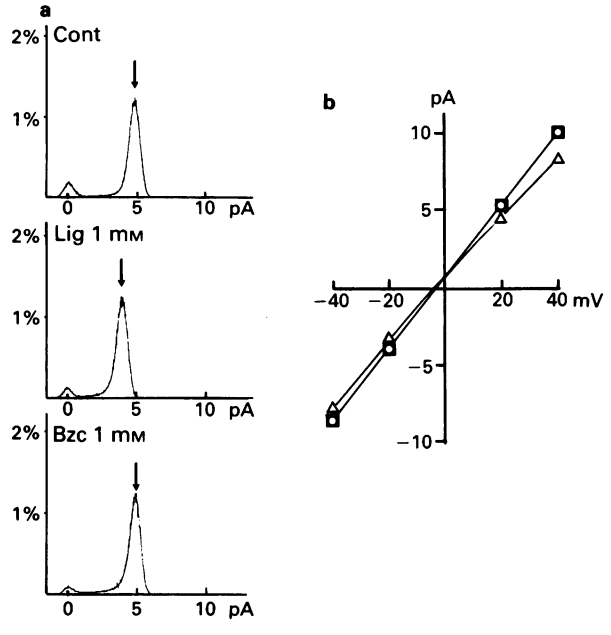


Figure 2 The amplitude histograms of the Ca^{2+}-activated K^+ channel currents recorded at +20 mV in the absence or the presence of internal lignocaine (Lig) or benzocaine (Bzc) (1 mM). Each histogram was determined from a 10–20 s segment of the current record. The arrows indicate the position of the distribution peaks, which were used to plot the I–V curve in (b). (b) (○) Control; (□) benzocaine and (△) lignocaine. The deviation of the reversal potential (−5 mV) from the expected K^+ equilibrium potential of 0 mV might be due to an experimental error in the voltage recording. Note that the density distribution at the closed current level was not much changed by the application of the local anaesthetics.

condition. The amplitude of the single channel current (i) was measured from the peak position, and were plotted against the membrane potential (V_m) in Figure 2a. Lignocaine reduced the channel conductance from 230 pS to 201 pS. The i–V_m relationship obtained in the presence of benzocaine was superimposable with the control.

Effects of QX-314 applied to the internal surface

The application of 1 or 2 mM QX-314 to the internal surface of the membrane reduced the current amplitude in a dose-dependent manner and increased noise in the open channel current (Figure 3). The voltage-dependent activation of the channel was not obviously modulated. The amplitude histograms of control and QX-314 records are compared at +20 mV in Figure 4a. The peak position of the open channel current was shifted to a lower level by the action of this drug, and the distribution of the open channel current was broader than in the control. The current amplitude at the distribution peak was plotted in Figure 4b. The channel inhibition was more marked for the outward current than for the inward current. The inhibition of the outward current increased with increasing depolarization. The current amplitude was decreased by 1 mM QX-314 to 80 ± 3, 69 ± 2, 65 ± 5, and 58 ± 5% control, at −40, −20, +20 and +40 mV, respectively. At 2 mM QX-314 the amplitude was 42 and 46% control at +40 mV and 68 and 68% control at −40 mV in two experiments. It should be noted that the conductance of single Na^+ channels is also apparently decreased by QX-314 for both the outward and inward currents, accompanied by an increase in noise (Wang, 1988).

The above findings with lignocaine and QX-314 might be explained in two alternative ways. Firstly, the channel conductance might be reduced by the drugs. In this case, the mean open probability (p_o) is given as follows:

$$p_o = I/(iN) \quad (1)$$

where I stands for mean patch current, i for the unit current amplitude, and N for channel number within the patch. In the present study N was 1 in every experiment analyzed. If i is measured from the distribution peak in the amplitude histogram, values of p_o are 0.90, 0.87 and 0.93 in the control, 1 mM, and 2 mM QX-314, respectively. However, the increase in the open channel noise induced by QX-314 makes an alternative mechanism more likely; that channel conductance remained constant in the open conformation, but the channel was blocked in a flickering manner by the drug, basically in the same manner as in the Na^+ channel, thereby producing a

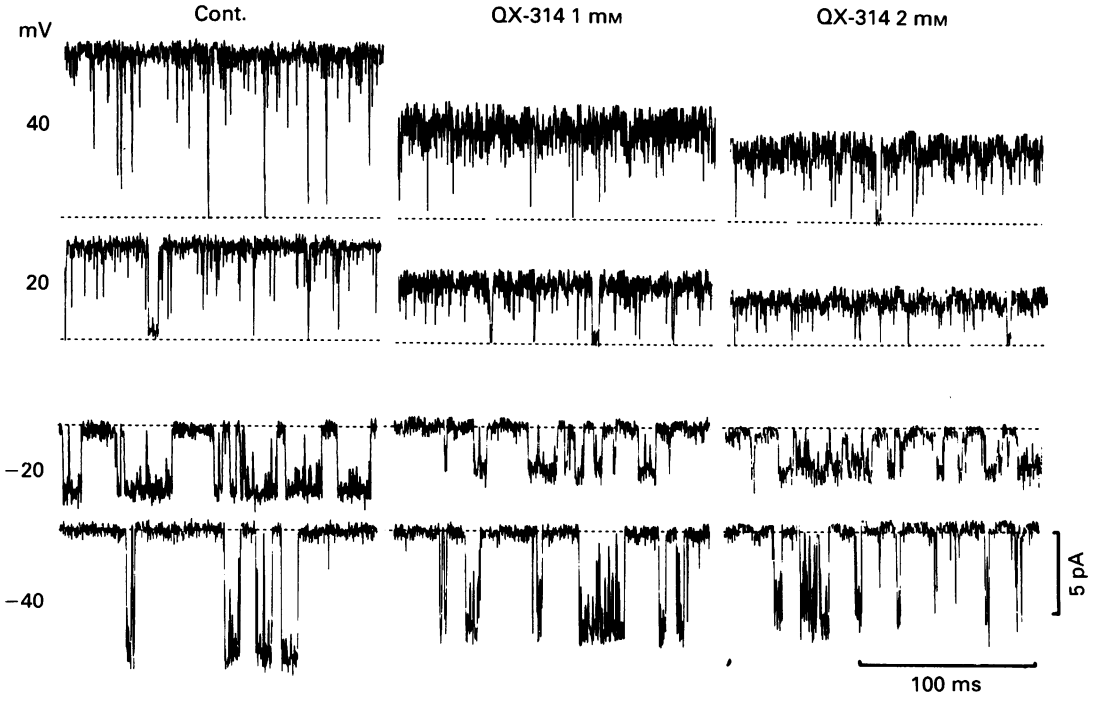


Figure 3 Effects of QX-314 1 mM or 2 mM applied to the internal surface of the inside-out patch. All records were obtained from the same Ca^{2+}-activated K^+ channel.

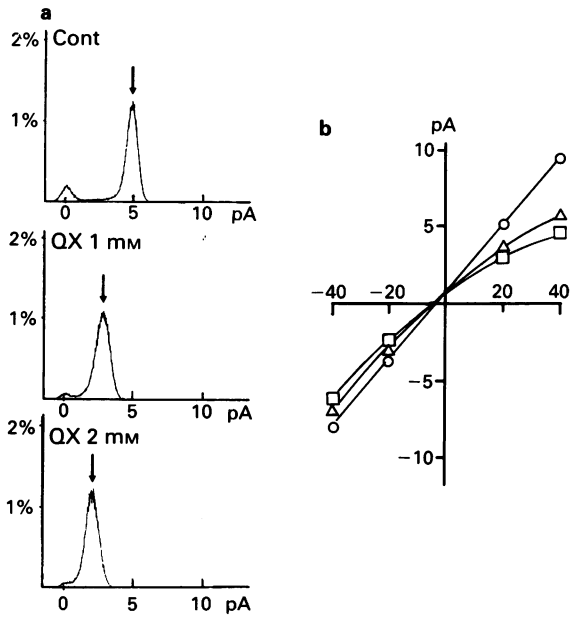


Figure 4 The amplitude histograms of the Ca^{2+}-activated K^+ channel current at +20 mV in the control and in the presence of internal QX-314 (QX) 1 mM or 2 mM. Each histogram was determined from a 10–20 s segment of the current record. The I-V curves in (b) are the control (○), QX-314 1 mM (△) and 2 mM (□). The reversal potential of −5 mV is due to an experimental error of unknown reason.

reduced open probability. The transition between the open and blocked states might be so fast that the recorded open channel current gave a kind of average current accompanied by increased noise. In this case, the i in Equation (1) is the amplitude of the current in the absence of the blocker. In Table 1, values of p_0 were decreased by lignocaine or by increasing QX-314 concentration. It is clear that benzocaine was ineffective on the Ca^{2+}-activated K^+ channel.

Drug actions at the external surface of the Ca^{2+}-activated K^+ channel

To examine the actions of the local anaesthetics from the external side of the membrane they were added in the pipette solution. As shown in Figure 5, no significant change was noticed in either the amplitude of the single channel current or in the open-close transitions. The measurement of the i–V_m relations confirmed no significant effect of the drugs on the channel conductance in 2 experiments each for 2 mM lignocaine (212 and 218 pS), benzocaine (233 and 228 pS), or QX-314 (226 and 229 pS). Although, p_0 varied between different patches in the control, we could not detect any consistent change in the p_0 during the application of the drugs. It might be concluded that the charged form of local anaesthetics blocks the Ca^{2+}-activated K^+ channel only from the internal side of the membrane.

The open-close kinetics under the blocking effect of the drug

We constructed open time and closed time histograms in the control condition and in the presence of the local anaesthetics. The channel was activated by 12.6 μM $[Ca^{2+}]_i$ added to the internal solution. In the control, the open time histogram showed a single exponential component, and the closed time histogram was fit by a sum of two exponential components as shown in a preceding study (Figure 6 of Yoshida *et al.*, 1991). The fraction of the slow component in the closed time histogram was very small, since the channel was maximally activated by 12.6 μM $[Ca^{2+}]_i$. Furthermore, the time constant of the distribution of the fast component was less than 1 ms (0.3–0.7 ms). Thus, it was difficult to detect obvious changes in the closed time histogram induced by the local anaesthetics.

In the presence of QX-314, the increased noise in the open channel current slightly interfered with the measurement of the open time and the closed time. We arbitrarily fixed the cut-off frequency to 2.5 kHz and defined the transition between the closed and open states by giving a threshold level at the half amplitude of the current deflection. The distribution of the open time was changed by the application of QX-314. Figure 6 shows representative histograms obtained before and during the application of 2 mM QX-314 at a holding potential of +40 mV. In the control, the channel frequently showed transitions from the open state to the closed states. QX-314 induced a marked reduction in the amplitude of the open channel currents and an increase in the high frequency noise, but the number of transitions to the closed level was decreased as shown in the inset current recordings. The open time histograms were well fitted with a single exponential curve. The time constant of the distribution (τ_{open}) was 12.6 ms in the control and 17.9 ms in the presence of 2 mM QX-314.

The values of τ_{open} obtained with different concentrations of QX-314 are compared with the control in Table 2. The fractional decrease in the current amplitude is also given as the current amplitude in the presence of the drug divided by the control amplitude (i_b/i_c). At 0.2 mM QX-314 the change in the mean open time was not significant. At higher concentrations, the value of τ_{open} was increased in every experiment (τ_{cont}/τ_{drug}).

The simple sequential model for open channel blockade (Neher, 1983) indicates a constant time integral of current during a burst, which is due to a train of transitions between the open state and the blocked state. To test this model, the relative amplitude was multiplied by the fractional mean open time (A × B in Table 2). The value is close to 1 at 0.5 mM QX-314, but tends to decrease with increasing drug concentration at +40 mV. This finding seems to be similar to that obtained in the acetylcholine (ACh)-induced single channel current of muscle cells on application of QX-222 (Neher,

Table 1 Effects of local anaesthetics on the current amplitude of the Ca^{2+}-activated K^+ channel

	i	*m.p.c.*	p_0 *I/iN*	p_0 *I/i*N*
Cont	6.33 ± 0.42 (23)	5.70 ± 0.64 (18)	0.90	
Bzc 1 mM	6.50 ± 0.26 (4)	6.46 ± 0.57 (4)	0.99	1.03
Lig 1 mM	5.41 ± 0.21 (7)	4.85 ± 0.30 (7)	0.90	0.77
QX-314 1 mM	4.00 ± 0.22 (9)	3.48 ± 0.51 (9)	0.87	0.54
QX-314 2 mM	3.24 (2)	3.01 (2)	0.93	0.48

The apparent unitary amplitude of the single channel current (i) was measured in the control or in the presence of 1 mM benzocaine (Bzc), lignocaine (Lig), QX-314, or 2 mM QX-314. The mean patch current (m.p.c.) is the time average of the current determined from record segments of 10–20 s in duration. The channel open probability (p_0) was calculated as m.p.c. divided by the apparent unit amplitude (m.p.c./i) or as m.p.c. divided by the unit amplitude obtained in the control (m.p.c./i_{cont}). Data are given as mean ± s.d. (number of experiments).

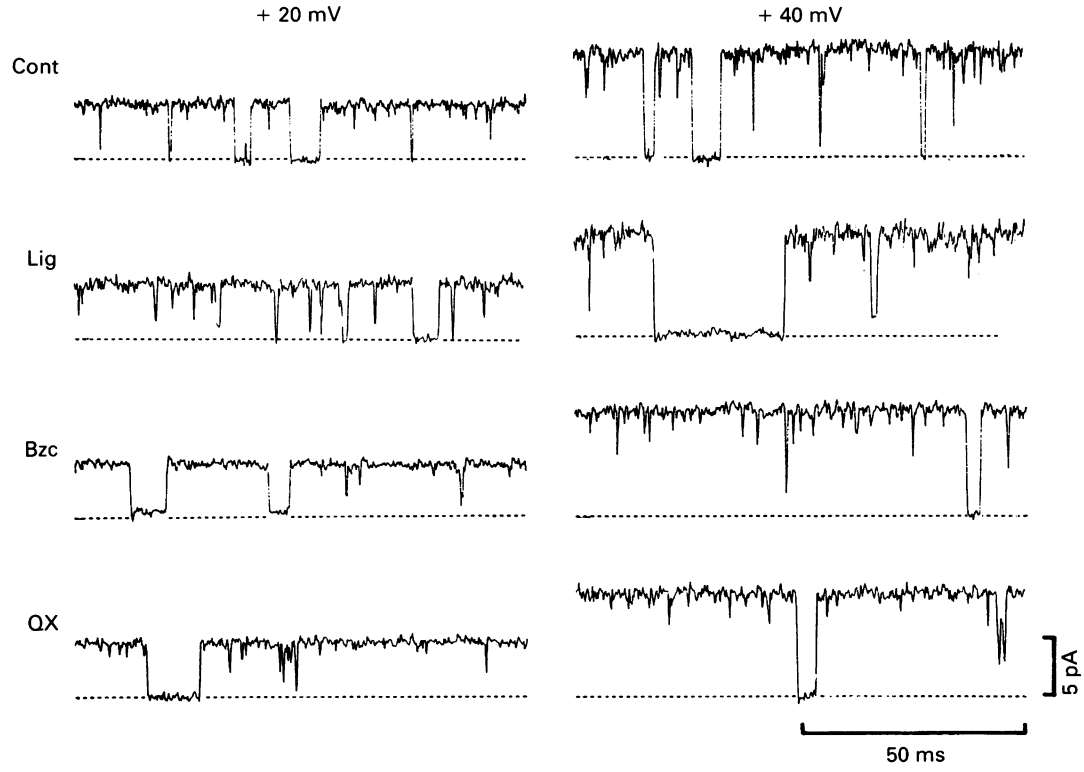


Figure 5 Effects of local anaesthetics applied in the external solution. The current records obtained at +40 mV and +20 mV were compared. The pipette solution contained 2 mM lignocaine (Lig), benzocaine (Bzc) or QX-314 (QX). The channel was activated by 10 μM Ca^{2+} in the standard internal solution.

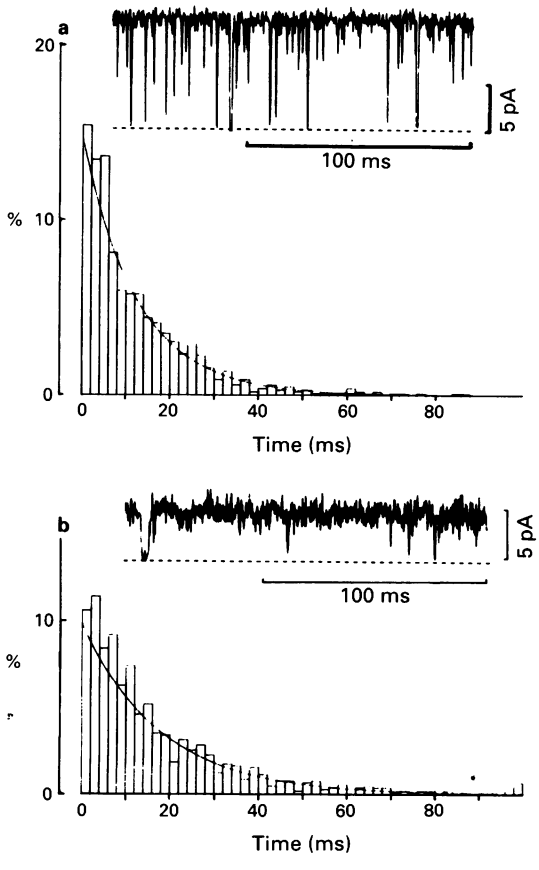


Figure 6 The open time histograms in the control (a) and presence of QX-314 (b) applied to the internal surface. The inset shows the original recordings obtained at +40 mV. The open time histogram was composed of a single exponential component with time constants of 12.6 ms and 17.9 ms in the control and 2 mM QX-314, respectively. The bin width was 2 ms and the superimposed curves were the best fits of a single exponential curve.

1983). We have no ready explanation for the large increase in τ_{open} produced by 1 mM QX-314 at +20 mV.

Based on the above findings, it might be speculated that the increased noise reflected the transitions between the open state and the blocked state with very fast kinetics, and that the apparent open time represented the 'bursting time' in the presence of the drug.

Dose-response relationships for local anaesthetics

Because of the difficulty in separating the blocked state from the closed states, the dose-response relationship could not be determined from simple measurements of p_0. The dissociation constant (K_D) can only be determined if a simple kinetic model is assumed to apply (Benham *et al.*, 1985; McCann & Welsh, 1987).

$$\text{Closed—Open—Block} \tag{2}$$

where,

$$K_D = [D]p_0\, p_b/(p_0 - p_b) \tag{3}$$

Here, [D] indicates the concentration of the blocker, and p_0 and p_b the open probabilities in the absence and presence of the blocker, respectively. It was assumed that channel conductance remained constant in the open state, and null in the blocked state. To get a linear relation Equation (3) is modified as,

$$(p_0 - p_b)/p_0 \cdot p_b = [D]/K_D \tag{4}$$

The value of p_b was determined from Equation (1). It seems that Equation (4) holds true in the present study as shown by the linear relationship in Figure 7a. It may indicate that the block is occurring on a basis of a one to one binding reaction. The values of K_D obtained from the slopes of the linear relationship were plotted against V_m in Figure 7b. The smooth curve superimposed is drawn according to the equation (Woodhull, 1973),

$$K_D = K_D(0) \cdot \exp(-\delta zFV_m/RT), \tag{5}$$

Table 2 Effects of QX-314 on the time constant in the open time histogram

	+20 mV					+40 mV				
	τ_{open}		A τ_b/τ_c	B i_b/i_c	A × B	τ_{open}		A τ_b/τ_c	B i_b/i_c	A × B
	Cont	Drug				Cont	Drug			
QX-314										
0.2 mM	9.84	10.27	1.04	0.91	0.95	13.78	14.20	1.03	0.85	0.88
0.5 mM	9.84	11.34	1.15	0.82	0.94	13.78	15.98	1.15	0.77	0.89
	12.02	15.23	1.26	0.81	1.02	14.67	18.43	1.25	0.69	0.86
1.0 mM	6.04	10.36	1.72	0.67	1.15	12.21	16.00	1.31	0.57	0.74
	5.72	11.50	2.01	0.62	1.25	12.76	16.30	1.27	0.58	0.74
	6.37	14.18	2.22	0.66	1.46	7.69	15.12	1.96	0.59	1.16
2.0 mM	13.59	15.41	1.13	0.50	0.57	15.51	18.66	1.20	0.48	0.58
						12.58	17.86	1.42	0.43	0.61
						12.52	21.75	1.73	0.46	

The open time histograms were constructed as shown in Figure 6, and their exponential time constant of distribution (τ_{open}) were determined in the control (cont) and in the presence of the QX-314 (drug) at the membrane potentials of +20 and +40 mV. The ratio of τ determined in the presence of the drug (τ_b) divided by the control τ (τ_c) was calculated (A). The ratio of the unit amplitudes (i_b/i_c) were also calculated (B). See text for the description of the value of A times B.

where $K_D(0)$ is the dissociation constant at 0 mV, σ a partition parameter, and z, F, R and T having their usual meanings in thermodynamics. The value of δ was 0.51, suggesting that the blocking site for QX-314 lies about half-way across the membrane electrical field.

Discussion

The present study demonstrates that the hippocampal Ca^{2+}-activated K^+ channel is not blocked from the external side of the membrane by lignocaine, QX-314 or benzocaine, but is blocked by lignocaine and QX-314 applied in the internal solution. These findings clearly indicate that the Ca^{2+}-activated K^+ channel is blocked by the drug through the 'hydrophilic pathway' but not via the 'hydrophobic pathway', which were suggested for the blocking of the Na^+ channel (Schwarz *et al.*, 1977). The positively charged quaternary amine may enter the channel pore through the internal mouth of the K^+ channel to block the channel. It has been shown that internal QX-314 produces an inhibition of K^+ currents in frog myelinated nerves (Strichartz, 1973), suggesting a similar blocking mechanism for the delayed rectifier K^+ channel. When QX-314 was applied internally to pyramidal neurones in the guinea-pig hippocampal slice preparation (Connors & Prince, 1982), the large K^+ conductance remained. It may be speculated that the blockade of the K^+ channel was relatively small compared with the block of the Na^+ channel.

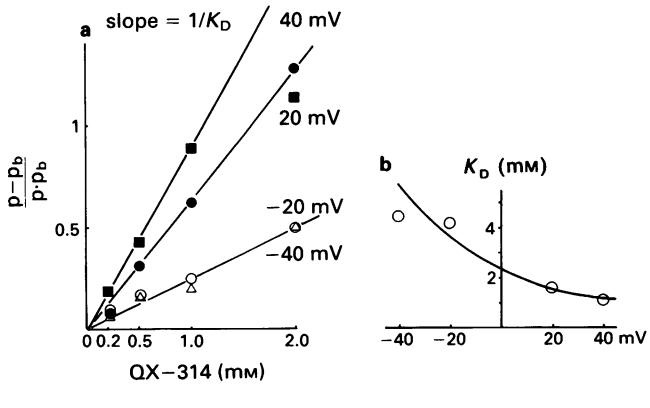


Figure 7 (a) Determination of the dissociation constant (K_D) for the blocking effect of QX-314 at +40 (■), +20 (●), −20 (○) and −40 mV (△). See text for explanation. (b) The voltage-dependency of the K_D, which were determined in (a).

A large and persistent K^+ current, activated by volatile general anaesthetics, was found in some neurones but not others (Franks & Lieb, 1988). In the squid axon clinical concentrations of general anaesthetics blocked a potassium conductance in the resting membrane (Haydon *et al.*, 1988). The former finding may explain the hyperpolarization recorded in the hippocampal pyramidal cells induced by the general anaesthetics (Nicoll & Madison, 1982). It might be speculated that the action of the local anaesthetics is different from that of the general anaesthetics.

The voltage-dependence of the action of QX-314 (Figures 4 and 7) indicates that the charged molecule indeed affects the channel. The effect of positively charged QX-314 on the Ca^{2+}-activated K^+ channel was increased when the outward driving force for the positive charge was increased by shifting the membrane potential progressively positive. This voltage-dependence was explained by assuming a binding site at an electrical distance of 0.5 across the membrane electrical field. The finding is in line with the fact that the effect of intracellular drug application on the Na^+ channel in planar lipid bilayers increases with increasing depolarizations, $\delta = 0.48$ from the internal side (Wang, 1988). Also the effect of extracellular drug application on ACh channels of endplate increases with increasing hyperpolarization, $\delta = 0.78$ from the external side (Neher & Steinbach, 1978).

The finding that benzocaine failed to affect the Ca^{2+}-activated K^+ channel is different from the blocking effect on the Na^+ channel (Hille, 1977b). If benzocaine shares a common binding site with other local anaesthetics (Schmidtmayer & Ulbricht, 1980), the molecular structure of the K^+ channel may prevent the invasion of benzocaine from the lipid bilayer into the channel complex (hydrophobic pathway). If the benzocaine-binding site is different from the binding site for other anaesthetics in the Na^+ channel (Mrose & Ritchie, 1978), this finding may suggest that the Ca^{2+}-activated K^+ channel does not have a binding site.

It is thought that the conductance of the blocked Na^+ channel is zero, and that the channel current fluctuates between the full open level and the blocked level in the presence of local anaesthetics. However, the on-off flickering block by QX-314 is too fast to be resolved by the present recording techniques, resulting in the apparent reduction in the unitary current flowing through open channels (Wang, 1988). In the present study, the current amplitude of the single Ca^{2+}-activated K^+ channel was also reduced by the application of local anaesthetics. We attribute the reduction in the amplitude to a very fast flickering block by the drug, since the current record shows a slight but significant increase in current fluctuations during the application of QX-314. It may be speculated that lignocaine may induce still faster block of

the Ca^{2+}-activated K^+ channel, so that the recorded current did not show an apparent increase in fluctuations. Clear transitions between the open and closed levels of the single channel current were demonstrated in the block of endplate channels of skeletal muscle (Neher & Steinbach, 1978; Neher, 1983). The blockade of a 47 pS K^+ channel in isolated turtle colon epithelial cells by lignocaine is also of the fast flickering type (Richard & Dawson, 1986). However, the present study does not completely exclude the possibility of a reduction in the single channel conductance.

The use-dependent block of the Na^+ channel also provides strong support for the hypothesis that local anaesthetics enter the ion pathway of the channel to induce the open channel block (Schwartz *et al.*, 1977; Strichartz, 1973). It will be necessary in the future to test whether the Ca^{2+}-activated K^+ channel is blocked by local anaesthetics before the channel is opened by Ca^{2+}.

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Neuropeptide Y (NPY) receptors in HEL cells: comparison of binding and functional parameters for full and partial agonists and a non-peptide antagonist

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1 We have compared the binding and Ca^{2+} mobilizing properties of various full agonists, partial agonists and a non-peptide antagonist at the neuropeptide Y (NPY) receptor of human erythroleukemia (HEL) cells.

2 [^{125}I]-NPY binding to intact HEL cells was rapid, saturable, of high affinity and with a specificity typical for the Y_1-like subtype: NPY, peptide YY (PYY) and [Pro^{34}]-NPY competed for [^{125}I]-NPY binding with high affinity whereas NPY_{13-36} and NPY_{18-36} had only low affinity.

3 NPY, PYY and [Pro^{34}]-NPY potently increased intracellular Ca^{2+} in HEL cells and had equal efficacy. NPY_{13-36}, vasoactive intestinal peptide (VIP) and pancreatic polypeptide (PP) increased intracellular Ca^{2+} only poorly.

4 Whereas VIP and PP did not significantly affect NPY-stimulated Ca^{2+} mobilization, NPY_{13-36} inhibited NPY-stimulated Ca^{2+} increases and shifted the NPY concentration–response curve to the right without altering its maximal effect.

5 The agonist (pEC_{50}) potencies of the various peptides corresponded well with the affinities of these compounds in the binding assay (pK_i), whereas the antagonist potencies (pK_b) of the peptide partial agonists and the pA_2 value of the non-peptide NPY antagonist (He 90481), calculated from functional data, were lower than the respective affinities determined in the binding studies.

6 A plot of the fractional Ca^{2+} response vs the fractional receptor occupancy did not reveal any non-linear receptor-effector coupling for NPY or [Pro^{34}]-NPY; a small receptor reserve might exist for PYY.

7 We conclude that the binding and functional properties of HEL cell NPY receptors are very similar. NPY, PYY and [Pro^{34}]NPY are full agonists at these receptors, whereas NPY_{13-36} is a partial agonist.

Keywords: Neuropeptide Y; peptide YY; binding; Ca^{2+}; HEL cell; receptor reserve

Introduction

Neuropeptide Y (NPY) is a putative neurotransmitter that was first isolated in 1982 from porcine brain (Tatemoto, 1982). NPY can mediate numerous central and peripheral effects including central regulation of blood pressure and food intake, presynaptic inhibition of transmitter release, and vasoconstriction (Gray & Morley, 1986; Edvinsson *et al.*, 1987; Potter, 1988; Walker *et al.*, 1991). Many of these effects have been characterized by studying structure-activity relationships for various peptide analogues of NPY (Wahlestedt *et al.*, 1986; Chang *et al.*, 1988; Danho *et al.*, 1988; Servin *et al.*, 1989; Boublik *et al.*, 1989; Michel *et al.*, 1990). Other investigators have characterized NPY binding sites in various tissues and cell lines using similar peptide analogues of NPY (Lundberg *et al.*, 1988; Walker & Miller, 1988; Sheikh *et al.*, 1989; Martel *et al.*, 1990). Such studies have revealed that multiple NPY receptor subtypes appear to exist but an exact definition of these subtypes remains to be established (Michel, 1991).

A direct side-by-side comparison of the pharmacological properties of NPY binding sites and physiological NPY effects has rarely been performed. Such comparison, however, is important to fulfill the criteria for the acceptance of a binding site as a receptor (Laduron, 1984). Moreover, the comparison of binding and functional receptor properties provides information about the efficacy of receptor–effector coupling. It should also be kept in mind that binding studies on NPY receptors presently rely on the agonist ligand, which may not accurately define receptor number since agonist binding to NPY receptors is subject to modulation by post-receptor events such as the number and functional status of G-proteins (Feth *et al.*, 1991). Thus, a comparison of binding and functional parameters should not only rely on agonists but rather include partial and pure antagonists.

We have previously introduced the human erythroleukemia (HEL) cell line as a model system to study human NPY receptors *in vitro*; HEL cell NPY receptors couple to two independent second messenger systems, inhibition of forskolin-stimulated adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation and mobilization of intracellular Ca^{2+} (Motulsky & Michel, 1988). In the present study we have directly compared the binding and Ca^{2+} mobilizing properties of HEL cell NPY receptors using full agonists, partial agonists as well as the non-peptide antagonist He 90481 (Michel & Motulsky, 1990).

Methods

Cell culture

HEL cells were originally obtained from Dr T. Papayannopoulou (Dept. of Medicine, University of Washington, Seattle, WA, U.S.A.). They were grown in suspension culture in RPMI 1640 medium supplemented with 2 mM glutamine, 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 10% foetal calf serum at 37°C with 95% air/5% CO_2. Cells were maintained at a density of 4–8 × 10^5 cells ml^{-1} by daily dilution with fresh medium.

Radioligand binding

HEL cells at a density of about 5 × 10^5 ml^{-1} were harvested by 10 min centrifugation at 450 *g* at room temperature and washed twice with phosphate-buffered saline. Thereafter they

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were resuspended at a density of 2×10^7 cells ml^{-1} in assay buffer of the following composition (mM): HEPES 10, NaCl 150, KCl 5, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and bovine serum albumin (10 mg ml^{-1}) at a pH of 7.4. The reaction was carried out in polystyrene tubes that had been precoated with Sigmacote and washed with 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 2 mg ml^{-1} BSA to reduce nonspecific binding. The reaction was started by adding 1.5×10^6 cells to assay buffer containing [^{125}I]-NPY at the indicated concentrations in a total volume of 500 μl and, after incubation at 25°C for 90 min, was terminated by centrifugation for 5 min at 3000 g. The cell pellet was washed twice with 1 ml phosphate-buffered saline at 4°C. The remaining radioactivity was quantified in a gamma-counter (Berthold, Wildbad, F.R.G.). Under these conditions specific [^{125}I]-NPY binding increased linearly with the number of HEL cells in the assay in a range from 0.2 to 2.5×10^6 cells per tube.

In order to achieve the various [^{125}I]-NPY concentrations, a stock solution of [^{125}I]-NPY was diluted with unlabelled NPY; thus, the total amount of c.p.m. remained constant and the specific activity decreased with increasing NPY concentrations. We defined non-specific binding as [^{125}I]-NPY binding in the presence of 200 nM unlabelled NPY.

Ca^{2+} measurements

The cytoplasmatic free Ca^{2+} concentration was quantified by use of the fluorescent indicator dye Fura-2 and a Hitachi F-2000 spectrofluorometer as previously described (Feth *et al.*, 1991). Briefly, experiments were performed in buffer of the following composition (mM): HEPES 20, NaCl 120, KH_2PO_4 5, Mg acetate 1, $CaCl_2$ 2, and 1 mg ml^{-1} glucose at pH 7.4 using approximately $6–8 \times 10^5$ cells ml^{-1}. After 1 h of loading with the dye the cells were washed twice and resuspended in fresh buffer and used for fluorescence measurements within the next hour. Fluorescence was converted to Ca^{2+} concentrations by use of a ratio method which does not take into account fluorescence coming from extracellular dye. However, we monitored such extracellular fluorescence by the Mn^{2+} ion quench method as previously described (Motulsky & Michel, 1988) and used cells only if fluoresence from extracellular dye was less than 15% of total fluoresence.

Chemicals

Human NPY and peptide YY (PYY) were from Bissendorf (Hannover, F.R.G.), human pancreatic polypeptide (PP) and vasoactive intestinal peptide (VIP) were from Bachem (Heidelberg, F.R.G.), He 90481 was kindly provided by Heumann Pharma (Nürnberg, F.R.G.), [Pro^{34}]-NPY was kindly provided by Dr T.W. Schwartz (Copenhagen, Denmark), and [^{125}I]-NPY (specific activity 74 TBq mmol^{-1}) was from Amersham (Braunschweig, F.R.G.).

Data analysis

Saturation binding experiments were analysed based on the law of mass action by fitting the experimental data to a rectangular hyperbolic function. Competition binding experiments were analysed by fitting the experimental data to a sigmoid function with a Hill-slope of -1. Maximum [^{125}I]-NPY binding was fixed at 100% and minimum [^{125}I]-NPY binding was fixed at 0%. Concentration–response relationships were analysed by fitting the experimental data to sigmoid functions; the bottom of the curve was fixed at 0 (i.e. no increase in intracellular Ca^{2+}) and the Hill slopes were fixed at 1 in order to allow optimal estimates of the EC_{50}. All of the above calculations were performed using iterative non-linear regression analysis with the InPlot program (GraphPAD Software, San Diego, U.S.A.). All data are expressed as mean ± s.e.mean.

The relationship of fractional response vs fractional receptor-occupancy was determined graphically. Percentage of the maximal response, calculated by the non-linear regression analysis of the concentration–response curves, was plotted against fractional receptor occupancy, calculated by the formula $100/(1 + K_d/[A])$. K_d is the affinity of the respective peptide as determined in the radioligand binding studies and [A] is the concentration of the peptide which elicited the corresponding fractional response.

Since the HEL cell responsiveness towards NPY varied considerably from day to day, we determined the response to 100 nM NPY in each set of experiments and expressed all other effects as % of this response (Michel *et al.*, 1990).

Results

Characterization of [^{125}I]-NPY binding sites

The binding of [^{125}I]-NPY to intact HEL cells was rapid and reached equilibrium conditions within 90 min (data not shown). Saturation binding isotherms under equilibrium conditions demonstrated saturable high affinity [^{125}I]-NPY binding to HEL cells (K_d 3.9 ± 1.0 nM, B_{max} 73.0 ± 18.8 fmol per 10^6 cells, $n = 4$, Figure 1) corresponding to approximately 44,000 binding sites per cell.

In competition experiments with NPY and related peptides we determined a rank order of potency of PYY (pK_i: 8.61) ⩾ human NPY (pK_i: 8.53) ⩾ [Pro^{34}]-NPY (pK_i: 8.49) ⩾ $NPY_{13–36}$ ($pK_i \approx 6$) ⩾ $NPY_{18–36}$ ($pK_i \approx 5$; Figure 2a and Table 1); PP and VIP did not compete for [^{125}I]-NPY binding in concentrations up to 1 μM (data not shown).

We have previously shown that He 90481 is a competitive, non-peptidic, low affinity antagonist for NPY-stimulated Ca^{2+} increases in HEL cells (Michel & Motulsky, 1990). In binding studies He 90481 competed for HEL cell [^{125}I]-NPY binding sites with a similar low affinity (pK_i 3.74 ± 0.03; Figure 2b, Table 1).

Mobilization of intracellular Ca^{2+}

In order to further characterize the NPY receptor mediating Ca^{2+} increases in HEL cells, we compared the Ca^{2+} mobilizing properties of various peptides with those of NPY. Whereas 100 nM NPY produced a significant, transient, increase in Fura-2 fluorescence, $NPY_{13–36}$, PP and VIP caused only very minor elevations which did not allow the establishment of reliable concentration–response curves. In contrast, low concentrations of PYY and [Pro^{34}]-NPY significantly increased intracellular Ca^{2+} (Figure 3, Table 1). The rank order of potency in elevation of Ca^{2+} was PYY (pEC_{50}: 8.73) ⩾ NPY (pEC_{50}: 8.30) ⩾ [Pro^{34}]-NPY (pEC_{50}: 8.21;

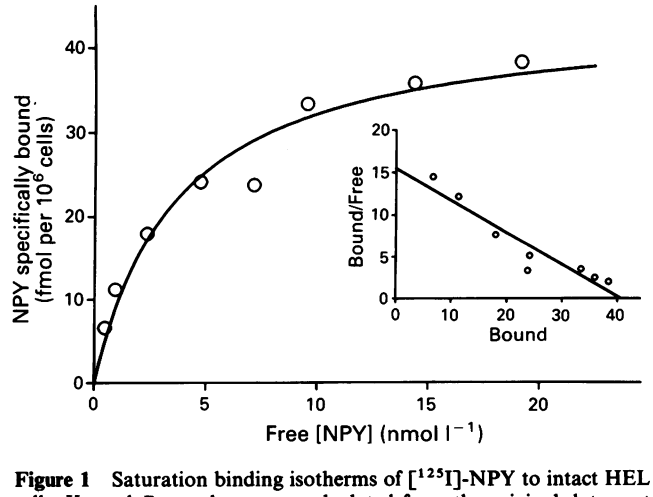


Figure 1 Saturation binding isotherms of [^{125}I]-NPY to intact HEL cells, K_d and B_{max} values were calculated from the original data, not from the Scatchard plot. Data are the average from triplicate determinations in a representative experiment. The insert shows the linearization of the data according to Scatchard (1949).

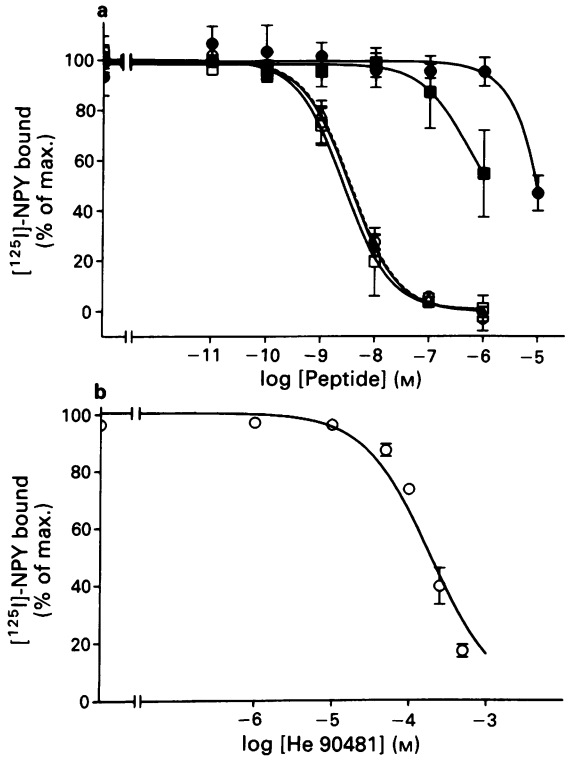


Figure 2 Competition of unlabelled peptides (a) and He 90481 (b) with [^{125}I]-neuropeptide Y (NPY) binding to HEL cells. Cells were incubated with 0.5 nM [^{125}I]-NPY in the presence of increasing concentrations of unlabelled peptides for 90 min at 25°C. In (a) effects of NPY (○), peptide YY (PYY, □), [Pro^{34}]-NPY (△), NPY_{13-36} (■), NPY_{18-36} (●) are shown. (b) Shows effects of He 90481 (○). Data are the mean of 3 experiments and vertical bars show s.d.

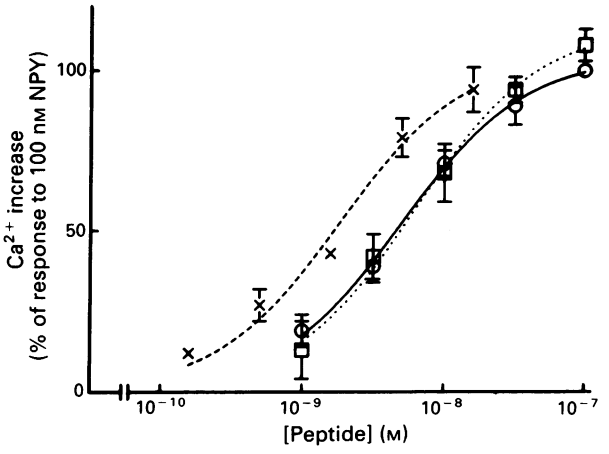


Figure 3 Concentration–response curves for peptide-mediated mobilization of intracellular Ca^{2+}. Data are the mean and vertical bars show s.e.mean of three experiments for neuropeptide Y (NPY) (○), [Pro^{34}]-NPY (□) and peptide YY (×).

Table 1), which corresponds to the potency obtained in our binding experiments. The maximum Ca^{2+} increase was similar for NPY, PYY and [Pro^{34}]-NPY (Table 1). Whereas NPY_{13-36} in concentrations up to 100 nM did not significantly elevate intracellular Ca^{2+}, 1 μM of NPY_{13-36} elevated Ca^{2+} by 9 ± 2% of the Ca^{2+} increase observed with 100 nM NPY in paired experiments (Table 2). Although VIP and PP did not inhibit NPY binding, 1 μM of these peptides elevated intracellular Ca^{2+} by 19 and 13% of the response to 100 nM NPY, respectively (Table 2).

The modest Ca^{2+} increase produced by NPY_{13-36}, VIP and PP could come from low intrinsic efficacy. Since partial agonists are, by definition, also competitive antagonists we tested the ability of these peptides to block NPY-stimulated Ca^{2+} elevations. Whereas 1 μM of either VIP or PP did not diminish the ability of NPY to raise intracellular Ca^{2+}, 1 μM of NPY_{13-36} slightly but significantly decreased the Ca^{2+} response to 100 nM NPY (Table 2). In order to test this apparent antagonism of NPY by NPY_{13-36} in more detail we compared paired concentration–response curves for NPY in the absence and presence of 1 μM NPY_{13-36}. NPY_{13-36} shifted the concentration–response curve for NPY to the right (Figure 4, Table 1) giving a pK_b value of 6.30 ± 0.16 (n = 3).

Studies on receptor reserve for Ca^{2+} mobilization in HEL-cells

Using the above data, we have plotted the fractional response for NPY, PYY and [Pro^{34}]-NPY vs the fractional receptor occupation by these peptides (Figure 5). The data points obtained for NPY and [Pro^{34}]-NPY did not differ significantly from the line of identity. The data points for PYY, however, were consistently above the line of identity and were

Table 2 Agonist and antagonist effects of pancreatic polypeptide (PP), vasoactive intestinal peptide (VIP) and NPY_{13-36} on Ca^{2+} mobilization in HEL cells

	Ca^{2+} rise by peptide	*Inhibition of Ca^{2+} rise induced by 100 nM NPY*
PP	19 ± 3%	3 ± 6%
VIP	13 ± 2%	−16 ± 6%
NPY_{13-36}	9 ± 2%	13 ± 4%*

All peptides were tested for agonist (left column) and antagonist effects (right column) at 1 μM. Agonist effects are expressed as % of the response elicited by 100 nM neuropeptide Y (NPY) in paired cuvettes. Antagonist effects are expressed as % inhibition of the response to 100 nM NPY. Data are the mean ± s.e.mean of 3–5 experiments (*P < 0.05 vs 100 nM NPY alone in paired experiments).

Table 1 Affinity of neuropeptide Y (NPY) and other peptides for [^{125}I]-NPY binding sites, together with their potency and efficacy at stimulating or inhibiting mobilization of intracellular Ca^{2+} in HEL cells

	Binding		*Ca^{2+} mobilization*	
	pK_i	*pK_b*	*pEC_{50}*	*Maximum (%)*
NPY	8.53 ± 0.09		8.30 ± 0.08	105 ± 1
PYY	8.61 ± 0.08		8.73 ± 0.17	108 ± 9
[Pro^{34}]-NPY	8.49 ± 0.04		8.21 ± 0.18	117 ± 10
NPY_{13-36}	≈6	6.30 ± 0.16		
NPY_{18-36}	≈5	6.53 ± 0.12[a]		
He 90481	3.74 ± 0.03	4.43[b]		

Data are the mean ± s.e.mean of 3–5 experiments as shown in Figures 2, 3 and 4. Maximal responses are expressed as % of the response to 100 nM NPY, as determined in the same set of cuvettes. For He 90481 a pA_2 value is shown instead of a pK_b value; only approximate pK_i values are shown for NPY_{13-36} and NPY_{18-36} since the complete competition was not achieved by the peptide concentrations used. [a] Data taken from Michel *et al.* (1990). [b] Data taken from Michel & Motulsky (1990).

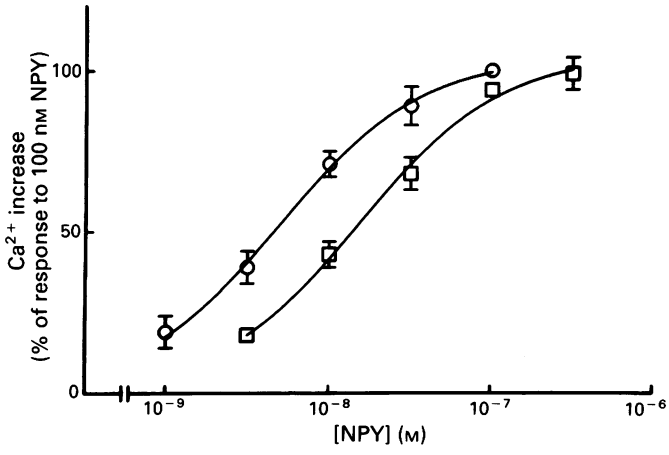


Figure 4 Antagonist properties of neuropeptide Y_{13-36} (NPY_{13-36}). Concentration–response curves for NPY-stimulated Ca^{2+} mobilization were obtained in the absence (○) or presence of 1 μM NPY_{13-36} (□). Data are the mean and vertical bars show s.e.mean of 3 experiments.

fitted better by a rectangular hyperbolic function, indicating the possible existence of a small receptor reserve for PYY-stimulated Ca^{2+} increases. Thus, 50% of maximal Ca^{2+} mobilization was achieved with approximately 30% receptor occupation.

Discussion

We have previously introduced HEL cells as a model to study human NPY receptors (Motulsky & Michel, 1988). The ability of 28 peptide analogues of NPY to stimulate Ca^{2+} elevations in this cell line (Michel *et al.*, 1990) correlates well with their ability to raise blood pressure in conscious unrestrained rats following intra-arterial injections (Boublik *et al.*, 1989). Moreover, the order of potency for Ca^{2+} increases by selected peptides from this group was similar to that for potentiating the field stimulation-induced vasoconstriction in the rabbit ear artery (Michel *et al.*, 1990). Thus, HEL cells appear to contain an NPY receptor subtype which is pharmacologically similar to that responsible for direct vasoconstriction in most vascular beds, as reflected by the blood pressure increases and for

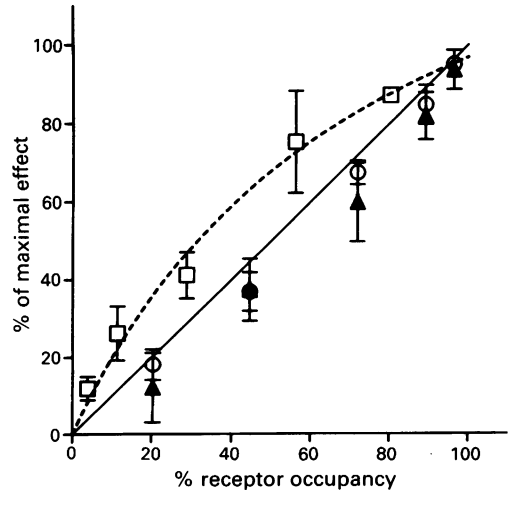


Figure 5 Receptor occupancy vs. fractional response of peptide-stimulated mobilization of intracellular Ca^{2+}. Neuropeptide Y (NPY) (○), peptide YY (□), [Pro^{34}]-NPY (▲). For assessment of the data see Methods.

potentiation of the vasoconstriction by other hormones. The value of HEL cells as a model system to study human NPY receptors has also been documented by the development of a peptide and a non-peptide antagonist of NPY receptors based on Ca^{2+} mobilization in HEL cells (Michel & Motulsky, 1990; Tatemoto, 1990). In order to evaluate further the value of this cell line for studies on human NPY receptors, we have now characterized HEL cell [^{125}I]-NPY binding sites and defined the NPY receptor subtype present in these cells; these data were used to assess the existence of a receptor reserve for Ca^{2+} mobilization via the HEL cell NPY receptor.

HEL cells exhibited high-affinity binding of [^{125}I]-NPY which had the pharmacological specificity to be expected from NPY receptors: VIP and PP had very low affinity, whereas human NPY and PYY had high affinity. Moreover, the affinity of the peptide analogues of NPY was in good agreement with their potency for mobilization of intracellular Ca^{2+}. Thus, [^{125}I]-NPY appears to label functional NPY receptors in HEL cells. The observation that HEL cells have more than 40,000 NPY receptors but less than 6000 α_2-adrenoceptors (McKernan *et al.*, 1987) might explain why NPY-stimulated Ca^{2+} increases in this cell line are consistently greater (Motulsky & Michel, 1988; Michel *et al.*, 1990) than those elicited by α_2-adrenoceptor agonists (Michel *et al.*, 1989; Motulsky & Michel, 1989).

We have previously shown that the C-terminal NPY fragment NPY_{18-36} possesses only low potency in HEL cells (Michel *et al.*, 1990), indicating the possible presence of a Y_1-like sub-type. The variable potency of C-terminal NPY fragments among assay systems, however, suggests that the definition of NPY receptor subtypes should not be based solely on a single C-terminal NPY-fragment (Michel, 1991). Therefore, we have now tested a second C-terminal fragment (NPY_{13-36}) as well as the Y_1-selective [Pro^{34}]-NPY (Krstenansky *et al.*, 1990). NPY_{13-36} and NPY_{18-36} had only very low affinity for [^{125}I]-NPY binding sites, whereas [Pro^{34}]-NPY had a similarly high affinity as NPY. This fits our functional data in which NPY_{13-36} (this study) and NPY_{18-36} (Michel *et al.*, 1990) had a low potency whereas [Pro^{34}]-NPY had a high potency. Moreover, all three peptides exhibited steep monophasic competition curves indicating interaction with a single homogeneous class of binding sites. Since PYY had a similar potency and affinity for HEL cell NPY receptors, they obviously do not belong to the PYY-insensitive Y_3-like subtype (Michel, 1991). Thus, we conclude that HEL cells express a homogeneous population of Y_1-like NPY receptors.

An additional finding of our binding experiments was that NPY_{18-36} had a ten fold lower affinity for HEL cell NPY receptors than the longer C-terminal fragment NPY_{13-36}. Since both fragments have similar affinities for the Y_2-like NPY receptors in rat brain and vas deferens (Martel *et al.*, 1990), it may be concluded that NPY_{18-36} has a greater selectivity for the Y_2-like subtype. This notion is supported by our recent literature survey which detected a greater discriminatory power of NPY_{18-36} in various assay systems (Michel, 1991).

Since the poor agonistic effects of NPY_{13-36}, VIP and PP could come from low affinity and/or from low efficacy and since NPY_{18-36} is a partial agonist at HEL cell NPY receptors (Michel *et al.*, 1990), we tested the possible antagonistic effects of these peptides. Since 1 μM VIP or PP did not inhibit the NPY-stimulated Ca^{2+} increases, they do not appear to have antagonistic effects at HEL cell NPY receptors. Since they also exhibited very low affinity in the binding studies, we conclude that their modest Ca^{2+} increasing effects are not mediated via NPY receptors but rather by a different (perhaps non-specific) mechanism. In contrast, 1 μM NPY_{13-36} produced a small but significant inhibition of NPY-stimulated Ca^{2+} increases and had detectable affinity for [^{125}I]-NPY binding sites. Similar to NPY_{18-36} (Michel *et al.*, 1990), NPY_{13-36} shifted the NPY concentration-response curve to the right without affecting the maximal response to NPY. These data

suggest competitive antagonism with a K_b value of approximately 500 nM, which is in a similar range as our previously observed K_b value of 297 nM for NPY_{18-36} (Michel *et al.*, 1990). In this context it should be noted that NPY_{18-36} behaves as a pure antagonist with low affinity at the putative Y_3-like PYY-insensitive NPY receptor in rat heart (Balasubramaniam & Sheriff, 1990). Thus, C-terminal NPY fragments apparently not only distinguished NPY receptor subtypes by their affinity, but also by their efficacy with low affinity and intermediate efficacy (partial agonism) at Y_1-like receptors (Wahlestedt *et al.*, 1986; Michel *et al.*, 1990), high affinity and efficacy (full agonism) at Y_2-like receptors (Wahlestedt *et al.*, 1986; Michel *et al.*, 1990), and intermediate affinity and low efficacy (pure antagonism) at Y_3-like receptors (Balasubramaniam & Sheriff, 1990).

We have previously demonstrated that He 90481 is a low affinity, competitive, non-peptidic antagonist for NPY-stimulated Ca^{2+} mobilization in HEL cells (Michel & Motulsky, 1990). Our binding studies confirm that He 90481 competitively binds to NPY receptors with low affinity. It should be noted, however, that the affinity of He 90481 in the binding studies was even lower than its previously obtained pA_2 value. Similarly, the affinity of the partial agonists NPY_{13-36} and NPY_{18-36} for the [^{125}I]-NPY binding sites was consistently lower than their presently (NPY_{13-36}) or previously (Michel *et al.*, 1990) described pK_b values. Moreover, the K_b value of NPY_{18-36} at the Y_3-like NPY receptor in rat heart was also significantly lower than its affinity for the NPY binding sites in this tissue (Balasubramaniam & Sheriff, 1990). In contrast, the potencies of the full agonists NPY, PYY and [Pro^{34}]-NPY agree well with their affinity in the binding studies. Taken together, these data suggest that competition for agonist binding might underestimate the potency of partial and pure antagonists.

Finally, we tested whether a receptor reserve exists for the Ca^{2+} increases elicited by NPY, PYY and [Pro^{34}]-NPY by comparing the fractional receptor occupancy and the fractional Ca^{2+} mobilization response. Since NPY receptors similar to many other G-protein coupled receptors exhibit high and low affinity states for agonists (Walker & Miller, 1988; Feth *et al.*, 1991), the question arises whether to use the high or the low affinity for calculation of receptor occupancy. On the one hand, agonists at G-protein coupled receptors are thought to act via the high affinity state of the receptor. On the other hand, steady state EC_{50} values for agonists at G-protein coupled receptors generally correlate better with their low affinity state. This is because the majority of receptors is in this state in intact cells, where the GTP concentration is high and the high-affinity state is induced following the agonist binding (Thomsen *et al.*, 1988). Thus, we used the low affinity state measurements obtained in the intact cells (where the GTP concentration is high) for our calculations of receptor occupancy. Our data do not reveal evidence for non-linear receptor–effector coupling for the full agonists NPY and [Pro^{34}]-NPY. Thus, no receptor reserve appears to exist for these two peptides. In contrast, the data points for PYY were consistently above the line of identity and were fitted well by a hyperbolic function indicating a possible small receptor reserve for this peptide. This idea is further supported by our previous observation that NPY was equally potent for inhibition of cyclic AMP accumulation and Ca^{2+} mobilization in HEL cells, whereas PYY was 18 fold more potent for inhibition of cyclic AMP accumulation than for Ca^{2+} mobilzation (Michel *et al.*, 1990). The requirement of a greater efficacy for receptor coupling to Ca^{2+} mobilization compared to coupling to inhibition of cyclic AMP accumulation was also documented by the greater efficacy of NPY_{18-36} and [D-Tyr^{36}]-NPY for cyclic AMP accumulation inhibition than Ca^{2+} mobilization (Michel *et al.*, 1990). Similar data were also found when comparing the efficacy of α_2-adrenoceptor agonists at HEL cell cyclic AMP accumulation inhibition and Ca^{2+} mobilization (Michel *et al.*, 1989). Taken together these data suggest that PYY has a greater intrinsic efficacy at HEL cell NPY receptors than NPY itself. If this would also be true for NPY receptors in other model systems, it might explain why different ratios of NPY/PYY potency have previously been obtained by other investigators in functional assay systems and raises the question as to whether PYY or NPY is the 'endogeneous' ligand at HEL cell NPY receptors.

In conclusion, our data demonstrate that [^{125}I]-NPY binding sites in HEL cells identify a homogeneous population of functional NPY receptors of the Y_1-like subtype. NPY_{13-36} is a partial agonist at these receptors. Although no receptor reserve exists for Ca^{2+} mobilization by NPY and [Pro^{34}]-NPY, a possible small receptor reserve for PYY indicates that this peptide might have a greater intrinsic efficacy at this receptor.

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Role of muscarinic receptor subtypes in central antinociception

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1 The ability to modify the pain threshold by the two M_1-muscarinic agonists: McN-A-343 and AF-102B and by the specific M_2-agonist arecaidine was examined in mice and rats by using three different noxious stimuli: chemical (writhing test), thermic (hot-plate test) and mechanical (paw pressure test).

2 In the mouse hot-plate test McN-A-343 (20–50 μg per mouse i.c.v.) and AF-102B (1–10 mg kg^{-1} i.p.) produced significant antinociception which was prevented by atropine (1 μg per mouse i.c.v.) and by the two selective M_1 antagonists: pirenzepine (0.01 μg per mouse i.c.v.) and dicyclomine (0.08 μg per mouse i.c.v. or 10 mg kg^{-1} i.p.) but not by the specific M_2-antagonist AFDX-116 (0.1 μg per mouse i.c.v.), naloxone (1 mg kg^{-1} i.p.) or by the acetylcholine (ACh) depletor hemicholinium-3 (HC-3) (1 μg per mouse i.c.v.). McN-A-343 and AF-102B were able to increase the pain threshold also in the mouse acetic acid writhing test and in rat paw pressure test. These antinociceptive effects were completely prevented by dicyclomine (0.08 μg per mouse i.c.v. or 10 mg kg^{-1} i.p.) but not by AFDX-116 (0.1 μg per mouse or rat i.c.v.).

3 In contrast with the M_1-agonists, the M_2-agonist arecaidine (0.1–2 μg per mouse or rat i.c.v.) did not induce antinociception in all three analgesic tests. However, arecaidine, at the same i.c.v. doses, was able to reduce the pain threshold in the hot-plate and paw pressure tests.

4 The site of muscarinic control of the pain threshold is localized in the CNS since drugs which do not cross the blood-brain barrier such as McN-A-343, pirenzepine and arecaidine exerted their effects only if injected i.c.v.

5 On the basis of the above findings and existing literature we suggest that the postsynaptic muscarinic receptors involved in antinociception belong to the M_1 subtype. Nevertheless, presynaptic autoreceptors (M_2 subtype) may play a role in pain regulation since they are involved in modulation of endogenous ACh release.

Keywords: Analgesia; antinociception; pain; muscarinic receptors; muscarinic agonists; muscarinic antagonists; acetylcholine

Introduction

Intracerebroventricularly (i.c.v.) administered acetylcholine (ACh) (Pedigo *et al.*, 1975) and both direct and indirect muscarinic agonists such as oxotremorine (George *et al.*, 1962; Bartolini *et al.*, 1980; 1987a) and its precursor tremorine (Lenke, 1958), arecoline (Herz, 1962), pilocarpine (Hendershot & Forsaith, 1959; Chernov *et al.*, 1967), physostigmine (Harris *et al.*, 1969; Ireson, 1970) and diisopropyl phosphorofluoridate (DFP) (Lentz *et al.*, 1969) induce antinociception in the laboratory animal. Since such antinociception is completely antagonized by atropine (Bartolini *et al.*, 1987a), a muscarinic receptor should be involved. The postsynaptic muscarinic receptor involved in antinociception should be of the M_1-subtype, since recently Malmberg-Aiello *et al.* (1988) and Ghelardini *et al.* (1990) have demonstrated that pirenzepine, a selective M_1 antagonist (Hammer *et al.*, 1980), is also able to block oxotremorine antinociception.

We thought it important to verify whether the two selective M_1-agonists: McN-A-343 (Hammer & Giachetti, 1982; Lambrecht & Mutschler, 1985; Lambrecht *et al.*, 1986) and AF-102B (Fisher *et al.*, 1986; Ono *et al.*, 1988; Fisher *et al.*, 1989; Nakahara *et al.*, 1990) and the M_2-agonist arecaidine (Mutschler & Hultzsch, 1973; Mutschler & Lambrecht, 1984; Barlow & Weston-Smith, 1985) were able to modify the pain threshold, since muscarinic antinociception was thus far obtained only by employing agonists devoid of selectivity for various muscarinic receptor subtypes.

Moreover, in order to strengthen the previous results concerning the antagonism of muscarinic antinociception by pirenzepine we investigated the antagonistic effect on antinociception of dicyclomine, another well known M_1-antagonist (Nilvebrant & Sparf, 1986). For comparison with the effect of the M_1-antagonists, the action of the selective M_2-antagonist AFDX-116 (Giachetti *et al.*, 1986a,b) was also tested.

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Preliminary data have been presented in XIth IUPHAR Congress, Amsterdam 1990.

Methods

Hot-plate test

Male Swiss albino mice (25–30 g) from the Morini breeding farm were used. The mice were placed inside a stainless steel container thermostatically set at 52.5 ± 0.1°C in a precision water-bath from KW Mechanical Workshops, Siena, Italy. The reaction times (s) were measured with a stop-watch before, and 15, 30, 45 and 60 min after treatment. The end-point used was the licking of the fore or hind paws. Those mice which scored below 12 or over 18 s in the pretest were rejected. An arbitrary cut-off time of 45 s was adopted.

Writhing test

Male Swiss albino mice (25–30 g) were injected i.p. with a 0.5% aqueous solution of acetic acid (10 ml kg^{-1}). The number of stretching movements was counted for 10 min, starting 5 min after acetic acid injection.

Paw pressure (Randall Selitto test)

The nociceptive threshold was determined with an analgesimeter (Ugo Basile, Varese, Italy) in rats. The instrument exerts a force which is applied at a constant rate (32 g per second) with a cone-shaped pusher on the upper surface of the rat hind paw. The force is continuously monitored by a pointer moving along a linear scale. The pain threshold is given by the force which induces the first struggling from the rat. Those pretested rats which scored below 30 g or over 90 g were rejected. An arbitrary cut-off value of 200 g was adopted.

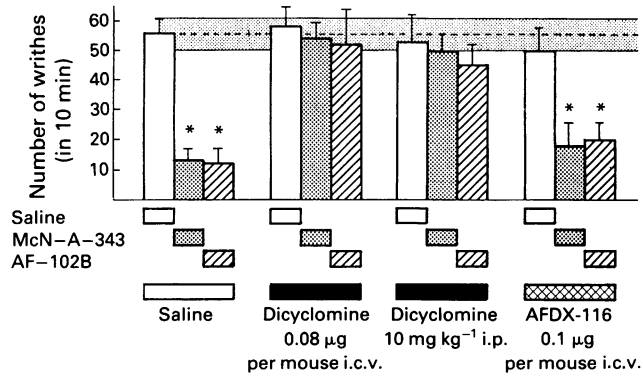


Figure 1 Effect of dicyclomine (0.08 μg per mouse – 10 mg kg^{-1} i.p.) and AFDX-116 (0.1 μg per mouse) pretreatments on antinociception induced by McN-A-343 (30 μg per mouse i.c.v.) and AF-102B (5 mg kg^{-1} i.p.) in the mouse writhing test. Dicyclomine and AFDX-116 were injected respectively 15 and 5 min before the administration of cholinomimetics. The nociceptive responses were recorded 15 min after administration of McN-A-343 and 30 min after AF-102B. Vertical lines show s.e.mean. * $P < 0.01$ in comparison with saline controls. Each column represents the mean of at least ten mice.

Drugs and reagents

The following drugs were used: atropine sulphate, hemicolinium-3 hydrobromide (HC-3) and naloxone hydrochloride (Sigma), dicyclomine hydrochloride (Lepetit), pirenzepine hydrochloride (Istituto Sieroterapico Milanese), AFDX-116 and McN-A-343 (De Angeli), AF-102B (Inst. for Neurobiol. Res. Bruxelles, Belgium), arecaidine propargyl ester (Dept. of Pharmacol. Univ. of Frankfurt, Germany). Other chemicals were of the highest quality commercially available. All drugs were dissolved in a physiological saline solution. Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 10 ml kg^{-1} by both s.c. and i.p. injection. The doses given in the text referring to atropine, HC-3, dicyclomine and pirenzepine are expressed as salts. I.c.v. administration was performed during brief ether anaesthesia according to the method described by Haley & McCormick (1957), by injecting mice and rats with the necessary dose dissolved in 5 and 10 μl of saline solution, respectively. In some experiments, mice but not rats received two i.c.v. injections performed at 5 and 15 min intervals. In such cases ether anaesthesia was repeated two times and each cerebral ventricle received only one injection.

Doses of muscarinic antagonist drugs were selected, by preliminary experiments, as the lowest ones able to prevent agonist effects.

Statistical analysis

Results are given as the mean ± s.e.mean. Dunnett's two-tailed test was used to verify the significance of differences between the means, and these were considered significant when P values were less than 0.05.

Results

Pain threshold effect of M_1- and M_2-selective agonists

McN-A-343 and AF-102B induced a significant increase in the pain threshold in both mice and rats in all three tests (Tables 1 and 2, Figures 1, 2 and 3). Antinociception from i.c.v. McN-A-343 reached a maximum after 15 min while that from i.p. AF-102B reached a maximum after 30 min. Both antinociceptive effects persisted unchanged for up to 45 min and then diminished 60 min after administration (Tables 1 and 2; Figures 2 and 3).

McN-A-343 exerted its antinociceptive activity by i.c.v. administration starting from 20 μg per mouse. Antinociception was dose-dependent up to a maximum of 50 μg per mouse, after which cholinergic symptoms (tremors and general prostration) invalidated a correct evaluation of the pain threshold. McN-A-343 (0.5 mg kg^{-1} s.c.), if injected parenterally, induced cholinergic symptoms approximately of the same intensity as those produced by oxotremorine (40 μg kg^{-1} s.c.). However, while parenterally administered oxotremorine induced antinociception (Ghelardini *et al.*, 1990), parenterally administered McN-A-343 did not.

AF-102B was able to induce antinociception when injected either intraperitoneally starting at 1 mg kg^{-1} i.p. or i.c.v. (1–5 μg per mouse) (data not shown). Weak cholinergic side-effects became visible after 10 mg kg^{-1} i.p. At higher i.p. doses side-effects did not permit testing.

In contrast to the antinociception induced by M_1-agonists, the selective M_2-agonist arecaidine produced a significant hyperalgesic action in the mouse hot-plate test (Table 3a) and rat paw pressure test (Table 3b). Arecaidine, although injected at the same i.c.v. doses used in the hot-plate and paw-pressure

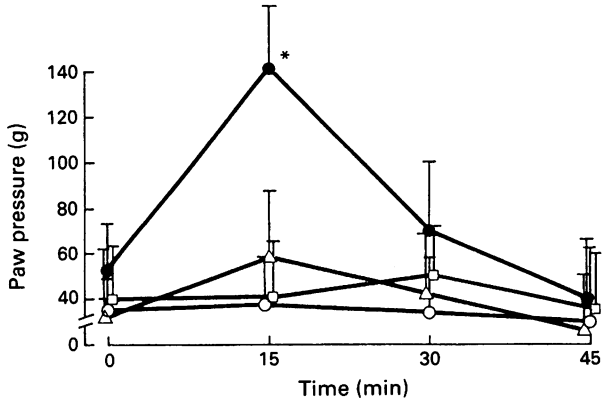


Figure 2 Time course of pain threshold after McN-A-343 in the rat paw pressure test. Antagonism by dicyclomine of antinociceptive effect induced by McN-A-343. Dicyclomine was injected 30 min before test. Vertical lines show s.e.mean. * $P < 0.01$ in comparison with saline controls. Each point is the mean of at least six rats. Saline (□); McN-A-343 30 μg per rat, i.c.v. (●); dicyclomine 10 mg kg^{-1}, i.p. (○); dicyclomine + McN-A-343 (△).

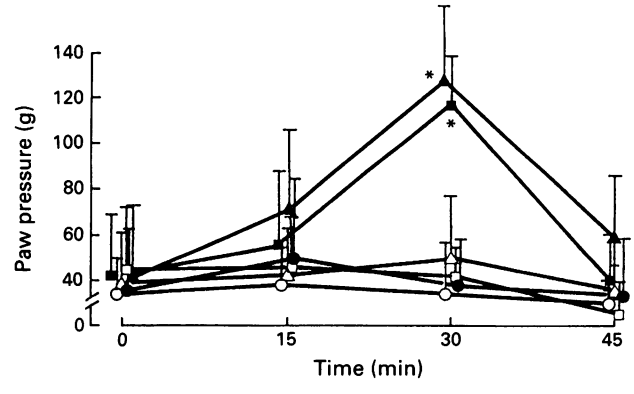


Figure 3 Time course of pain threshold after AF-102B in rat paw pressure test. Antagonism by dicyclomine occurred but not by AFDX-116 on AF-102B antinociception. Dicyclomine and AFDX-116 were injected respectively 30 min and 20 min before test. Vertical lines show s.e.mean. * $P < 0.01$ in comparison with saline controls. Each point represents the mean of at least six rats. Saline (□); AF-102B 5 mg kg^{-1}, i.p. (▲); dicyclomine 10 mg kg^{-1}, i.p. (○); dicyclomine + AF-102B (△); AFDX-116 0.1 μg per rat, i.c.v. (●); AFDX-116 + AF-102B (■).

Table 1 Effect of atropine, pirenzepine, dicyclomine, HC-3, naloxone and AFDX-116 on antinociception caused by McN-A-343

			Hot-plate *Licking latency in mice* (s)				
	Treatment	*μg per*	*Before*	*After treatment*			
Pretreatment	(i.c.v.)	*mouse*	*treatment*	*15 min*	*30 min*	*45 min*	*60 min*
Saline	Saline		15.6 ± 0.8	16.2 ± 1.0	15.8 ± 0.9	15.3 ± 1.1	16.1 ± 1.3
(i.p., 10 ml kg^{-1}	McN-A-343	10	15.0 ± 0.7	14.6 ± 1.3	15.7 ± 1.2	13.6 ± 1.1	14.9 ± 1.7
or i.c.v.,	McN-A-343	20	14.5 ± 0.5	21.1 ± 2.2*	18.2 ± 2.1	15.1 ± 1.9	16.3 ± 1.5
5 μl per mouse)	(x) McN-A-343	30	15.1 ± 0.4	27.6 ± 1.1**	26.6 ± 1.8**	22.6 ± 1.5*	17.5 ± 1.6
	McN-A-343	50	15.3 ± 0.5	42.1 ± 4.3**	40.5 ± 3.7**	35.5 ± 2.5**	22.8 ± 2.1*
Atropine	Saline		14.7 ± 0.8	13.9 ± 1.7	16.1 ± 2.2	15.7 ± 2.1	16.1 ± 2.3
(i.c.v.	McN-A-343	30	15.5 ± 0.7	17.3 ± 1.6	15.6 ± 1.9	14.2 ± 1.7	15.4 ± 1.9
1 μg per mouse)							
Pirenzepine	Saline		14.7 ± 1.3	14.1 ± 1.6	15.7 ± 1.3	16.1 ± 1.8	15.9 ± 1.7
(i.c.v.,	McN-A-343	30	13.7 ± 1.0	16.8 ± 1.7	17.1 ± 1.6	15.8 ± 1.3	14.0 ± 1.0
0.01 μg per mouse)							
Dicyclomine	Saline		14.6 ± 1.1	13.6 ± 1.8	15.9 ± 2.1	16.8 ± 2.0	14.8 ± 1.3
(i.c.v.,	McN-A-343	30	15.7 ± 0.8	17.7 ± 1.6	16.8 ± 1.9	15.4 ± 1.8	13.9 ± 2.1
0.08 μg per mouse)							
HC-3	Saline		15.3 ± 0.8	16.2 ± 1.5	16.0 ± 2.2	14.4 ± 2.3	13.6 ± 2.1
(i.c.v.,	McN-A-343	30	15.5 ± 1.0	26.9 ± 2.9**	24.6 ± 1.3**	22.5 ± 2.4**	17.1 ± 1.8
1 μg per mouse)							
Naloxone	Saline		14.9 ± 1.3	14.4 ± 1.6	15.6 ± 2.1	15.5 ± 1.8	16.3 ± 1.7
(i.p.,	McN-A-343	30	14.5 ± 1.2	25.5 ± 2.0**	23.8 ± 1.8**	25.5 ± 2.7**	14.4 ± 1.8
1 mg kg^{-1})							
AFDX-116	Saline		14.9 ± 1.0	16.1 ± 2.2	16.3 ± 1.7	14.7 ± 1.9	13.0 ± 1.2
(i.c.v.,	McN-A-343	30	15.1 ± 1.2	25.9 ± 2.9**	24.1 ± 2.3*	20.1 ± 2.1*	14.8 ± 2.5
0.1 μg per mouse)							

The number of mice ranged from 8–20 with the exception of (x) where $n = 50$.
* $P < 0.05$, ** $P < 0.01$ in comparison with saline-saline.

tests, did not modify either the number of writhes induced by i.p. acetic acid in mice (Figure 4), or the hyperalgesia induced by naloxone (1 mg kg^{-1} i.p.) in the same test (Figure 4).

Arecaidine showed its hyperalgesic effect from 15 to 30 min after i.c.v. administration in the hot-plate test and from 30 to 60 min in the rat paw pressure test (Table 3a,b). The decrease in the pain threshold was dose-dependent starting in mice at the dose of 0.1 μg and in rats at the dose of 0.5 μg. Higher doses than 2 μg per mouse or rat induced a general state of prostration. No cholinergic symptoms were present in the range of i.c.v. doses used.

Arecaidine, i.p. was also able to induce an equipotent hyperalgesic effect either in rats (10 mg kg^{-1}) or in mice (20 mg kg^{-1}) (data not shown). Hyperalgesia began after 15 min and persisted up to 30 min. By using these doses no cholinergic symptoms were observed. However, higher doses

Table 2 Effect of atropine, pirenzepine, dicyclomine, HC-3, naloxone, AFDX-116 on antinociception caused by AF 102B

			Hot-plate *Licking latency in mice* (s)				
	Treatment		*Before*	*After treatment*			
Pretreatment	(i.p.)	mg kg^{-1}	*treatment*	*15 min*	*30 min*	*45 min*	*60 min*
Saline	Saline		16.4 ± 0.9	15.1 ± 1.3	16.0 ± 1.0	14.5 ± 1.0	15.3 ± 0.7
(i.p.,	AF 102B	0.1	14.9 ± 1.1	14.1 ± 1.6	15.6 ± 1.9	16.3 ± 1.2	14.8 ± 1.9
10 ml kg^{-1})	AF 102B	1	15.1 ± 0.9	20.7 ± 1.3*	21.5 ± 2.8*	22.3 ± 2.4*	16.9 ± 1.8
	(x) AF 102B	5	15.6 ± 0.7	21.3 ± 1.1*	27.1 ± 1.7**	26.8 ± 1.9**	18.2 ± 1.3
	AF 102B	10	15.8 ± 0.8	25.8 ± 2.3**	30.1 ± 2.0**	25.9 ± 2.1**	19.0 ± 1.7
Atropine	Saline		15.6 ± 0.8	14.2 ± 1.4	16.3 ± 1.8	15.1 ± 1.0	14.3 ± 1.3
(i.c.v.,	AF-102 B	5	14.2 ± 0.7	15.4 ± 1.8	15.3 ± 1.9	14.6 ± 2.1	15.1 ± 1.6
1 μg per mouse)							
Firenzepine	Saline		15.6 ± 0.9	16.3 ± 1.0	13.6 ± 1.9	16.1 ± 1.5	13.8 ± 1.7
(i.c.v.,	AF 102B	5	15.4 ± 0.6	15.5 ± 1.6	17.3 ± 1.6	16.9 ± 1.4	15.7 ± 1.7
0.01 μg per mouse)							
Dicyclomine	Saline		15.6 ± 1.0	14.2 ± 1.6	15.8 ± 1.8	14.8 ± 1.3	15.2 ± 1.6
(i.c.v.,	AF 102B	5	14.3 ± 0.9	17.3 ± 2.1	16.2 ± 1.4	16.9 ± 1.7	16.1 ± 1.7
0.08 μg per mouse)							
HC-3	Saline		14.8 ± 0.9	13.8 ± 1.5	14.4 ± 1.6	15.5 ± 1.3	16.0 ± 1.1
(i.c.v.,	AF 102B	5	15.3 ± 1.0	19.0 ± 0.7*	26.4 ± 2.0**	25.8 ± 1.2**	17.4 ± 1.7
1 μg per mouse)							
Naloxone	Saline		14.1 ± 1.1	15.3 ± 1.0	16.9 ± 1.3	14.4 ± 1.2	15.8 ± 1.0
(i.p.,	AF 102B	5	15.1 ± 0.9	19.0 ± 2.6	25.8 ± 1.9**	27.0 ± 2.1**	18.1 ± 2.0
1 mg kg^{-1})							
AFDX-116	Saline		14.9 ± 1.0	16.1 ± 2.2	16.3 ± 1.7	14.7 ± 1.9	15.0 ± 1.2
(i.c.v.,	AF 102B	5	15.2 ± 1.1	20.9 ± 2.3*	26.6 ± 1.8**	25.3 ± 1.7**	17.3 ± 2.1
0.1 μg per mouse)							

The number of mice ranged from 8–20 with the exception of (x) wehre $n = 45$.
* $P < 0.05$, ** $P < 0.01$ in comparison with saline-saline.

Table 3 Effect of HC-3 (a and b) and AFDX-116 (a) on hyperalgesia caused by arecaidine in (a) mouse hot plate test and (b) rat paw pressure test

a

			Hot-plate Licking latency in mice (s)			
Pretreatment (i.c.v.)	*Treatment* (i.c.v.)	*μg per mouse*	*Before treatment*	*After treatment* 15 min	30 min	45 min
Saline	(x) Saline		14.6 ± 0.7	14.8 ± 1.1	14.9 ± 1.2	14.7 ± 0.9
5 μl	Arecaidine	0.01	15.0 ± 0.9	13.8 ± 1.3	14.8 ± 1.6	15.6 ± 1.6
	Arecaidine	0.1	14.7 ± 0.9	10.2 ± 1.1*	11.3 ± 1.4*	13.9 ± 0.8
	Arecaidine	1	14.7 ± 1.3	10.6 ± 1.0*	10.5 ± 0.9*	13.8 ± 1.3
	(x') Arecaidine	2	14.9 ± 0.9	11.3 ± 1.1*	12.0 ± 0.7*	14.7 ± 1.1
HC-3	Saline		15.1 ± 1.0	15.8 ± 1.6	16.3 ± 1.9	17.1 ± 2.5
1 μg per mouse	Arecaidine	0.1	14.5 ± 0.6	13.6 ± 1.5	14.5 ± 1.6	15.9 ± 1.3
AFDX-116	Saline		14.9 ± 1.1	15.5 ± 1.6	16.4 ± 2.1	15.8 ± 1.7
0.1 μg per mouse	Arecaidine	0.1	15.6 ± 1.3	16.1 ± 1.8	14.0 ± 1.6	13.9 ± 1.9

b

			Paw-pressure in rats (g)			
	Treatment (i.c.v.)	*μg per mouse*	*Before treatment*	*After treatment* 30 min	45 min	60 min
Saline	Saline		70 ± 4.0	66 ± 6.0	74 ± 4.0	72 ± 4.0
5–10 μl	Arecaidine	0.1	70 ± 4.0	56 ± 8.0	60 ± 10	44 ± 6.0
	Arecaidine	0.5	70 ± 6.0	32 ± 2.0*	32 ± 2.0*	42 ± 2.0*
	Arecaidine	1	72 ± 6.0	38 ± 2.0*	32 ± 4.0*	32 ± 2.0*
	Arecaidine	2	76 ± 4.0	40 ± 6.0*	36 ± 4.0*	32 ± 2.0*
HC-3	Saline		70 ± 4.0	68 ± 4.0	70 ± 4.0	66 ± 6.0
1 μg per rat	Arecaidine	1	70 ± 4.0	68 ± 4.0	62 ± 6.0	54 ± 2.0

The number of animals ranged from 5–18 with the exception of (x) where $n = 35$ and (x') where $n = 25$.
* $P < 0.01$ in comparison with saline-saline.

(50 mg kg^{-1}, i.p.) induced hyperalgesia together with sialorrhea and lacrimation.

Antagonism of antinociception by muscarinic blockers

Tables 1 and 2 show that atropine (1 μg per mouse i.c.v.) administered 15 min before both McN-A-343 and AF-102B, antagonized the antinociception induced in mice by the two drugs. Similarly, Tables 1 and 2 show that i.c.v. pirenzepine (0.01 μg per mouse) and i.c.v. dicyclomine (0.08 μg per mouse) administered respectively 5 and 15 min before both McN-A-343 and AF-102B, were able to abolish their antinociceptive effect in the hot-plate and writhing tests. Moreover, dicyclomine administered i.p. (10 mg kg^{-1}) also antagonized the antinociception induced by the M_1-agonist in the rat paw pressure test (Figures 2 and 3).

Neither naloxone (1 mg kg^{-1} i.p.), nor HC-3 (1 μg per mouse i.c.v.), administered 15 min and 5 h respectively before treatment, modified antinociception induced by McN-A-343 or AF-102B (Tables 1 and 2). However, HC-3 pretreatment prevented arecaidine hyperalgesia (Table 3a and b).

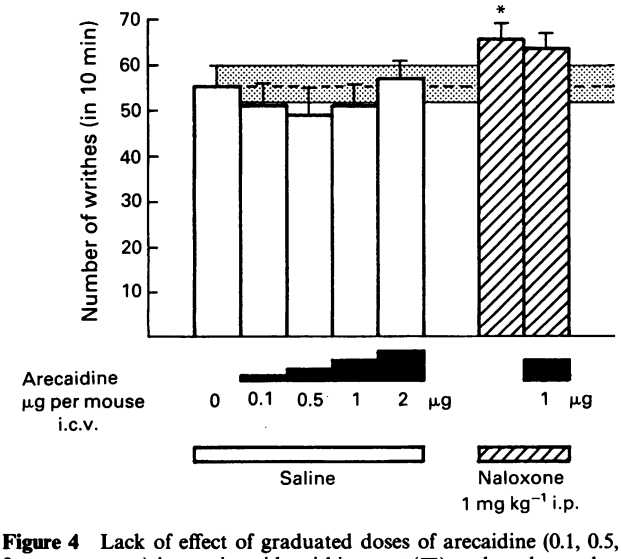


Figure 4 Lack of effect of graduated doses of arecaidine (0.1, 0.5, 1, 2 μg per mouse) in acetic acid writhing test (□) and on hyperalgesia induced by naloxone (1 mg kg^{-1} i.p.) (▨) in the same test. Arecaidine was injected 30 min before test and naloxone 5 min before arecaidine. Vertical lines show s.e.mean. * $P < 0.01$ in comparison with saline controls. Each column is the mean of at least 10 mice.

Lack of antagonism by the M_2-muscarinic blocker AFDX-116

Tables 1 and 2 and Figures 1 and 3 show that AFDX-116 did not reduce the antinociceptive activity of McN-A-343 and AF-102B. AFDX-116 was injected i.c.v. 5 min before the agonists with a dose of 0.1 μg which was the maximum usable without interfering with normal behaviour. However, at the same dose, AFDX-116 abolished arecaidine induced hyperalgesia (Table 3a).

Discussion

Our results, obtained with three different noxious stimuli (chemical, thermic and mechanical) clearly show that both the specific M_1-muscarinic agonists McN-A-343 and AF-102B are able to induce antinociception in mice and rats. Conversely, the specific M_2-agonist arecaidine did not increase the pain threshold, which was decreased in the mouse hot-plate and rat paw pressure tests.

Antinociception induced by McN-A-343 and AF-102B was antagonized by the two selective M_1-antagonists pirenzepine and dicyclomine but not by the selective M_2-antagonist AFDX-116. Therefore, it appears reasonable to suggest that only M_1-receptors are responsible for postsynaptic muscarinic antinociception. As a consequence, antinociception induced by non-specific cholinomimetics such as oxotremorine, arecoline, pilocarpine, physostigmine and DFP (see Introduction), which are able to stimulate simultaneously a variety of muscarinic receptor subtypes, appear to be due to M_1-receptor activation. Similarly, antagonism of antinociception induced by non-specific muscarinic blockers such as atropine (Bartolini *et al.*,

1987a; Ghelardini *et al.*, 1990) was due to M_1 antagonism only.

The antinociceptive effects of these drugs take place in the CNS since both agonists (McN-A-343) and antagonists (pirenzepine) which are unable to cross the blood-brain barrier exert their effects only when injected i.c.v.

The very low i.c.v. doses of pirenzepine and dicyclomine which are able to antagonize antinociception, doses respectively 100 and 12.5 times lower than that of atropine (Tables 1 and 2), show that the antagonism obtained at such low doses is selective for M_1-receptors. On the other hand, it is very difficult to extrapolate literature data concerning concentrations used in *in vitro* binding experiments, to doses employed *in vivo*. Giraldo *et al.* (1988) showed that concentrations of pirenzepine 72 times higher are necessary to displace [^{3}H]-pirenzepine from (M_2) atrial receptors compared to forebrain ($M_1 + M_2$) receptors. These authors stated that to displace ligands from M_2 (cardiac)-receptors pirenzepine concentrations above 1×10^{-6} M are necessary while to displace ligands from M_1-receptors, concentrations of pirenzepine higher than 1.4×10^{-8} M are sufficient. It is interesting to note that, the calculated concentration of the drug present in the cerebral tissue after i.c.v. injection of 10 ng (the dose necessary to antagonize muscarinic antinociception) should be about 8×10^{-8} M, assuming a uniform distribution of pirenzepine in the brain. The pirenzepine doses used therefore seem to be in the range of M_1-selectivity. Moreover the specificity of pirenzepine and dicyclomine against muscarinic receptors is further validated by the findings that these drugs, injected at the same doses needed to block antinociception caused by muscarinic agonists, are not able to modify analgesia induced by morphine and by the γ-aminobutyric acid$_B$-agonist baclofen (Ghelardini *et al.*, 1990).

AFDX-116 did not modify antinociception induced by M_1-agonists. This lack of effect might be caused by a dose of AFDX-116 inadequate to block M_2-receptors effectively. However, this possibility should be rejected since AFDX-116, at the same i.c.v. doses, was able to prevent the hyperalgesia induced by the M_2-agonist arecaidine.

On the bases of the above results (no antinociception by arecaidine and lack of antagonism by AFDX-116) an M_2 receptor involvement in postsynaptic antinociception should be ruled out. Although postsynaptic M_2-receptors are not implicated in muscarinic analgesia, presynaptic M_2 receptors play an important role in pain regulation.

There is much evidence from previous studies (Potter *et al.*, 1984; Raiteri *et al.*, 1984; Mash *et al.*, 1985; Mash & Potter, 1986; Spencer *et al.*, 1986; Pohorecki *et al.*, 1988) showing that in the CNS cholinergic inhibitory autoreceptors are of M_2-subtype. We have recently demonstrated (Bartolini *et al.*, 1987a,b; 1989; Malmberg-Aiello *et al.*, 1988; Gualtieri *et al.*, 1989; Ghelardini *et al.*, 1990) that it is possible to induce antinociception by increasing central ACh release by blocking only the presynaptic muscarinic autoreceptors.

In fact, autoreceptors could regulate pain by modulating ACh release via a negative feedback mechanism. In agreement with this hypothesis, the M_2-agonist arecaidine induced hyperalgesia which was prevented following ACh depletion with HC-3. Bartolini *et al.* (1989), Gualtieri *et al.* (1989), Ghelardini *et al.* (1990) and Iwamoto (1989) have shown that the two M_2-antagonists AFDX-116 (2–10 ng per mouse i.c.v.) and methoctramine (starting at 5 ng per mouse i.c.v.) are both able to induce antinociception in rodents.

AFDX-116 and methoctramine analgesias are prevented not only by M_1-antagonists but also by the two ACh depletors: HC-3 (Bartolini *et al.*, 1989; Gualtieri *et al.*, 1989) and vesamicol (Iwamoto, 1989). These findings suggest that both the decrease and increase in pain threshold induced by M_2 agonists and antagonists derives from decreased and increased ACh release respectively which, in turn, modulate pain via M_1-muscarinic receptors.

The observation that McN-A-343 and AF-102B preserve their antinociceptive effect even after depletion of cerebral ACh (mice pretreated with HC-3) further supports the involvement of M_1-postsynaptic receptors in analgesia.

Hyperalgesia induced by arecaidine in the hot-plate and paw pressure tests was not confirmed in the acetic acid writhing test. Since the lack of hyperalgesic effect of arecaidine in the writhing test was not removed even with naloxone pretreatment of mice, we can rule out the possibility that a simultaneous activation of the enkephalinergic system by chemical peritonitis masks arecaidine hyperalgesia.

Therefore, although a presynaptic autoreceptor hypothesis for arecaidine hyperalgesia appears to be attractive, further research is necessary in order to understand not only why arecaidine does not induce hyperalgesia in the writhing test but also why i.c.v. pirenzepine, dicyclomine and i.c.v. HC-3 do not decrease pain threshold in all three analgesic tests.

In conclusion, although M_2 presynaptic muscarinic receptors seem to play a role in cholinergic antinociception, our results have shown that the postsynaptic muscarinic receptors involved in antinociception belong to the M_1 subtype.

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Effects of acromelic acid A on the binding of [^{3}H]-kainic acid and [^{3}H]-AMPA to rat brain synaptic plasma membranes

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1 The ability of acromelic acid A to inhibit [^{3}H]-kainic acid and [^{3}H]-(**RS**)-α-amino-3-hydroxy-5-methyloxazole-4-propionic acid ([^{3}H]-AMPA) binding to rat brain synaptic plasma membranes was investigated by equilibrium radioligand binding assay.

2 Kinetic analysis of [^{3}H]-kainic acid binding demonstrated the existence of two kainate binding sites in this tissue preparation and yielded equilibrium dissociation constants for [^{3}H]-kainic acid of $K_D = 0.4$ nM and $K_D = 20.8$ nM.

3 Kainic acid and domoic acid both appeared to displace [^{3}H]-kainic acid from a single binding site with equilibrium binding constants of $K_D = 19.4$ nM and $K_i = 14.5$ nM respectively. Acromelic acid A exhibited a biphasic inhibition of [^{3}H]-kainic acid binding to synaptic membranes with binding affinities of $K_i = 15.1$ nM and $K_i = 1.49$ μM.

4 In the absence of chaotropic ions, the order of potency of inhibition of [^{3}H]-AMPA binding was acromelic acid A ($K_i = 26$ nM) > AMPA ($K_D = 184$ nM) > domoic acid ($K_i = 499$ nM).

5 The inclusion of 100 mM thiocyanate ion in the [^{3}H]-AMPA binding assay resulted in a change in the order of potency to: AMPA ($K_D = 160$ nM) > acromelic acid A ($K_i = 289$ nM) > domoic acid ($K_i = 9.02$ μM).

6 These results show that acromelic acid A distinguishes two kainate binding sites in rat brain synaptic plasma membranes and in addition, that in the absence of chaotropic ions, acromelic acid A is the most potent displacer of [^{3}H]-AMPA binding yet described.

Keywords: Kainate; AMPA; thiocyanate; excitatory amino acid; acromelic acid A; domoic acid

Introduction

Excitatory amino acids are thought to be the major excitatory transmitters in mammalian brain (Cotman *et al.*, 1987) and have been shown by electrophysiological and pharmacological means to act at least four classes of glutamate receptor termed the N-methyl-D-aspartate (NMDA), kainate, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and metabotropic quisqualate receptors (Watkins *et al.*, 1990). Of all the agonists for these receptors, the most potent are the neurotoxins which are thought to act specifically at the kainate receptor and are used in neurobiology as cytotoxins (McGeer *et al.*, 1978). Two of these agonists, kainic acid and its more potent analogue domoic acid, have been known for some time but recently another kainic acid analogue, acromelic acid, was extracted from a poisonous mushroom (Konno *et al.*, 1983). Natural supplies of the two isomers of this compound, acromelic acids A and B, were extremely limited but syntheses of acromelic acid A (Konno *et al.*, 1986; Baldwin & Li, 1988) have enabled its electrophysiological characterization in the isolated spinal cord of the rat revealing it to be the most potent excitatory amino acid yet described (Ishida & Shinozaki, 1988).

To date, there has been only limited investigation of the pharmacology of domoic acid and acromelic acid A by radioligand binding studies. Domoic acid has been reported to bind with high affinity ($K_i = 13$ nM) to a kainate binding site in rat cerebellar membranes (Slevin *et al.*, 1983) and the only study on acromelic acid A was conducted on synaptic membranes from frog spinal cord where it recognized a single kainate binding site with an inhibition constant of $IC_{50} = 3$ nM (Maruyama & Takeda, 1989). Since both acromelic acid A and domoic acid are potentially of some importance as affinity chromatography ligands for the purification of the kainate receptor and as tools for the selective lesioning of brain, the binding characteristics of these compounds to rat brain synaptic membranes have been investigated. The results show that acromelic acid A binds to two different rat brain kainate binding sites and that both acromelic acid A and domoic acid have high affinity for the AMPA binding site in the rat brain.

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Methods

Membrane preparation

The method was essentially that of Jones & Matus (1974). Female Wistar rats (200–250 g) were stunned and killed by decapitation and the whole brains rapidly removed and stored on ice. The following isolation was performed at 4°C unless otherwise stated. The tissue was homogenized in 9 volumes of 0.32 M sucrose in 10 mM Na-phosphate buffer (pH 7.1) in a glass homogenizer with a motorized teflon pestle. The homogenate was centrifuged (2000 g_{av}, 10 min), the pellet discarded and the supernatant spun (39,000 g_{av}, 20 min). The pellet was resuspended in distilled water with a hand-held homogenizer and the suspension incubated at 37°C for 30 min. After centrifugation (39,000 g_{av}, 20 min) the pellet was resuspended in 0.32 M sucrose and overlaid upon 0.8 M sucrose above 1.2 M sucrose for density gradient centrifugation. Centrifugation (60,000 g_{av}, 120 min) yielded a layer of synaptic plasma membrane at the interface of the 1.2 M and 0.8 M sucrose layers which was then washed three times by suspension in 50 mM Tris-acetate buffer (pH 7.0) and centrifugation (39,000 g_{av}, 20 min). After resuspension in the minimum volume of 50 mM Tris-acetate the membrane suspension was rapidly frozen in liquid nitrogen and stored at −70°C for up to two months before use. After thawing, the membranes were washed three times by resuspension in 50 mM Tris-acetate (pH 7.0) and centrifugation (39,000 g_{av}, 20 min). Finally the pellet was

resuspended in 50 mM Tris-acetate (pH 7.0) to give a protein concentration of 2.5–3.5 mg ml^{-1}.

Protein determination

Protein determinations were performed by BCA protein assay (Pierce Chemical Corporation, Rockford, Illinois, U.S.A.), based on the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Binding experiments

Membrane suspensions (0.25–0.35 mg protein) were incubated in triplicate with [^{3}H]-kainic acid (5 nM) or [^{3}H]-AMPA (15 nM) in the presence or absence of competitive ligands. The incubations were conducted in Tris-acetate (pH 7.0) in a final volume of 0.2 ml for 30 min at 4°C. Bound radioactivity was separated from free radioactivity either by rapid filtration through Whatman GF/C glass fibre filters followed by 2 × 2 ml washes with ice-cold distilled water, or by centrifugation (12,000 g_{av}, 3 min) followed by superficial rinsing of the pellets twice with ice-cold distilled water (0.2 ml). The pellets were dissolved in SDS (1%, 1 ml) and the radioactivity in the solutions, or that retained by the filters in the case of filtration assays, was determined by liquid scintillation spectrometry after equilibration (3 h) in Liquiscint (National Diagnostic). Specific [^{3}H]-kainic acid binding (approximately 8000 d.p.m. mg^{-1}) or [^{3}H]-AMPA binding (approximately 1700 d.p.m. mg^{-1}) was obtained by subtracting from the total bound radioactivity the amount not displaced by an excess (0.1 mM) of the appropriate unlabelled ligand: kainic acid or AMPA. Both filtration and centrifugation procedures gave the same results, although non-specific binding values obtained by the centrifugation method were typically 40% of the total, compared to 10% non-specific binding found with the filtration method. In some [^{3}H]-AMPA binding experiments sodium thiocyanate was added to the incubation to give a final concentration of 100 mM and this caused the specific binding to rise to approximately 6000 d.p.m. mg^{-1}.

Kinetic analysis of binding

[^{3}H]-kainic acid (7.5 nM) was incubated with membrane suspensions as described above and association rates were determined by termination of the assay either by rapid filtration or centrifugation. Dissociation rates were obtained by adding 0.1 ml of 0.3 mM kainic acid to membrane suspensions that had been incubated with 7.5 nM [^{3}H]-kainic acid for 30 min and the incubations were terminated either by filtration or centrifugation.

Data analysis

Data from the equilibrium binding assays and kinetic experiments were analysed by the non-linear curve fitting programmes Ligand (Munson & Rodbard, 1980) and Kinetic respectively. These programmes form part of the package Kinetic, EBDA, Ligand, Lowry (Biosoft, Cambridge).

Materials

Samples of synthetic (3S,4S,5S)-5-[5-carboxy-4-(carboxymethyl)-3-pyrrolidinyl]-1,6-dihydro-6-oxo-pyridine-carboxylic acid (acromelic acid A) were obtained as gifts from Prof. J.E Baldwin and Prof. H. Shinozaki. [Vinylidene-^{3}H]-kainic acid (specific activity 58.0 Ci mol^{-1}) and [^{3}H]-(RS)- α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid ([^{3}H]-AMPA) (specific activity 29.2 Ci $mmol^{-1}$) were obtained from New England Nuclear (Boston, MA, U.S.A.). (RS)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) was purchased from Tocris Neuramin (Buckhurst Hill, Essex). All other compounds were obtained from Sigma (St. Louis, MO, U.S.A.).

Results

A detailed kinetic analysis of [^{3}H]-kainic acid binding to synaptic plasma membranes (SPMs) was conducted. The dissociation curve is as shown in Figure 1(a) and computer-assisted fitting revealed a single dissociation rate constant (k_{-1}) of $0.00136 \pm 0.00052\,s^{-1}$ (s.e.mean, $n = 3$). In contrast, the association curve (Figure 1b) appears biphasic with observed association rates (k_{OBS}) of $0.0245 \pm 0.0083\,s^{-1}$ and $0.00185 \pm 0.0008\,s^{-1}$ ($n = 3$) at two sites occurring in the ratio $28 \pm 9\%:72 \pm 9\%$ ($n = 3$) respectively. From these data, association rates (K_{+1}) can be calculated using the relationship $K_{+1} = (K_{OBS} - K_{-1})/L$ where L represents the radioligand concentration (7.5 nM). Thus K_d values for the two sites of 0.44 nM and 20.8 nM were derived from the relationship $K_D = K_{-1}/K_{+1}$.

However, equilibrium binding data obtained on the binding of [^{3}H]-kainic acid to SPMs, using a radioligand concentration of 5 nM, demonstrated a single population of kainate binding sites with $K_D = 19.4 \pm 1.7$ nM ($n = 13$) and $B_{max} = 288 \pm 42$ fmol mg^{-1} ($n = 13$) (see also Figure 2). The kainic acid analogue domoic acid also bound to a single site with $K_i = 14.5 \pm 0.7$ nM (Hill slope $= 0.86 \pm 0.06$) ($n = 3$) (Figure 2).

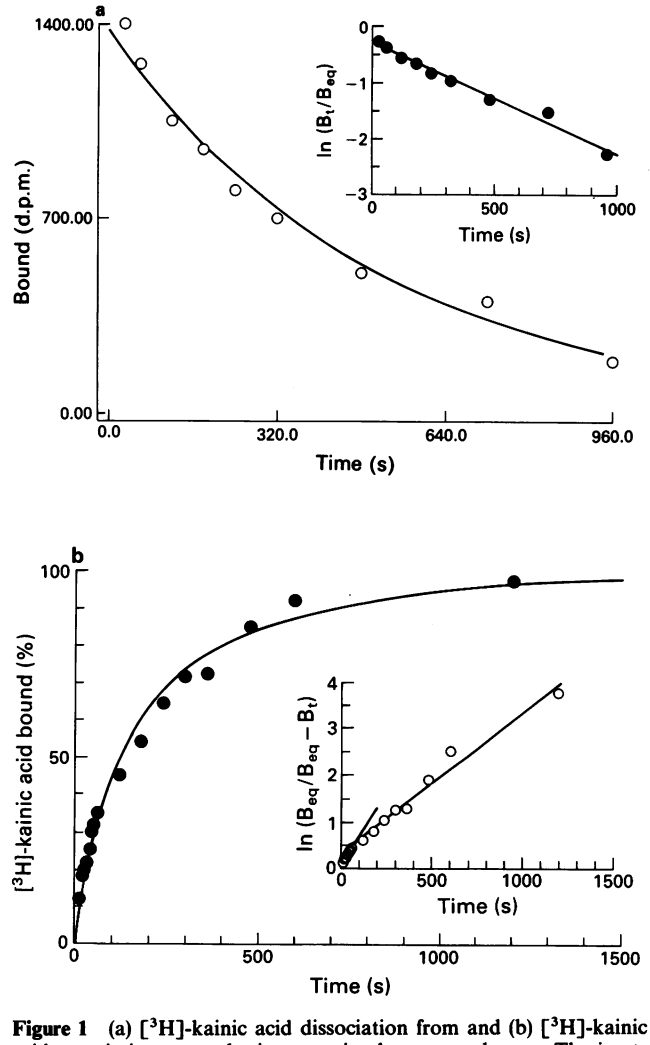


Figure 1 (a) [^{3}H]-kainic acid dissociation from and (b) [^{3}H]-kainic acid association to rat brain synaptic plasma membranes. The insets display linear transformations of the data, which are from single experiments, each representative of three separate experiments.

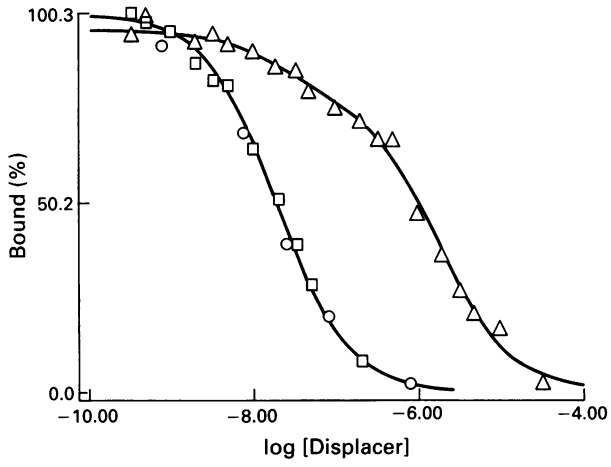


Figure 2 Inhibition of [^{3}H]-kainic acid binding to rat brain synaptic membranes by kainic acid (□), domoic acid (○) and acromelic acid A (△). The data shown are from single experiments representative of at least three separate experiments, each performed in triplicate.

However when acromelic acid A was used to inhibit the binding of [^{3}H]-kainic acid the shallow binding curve obtained (Hill slope = 0.61 ± 0.02) was biphasic (Figure 2) showing that acromelic acid A recognizes two kainate binding sites with K_is of 15.1 ± 4.6 nM and 1.49 ± 0.20 μM (n = 5) representing 28.6 ± 6% and 72 ± 6% (n = 5) of the total kainate binding sites respectively.

The ability of acromelic acid A and domoic acid to displace [^{3}H]-AMPA from SPMs was also investigated. In the absence of chaotropic ions, AMPA bound to a single site in SPMs with a K_D = 184.5 ± 21 nM (n = 7) and B_{max} = 265 ± 32 fmol mg^{-1} (n = 7). Under the same conditions, acromelic acid A displaced [^{3}H]-AMPA with a K_i = 15.6 ± 5.6 nM (n = 4) (Figure 3). [^{3}H]-AMPA binding to SPMs was inhibited by domoic acid with a K_i = 499 ± 64 nM (n = 3) (Figure 3) and by kainic acid with a K_i = 11.5 ± 2.8 μM (n = 4) (data not shown).

Since [^{3}H]-AMPA binding assays are normally performed in the presence of chaotropic ions to enhance binding (Young & Fagg, 1990) the effect of the inclusion of 100 μM potassium thiocyanate was determined. This caused no change in the affinity of AMPA for the [^{3}H]-AMPA binding site, K_D = 160.5 ± 28 nM (n = 7), but caused the number of sites to rise dramatically to B_{max} = 995 ± 130 fmol mg^{-1} (n = 7). However, the addition of 100 mM potassium thiocyanate also had a profound effect on the binding of acromelic acid A and domoic acid to the [^{3}H]-AMPA binding site. The affinity of acromelic acid A fell to K_i = 289 ± 34 nM (n = 3) and that of domoic acid to K_i = 9.02 ± 1.07 μM (n = 3) (Figure 3). The affinity of kainic acid for the [^{3}H]-AMPA binding site appeared to be unaffected by the inclusion of thiocyanate.

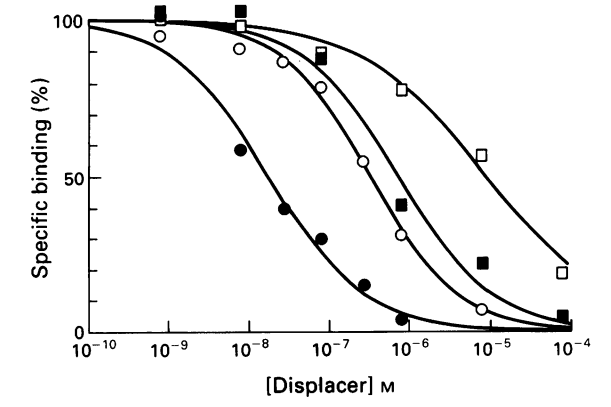


Figure 3 Inhibition of [^{3}H]-AMPA binding to rat brain synaptic plasma membranes by domoic acid (squares) and acromelic acid A (circles) in the presence (open symbols) and absence (closed symbols) of 100 mM potassium thiocyanate. The data are from single experiments, respresentative of at least three separate experiments.

Discussion

The results of our experiments demonstrate that acromelic acid A exhibits a complex pattern of binding to rat brain synaptic plasma membrane (SPMs). Furthermore, the inclusion of chaotropic ions in [^{3}H]-AMPA binding assays can drastically alter the binding affinities of certain glutamate receptor agonists.

A kinetic analysis of [^{3}H]-kainic acid binding to SPMs revealed the presence of a high affinity site with K_D = 0.44 nM and a lower affinity site of K_D = 20.8 nM in the ratio of 28%:72% respectively. Previous studies on the binding of kainic acid to rat brain have shown the existence of two binding sites in rat brain membrane preparations (London & Coyle, 1979; Honoré *et al.*, 1986) but these were of lower affinity than the sites found in this study. The discrepancy might be accounted for by the fact that an enriched synaptosomal membrane preparation was used in our binding studies, rather than the crude P2 membrane preparation used elsewhere. However, Scatchard analysis of [^{3}H]-kainic acid equilibrium binding data indicated that kainic acid recognized only a single binding site in SPMs and it was only when acromelic acid A was used to displace [^{3}H]-kainic acid from synaptosomal membranes that a biphasic displacement curve was obtained and the presence of two kainate binding sites, with differing affinities for acromelic acid A, was confirmed. The proportion of high and low affinity kainate binding sites recognized by acromelic acid A matches that found by kinetic analysis of [^{3}H]-kainic acid binding while the higher of the kinetically-derived K_Ds is almost the same as that derived from the equilibrium binding assays.

It has been suggested that acromelic acid might exert its depolarizing action in immature rat spinal cord mainly via AMPA receptors (Watkins *et al.*, 1990) and so the ability of this compound and other 'kainate agonists' to displace specifically bound [^{3}H]-AMPA was investigated. The finding that in the absence of thiocyanate, acromelic acid A bound to the AMPA binding site with a dissociation constant of K_i = 15.6 nM means that acromelic acid A has a higher affinity for this site than any compound yet described, more than ten times higher than AMPA itself. Domoic acid, with a dissociation constant of K_i = 499 nM, also exhibited a surprisingly high affinity for the AMPA binding site for a compound which has hitherto been thought to be a pure kainate receptor agonist. Domoic acid has also been reported to display high affinity for an AMPA binding site recently solubilized from frog brain (Henley *et al.*, 1989) but kainic acid had an even higher affinity for this site. Kainic acid itself exhibited only micromolar affinity for the AMPA binding site, but it has been pointed out (Young & Fagg, 1990) that micromolar concentrations of kainic acid are generally required to elicit an electrophysiological response, in marked contrast to the low nanomolar dissociation constant kainic acid exhibits at the kainate binding site. Indeed the electrophysiological potencies of acromelic acid A, domoic acid and kainic acid in rat spinal cord (Ishida & Shinozaki, 1988) are much more in keeping with their relative affinities for the AMPA binding site than for the kainate binding site. The recent evidence that kainic acid is able to evoke responses from functionally expressed AMPA receptors derived from cloned DNAs (Keinanen *et al.*, 1990) supports the idea that AMPA receptors are capable of mediating the effects of kainic acid. While at least one AMPA binding site has been isolated which does not recognize kainic acid (Wenthold *et al.*, 1990) it seems possible that all these

compounds with a high affinity for the kainate binding site may sometimes elict their electrophysiological responses through the AMPA receptor. The inclusion of thiocyanate ion in [^{3}H]-AMPA binding assays is now commonplace and in a recent review the practice was described as being essential (Young & Fagg, 1990). Although in this study the assay counts obtained in the absence of thiocyanate were quite adequate for routine measurements, we have found that the presence of thiocyanate ion enhances the specific binding of [^{3}H]-AMPA by more than three times, apparently by unmasking occluded AMPA binding sites as previously suggested (Olsen *et al.*, 1987). However, the reduction in the affinity of acromelic acid A and domoic acid for the AMPA binding site that was observed in this study due to the presence of 100 mM thiocyanate ion was eighteen fold. It is worth noting that electrophysiological studies on AMPA receptors are generally conducted in the absence of thiocyanate, in marked contrast to membrane binding studies on the [^{3}H]-AMPA binding site.

The finding that acromelic acid A and domoic acid exhibit sub-micromolar affinities for both the kainate and AMPA binding sites would suggest that affinity columns based on these compounds could be used to purify both sites. In this context it is interesting to note that in a recent study the use of a domoic acid affinity resin to purify kainate binding sites from *Xenopus* brain did indeed result in the co-purification of both the kainate and the AMPA binding sites (Ambrosini *et al.*, 1990). Since they were purified in a 1:1 ratio it is probable that in this tissue the kainate and AMPA binding sites are present on a single protein. The affinity-purified kainate binding site from frog (Wada *et al.*, 1989) and chick brain (Gregor *et al.*, 1989) is reported to be a protein of around M_r = 48 kD, but that cloned from rat brain has a molecular weight of 100 kD (Hollmann *et al.*, 1989). Thus if kainate and AMPA binding sites are present on the same protein in non-mammalian brains this may not be the case in mammalian brain. The use of affinity columns based on acromelic acid A, domoic acid and other kainate agonists such as those recently described (Smith & McIlhinney, 1990) may help to resolve the nature of the kainate and AMPA binding sites in rat brain.

In conclusion we have shown that acromelic acid A is a high affinity ligand for both the kainate and AMPA binding sites in rat brain SPMs. The existence of two kainate binding sites has been demonstrated and it has been shown that acromelic acid A exhibits markedly different affinities for the two sites. Finally we offer a caveat to those conducting [^{3}H]-AMPA binding assays that the inclusion of thiocyanate ion can significantly affect the binding affinities of some compounds and may therefore result in a misinterpretation of the rank order of potency obtained in binding studies.

We would like to thank Prof. J.E. Baldwin and Prof. H. Shinozaki for their very generous gifts of acromelic acid A and Prof. J.C. Watkins for his advice and encouragement.

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Platelet-activating factor synthesis by peritoneal mast cells and its inhibition by two quinoline-based compounds

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1 Peritoneal mast cells from rat were co-incubated *in vitro* in a platelet aggregometer cuvette with washed rabbit platelets. In response to stimulation with calcium ionophore (A23187; 1–5 μM), the mast cells released a substance which stimulated the platelets to aggregate. These concentrations of ionophore did not stimulate platelet aggregation in the absence of mast cells, nor affect the responsiveness of the platelets to aggregation induced by thrombin or PAF. Release of a PAF-like substance was also observed in response to stimulation of the mast cells with antigen.

2 This pro-aggregatory activity is attributable to the release of PAF by the mast cells, since the activity could be abolished by preincubating the platelets with a specific PAF receptor antagonist (WEB 2086; 10 μM). Furthermore, the platelet-aggregating factor co-migrated with PAF on thin-layer chromatographs and could be abolished by incubation with phospholipase A_2 (20 $\mu g\,ml^{-1}$) or a specific antibody directed against PAF.

3 The release of PAF by peritoneal mast cells could be inhibited, in a concentration-dependent manner, by PF-5901 (IC_{50} of 3.9 μM) or Wy-50,295 (IC_{50} of 1.2 μM), two structurally similar compounds with inhibitory effects on leukotriene synthesis, as well as leukotriene D_4 (LTD_4) receptor antagonist properties.

4 Inhibition of PAF synthesis was not observed when the mast cells were incubated with a structurally unrelated 5-lipoxygenase inhibitor (A-64077), a structurally dissimilar inhibitor of 5-lipoxygenase activating protein (MK-886) or with a structurally related LTD_4 receptor antagonist (MK-571) which lacks inhibitory effects on leukotriene synthesis, each at concentrations of up to 100 μM.

5 Neither PF-5901 nor Wy-50,295 (1 or 10 μM) significantly affected histamine release or prostaglandin D_2 synthesis by peritoneal mast cells in response to calcium ionophore stimulation.

6 These results demonstrate the ability of a class of quinoline-based compounds to inhibit PAF synthesis by peritoneal mast cells. This activity does not appear to be related to effects of these compounds on leukotriene synthesis or LTD_4 receptors. The ability of these compounds to inhibit PAF synthesis may contribute to their anti-inflammatory properties.

Keywords: PAF; mast cells; leukotrienes; inflammation; anti-inflammatory; allergy; quinolines

Introduction

Inflammatory mediator release from activated mast cells represents an important early event in many disease conditions associated with chronic inflammation (Gordon *et al.*, 1990). Because of this, the mast cell and the mediators these cells release have been identified as potential targets for the treatment of inflammatory conditions and allergic reactions. One mediator which has been implicated in a variety of inflammatory disorders is platelet-activating factor, or PAF (reviewed by Braquet *et al.*, 1987). Such a role for PAF has been supported by numerous studies demonstrating that treatment with PAF antagonists is beneficial in a variety of animal models of inflammation and allergy. While the biosynthesis of PAF has been studied extensively, potent and specific inhibitors have not been identified. Ketotifen has been shown to inhibit PAF release from mouse mast cells, but similar concentrations of this compound also inhibited calcium influx and release of β-hexosaminidase (Joly *et al.*, 1987).

Mast cells derived from a variety of sources synthesize PAF (Mencia-Heurta *et al.*, 1983; Schleimer *et al.*, 1986; Musch *et al.*, 1987; Triggiani *et al.*, 1990), but little is known of the regulation of PAF synthesis and release by these cells. Products of the lipoxygenase pathway of arachidonic acid metabolism appear to play a role in modulating histamine release by rat peritoneal mast cells (Chand *et al.*, 1987; Mansini *et al.*, 1987), although some caution must be exercised in accepting these results, since the lipoxygenase inhibitors used in these studies (e.g. nordihydroguaretic acid) are known to affect other enzyme systems. Furthermore, there is no direct evidence that rat peritoneal mast cells are capable of producing 5-lipoxygenase products of arachidonic acid. Heavy *et al.* (1988) were unable to detect leukotriene B_4 (LTB_4) or LTC_4 release from rat peritoneal mast cells stimulated with anti-IgE antibody, while intestinal mucosal mast cells released significant quantities of LTB_4 and LTC_4 in response to similar stimulation.

The present study was performed to determine if quinoline-based inhibitors of leukotriene synthesis affect PAF synthesis by rat peritoneal (connective tissue-type) mast cells. In order to do these studies, we first had to establish a model in which PAF synthesis by these cells could be detected. An *in vitro* assay was established, based on that of Salvemini *et al.* (1990), in which peritoneal mast cells were co-incubated with rabbit platelets. Stimulation of the mast cells with calcium ionophore caused release of a pro-aggregatory substance which co-migrated with PAF on thinlayer chromatography, was inactivated by preincubation with phospholipase A_2 or an antibody directed against PAF, and the effects of which could be inhibited by a specific PAF receptor antagonist.

Methods

Animals

Male, Sprague Dawley rats (300–400 g; Charles River Canada Inc., St. Constant, Canada) and male, New Zealand white

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rabbits (2–3 kg; Reimans Fur Ranches Ltd., St. Agatha, Canada) were used in this study. Rats were housed in groups of four at a temperature of 25°C on a 12 h light/dark cycle. Rabbits were individually housed in standard cages under similar conditions. All animals had free access to both food and water prior to the beginning of the study. The following experimental procedures were approved by the Animal Care Committee of the University of Calgary and are in accordance with the guidelines of the Canadian Council on Animal Care.

Isolation of rat peritoneal mast cells

Rats were anaesthetized with ether, then killed by cervical dislocation prior to intraperitoneal injection of 15 ml cold (4°C) HEPES-buffered Tyrode solution (pH 7.2) containing 0.1% bovine serum albumin (BSA). The abdomen of the animal was massaged for 30 s and then the peritoneal cavity was exposed through a ventral incision. The peritoneal fluid was aspirated and layered upon a two-step discontinuous Percoll gradient as described previously (Lee *et al.*, 1985). The peritoneal mast cells (PMC; 97–99% pure, 97% viability) were resuspended in RPMI-HEPES solution (1×10^6 ml^{-1}) and kept on ice until used in the platelet aggregation assay.

Platelet aggregation bioassay

Platelets were isolated from rabbit arterial blood (collected under pentobarbitone anaesthesia) and 'washed' in the presence of prostacyclin (300 ng ml^{-1}), as previously described by Radomski & Moncada (1983). Aliquots (500 μl) of the platelet-rich solution (PRS) were incubated in a cuvette at 37°C for 1 min in a Payton dual-channel aggregometer while continuously stirred at 800 r.p.m. Light transmission through the suspension of platelets was recorded on a chart recorder. Aggregation of the platelets leads to an increase in light transmission through the cuvette. Prior to the start of the study, the PRS was tested for its responsiveness to synthetic PAF and a standard curve was established. PMC (1×10^3–5×10^4) were then added to the PRS and the cell suspension was allowed to equilibrate for 1 min. Calcium ionophore (A23187) was then added to the cells and the resulting aggregation was recorded on a Linear dual channel aggrecorder for 2 min. When added to a suspension of platelets in the absence of PMC, the concentrations of A23187 used (1–5 μM) failed to cause platelet aggregation and did not significantly affect the responsiveness of the platelets to thrombin (50–175 mu ml^{-1}) or PAF (10–50 pg ml^{-1}). Experiments of this type were repeated in 7 separate preparations of platelets. A number of experiments were performed to verify that the platelet aggregation was a consequence of release of PAF by the PMC. Firstly, in each assay, PMC (1×10^3 to 5×10^4) were added to platelets which had been pre-incubated for 2 min with a specific PAF receptor antagonist (WEB 2086; 10–20 μM; Casals-Stenzel *et al.*, 1987) and then stimulated as above. These concentrations of WEB 2086 did not significantly affect the aggregatory response of the platelets to thrombin, and totally prevented platelet aggregation induced by 100 pg ml^{-1} of PAF. In a second series of experiments ($n = 3$), PMC (1×10^3–1.25×10^5) were added to 0.5 ml of 0.25% BSA/0.9% saline and stimulated with 5 μM A23187. One minute later, 1.0 ml of −20°C acetone was added to the PMC suspension. After a 5 min centrifugation at 1000 *g*, PAF was extracted in equal volumes of chloroform and methanol from both the supernatant and pellet and the organic phase from each sample was applied to thin-layer chromatography (t.l.c.) plates (Whatman silica gel 60A). TLC was then performed as described previously (Parente & Flower, 1985). The portion of the chromatograph co-migrating with authentic PAF was scraped and resuspended in 0.25% BSA/0.9% saline. Each sample was tested for platelet aggregating activity by the assay described above. Duplicate samples were incubated in the presence of phospholipase A_2 (bovine pancreatic; 20 μg ml^{-1}) for 5 min prior to testing in the platelet aggregation assay. Finally, experiments ($n = 2$) were performed in which PMC (1×10^3–5×10^4) were stimulated with A23187 (1–5 μM). An aliquot of the PMC suspension was then added to a suspension of platelets in the platelet aggregometer and the response of the platelets was monitored. The experiments were then repeated, but immediately after challenging the PMC with A23187, 10 μl of an antibody directed against PAF was added to the suspension of PMC. The antibody was used at a dilution of 1:2000. Appropriate controls were performed to determine if the antibody itself or non-immune, heat-inactivated rabbit serum modified the responsiveness of the platelets to PAF.

Immunological stimulation of PAF synthesis by peritoneal mast cells

In order to determine if PAF synthesis by PMC could be stimulated through immunological activation of the cells, PMC were harvested from rats infected >15 days previously with *Nippostrongylus brasiliensis*, as described in detail previously (Befus & Bienenstock, 1979). Experiments were then performed in which the PMC were co-incubated with platelets, as described above, except that the mast cells were stimulated with either antigen (supernatant of the homogenate of 20 adult *N. brasiliensis* worms in saline) or anti-IgE (1/40 dilution of rabbit anti-rat IgE; Befus & Bienenstock, 1979). Control experiments were performed to determine if either of these stimuli affected the ability of the platelets to aggregate in response to exogenous PAF or thrombin. In addition, the ability of PMC from infected rats to release PAF in response to stimulation with A23187 (1–5 μM) was compared to that from PMC from non-infected rats. The ability of the immunological stimuli to elicit histamine release from PMC was also assessed (see below).

Effects of various drugs on ionophore-stimulated PAF release by peritoneal mast cells

Peritoneal mast cells (4×10^4) were incubated with various concentrations (0.1–100 μM) of one of the following compounds for 5 min at 20°C: PF-5901, Wy-20,595, MK-571, A-64077 or MK-886 (Figure 1). PF-5901 and Wy-50,295 are inhibitors of 5-lipoxygenase activating protein (FLAP) and have LTD_4 receptor antagonist properties (Van Inwegen *et al.*, 1987; Kreft *et al.*, 1990; Evans *et al.*, 1991). MK-886 is also an inhibitor of FLAP (Rouzer *et al.*, 1990). MK-571 is a potent LTD_4 receptor antagonist (Jones *et al.*, 1989). A-64077 is a 5-lipoxygenase inhibitor (Laursen *et al.*, 1990). The cells were then added to fresh suspensions of washed platelets which had been incubated at 37°C in the aggregometer for 1 min. The cells were then stimulated with an appropriate concentration of A23187, as above, and the response to this stimulation was recorded. The 5 min incubation of PMC did not interfere with the ability of these cells to produce PAF, nor did the vehicles used for the various drugs (dimethylsulphoxide (DMSO) and methanol; final concentrations of 1%) alter PAF release by the PMC or the ability of platelets to aggregate in response to thrombin or PAF.

Histamine and prostaglandin D_2 assays

In order to determine whether compounds exhibiting inhibitory effect on PAF release by PMC also affected other responses of PMC, the effects of these compounds on histamine and prostaglandin D_2 (PGD_2) release were assessed. The PMC were isolated as outlined above and histamine release was assessed as described previously (Bissonnette & Befus, 1990). Briefly, PMC (5×10^4) were incubated for 5 min at 20°C with PF-5901 or Wy-50,295 (1–10 μM) or the appropriate vehicle. The PMC were then stimulated with A23187 (1 or 5 μM) and incubated for 1 min. After incubation, the PMC were transferred to a tube containing 3 ml of HEPES-buffered Tyrode solution (pH 7.2) containing 0.1% BSA and centrifuged at 150 *g* for 3 min. The fractions were separated, and the

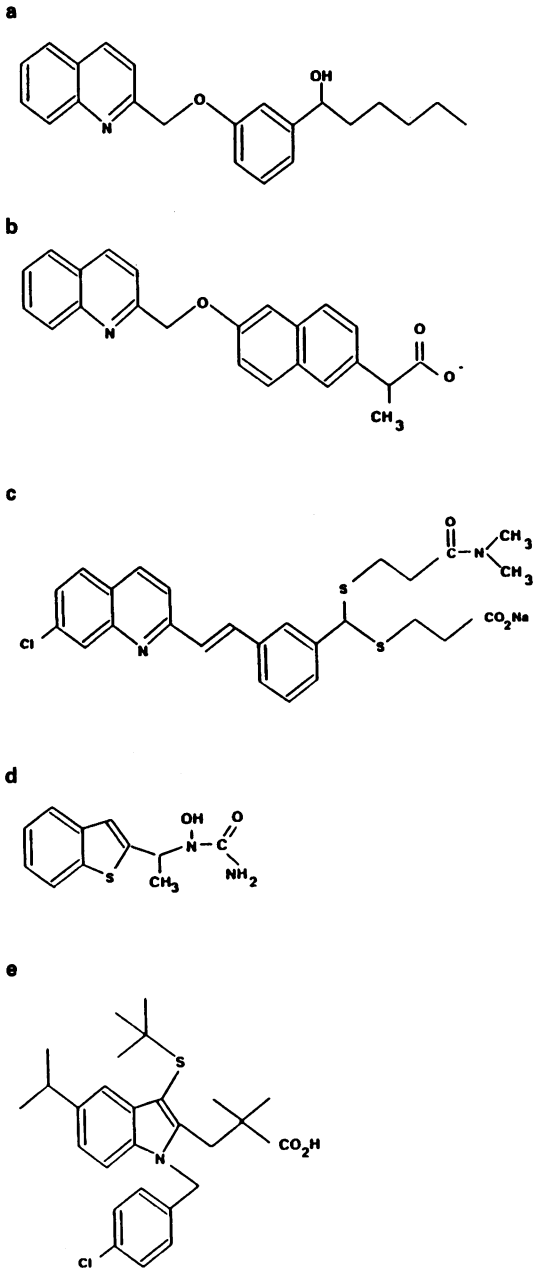


Figure 1 Chemical structures of the pharmacological compounds used in this study: (a) PF-5901; (b) Wy-50,295, (c) MK-571, (d) A-64077, and (e) MK-886.

pellet fractions were boiled for 10 min to release the remaining cell-associated histamine. Following protein precipitation with 0.25% trichloroacetic acid, histamine concentrations were determined in the supernatants and pellets by a fluorometric assay (Shore *et al.*, 1959). Histamine release into the supernatant was expressed as a percentage of total cellular histamine content less the spontaneous background histamine release (< 2% of total histamine).

For PGD_2, 100 μl aliquots containing 3×10^5 PMC were added to 400 μl of HEPES-buffered Tyrode solution (pH 7.2). The cells were incubated for 5 min with vehicle, PF-5901 (1 or 10 μM) or Wy-50,295 (1 or 10 μM) and were then stimulated with 4 μM A23187. After a 10 min incubation in a shaking water bath (37°C), the samples were centrifuged at 200 *g* (8 min; 4°C). The supernatants were aspirated and the pellets resuspended in 500 μl of the HEPES-buffered Tyrode solution and sonicated for 1 min. PGD_2 concentrations in the samples of supernatants and pellets were determined with a commercially available ELISA (Caymen).

Materials

PF-5901 (α-pentyl-3-(2-quinolinylmethoxy)-benzene-methanol) was supplied by the Purdue Frederick Company (Norwalk, CT, U.S.A.). It should be noted that PF-5901 has previously been named 'REV-5901' and 'RG-5901'. The tromethamine salt of Wy-50,295 (S-(+)-α-methyl-6-(2-quinolinylmethoxy)-2-naphthaleneacetic acid was a gift from Wyeth-Ayerst Research (Princeton, NJ, U.S.A.). A-64077 (N-(1-benzo(b)thien-2-ylethyl)-N-hydroxy-urea) was obtained from the R.W. Johnson Pharmaceutical Research Institute (Raritan, NJ, U.S.A.). MK-571 (3-(3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl) ((3-dimethyl amino-3-oxopropyl)-thio)methyl)thio propanoic acid and MK-886 (3-[1-(4-chlorobenzyl)-3- t-butyl-thio-5- isopropylindol-2- yl]-2,2-dimethyl propanoic acid) were supplied by Merck-Frosst Research Laboratories (Kirkland, Canada). Sodium prostacyclin was a gift from Burroughs-Wellcome Canada (Montreal, Canada). The prostacyclin was dissolved in 0.25 M Tris buffer (pH 8.4) and was stored in aliquots at −20°C. Solutions of PF-5901, Wy-50,295 and A-64077 were prepared in either DMSO and methanol for these studies. MK-571 was dissolved in 0.9% saline. WEB 2086 was a gift from Boehringer Ingelheim (Germany). The rabbit anti-PAF antibody was obtained from Amersham Canada (TRK 990; Oakville, Canada) and was used at a dilution of 1:2000 in phosphate buffered saline (pH 7.4). All other reagents were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.) or Fisher Scientific (Edmonton, Canada).

Results

Characterization of PAF release by peritoneal mast cells

PMC co-incubated with washed rabbit platelets and stimulated with A23187 released a pro-aggregatory substance within 10–20 s (Figure 2). Maximal platelet aggregation was normally seen within 2–3 min following stimulation of the PMC, but the amount of platelet aggregation was dependent upon the number of PMC added to the platelet suspension. When compared to a standard curve constructed by testing the effects of synthetic PAF on platelets, the PMC produced the equivalent of 20.4 ± 9.2 pg PAF per 1×10^4 cells (mean of 7 separate aggregation assays). The pro-aggregatory activity of ionophore-stimulated PMC was not the result of effects of A23187 on the platelets, since the ionophore did not stimulate platelet aggregation in the absence of PMC, nor did it affect the responsiveness of platelets to PAF or thrombin.

There are several lines of evidence to support the hypothesis that the aggregation of platelets observed following stimulation of the PMC/platelet suspension with ionophore was attributable to the release of PAF by the PMC. Firstly, in each assay, this activity could be completely inhibited by preincubating the platelets with WEB 2086. Secondly, the pro-aggregatory activity released from PMC in response to ionophore was found to co-migrate with synthetic PAF on thin-layer chromatography and could be eliminated by preincubating the samples with 20 $\mu g\,ml^{-1}$ phospholipase A_2 or excess amounts of antibody directed against PAF ($n = 3$). Interestingly, all of the platelet activating substance produced by the PMC could be recovered in the supernatant (i.e. extracts of the pelleted PMC contained no detectable platelet activating activity).

PAF synthesis in response to immunological stimulation

Ionophore-stimulated PAF release from PMC harvested from rats previously infected with *N. brasiliensis* (24.4 ± 3.5 pg/10^4 cells) did not significantly differ from that observed from PMC harvested from non-infected rats (20.4 ± 9.2 pg/10^4 cells). Stimulation of sensitized PMC with antigen resulted in a mean release of 2.6 ± 1.1 pg/10^4 cells, markedly less than was observed with ionophore stimulation. However, this amount of antigen also stimulated markedly less histamine release by

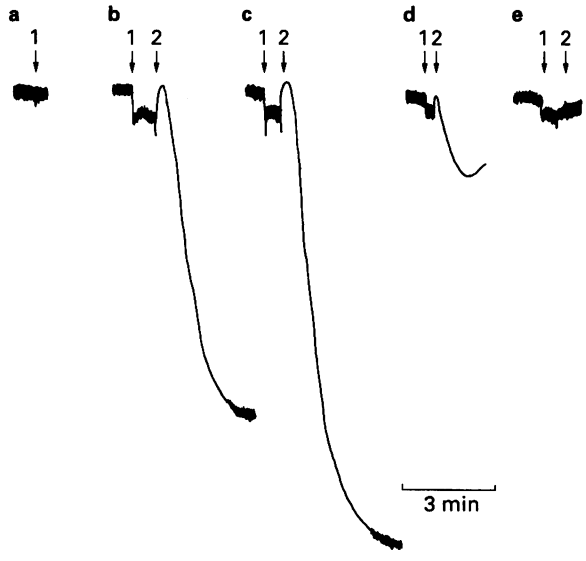


Figure 2 Representative platelet aggregometry tracings. Light transmission through a suspension of platelets was recorded. A downward deflection of the pen is indicative of increased light transmission, which occurs when the platelets aggregate. In (a) stimulation (arrow 1) of platelets with A23187 (1.5 μM) failed to elicit aggregation. In (b) and (c), suspensions of peritoneal mast cells (2×10^4 and 5×10^4, respectively) were added to the platelet suspension at arrow 1. Stimulation (arrow 2) with A23187 (1.5 μM) resulted in platelet aggregation, the magnitude of which was related to the number of mast cells added. In (d), the protocol was identical to that in (b), except that the mast cells were preincubated with PF-5901 (10 μM) for 5 min before being added to the platelet suspension (mast cells added at arrow 1, A23187 added at arrow 2). In (e) the protocol was identical to that of panel (b), except that the platelets were preincubated with WEB 2086 (10 μM) for 1 min before the addition of mast cells (arrow 1); A23187 (1.5 μM) was added at arrow 2.

PMC than did ionophore. Antigen stimulation (supernatant from 20 worm equivalents) resulted in a specific release of histamine of $9 \pm 2\%$, while a 1 μM concentration of A23187 stimulated specific histamine release of $34 \pm 3\%$. Unfortunately, higher concentrations of the antigen (i.e. >20 worm equivalents), as well as anti-IgE, interfered with the responsiveness of the platelets to exogenous PAF.

Pharmacological modulation of PAF synthesis by peritoneal mast cells

Preincubation of PMC with either Wy-50,295 or PF-5901, both of which are quinoline-based FLAP inhibitors, resulted in a concentration-dependent inhibition of PAF synthesis by ionophore-stimulated PMC (Figure 3). The IC_{50} of Wy-50,295 was 1.2 μM, while that of PF-5901 was 3.9 μM. Conversely, a structurally dissimilar 5-lipoxygenase inhibitor, A-64077, had no significant effect on PAF synthesis by PMC at concentrations of up to 100 μM (Figure 3). MK-571, a quinoline-based LTD_4 antagonist, also did not affect PAF release by PMC when tested at concentrations of up to 1000 μM (Figure 3). Furthermore, MK-886, an inhibitor of FLAP which is not quinoline-based, failed to affect significantly synthesis of PAF by PMC at concentrations of up to 100 μM (data not shown). None of the compounds tested significantly affected the responsiveness of platelets to stimulation by synthetic PAF or thrombin.

Histamine and prostaglandin D_2 release from stimulated peritoneal mast cells

Stimulation of 5×10^4 PMC with A23187 (1 or 5 μM) resulted in the specific release of $34 \pm 3\%$ and $42 \pm 3\%$ of total cellular histamine content, respectively. Preincubation of PMC with PF-5901 or Wy-50,295 at concentrations of 1 or 10 μM

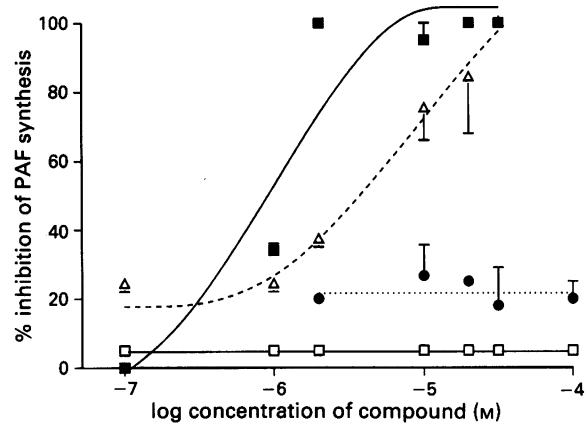


Figure 3 Inhibition of PAF release from rat peritoneal mast cells by Wy-50,295 (■), PF-5901 (△), A-64077 (●) and MK-571 (□). The effects of A-64077 and MK-571 did not differ significantly from those of the respective vehicles. MK-886 also did not significantly affect PAF release. The results obtained with MK-886 were virtually identical to that shown for MK-571. Each point represents the mean of 4–7 separate experiments; vertical bars show s.e.mean. Percentage inhibition was calculated relative to the amount of PAF release observed in ionophore-stimulated PMC incubated in the presence of the vehicles for each compound.

did not significantly affect the release of histamine in response to stimulation with ionophore.

In response to ionophore stimulation, PMC released an average of 73.8 ± 25.3 ng of PGD_2 per 10^6 cells. Preincubation of PMC with either PF-5901 or Wy-50,295 (each at 1 or 10 μM) did not significantly affect PGD_2 release.

Discussion

These results demonstrate that peritoneal mast cells, when stimulated with a calcium ionophore, release a platelet-activating substance with similar biological, chemical and immunoreactive characteristics to PAF. Unlike the case with some other cell types (Lynch *et al.*, 1986; Zimmerman *et al.*, 1990), the PAF produced by rat peritoneal mast cells appears to be released, rather than remaining associated with the cell. PAF release could also be stimulated through immunological activation of sensitized PMC. The present study further demonstrates that PAF release by peritoneal mast cells can be inhibited by micromolar concentrations of two quinoline-based compounds (PF-5901 and Wy-50,295).

The two compounds which affected PAF synthesis have each been shown to be potent inhibitors of leukotriene synthesis, through effects on FLAP, as well as LTD_4 receptor antagonists (Van Inwegen *et al.*, 1987; Kreft *et al.*, 1990; Evans *et al.*, 1991). However, the effects of these compounds on PAF synthesis did not appear to be related to these properties, since inhibition of PAF synthesis was not observed with a LTD_4 receptor antagonist (MK-571; Jones *et al.*, 1989), with a structurally unrelated 5-lipoxygenase inhibitor (A-64077; Laursen *et al.*, 1990), or with a structurally dissimilar (i.e. non-quinolinyl) FLAP inhibitor. A separation of effects of Wy-50,295 on leukotriene and PAF synthesis by human neutrophils has been reported by Lock *et al.* (1990). They demonstrated that at a concentration of 5 μM, Wy-50,295 did not significantly affect PAF synthesis, but did reduce LTB_4 synthesis by >95%.

The effects of PF-5901 and Wy-50,295 on PAF release by rat peritoneal mast cells may have occurred as a consequence of a non-specific inhibition of mast cell activation. To test this hypothesis, the effects of these compounds on ionophore-stimulated histamine and PGD_2 release were assessed. The observation that concentrations of these two compounds which markedly inhibited PAF release failed to affect significantly the release of these two mediators suggests a more specific mechanism of action. The precise nature of that

mechanism has yet to be established. The present results are in agreement with those of Warner *et al.* (1988) who, using human lung mast cells, demonstrated that a 5-lipoxygenase inhibitor (L651,392) failed to modify histamine release. The ability of PF-5901 and Wy-50,295 to inhibit PAF release by mast cells can also be distinguished from the previously reported ability of ketotifen to inhibit PAF synthesis by mouse bone marrow-derived mast cells (Joly *et al.*, 1987). In that study, ketotifen was also found to inhibit the release of β-hexosaminidase, a preformed granular enzyme. Release of β-hexosaminidase has been shown to correlate well with histamine release (Mencia-Huerta *et al.*, 1983).

PAF synthesis by mast cells from various sources has been demonstrated previously (Mencia-Huerta *et al.*, 1983; Schleimer *et al.*, 1986; Musch *et al.*, 1987; Triggiani *et al.*, 1990). A striking feature of the present study was the rapidity with which PAF is released following stimulation of peritoneal mast cells. PAF release was detectable within seconds of stimulation with ionophore. The quantity of PAF released in response to ionophore challenge was also significant. Indeed, the amount of PAF released from rat peritoneal mast cells (~2 ng per million cells) was an order of magnitude lower than the levels of prostaglandin D_2 released by these cells in response to stimulation with ionophore. The concentrations of ionophore required to elicit this amount of PAF release from PMC was sufficient to induce specific histamine release in the 34–42% range.

The mechanism through which PF-5901 and Wy-50,295 inhibit PAF synthesis is not known. Since these compounds do not interfere with ionophore-stimulated histamine or PGD_2 release, it is unlikely that they significantly modify the calcium influx in response to this challenge. It is conceivable that these compounds inhibit the activity of one of the two enzymes involved in the biosynthesis of PAF, namely phospholipase A_2 and acetyl CoA transferase. However, since neither of these compounds affected PGD_2 release in the present study, or exerted effects on prostaglandin synthesis in various other systems (Van Inwegen *et al.*, 1987; Kreft *et al.*, 1990), effects on phospholipase A_2 seem unlikely. The possibility that PF-5901 and Wy-50,295 alter acetyl CoA transferase activity requires further investigation. Since these two compounds inhibit leukotriene synthesis by binding to FLAP (Evans *et al.*, 1991), the extent to which inhibition of PAF synthesis can be extended to other FLAP inhibitors is of interest. However, MK-886, the prototype FLAP inhibitor, failed to affect significantly PAF synthesis by PMC in the present study. In a preliminary report, however, Lock *et al.* (1990) showed that MK-886 inhibited PAF synthesis by human neutrophils, albeit at concentrations well above those required for inhibition of leukotriene synthesis.

In summary, two quinoline-based compounds were found to inhibit PAF release by peritoneal mast cells at concentrations in the micromolar range. These compounds may therefore be useful pharmacological probes for investigations of the role of PAF in various inflammatory and allergic conditions. Moreover, inhibition of PAF release may contribute to the anti-inflammatory effects of these compounds.

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Inhibition of neuropeptide Y-induced potentiation of noradrenaline-induced vasoconstriction by PP56 (D-*myo*-inositol 1,2,6-tris-phosphate)

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1 Although neuropeptide Y (NPY) is a potent vasoconstrictor in many vascular beds, nanomolar concentrations of this peptide potentiate the noradrenaline-induced contractions in rabbit gastroepiploic and femoral arteries, and guinea-pig mesenteric and uterine arteries.

2 The potentiating effect of NPY on noradrenaline-induced contraction was present in endothelium-denuded femoral arteries.

3 The potentiating effect of NPY on noradrenaline-induced contraction was antagonized by PP56 (D-*myo*-inositol 1,2,6-trisphosphate) in low concentrations (down to 0.1 nM). This antagonistic effect was observed in all four types of vessels studied. Contractions induced by noradrenaline, histamine, endothelin-1 and potassium were not altered by PP56 in concentrations upto 1 μM in femoral artery of rabbit.

4 We provide evidence that a non-peptide (PP56) can selectively antagonize NPY-induced effects in rabbit and guinea-pig peripheral arteries without affecting the vasoconstrictor response to noradrenaline.

Keywords: Neuropeptide Y; vasoconstriction; D-*myo*-1,2,6-inositol trisphosphate; inhibition of potentiation

Introduction

Sympathetic neurotransmission is now known to be mediated not by a single neurotransmitter, but may rather involve the interaction of several substances released from the nerve terminal. Whereas noradrenaline (NA) is recognized to be the primary neurotransmitter for the sympathetic nervous system, recent attention has focused on the importance of neuropeptide Y (NPY) in influencing sympathetic nerve activity (Edvinsson *et al.*, 1987). NPY is co-localized with NA in perivascular sympathetic nerve fibres and is co-released with NA following sympathetic nerve activation, suggesting that NPY acts as a co-transmitter (for references see Edvinsson *et al.*, 1987).

The vascular effects of NPY vary with species and tissue. The effects of NPY on blood vessels include direct vascular smooth muscle contraction (Edvinsson *et al.*, 1983; 1984), potentiation of agonist-evoked responses (Ekblad *et al.*, 1984; Edvinsson *et al.*, 1984), inhibition of relaxation (Han & Abel, 1987; Fallgren *et al.*, 1989) and inhibition of the release of NA (Pernow *et al.*, 1986). NPY causes direct contraction in some tissues, but not all, and this effect occurs at relatively high concentrations of NPY. However, although a number of arteries fail to contract upon NPY administration, a potentiation of contractile responses to exogenous NA and transmural nerve stimulation occurs at nanomolar concentrations of NPY (Ekblad *et al.*, 1984; Edvinsson *et al.*, 1984). This response seems to prevail in the peripheral circulation, since coronary arteries and cerebral arteries preferably contract upon NPY administration (Edvinsson *et al.*, 1983; Clarke *et al.*, 1987).

The mechanism responsible for the potentiating effect of NPY is unclear. However, it seems to depend on calcium released from intracellular stores (Wahlestedt *et al.*, 1985). In parallel, NPY appears to facilitate the refill of the intracellular calcium stores from the extracellular medium, conceivably due to its ability to evoke membrane depolarization (Fallgren *et al.*, 1990). Other mechanisms proposed to explain the potentiating effect include inhibition of adenylate cyclase activity (Fredholm *et al.*, 1985) and enhanced accumulation of inositol trisphosphate (Häggblad & Fredholm, 1987).

To understand further the functional role of NPY in autonomic transmission there is a need of a selective NPY-antagonist. PP56 is a new chemical entity with NPY-blocking properties (Edvinsson *et al.*, 1990). It is at present being developed for therapeutic use in cardiovascular and inflammatory disorders by Perstorp Pharma, Sweden. The purpose of the present study was to characterize the effect of NPY on rabbit and guinea-pig arteries and to examine the NPY-antagonistic effects of PP56 (D-*myo*-inositol 1,2,6-trisphosphate; U.S. Pat. No. 4735963, EPO Pat. No. 179439, Dr M.J. Sirén, 1984). The initial observations indicated that NPY had no direct contractile effect but preferentially potentiated the responses to NA both in the presence and absence of endothelium. Furthermore, PP56 was found to be a very potent NPY blocker.

Methods

Adult male rabbits (2–3 kg) were anaesthetized with pentobarbitone (30 mg kg^{-1}) and exsanguinated. The gastroepiploic and femoral arteries were rapidly dissected out and placed in an ice-cold oxygenated buffer solution of the following composition (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.5 and glucose 11. Adult female guinea-pigs (300–400 g) were anaesthetized with pentobarbitone (80 mg kg^{-1}) and decapitated. The mesenteric and uterine arteries were rapidly removed and placed in the same buffer solution as above. Ring segments of the arteries, 2–3 mm long, were immersed in temperature controlled tissue baths (2.5 ml in volume) containing the buffer solution aerated with 5% CO_2 in O_2, giving a pH of 7.4. The specimens were mounted on two L-shaped metal holders (0.1 or 0.2 mm in diameter depending on vessel diameter). The position of one of the holders could be changed by means of a movable unit allowing fine adjustments of the vascular tension. The other holder was connected to a force displacement transducer (Grass FT03C). The transducer signals were amplified by a Transbridge TBM4 amplifier, digitalized by Maclab TM analog-digital converter and recorded by a MacIntosh Plus computer. Increase in isometric tension was expressed in mN. The arteries were given an initial tension of 2–4 mN depending on vessel type and allowed to maintain this level of tension for a resting period of 90 min. After the resting period

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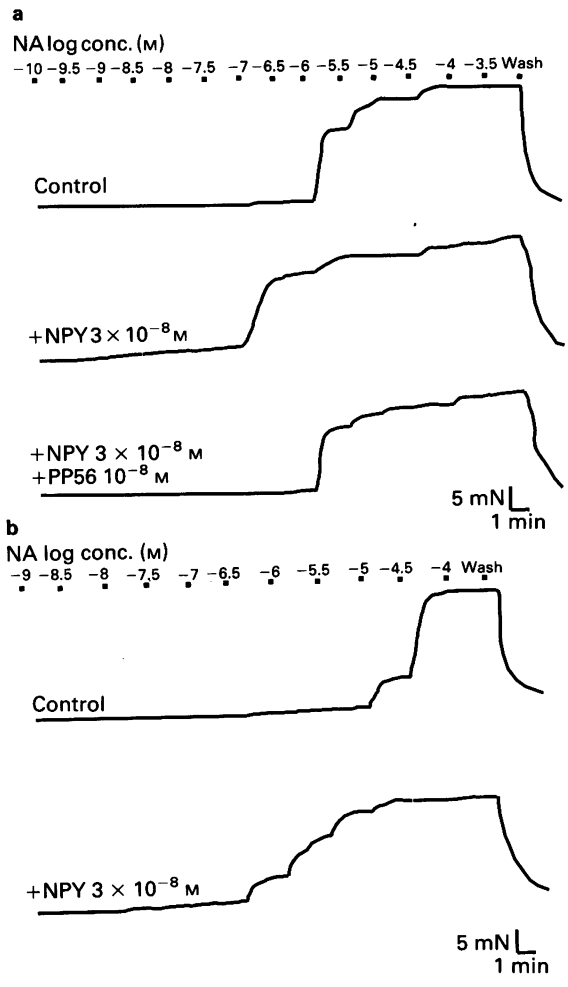


Figure 1 (a) Typical tracings showing the effect of noradrenaline (NA), NA in the presence of neuropeptide Y (NPY) and NA in the presence of both NPY and D-*myo*-inositol 1,2,6-trisphosphate (PP56) in rabbit femoral artery. Calibration bars are inserted. (b) NPY-induced potentiation occurs in the absence of endothelium in the rabbit femoral artery. The endothelium was removed by gentle rubbing of the vessel with a wooden stick and verified by lack of dilator response to acetylcholine of arteries precontracted with NA. Calibration bars are inserted.

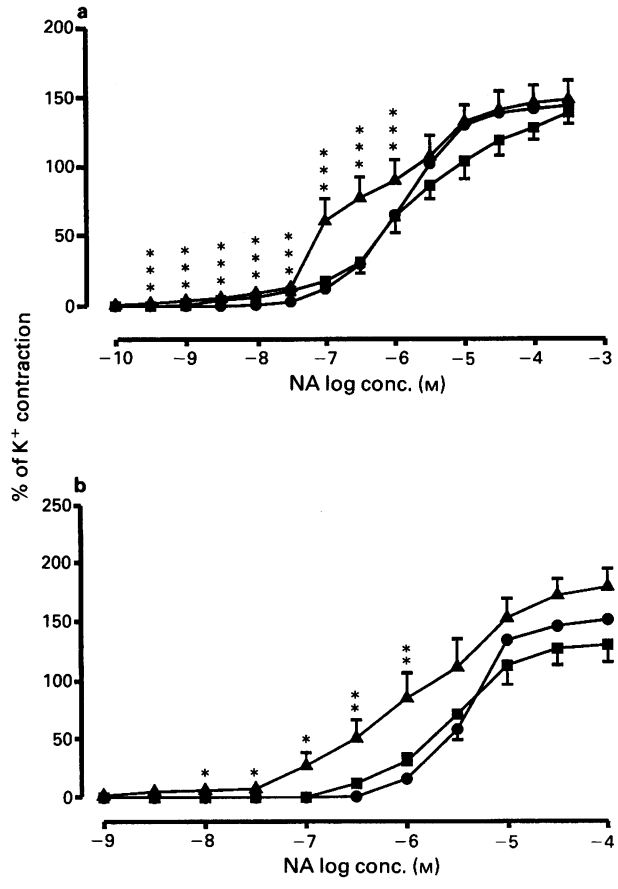


Figure 2 Effect of neuropeptide Y (NPY) on contraction elicited by noradrenaline in the absence and presence of D-*myo*-inositol 1,2,6-trisphosphate (PP56) in the rabbit (a) femoral and (b) gastroepiploic arteries. Control experiments [●]; tests in the presence of 30 nM NPY [▲]; and tests in the presence of 30 nM NPY and 10 nM PP56 [■]. Values given as the mean with the error bars representing s.e.mean. Number of experiments, $n = 6$–8. Unpaired Student's t test for control versus NPY treatment; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

the contractile capacity of each specimen was examined by exposure to a potassium-rich buffer solution (60 mM) obtained by equimolar substitution of NaCl by KCl in the above buffer. After two reproducible tests (variation less than 10%), concentration-response curves for NA (1 nM–0.1 mM) were obtained. The experiments were performed in 4 tissue baths, with 4 neighbouring arterial segments. One preparation served as control and received NA only. The enhancing effect of NPY was tested in the other three preparations by addition of NPY (30–100 nM) 2–3 min before the first concentration of NA. In two of the preparations receiving NPY, the NPY-antagonistic property of D-*myo*-inositol 1,2,6-trisphosphate (10 pM–10 μM) was examined by incubation of the vessels with PP56 for 20 min before NPY was added. In this manner each arterial segments were used for experimentation only once, i.e. the vessels were never exposed to NPY and PP56 on more than one occasion and, therefore, the risk of desensitization of the preparations was unlikely. This schedule was repeated from the different arteries of several animals in order to obtain reliable and statistically significant results.

In separate experiments on rabbit femoral arteries the endothelium was removed mechanically by gently rubbing the internal surface of the vascular segment with a wooden stick. The absence of endothelium was verified by loss of the dilator response to acetylcholine (Furchgott & Zawadski, 1980). In ring segments of femoral arteries a modulatory effect of PP56 was examined on contraction induced by cumulatively applied histamine, endothelin-1 and potassium.

Data analysis

Statistical analysis was performed by comparing the concentration-response curves to NA obtained alone, in the presence of NPY, or in the presence of NPY and PP56. Results are expressed as the means ± s.e.mean. Student's unpaired t test was used to determine statistical differences between means.

Drugs

Noradrenaline, acetylcholine and histamine (Sigma, U.S.A.), endothelin-1 (Peninsula, U.S.A.), neuropeptide Y (Porcine, Sigma, U.S.A.) and cocaine (ACO, Sweden) were dissolved and diluted in saline immediately before the *in vitro* tests in order to avoid possible breakdown or adsorption to walls of containers. D-*myo*-Inositol 1,2,6-trisphosphate (PP56, Perstorp Pharma, Sweden) was dissolved in 0.9% saline with 1% bovine serum albumin. A fresh solution of PP56 was made every day to avoid breakdown. All concentrations given below are the final molar concentration in the tissue bath during the experiments.

Results

Noradrenaline (NA) evoked a concentration-dependent contraction of rabbit and guinea-pig arteries. This response was sensitive to prazosin but not rauwolscine (Wahlestedt *et al.*,

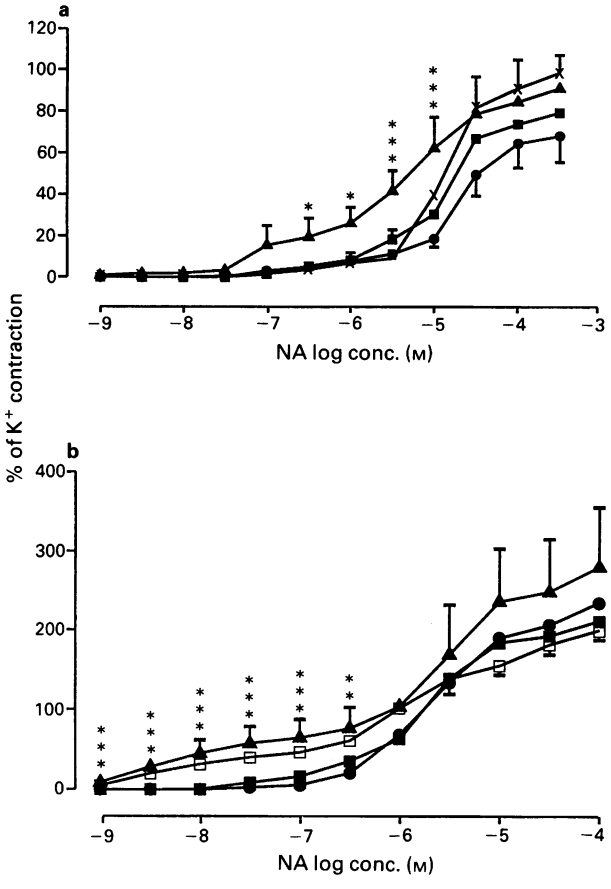


Figure 3 Effect of neuropeptide Y (NPY) on contraction elicited by noradrenaline in the absence and presence of D-*myo*-inositol 1,2,6-trisphosphate (PP56) in the guinea-pig (a) mesenteric and (b) uterine arteries. Control experiments [●]; tests in the presence of 0.1 μM NPY [▲] and tests in the presence of 0.1 μM NPY and 1 μM [×], 10 nM [■] or 1 nM [□] PP56. Values given as the mean with error bars representing s.e.mean. Number of experiments, $n = 6$–8. Unpaired Student's t test for control versus NPY: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

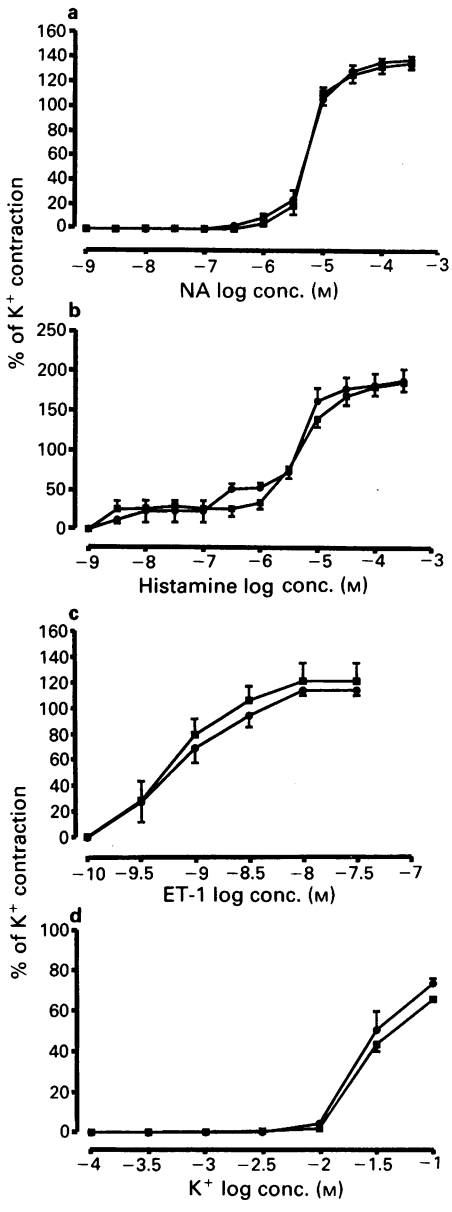


Figure 4 Effect of D-*myo*-inositol 1,2,6-trisphosphate (PP56) on contractions induced by (a) noradrenaline (NA), (b) histamine, (c) endothelin-1 (ET-1) and (d) potassium (K^+) in the rabbit femoral artery. Control experiments [●] and tests in the presence of 1 μM PP56 [■]. Values given as the mean with error bars representing s.e.mean. Number of experiments, $n = 6$.

1985; Fallgren & Edvinsson, 1986), demonstrating an action predominantly mediated via α_1-adrenoceptors. The experiments were performed in the presence of 1 μM cocaine to inhibit prejunctional uptake of NA. In the previous studies propranolol was found not to modify the contractile response to NA. NPY *per se* did not induce any constriction in concentrations up to 0.1 μM.

The effect of NPY on NA-evoked constriction was manifested in a left-ward shift of the lower half of the concentration-response curve for NA without any change in maximum response (Figures 1, 2 and 3). Removal of the endothelium in the rabbit femoral artery abolished acetylcholine-induced relaxation in arteries precontracted with NA (not shown). NA caused a concentration-dependent contraction which was potentiated in the same manner by NPY in endothelium-denuded vessels (Figure 1b) as were seen in intact vessels (Figure 1a).

PP56 selectively blocked the NPY-evoked potentiation of the NA-induced constriction without further affecting the concentration-response curve of NA (Figures 1a, 2 and 3). As can be seen in Figure 3 the NPY-blocking effect of PP56 was concentration-dependent. Inhibition started to appear in concentrations of around 0.1 nM (not shown). Total inhibition was obtained at 10 nM PP56 (Figures 2 and 3), and higher concentrations of PP56 (up to 1 μM) equally inhibited the NPY effect without altering the shape of the NA-evoked concentration-response curve (Figure 3).

As shown by the lack of effect on vascular tension during the 20 min incubation, PP56 did not show any effect *per se* on resting artery segments in concentrations up to 1 μM and it did not significantly change the NA-induced contraction in any of the vessels studied. In addition, PP56 was without effect on contractions induced by NA, histamine, endothelin-1 or potassium, respectively (Figure 4 and data not shown).

Discussion

The present results are in favour of the view that the NPY-induced potentiation involves a direct stimulation of smooth muscle cell-surface receptors (Gustafsson & Nilsson, 1990), rather than activation of an endothelium-dependent mechanism (Daly & Hieble, 1987). Receptor stimulation in general may initiate hydrolysis of a membrane-bound inositol lipid with subsequent formation of two second messengers, one of which is inositol 1,4,5-trisphosphate (1,4,5-IP_3) and the other diacylglycerol (DAG) (Berridge & Irvine, 1989). This may be particularly relevant for the NPY-induced potentiation of contraction in smooth muscle since Häggblad & Fredholm

(1987) found an enhanced accumulation of inositol trisphosphate after NPY administration in the vas deferens. 1,4,5-IP_3 regulates intracellular calcium both by mobilizing calcium from internal stores and, perhaps indirectly, by stimulating calcium entry. It has been postulated that NPY causes potentiation by facilitating the mobilization of intracellularly stored calcium ions (Wahlestedt *et al.*, 1985). Indeed, the potentiation was most prominent in low NA concentrations which putatively are associated with second messenger-mediated increase of intracellular calcium levels (for reference see Fallgren, 1988).

Recently we found evidence for two calcium-dependent mechanisms involved in the NPY-induced potentiation (Fallgren *et al.*, 1990 and unpublished): it was hypothesized that NPY first evoked intracellular release of calcium which may act as a second (or third) messenger that signals an increased sensitivity to the membrane-bound adrenoceptors. Accordingly, chelation of intracellular calcium pools with quin-2 blunted the potentiation without depressing the NA-induced concentration-response curve (Fallgren *et al.*, 1990), an effect similar to that of PP56 in the present study. Second, extracellular calcium influx seems to be a prerequisite for the observed effect of NPY, as evidenced by an inhibitory effect of different blockers of calcium entry (Fallgren *et al.*, 1990). PP56 does not inhibit extracellular calcium influx, as shown in the guinea-pig basilar artery (Edvinsson *et al.*, 1990, and unpublished). Hence, from a functional point of view, PP56 mimics the effect of quin-2, obviously by obstruction of the subtle intracellular calcium fluxes mediating the enhanced contractile response to NA in the presence of NPY.

The exact mechanism of action of D-*myo*-inositol 1,2,6-trisphosphate (PP56) is not known and further studies are required to elucidate its NPY-antagonistic property. However, due to the molecular structure of PP56 an interaction with 1,4,5-IP_3 is possible, although the weak lipid solubility of PP56 would contradict a direct effect on endogenous 1,4,5-IP_3. If so, the demonstration that PP56 inhibited the NPY effect would be in agreement with a suggestion that its NPY-blocking effect occurs via activation of the recently identified cell surface receptors for inositol phosphates (Vallejo *et al.*, 1987). Such inositol phosphate receptors may functionally be linked to the NPY-receptors due to the rather selective inhibition of NPY evoked by PP56 (Edvinsson *et al.*, 1990).

In conclusion, the present results support the view that PP56 antagonizes NPY by interference with an inositol phosphate-sensitive mechanism, conceivably associated with cell surface receptors coupled to intracellular calcium fluxes.

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Binding properties of nine 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP) analogues to M_1, M_2, M_3 and putative M_4 muscarinic receptor subtypes

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1 We compared the binding properties of 4-diphenyl-acetoxy-N-methyl-piperidine methiodide (4-DAMP) and nine analogues of this compound on muscarinic receptors of human neuroblastoma NB-OK1 cells (M_1 subtype), rat heart (M_2 subtype), rat pancreas (M_3 subtype) and to the putative M_4 subtype in striatum.

2 The requirements for high affinity binding were somewhat different for the four receptor subtypes. In general, the requirements of M_3 receptors were more stringent than for M_1, M_2 or putative M_4 receptors.

3 The abilities of the compounds to discriminate muscarinic receptor subtypes were not correlated with their affinities at any subtype.

4 The temperature-dependence of binding of 4-DAMP analogues to M_2 receptors varied with the drug structure. In particular, the increased affinity of the α-methyl derivative of 4-DAMP could be ascribed to van der Waals interactions.

5 The affinities of most 4-DAMP analogues for M_2 and M_3 receptors were similar to their pharmacological potencies on atrial and ileum preparations, respectively.

6 At concentrations above 1 μM, all 4-DAMP analogues as well as atropine, reduced the [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) dissociation rate from cardiac muscarinic receptors, with no obvious structure-activity relationship.

Keywords: 4-Diphenyl-acetoxy-N-methyl-piperidine (4-DAMP) analogues; atropine; pirenzepine; AF-DX 116; muscarinic receptor subtypes; structure-activity relationship; binding/functional correlations

Introduction

Three muscarinic receptor subtypes have recently been described, based on binding and pharmacological studies *in vitro* and *in vivo* (see Birdsall *et al.*, 1989) and there are indications of the existence of a fourth (Lazareno *et al.*, 1990; Waelbroeck *et al.*, 1990; Dörje *et al.*, 1991). M_1 receptors in the nervous system are characterized by high affinity for pirenzepine (Hammer *et al.*, 1980; Brown *et al.*, 1980). The other muscarinic receptors (with a low affinity for pirenzepine), have been subdivided using the cardioselective muscarinic antagonists methoctramine (Giraldo *et al.*, 1988) and AF-DX 116 [11-({2-[diethylamino)-methyl]-1-piperidinyl}acetyl)-5,11-dihydro-6H-pyrido (2,3b)(1,4)benzodiazepin-6-one] (Hammer *et al.*, 1986; Giachetti *et al.*, 1986). Cardiac M_2 receptors from mammalian heart show high affinity for methoctramine and AF-DX 116 and low affinity for pirenzepine. By contrast, secretory gland and smooth muscle M_3 receptors have a low affinity for methoctramine, AF-DX 116, and pirenzepine (Batink *et al.*, 1987; Doods *et al.*, 1987; Birdsall *et al.*, 1980; Waelbroeck *et al.*, 1987; Lambrecht *et al.*, 1987). About 40% of the muscarinic binding sites in striatum, like the functional receptors in uterus smooth muscle, have a high affinity for methoctramine and a low affinity for AF-DX 116 and for pirenzepine (Waelbroeck *et al.*, 1990; Dörje *et al.*, 1991). Since their binding/pharmacological properties are different from the previously identified M_1, M_2 and M_3 receptors, and very similar to cloned M_4 receptors, we shall hereafter call these binding sites 'putative M_4 sites'.

We previously demonstrated that these four muscarinic binding sites can be studied using [^{3}H]-N-methyl scopolamine ([^{3}H]-NMS) binding to, human neuroblastoma NB-OK 1 cells (M_1), rat heart (M_2), rat pancreas (M_3), and rat striatum (putative M_4 sites having slow [^{3}H]-NMS dissociation rate) respectively (Waelbroeck *et al.*, 1987; 1988*a*,*b*; 1990).

4-Diphenyl-acetoxy-N-methyl-piperidine (4-DAMP) (Barlow *et al.*, 1976) was one of the first compounds able to discriminate two receptor subclasses sharing low affinity for pirenzepine (the M_2 and M_3 receptors). It also has high affinity for muscarinic receptors (of the order of 10^9 to 10^8 M^{-1} depending on the subtype studied) (Barlow *et al.*, 1976; Waelbroeck *et al.*, 1987; Doods *et al.*, 1987), suggesting that its discriminatory capacity relies on good fit to high affinity receptors rather than on steric hindrance in low affinity receptors. We have compared the binding properties of nine 4-DAMP analogues to the 4 muscarinic receptor subtypes, in order to define further the structural requirements of the binding sites.

The synthesis and the pharmacological properties, in ileum (M_3 receptors) and atria (M_2 receptors), of the nine 4-DAMP analogues studied have been described by Barlow & Shepherd (1985, 1986).

Methods

NB-OK 1 cells: Culture and homogenates preparation

NB-OK 1 human neuroblastoma cells (a generous gift from Dr N. Yanaihara, Shizuoka, Japan) were maintained in RPMI 1640 medium enriched with 100 unit ml^{-1} penicillin and

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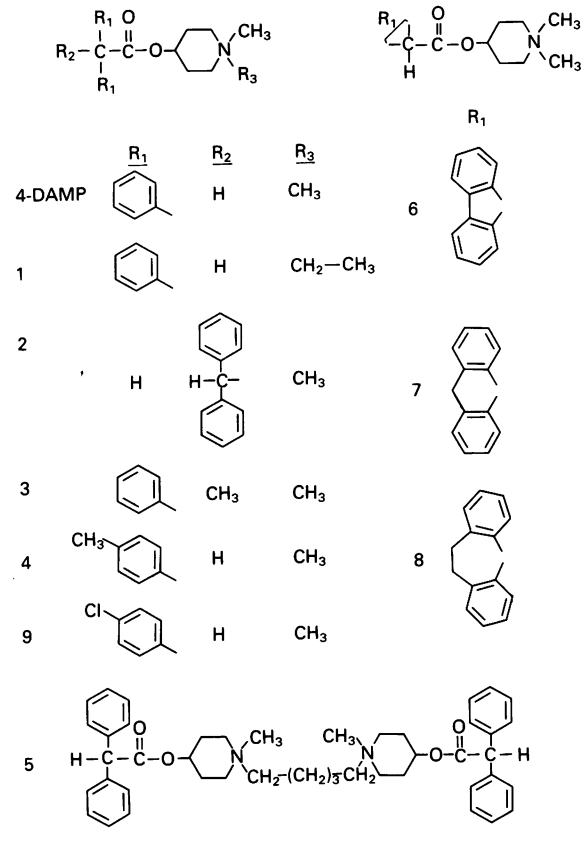


Figure 1 Structure of 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP) and of analogues 1 to 9: 4-diphenylacetoxy-N-methyl piperidine *etho* bromide (1), 4-diphenylpropionyl-N-methyl piperidine methobromide (homo-4-DAMP) (2), 4-(α-methyl-4)-DAMP methobromide (3), 4-(di-*p*-tolyl)acetoxy-N-methyl piperidine methobromide (4), pentamethylene-*bis*-4-(diphenyl-acetoxy)-N-methylpiperidinium bromide (5), 4-(fluoren-9-carboxy)-N-methyl piperidine methobromide (6), 4-(xanthen-9-carboxy)-N-methyl piperidine methobromide (7), 4-(dibenzo cycloheptane-carboxy)-N-methylpiperidine methobromide (ethane-bridged 4-DAMP) (8), 4-(di-*p*-chlorophenyl)acetoxy-N-methylpiperidine methobromide (9).

100 μg ml^{-1} streptomycin, and 10% foetal calf serum at 37°C, as previously described (Waelbroeck *et al.*, 1988a). For [^{3}H]-NMS binding studies, cells were harvested in a 20 mM sodium phosphate buffer enriched with 1 mM EDTA and 150 mM NaCl (pH 7.4); centrifuged at 500 *g* for 5 min, then resuspended, and homogenized in 20 mM Tris-HCl buffer enriched with 5 mM $MgCl_2$ (pH 7.5) and stored in liquid nitrogen until use. Samples (80 μl), containing 160 to 200 μg protein per tube, were used. Protein concentration was determined according to Lowry *et al.* (1951).

Cardiac homogenate preparation

Male Wistar albino rats (200–250 g) were killed by decapitation, the heart removed and rinsed in isotonic (150 mM) NaCl. The homogenate was prepared as previously described (Waelbroeck *et al.*, 1990) and was used either fresh or stored in liquid nitrogen until use; 80 μl of this homogenate (400 to 500 μg protein per tube) were used.

Pancreas homogenate preparation

The pancreases were removed, minced with scissors and homogenized as previously described, in a buffer containing 500 kallikrein inhibitor units ml^{-1} Trasylol, 0.2 mg ml^{-1} bacitracin and 1% bovine serum albumin (Waelbroeck *et al.*, 1987). Samples (1 ml), 800 to 1000 μg pancreas protein per tube, were used.

Striatum homogenate preparation

The striatum was removed, homogenized as previously described (Waelbroeck *et al.*, 1990) and stored in liquid nitrogen until use.

[^{3}H]-N-methyl scopolamine binding experiments

[^{3}H]-NMS binding was measured at 25°C in a total volume of 1.2 ml, in the following incubation buffer: 50 mM sodium phosphate (pH 7.4), 2 mM $MgCl_2$ and 1% (w/v) bovine serum albumin. For binding studies to pancreas receptors, we also added Trasylol and bacitracin (see above) to inhibit further proteolytic activity.

The [^{3}H]-NMS concentration used (0.25 nM for binding studies to NB-OK 1 and pancreas homogenates, 1.0 nM for binding studies to cardiac homogenates) corresponded to approximately twice the K_D values in the systems used. Receptor concentrations in the assays were 50 to 200 pM. Non-specific binding was defined as [^{3}H]-NMS binding in the presence of 1 μM atropine in the three systems.

The incubation period was 2 h, when using NB-OK 1 and cardiac homogenates, and 4 h when using pancreas homogenates. In striatum homogenates, [^{3}H]-NMS (at 240 pM) labels approximately 27% M_1, 9% M_2, 9% M_3 and 56% M_4 sites (Waelbroeck *et al.*, 1990). We preincubated the homogenates with tracer and unlabelled drug for 2 h at 25°C to monitor [^{3}H]-NMS binding. We then added 1 μM atropine to each sample to prevent reassociation of the tracer, and allowed [^{3}H]-NMS dissociation to proceed for 35 min. After this dissociation period, 85% of the residual [^{3}H]-NMS was bound to M_4 sites, 15% to M_3 sites.

To terminate the incubation, each sample was diluted with 2 ml of ice-cold 50 mM sodium phosphate buffer (pH 7.4) and filtered on GF/C glass-fibre filters (Whatman), presoaked overnight in 0.05% polyethyleneimine. The filters were rinsed 3 times with the filtration buffer, dried, and the radioactivity (bound tracer) counted by liquid scintillation.

[^{3}H]-N-methyl scopolamine dissociation from cardiac sites

[^{3}H]-NMS was preincubated with cardiac homogenates for 15 min at 25°C. Tracer dissociation was induced either by: (1) addition of 1 μM atropine (control); (2) simultaneous addition of 1 μM atropine and the indicated 4-DAMP analogue concentrations or (3) addition of the indicated atropine concentrations (1 μM to 1 mM), and allowed to proceed for 5 min at 25°C before filtration. The results are expressed as a fraction of the initial tracer binding (before dissociation) on a logarithmic scale.

Temperature-dependence of drug binding to cardiac sites

[^{3}H]-NMS binding to cardiac homogenates was measured as described above, after an incubation of 4 h at 4°C, 2 h at 12°C, 1 h at 25°C and 20 min at 37°C. The tracer concentration used for competition curves was 1.0 nM at all temperatures. [^{3}H]-NMS saturation curves, obtained with tracer concentrations from 0.25 nM to 4.0 nM at each temperature were used to determine the [^{3}H]-NMS K_D value and calculate the unlabelled drug K_i values as explained below. To calculate the enthalpy and entropy changes associated with drug binding, we plotted the $\ln K_a = -\ln K_i$ values as a function of 1/T:

$$\ln K_a = \left[-\frac{\Delta H^{o\prime}}{R}\frac{1}{T} + \frac{\Delta S^{o\prime}}{R} \right]$$

Mathematical analysis

The competition curves were analysed with the competition-assisted curve fitting procedure described by Richardson & Humrich (1984). The K_i values were calculated with the equation of Cheng & Prusoff (1973), using the [^{3}H]-NMS K_D value in the same tissue.

Compounds

[^{3}H]-NMS (1-[N-methyl ^{3}H]-scopolamine methyl chloride) (74 Ci mmol^{-1}) was obtained from Amersham International (Bucks, England). Atropine sulphate was from Sigma Chemical Co. (St Louis, MO, U.S.A.). Tissue culture material and media were obtained from Gibco (Gent, Belgium). All other reagents were of the highest grade available. 4-Diphenyl-acetoxy-N-methyl-piperidine methobromide (4-DAMP) and the nine 4-DAMP analogues were generous gifts from Dr R.B. Barlow. The structure of these analogues is given in Figure 1.

Results

We have previously demonstrated, using selective antagonists, that muscarinic receptor sites from NB-OK 1 cells are 80% M_1; from heart, 100% M_2; from pancreas, 100% M_3 and that the 'slow-dissociation sites' in striatum are 85% M_4 sites.

The competition curves for *bis*-pentamethylene 4-DAMP were steep (n_H 1.5 ± 0.10); all others were compatible with competitive inhibition to a single receptor type (with Hill coefficients varying from 0.95 to 1.05 ± 0.10). We tested the hypothesis that all 4-DAMP analogues and the tracer interacted competitively with the same receptor population by comparing competition curves obtained with 0.3 nM, 1.0 nM and 3.0 nM [^{3}H]-NMS, in each of the 4 systems. Since this was apparently true, including for *bis*-pentamethylene 4-DAMP, we used the Cheng-Prusoff equation (Cheng & Prusoff, 1973) to calculate the K_i (inhibition constant) for all antagonists.

Binding to four muscarinic receptor subtypes at 25°C

The pK_i values for binding of the nine 4-DAMP analogues to the four muscarinic receptor subtypes are given in Table 1, and their selectivities for each pair of sites, in Table 2. The standard deviation of the pK_i values (4 to 6 determinations)) was typically 0.10 log units, and always below 0.15 log units. The affinities of the 4-DAMP analogues varied by over 1000

Table 2 Selectivity of the 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP) analogues at each pair of sites

Analogue number	M_1/M_2	M_1/M_3	M_1/M_4	M_2/M_3	M_2/M_4	M_3/M_4
4-DAMP	10	1.3	1	−8	−10	−1.3
1	13	1.6	1	−8	−13	−1.6
2	8	3	1.6	−2.5	−5	−2
3	4	2.5	1.3	−1.6	−5	−3
4	1.6	5	5	3	3	1
5	2.5	3	−2.5	1.2	−6	−8
6	3	1.6	2	−2	−1.6	1.3
7	8	3	1.2	−2	−10	−4
8	5	3	1.6	−1.6	−3.2	−2
9	3	6	6	2	2	1

Ratio of the larger K_i value to the smaller: where the first K_i in the comparison is the larger (i.e. the antagonist had a lower affinity for the first site), the ratio is preceded by a minus (−) sign.

Table 1 pK_i values obtained in binding studies for NB-OK 1 (M_1 receptors), heart (M_2 receptors) and pancreas (M_3 receptors) homogenates and to the slowly-dissociating (M_4) sites in striatum[a]

Drug	*Analogue number*	M_1 *NB-OK 1*	M_2 *Heart*	M_3 *Pancreas*	*Striatum* M_4 *sites*
Atropine[b]		9.6	9.0	9.4	9.6
Pirenzepine[b]		8.2	6.6	6.6	7.1
AF-DX 116[b]		6.3	7.3	5.8	6.3
Himbacine[b]		7.1	8.1	6.5	8.0
Methoctramine[b]		7.3	7.9	6.0	7.6
4-DAMP[b]		9.1	8.1	9.0	9.1
4-Diphenylacetoxy-N-methyl piperidine *etho* bromide	1	8.9	7.8	8.7	8.9
4-Diphenylpropionyl-N-methyl piperidine methobromide (homo-4-DAMP)	2	6.9	6.0	6.4	6.7
4-(α-methyl-4-)DAMP methobromide	3	9.8	9.2	9.4	9.9
4-(Di-*p*-tolyl)acetoxy-N-methyl piperidine methobromide	4	8.0	7.8	7.3	7.3
Pentamethylene-*bis*-4-(diphenyl-acetoxy)-N-methylpiperidinium bromide	5	8.0	7.6	7.5	8.4
4-(Fluoren-9-carboxy)-N-methyl piperidine methobromide	6	8.9	8.4	8.7	8.6
4-(Xanthen-9-carboxy)-N-methyl piperidine methobromide	7	9.9	9.0	9.4	10.0
4-(Dibenzo cycloheptane-carboxy)-N-methylpiperidine methobromide (ethane-bridged 4-DAMP)	8	6.8	6.1	6.3	6.6
4-(Di-*p*-chlorophenyl)acetoxy-N-methylpiperidine methobromide	9	7.8	7.3	7.0	7.0

[a] The standard deviation of the pK_i values was typically ±0.1 log unit, always below 0.15 log unit. The Hill coefficients varied between 0.95 and 1.05 (standard deviation: ±0.1) except for compound 5: Hill coefficients of 1.5 ± 0.10 at the four receptors were observed for this compound.

[b] Values taken from Waelbroeck *et al.*, 1987, or Waelbroeck *et al.*, 1990.

fold. The values obtained at M_2 and M_3 receptors were in good agreement with their potency in pharmacological assays using, respectively, cardiac atria and ileum (Barlow *et al.*, 1976; Barlow & Shepherd, 1985; 1986). The only exception was *bis*-pentamethylene 4-DAMP, which had a much lower affinity for pancreas M_3 sites than expected from pharmacological studies in the ileum (Barlow & Shepherd, 1986).

'Allosteric' interactions with cardiac receptors

To examine whether these analogues interacted with allosteric sites, in addition to binding sites in muscarinic receptors, we measured the effect of atropine, 4-DAMP and 4-DAMP analogues on [^{3}H]-NMS dissociation from cardiac muscarinic receptors (Figure 2). When tested at high concentration, atropine and 4-DAMP decreased the [^{3}H]-NMS dissociation rate with a $-\log EC_{50}$ of 3.7 to 3.5. Analogues 1 to 4 and 7 to 9 had a slightly (3 to 5 fold) greater potency with a $-\log EC_{50}$ value of 4.2 (Figure 2, superimposable curves). Analogues 6 (fluorenyl 4-DAMP) ($-\log EC_{50}$:5.2) and 5 (*bis*-pentamethylene 4-DAMP) ($-\log EC_{50}$:5.7) were the most potent analogues in this respect but, even so, the effects of these analogues on [^{3}H]-NMS dissociation from cardiac receptors were observed at concentrations 100 to 1,000,000 fold above their K_i value.

Thermodependence of 4-DAMP analogues binding to cardiac receptors

We measured the affinity of atropine, 4-DAMP and eight analogues at four temperatures to calculate the enthalpy and entropy changes associated with binding to cardiac M_2 receptors. The van't Hoff plots were linear: $\Delta H^{o\prime}$ and $\Delta S^{o\prime}$ values were constant.

As shown in Table 3, binding of 4-DAMP and seven 4-DAMP analogues was associated with a negative (favourable) enthalpy change and positive (favourable) entropy change. Binding of one 4-DAMP analogue (compound 8), was entropy driven only, the enthalpy change associated with binding being unfavourable. The enthalpy and/or entropy change associated with the binding of four analogues were significantly different from 4-DAMP values: decreased for homo 4-DAMP 2, (di-*p*-tolyl) and (di-*p*-chloro)-4-DAMP (4 and 9) and increased for the ethane bridged 4-DAMP analogue (8).

Table 3 Changes in enthalpy ($\Delta H^{o\prime}$ in kJ mol^{-1}) and entropy ($\Delta S^{o\prime}$ in J mol^{-1} K^{-1}) associated with the binding of 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP) or 4-DAMP analogues to cardiac M_2 receptors[a]

Analogue number	$\Delta H^{o\prime}$	$\Delta S^{o\prime}$
4-DAMP	−5.987	+136
1	−8.406	+122
2	−11.615	+77[d]
3	−12.970	+136
4	−15.430[b]	+98[c]
6	−9.230	+133
7	−11.113	+136
8	+5.674[c]	+136
9	−16.707[b]	+84[b]

a We estimated the standard deviation of the enthalpy and entropy changes at ±5.51 kJ mol^{-1} and ±18.6 J mol^{-1} K^{-1}, respectively.
[b] Significantly different ($P > 0.9$) from the corresponding value for 4-DAMP.
[c] Significantly different ($P > 0.95$) from the corresponding value for 4-DAMP.
[d] Significantly different ($P > 0.98$) from the corresponding value for 4-DAMP.

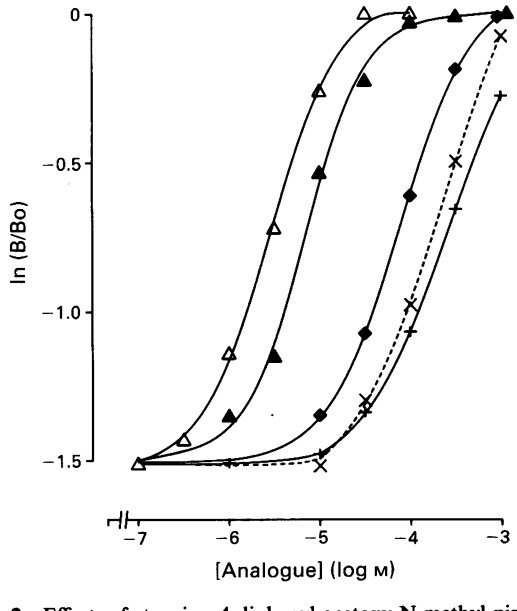


Figure 2 Effects of atropine, 4-diphenyl-acetoxy-N-methyl-piperidine (4-DAMP) and 4-DAMP analogues on the [^{3}H]-N-methyl-scopolamine ([^{3}H]-NMS) dissociation rate from cardiac muscarinic receptors. [^{3}H]-NMS dissociation was induced by isotopic dilution, using 1 μM atropine together with the indicated 4-DAMP or 4-DAMP analogues concentrations, or using the indicated atropine concentrations. Residual [^{3}H]-NMS binding was measured 5 min after the addition of atropine alone (+), or 1 μM atropine and the indicated concentrations of 4-DAMP (×---×), 4-DAMP analogues 1, 2, 3, 4, 7, 8, or 9 (◆), analogue 6 (▲) or analogue 5 (△). This experiment was representative of 3 experiments performed in duplicate.

Discussion

Binding patterns of 4-DAMP analogues to neuronal, cardiac and pancreatic muscarinic receptors

The binding of 4-DAMP and nine analogues was measured in four different systems but under comparable conditions.

The receptor-selectivities of 4-DAMP analogues (Table 2) were independent of their affinities for any given subtype (Table 1): for example, homo-4-DAMP (2) and the xanthene-derivative (7) had the same $M_4 > M_2$ selectivity as 4-DAMP but, 100 fold lower and 10 fold higher affinities respectively, for these subtypes. The requirements for high affinity binding were somewhat different at the four subtypes. For example: modification of the phenyl rings by *para*-chloro or *para*-methyl substituents (4, 9) decreased 50 or 100 fold the analogue's affinity for M_3 and M_4 receptors, 10 or 16 fold for M_1 receptors and only 1.6 or 5 fold for M_2 receptors. Addition of an α-methyl group (3) increased the receptor affinities 2 to 15 fold depending on the subtype.

The binding properties of the various 4-DAMP analogues gave some indications on binding site conformation. Indeed, on theoretical grounds, when 4-DAMP methobromide binds to muscarinic receptors, its two phenyl rings, instead of rotating freely in the solvent, are presumably blocked by their interaction with the receptor. This must correspond to an entropy decrease, which ought to be compensated to allow high affinity binding. Therefore, the affinity of a 'rigid' analogue should be greater than that of the parent molecule if the two phenyl rings are maintained in the correct position. Calculations by Andrews (1986) indicated that the K_b increase

obtained when preventing two bonds from freely rotating is, on average, of the order of 10 fold: it should be much greater if the groups are maintained in the optimal position. Three of the analogues tested in this work (analogues 6, 7 and 8) can be considered as 'rigid' in the sense that the two phenyl residues are bridged by a covalent bond. The four subtypes of muscarinic receptors showed greater affinities for the xanthene derivative (analogue 7) than for the fluorene (analogue 6) and ethane-bridged (analogue 8) derivatives. This suggests that the two phenyl groups of 4-DAMP were normally accepted in a nearly planar conformation in the muscarinic binding site. The xanthene derivative (analogue 7) had the highest affinity for the three subtypes: either it is closest to the optimal 4-DAMP conformation (in the binding site) or the added oxygen bridge allows favourable interaction(s) with muscarinic receptors.

The affinity increase, when comparing analogue 7 with 4-DAMP, was 10 fold in NB-OK1 (M_1), heart (M_2) and striatum (M_4) and only 3 fold in pancreas, suggesting that position of the two phenyl groups of 4-DAMP in the pancreas (M_3) binding site is different from their position in the three other receptor subtypes.

Thermodependence of drug binding to cardiac M_2 receptors

It has been suggested that hydrophobic association and ionic bond formation are associated with positive enthalpy and entropy changes, whereas hydrogen bond formation and van der Waals interactions are associated with negative enthalpy and entropy changes (Ross & Subramanian, 1981).

In this work we have measured the enthalpy and entropy changes associated with the binding of 4-DAMP and several analogues to cardiac M_2 receptors (Table 3). This subtype was chosen because drug binding to M_2 sites is much faster than binding to M_1, M_3 or M_4 sites: it is therefore easier to achieve equilibrium at low temperatures (Waelbroeck *et al.*, 1987; 1988a,b). The thermodynamic parameters associated with 4-DAMP binding to M_2 receptors can be explained by good hydrogen bond and van der Waals interactions (negative $\Delta H°'$) and ionic bond and hydrophobic interactions (positive $\Delta S°'$). The higher affinity of analogues 6 and 7 and the lower affinity of analogue 8 were associated, with more negative and with positive enthalpy changes, respectively. This is compatible with, respectively, more- and less favourable van der Waals interactions between analogues 6, 7 and 8 and the M_2 receptor, as compared to 4-DAMP, and supports the hypothesis that the position of the two phenyl groups are, respectively, closer (6, 7) or further away (8) from the optimal conformation required to recognize the M_2 receptor. We also measured the enthalpy and entropy changes associated with the binding of the N-methyl-(1), homo-(2), α-methyl(3), (di-*p*-tolyl)-(4) and (di-*p*-chloro)-(9) analogues of 4-DAMP to cardiac M_2 receptors.

The affinity, binding enthalpy and entropy changes of 4-DAMP and its N-ethyl analogue (1) were not significantly different. The lower affinity of homo-4-DAMP for cardiac M_2 sites is explained by a significantly smaller entropy change. The results suggest that homo-4-DAMP might induce less favourable ionic or hydrophobic interactions. α-methyl-4-DAMP (analogue 3) induced the same entropy change and a slightly lower enthalpy change than 4-DAMP when binding to M_2 sites: the α-methyl group is probably in close contact with the receptor. Modification of the phenyl rings by a *para*-chloro or *para*-methyl substituent decreased significantly the analogues' affinities for M_2 muscarinic receptors, as well as the enthalpy and entropy changes associated with drug binding. These effects might be explained by differences in the electron distribution of 4-DAMP, analogues 4 and 9, inducing differences in van der Waals interactions between the drugs and the M_2 receptor.

We did not attempt to evaluate the enthalpy and entropy changes associated with *bis*-penta-methylene 4-DAMP (5) binding because competition curves obtained with this analogue gave an anomalous (high) Hill coefficient and because this analogue was the most potent inhibitor of [^{3}H]-NMS dissociation from cardiac receptors (see below).

Allosteric effects of 4-DAMP analogues

To verify whether 4-DAMP and its analogues recognized an allosteric site on the muscarinic receptors in addition to the muscarinic binding site, we measured the effect of these molecules on [^{3}H]-NMS dissociation from cardiac M_2 receptors, a subtype more sensitive in our experience than neuroblastoma M_1 or pancreas M_3 receptors to allosteric effects.

As shown in Figure 1, all analogues tested, like atropine (Birdsall *et al.*, 1987), were capable of decreasing [^{3}H]-NMS dissociation from cardiac membranes provided that very high drug concentrations were used. For [^{3}H]-NMS binding inhibition to the four subtypes, the potency of 4-DAMP analogues varied by over 10^4 fold (from 0.1 nM to 1 μM) and demonstrated a marked structure-dependence compatible with a specific binding site. By contrast, all analogues showed a very similar (low) potency in decreasing [^{3}H]-NMS dissociation. Fluorenyl-4-DAMP (analogue 6) and *bis*-pentamethylene 4-DAMP (analogue 5) alone were active at 30 to 100 fold lower concentrations than atropine or the other 4-DAMP analogues tested. In fact the drug concentration inducing a half-maximal effect on tracer dissociation seemed to depend mostly on the size of the drug molecule rather than on a particular structure. It has been suggested (Birdsall *et al.*, 1987) that the effect of atropine on the off-rate of [^{3}H]-NMS is due to membrane perturbation rather than to a specific drug-receptor interaction. Indeed, atropine binding is competitive with [^{3}H]-NMS but affected allosterically by gallamine (Birdsall *et al.*, 1987), suggesting that it recognises the muscarinic as opposed to the allosteric binding site.

Since all drugs tested here are amphiphilic molecules with at least one positive charge and a large hydrophobic moiety, we suspect that this 'allosteric' effect might reflect alterations of the lipid environment of the receptor, rather than drug binding to the gallamine (allosteric) site on the receptor protein. At any rate, for all drugs tested in this work, the concentrations necessary to inhibit [^{3}H]-NMS binding were at least 1000 fold lower than the concentrations affecting tracer dissociation, so that this non-specific effect did not affect the competition curves described above.

Comparison with pharmacological data

The 4-DAMP analogues studied in this work all behave as muscarinic antagonists in isolated cardiac atria and in isolated ileum from guinea-pig (Barlow & Shepherd, 1985; 1986). A comparison of the pK_i values in binding studies (this work) and pA_2 values in pharmacological studies (Barlow *et al.*, 1976; Barlow & Shepherd, 1985; 1986) supports the hypothesis that the atrial receptors belong to the M_2 cardiac subtype, whereas the ileum receptors belong to the M_3 pancreatic subtype. The only exception to this rule was *bis*-pentamethylene 4-DAMP, which had a 10 fold lower potency in pancreas binding studies than expected from previous pharmacological data. The significance of this discrepancy is, however, not clear, as: (1) *bis*-pentamethylene 4-DAMP competition curves had a Hill coefficient greater than 1.0 and (2) this compound was the most potent inhibitor of [^{3}H]-NMS dissociation from cardiac receptors; two results which suggest that it might interfere with muscarinic receptors in a 'non classical' manner.

In conclusion, our results confirm that the requirements for high affinity binding to the four muscarinic receptor subtypes are different.

The structural requirements of M_3 receptors were more stringent when compared to M_2, M_1 or M_4 receptors. As a

result, most of the 4-DAMP analogues studied in this work had a different selectivity pattern from 4-DAMP itself.

At very high concentration, all 4-DAMP analogues modified the [^{3}H]-NMS dissociation rate from cardiac membranes, their potency correlating with their size, not with a specific structure, suggesting membrane disturbance rather than binding to a genuine allosteric site.

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Endothelium-dependent relaxation of rabbit middle cerebral artery to a histamine H_3-agonist is reduced by inhibitors of nitric oxide and prostacyclin synthesis

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1 The possible involvement of prostanoids and endothelium-derived relaxing factor (EDRF) in the vasodilatation induced by a histamine H_3-agonist was examined in the rabbit perfused middle cerebral artery preconstricted with K^+ (50 mM).

2 The endothelium-dependent relaxation to (**R**)-α-methylhistamine [(**R**)-α-MeHA] was competitively antagonized by thioperamide (an H_3-antagonist) with a pA_2 of 9.05, but unaffected by propranolol, atropine, L- and D-sulpiride. This effect was stereoselective since the (**S**)-isomer was 100 times less potent than the (**R**)-isomer.

3 Two inhibitors of nitric oxide synthesis, N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA), inhibited the relaxation induced by (**R**)-α-methylhistamine. The inhibitory effects of 10^{-5} M N^G-nitro-L-arginine methyl ester and 10^{-5} M N^G-monomethyl-L-arginine were reversed by equimolar concentrations of L-arginine, but strongly enhanced by 10^{-4} M tranylcypromine. Tranylcypromine alone (10^{-5} M–10^{-4} M) partially reduced the (**R**)-α-methylhistamine-induced relaxation. Both dexamethasone and indomethacin also inhibited this relaxation.

4 The results suggest that the H_3-mediated relaxation of the rabbit middle cerebral artery may involve release of both a prostanoid, probably prostacyclin, and endothelium-derived relaxing factor. The relaxant effects of these two endogenous compounds appear to be synergistic.

Keywords: Endothelium; relaxation; cerebral arteries; histamine H_3-receptor; prostacyclin; endothelium-derived relaxing factor

Introduction

Classical histamine receptors (H_1 and H_2) are found in vascular endothelial and smooth muscle cells (Levi *et al.*, 1982; Ea Kim *et al.*, 1986; Sercombe *et al.*, 1986). Recently, a novel H_3-autoreceptor has been recognised in rat brain (Arrang *et al.*, 1987; Van der Werf *et al.*, 1987), in nerves of the guinea-pig ileum (Trzeciakowski, 1987), mesenteric artery (Ishikawa & Sperelakis, 1987) and human airways (Ichinose & Barnes, 1989). The H_1- and H_2-mediated actions of histamine on effector cells are linked with the accumulation of guanosine 3′ : 5′-cyclic monophosphate (cyclic GMP) and adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP), respectively (Schwartz *et al.*, 1986; Green, 1987). In blood vessels, cyclic GMP formation is activated by endothelium-derived relaxing factor (EDRF) (Rapoport & Murad, 1983), while cyclic AMP formation is stimulated by prostacyclin (PGI_2) (Gorman *et al.*, 1977; Tateson *et al.*, 1977). The identification of nitric oxide (NO) (or a compound containing the NO ligand) as EDRF (Palmer *et al.*, 1987; Long & Berkowitz, 1989) and the finding that N^G-monomethyl-L-arginine (L-NMMA) or N^G-nitro-L-arginine methyl ester (L-NAME) are inhibitors of NO synthesis in vascular endothelium (Rees *et al.*, 1990; Woolfson & Poston, 1990) have emphasized the importance of local control of vasomotor tone.

In a previous paper, we indicated that a potent and selective H_3-agonist, (**R**)-α-methylhistamine [(**R**)α-MeHA], relaxed rabbit middle cerebral artery (MCA) by an action on H_3- but not H_1- or H_2-receptors (Ea Kim & Oudart, 1988). In the present study, we show that the (**R**)α-MeHA-induced dilatation of rabbit MCA is dependent on the endothelium and is reduced by inhibitors of NO and PGI_2 synthesis.

Method

Isolated artery segment

The MCA was excised from 'Fauve de Bourgogne' rabbits (2–3 kg) after death. The artery segment (about 8 mm long) was cannulated with a short polished hypodermic needle (0.4 mm o.d.), mounted in an organ bath at 37°C and perfused at a constant rate (0.8–1.2 ml min^{-1}). The buffer solution contained (in mM): NaCl 126, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1.3, $NaHCO_3$ 18, NaH_2PO_4 1.2 and glucose 5.5, was gassed with 95% O_2:5% CO_2 giving a pH of 7.30–7.45 and was maintained at 37°C. Further details of the experimental arrangement have been published previously (Oudart *et al.*, 1981). In some arteries, the endothelium was removed by gently rubbing the luminal surface with a pipe cleaner. The failure of acetylcholine (3×10^{-5} M) to induce relaxation of these arteries was taken as an indication of endothelium removal.

Protocol

After equilibration for 45 min, the buffer solution was changed to a high-K^+ solution (50 mM K^+, Na^+ reduced by 50 mM) to produce a maximal preconstriction for testing the relaxant effects of drugs. The mean initial pressure (± s.e.mean) in high-K^+ solution was (32.6 ± 2.5) mmHg ($n = 76$). In some experiments, the effects of agonists were tested directly on resting arteries.

Increasing concentrations of (**R**)α-MeHA or its enantiomer (**S**)-α-methylhistamine [(**S**)α-MeHA] were then added to the perfusate reservoir. When an antagonist (thioperamide, propranolol, L- or D-sulpiride, atropine), an inhibitor of prostaglandin synthesis (dexamethasone), an inhibitor of cyclo-oxygenase (indomethacin) or a PGI_2-synthesis inhibitor (tranylcypromine) was to be tested, it was added to the perfusate reservoir 30 min before concentrations of (**R**)α-MeHA. This pre-incubation time was 10–15 min in the case of an inhibitor of NO synthesis (L-NAME or L-NMMA). The reversibility of the inhibition by L-NAME or L-NMMA was tested by combined incubation with equimolar concentrations of L- and D-arginine. Papaverine (10^{-4} M) was added at the end of each experiment in order to define 100% relaxation. One series of concentrations of the agonist was tested on each artery.

Data analysis

All responses (relaxations and contractions) were expressed as a percentage of the maximum papaverine-induced dilatation.

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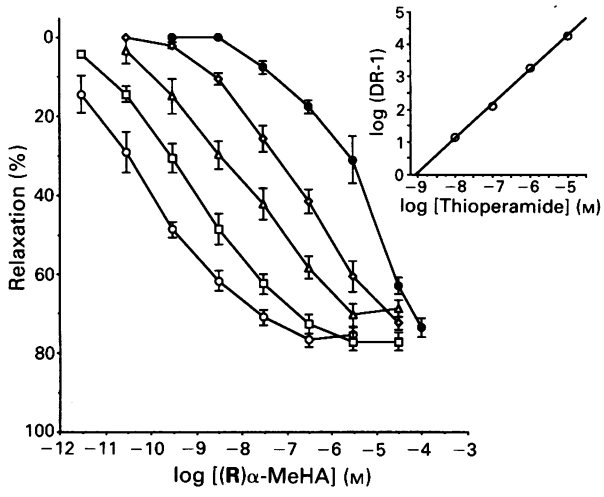


Figure 1 Effects of (R)-α-methylhistamine ((R)α-MeHA) alone (○) and with 10^{-8} M (□), 10^{-7} M (△), 10^{-6} M (◇) or 10^{-5} M (●) thioperamide in rabbit middle cerebral artery (MCA) preconstricted with 50 mM K^+. The EC_{50} values were $(9.4 \pm 2.3) \times 10^{-11}$ M, $(1.4 \pm 0.4) \times 10^{-9}$ M, $(1.6 \pm 0.6) \times 10^{-8}$ M, $(1.9 \pm 0.5) \times 10^{-7}$ M and $(3.0 \pm 0.8) \times 10^{-6}$ M, respectively. Each point represents the mean and vertical bars the s.e.mean, $n = 6$. The insets represent a Schild plot of the data, dose-ratio (DR) being determined from EC_{50} values for (R)α-MeHA. The slope for the regression of log (DR-1) versus log [thioperamide] was 1.1 ± 0.1, the coefficient of correlation was 1 and the pA_2 was 9.05.

Median effective concentration (EC_{50}), maximum relaxation E_m) and slope of regression lines were calculated from the concentration-response curve plotted for each agonist in each artery. The comparison of values was performed by Student's t test. Differences were considered significant when $P < 0.05$. Dispersion of the values about the mean was indicated by the standard error of the mean (s.e.mean).

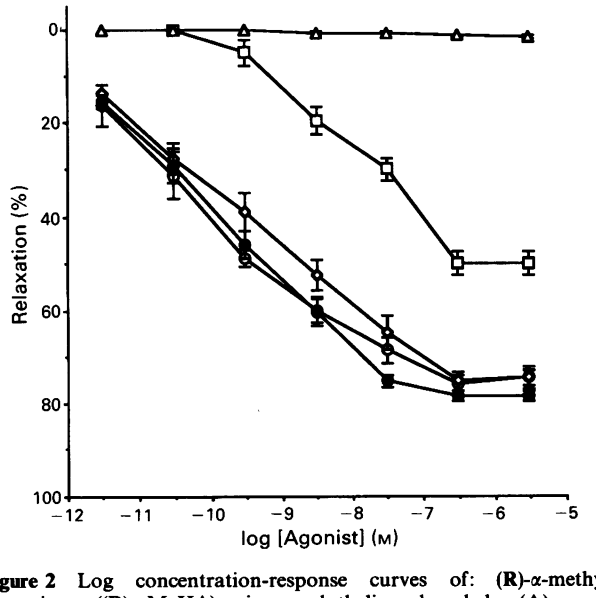


Figure 2 Log concentration-response curves of: (R)-α-methylhistamine ((R)α-MeHA) in endothelium-denuded (△), and endothelium-containing (○) K^+-preconstricted rabbit middle cerebral artery (MCA); (R)α-MeHA in the presence of 3×10^{-7} M atropine (◇) or 3×10^{-6} M propranolol (●) in endothelium-containing K^+-preconstricted rabbit MCA; (S)α-MeHA (□) [$EC_{50} = (1.0 \pm 0.6) \times 10^{-8}$ M] in endothelium-containing K^+-preconstricted rabbit MCA. Each point represents the mean and vertical bars the s.e.mean, $n = 6$.

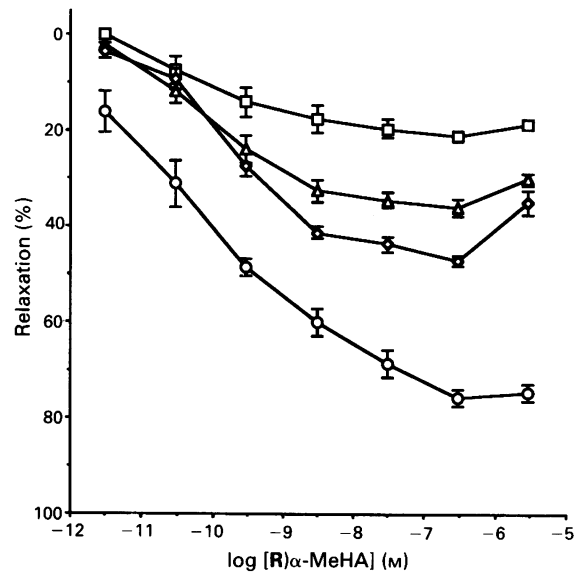


Figure 3 Effects in K^+-preconstricted rabbit middle cerebral artery (MCA) of (R)-α-methylistamine ((R)α-MeHA) alone (○) and in the presence of 10^{-6} M(△), and 10^{-5} M (□) N^G-nitro-L-arginine methyl ester (L-NAME), and 10^{-5} M L-NAME plus 10^{-5} M L-arginine (◇). Each point represents the mean and vertical bars the s.e.mean, $n = 6$.

Drugs

The compounds were kindly donated by: Dr J.C. Schwartz, France ((R)- and (S)-α-methylhistamine dihydrochloride, thioperamide); Merck Sharp et Dohme-Chibret, France (indomethacin); ICI. pharma, France (propranolol hydrochloride); Delagrange, France (L- and D-sulpiride); Houdé, France (papaverine hydrochloride). The following compounds were purchased from: Research Biochemicals Inc., U.S.A. (N^G-monomethyl-L-arginine acetate); Sigma Chemical Co., U.S.A. (dexamethasone, atropine, tranylcypromine hydrochloride, N^G-nitro-L-arginine methyl ester hydrochloride, L- and D-arginine hydrochloride). Thioperamide and indomethacin were dissolved in dimethylsulphoxide and ethanol (10^{-3} M stock solution), respectively.

Results

Responses to (R)-α-methylhistamine

(R)α-MeHA (3×10^{-12}–3×10^{-6} M) induced concentration-dependent relaxation of high K^+-preconstricted rabbit MCA, reaching approximately 75% of the papaverine-induced maximum dilatation. When 10^{-8} M, 10^{-7} M, 10^{-6} M or 10^{-5} M thioperamide was present, the concentration-response curve for (R)α-MeHA-induced relaxation was shifted to the right without a significant change of the E_m (Figure 1). Schild plot analysis indicated that antagonism by this compound was competitive, with a pA_2 of 9.05 (Figure 1). Thioperamide alone (3×10^{-12}–10^{-5} M) elicited a slight relaxation reaching $9 \pm 5\%$ ($n = 6$). Neither 3×10^{-7} M atropine, 3×10^{-6} M propranolol (Figure 2) nor 3×10^{-6} M L- or D-sulpiride (not shown, $n = 6$) significantly reduced the (R)α-MeHA-induced vasodilatation. Removal of the endothelium did, however, abolish the relaxation to (R)α-MeHA (Figure 2). (R)α-MeHA (3×10^{-12}–3×10^{-6} M) did not significantly affect resting tone in arteries (not shown, $n = 6$). The (S)-isomer (3×10^{-11}–3×10^{-6} M) also induced relaxation of the endothelium-containing K^+-preconstricted MCA; the concentration-response curve was parallel to that of the (R)-isomer (slope: (R)α-MeHA, 16.2 ± 2.4; (S)α-MeHA, 14.5 ± 0.9; difference not significant), the E_m was significantly smaller ($P < 0.001$) (Figure 2), and the EC_{50} was about 100 times higher than that of (R)α-MeHA (Figures 1 and 2).

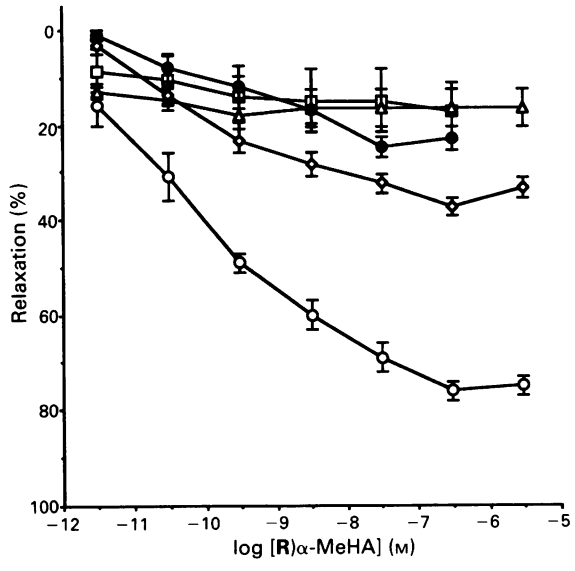


Figure 4 Effects in K^+-preconstricted rabbit middle cerebral artery (MCA) of (R)-α-methylhistamine ((R)α-MeHA) alone (○) and with 10^{-5} M dexamethasone (□), 10^{-5} M indomethacin (△), 10^{-5} M (◇) and 10^{-4} M (●) tranylcypromine. Each point represents the mean and vertical bars the s.e.mean, $n = 6$.

Inhibition by L-*NAME and* L-*NMMA*

Both inhibitors antagonized the (R)α-MeHA-induced relaxation in a concentration-dependent manner and this effect was maximal (approximately 72% and 58% inhibition) after incubation with 10^{-5} M L-NAME (Figure 3) and 10^{-5} M L-NMMA, respectively. Higher concentrations ($>10^{-5}$ M) of inhibitors induced no further reduction of relaxation. L-NAME or L-NMMA alone caused a weak and not significant contraction of the endothelium-containing K^+-preconstricted arteries ($5 \pm 2\%$, $n = 6$, L-NAME and $3 \pm 1\%$, $n = 6$, L-NMMA of the maximum papaverine-induced dilatation). The addition of L- but not D-arginine (10^{-5} M) reversed the L-NAME- (Figure 3) and L-NMMA-induced inhibition of the response to (R)α-MeHA (about 58%, L-NMMA).

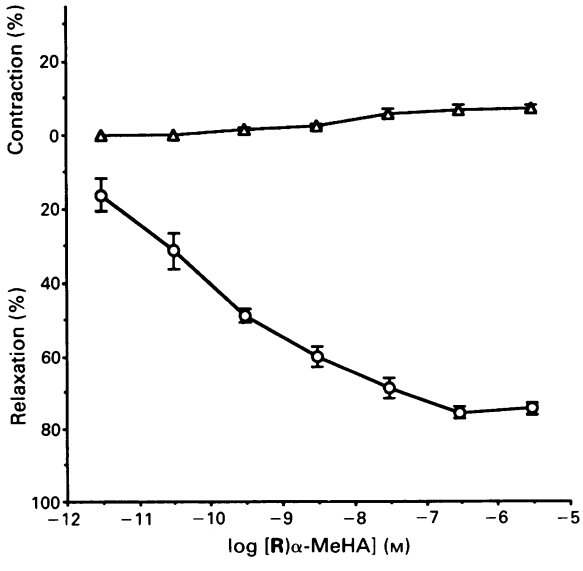


Figure 5 Reversal of (R)-α-methylhistamine ((R)α-MeHA)-induced relaxation of K^+-preconstricted rabbit middle cerebral artery (MCA) to contraction following treatment with 10^{-4} M tranylcypromine plus 10^{-5} M N^G-nitro-L-arginine methyl ester (L-NAME) (△); control is (○). Each point represents the mean and vertical bars the s.e.mean, $n = 6$.

L- but not D-arginine (3×10^{-11}–10^{-5} M) by itself induced a slight relaxation of the endothelium-containing K^+-preconstricted rabbit MCA, reaching $4 \pm 0.3\%$, $n = 7$, $P < 0.05$.

Effects of dexamethasone, indomethacin and tranylcypromine

Dexamethasone (10^{-5} M), indomethacin (10^{-5} M) and tranylcypromine (10^{-5} and 10^{-4} M) each inhibited the relaxation induced by (R)α-MeHA in high-K^+-preconstricted arteries (Figure 4). At 10^{-5} M tranylcypromine, the maximum vasodilatation was decreased by approximately 50% and at 10^{-4} M by approximately 68%. Higher concentrations ($>10^{-4}$ M) of tranylcypromine did not induce further reduction of relaxation (not shown, $n = 6$).

Effects of combined blockade with tranylcypromine and L-*NAME or* L-*NMMA*

The simultaneous application of 10^{-4} M tranylcypromine and 10^{-5} M L-NAME (Figure 5) or L-NMMA abolished and even slightly reversed the relaxation to (R)α-MeHA to a contraction.

Discussion

A major new finding of this study is that (R)α-MeHA, a potent and specific H_3-agonist, induces relaxation of high-K^+-preconstricted rabbit MCA. In a previous paper, we indicated that the relaxant effect of (R)α-MeHA in rabbit MCA was not mediated by H_1- or H_2-receptor activation (Ea Kim & Oudart, 1988), and probably arose through an action on H_3-receptors. This is supported by the present finding that the vasodilatation was competitively antagonized by thioperamide (a specific H_3-antagonist according to our own unpublished results and those of Arrang *et al.*, 1987) with a pA_2 value of 9.05. This pA_2 value is close to that (8.96) found for blockade of H_3-mediated inhibition of release of [^{3}H]-histamine in rat brain slices (Arrang *et al.*, 1987). Furthermore, the (R)α-MeHA-mediated relaxation was not affected by β-adrenoceptor, muscarinic or dopamine receptor antagonists. (R)α-MeHA was stereoselective in our model since the EC_{50} value for the (R)-isomer was about 100 times less than that of the (S)-isomer. This is in agreement with their relative effects on the release of [^{3}H]-histamine (Arrang *et al.*, 1987).

Although we do not provide direct evidence that (R)α-MeHA releases dilator substances from the endothelium, two lines of evidence suggest that the relaxation in the rabbit MCA may be endothelium-dependent. Firstly, mechanical removal of the endothelium abolished (R)α-MeHA-induced vasodilatation. Secondly, L-NAME and L-NMMA, which have been shown to inhibit competitively and in an enantiomerically specific manner endothelial NO synthesis from L-arginine (Knowles *et al.*, 1990), antagonized in a concentration-dependent manner the effect of the amine. In addition, the blockade by L-NAME and L-NMMA was reversed by L- but not D-arginine as previously described (Palmer *et al.*, 1988; Moore *et al.*, 1990). Furthermore, L- but not D-arginine induced slight but significant endothelium-dependent relaxation of rabbit MCA, and this is in agreement with the findings of Palmer *et al.* (1988) and Thomas *et al.*, (1986) in rat and rabbit aorta. We also found that the inhibitory effect of L-NAME was greater than that of L-NMMA, and this is consistent with the findings of Mülsch & Busse (1990) and Moore *et al.* (1990). The concentrations of L-NAME and L-NMMA used to produce maximal inhibition of (R)α-MeHA-induced relaxation (10^{-5} M) are lower than those used by other workers to inhibit NO synthesis (up to 10^{-4} M) (Rees *et al.*, 1990; Woolfson & Poston, 1990). One possible explanation is that in rabbit MCA, the endothelial

enzyme responsible for the synthesis of NO is more sensitive to inhibition by L-NAME and L-NMMA than those previously described. These results suggest that endothelial cells possess a biochemical pathway converting L-arginine to NO which might participate in the relaxation of rabbit MCA to (R)α-MeHA.

The maximum inhibition of the endothelium-dependent relaxation induced by (R)α-MeHA in rabbit MCA was 60–70%. In rings of rabbit aorta, L-NMMA inhibited acetylcholine-induced relaxation by approximately 65% (Rees *et al.*, 1989), while L-NAME inhibited it by approximately 90% (Kobayashi & Hattori, 1990). In the rabbit femoral artery, L-NAME inhibited vasodilatation to acetylcholine by about 80% (Mülsch & Busse, 1990). These authors suggested that the difference in the maximum effect in different preparations is probably due to variations in the uptake and metabolism of these compounds by endothelial cells rather than the involvement of another mediator. We found, however, that (R)α-MeHA-induced vasodilatation was also antagonized by dexamethasone (an inhibitor of prostaglandin synthesis probably at the level of phospholipase A_2 according to the results of Floman & Zor (1976), Gryglewski *et al.* (1975), and Russo-Marie *et al.* (1981)), indomethacin and tranylcypromine. In preliminary experiments, we observed similar inhibition of relaxation induced by (R)α-MeHA in the presence of quinacrine, another phospholipase A_2 inhibitor (Ea Kim, unpublished results). Furthermore, the combination of L-NAME or L-NMMA with tranylcypromine abolished and even slightly reversed the relaxation to contraction. It is possible, therefore, that a prostanoid may also be involved in the relaxation to (R)α-MeHA. Shimokawa *et al.* (1988) demonstrated that exogenous prostacyclin releases EDRF by increasing cyclic AMP production, and thus potentiates its action in coronary arteries of the pig. It is therefore possible that the apparent synergy between EDRF and prostacyclin underlying (R)α-MeHA-induced relaxation could arise either by the independent action of these two vasodilators or by a more complex interaction where they affect the release of each other.

In perfused rabbit MCA, stimulation of H_3-receptors by (R)α-MeHA promotes the biosynthesis of a prostanoid, possibly PGI_2, and EDRF. These two agents appear to induce vasodilatation synergistically by a mechanism that remains obscure.

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Actions of methoctramine, a muscarinic M_2 receptor antagonist, on muscarinic and nicotinic cholinoceptors in guinea-pig airways *in vivo* and *in vitro*

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1 The effects of the muscarinic M_2 receptor antagonist methoctramine, on contractions of airway smooth muscle induced by cholinergic nerve stimulation and by exogenously applied acetylcholine (ACh), have been investigated *in vivo* and *in vitro* in guinea-pigs.

2 Stimulation of the preganglionic cervical vagus nerve in anaesthetized guinea-pigs, caused bronchoconstriction and bradycardia which were mimicked by an intravenous dose of ACh. The muscarinic M_2 antagonist, methoctramine (7–240 nmol kg^{-1}), inhibited the bradycardia induced by both vagal stimulation and ACh (ED_{50}: 38 ± 5 and 38 ± 9 nmol kg^{-1}, respectively). In this dose-range, methoctramine facilitated vagally-induced bronchoconstriction (ED_{50}: 58 ± 5 nmol kg^{-1}), despite some inhibition of ACh-induced bronchoconstriction (ED_{50}: 81 ± 11 nmol kg^{-1}). The inhibition of ACh-induced bronchoconstriction and hypotension was dose-dependent, but was not statistically significant until doses of 120 nmol kg^{-1} and 240 nmol kg^{-1} respectively.

3 In the guinea-pig isolated, innervated tracheal tube preparation, methoctramine (0.01–1 μM) caused facilitation of contractions induced by both pre- and postganglionic nerve stimulation, whereas contractions induced by exogenously applied ACh were unaffected. Higher concentrations of methoctramine ($\geq$ 10 μM), reduced responses to both nerve stimulation and exogenous ACh, indicating blockade of postjunctional muscarinic M_3 receptors.

4 ACh caused a slow maintained increase in tone of the tracheal tube and at the same time reduced the contractions induced by nerve stimulation. This inhibitory effect of ACh on neuronally mediated responses was antagonized by methoctramine (0.01–1 μM) in a concentration-dependent manner. However, the ACh-induced tone change was unaffected by methoctramine in this concentration-range, indicating a lack of muscarinic M_3 receptor antagonist activity in this concentration-range.

5 The effect of methoctramine on responses induced by pre- and postganglionic nerve stimulation was not identical. At concentrations of methoctramine of 1 μM and greater, preganglionic stimulation-induced contractions were reduced when compared to those induced by postganglionic stimulation, suggesting an inhibitory effect of methoctramine on ganglionic transmission. This ganglion blocking action of methoctramine was not due to its reported M_1 receptor antagonist activity (blocking facilitatory M_1 receptors in the ganglia) since pirenzepine was without effect in this preparation. We believe that the ganglionic blocking action of metoctramine is due to its nicotinic receptor antagonist properties, since the concentration of methoctramine inhibiting ganglionic transmission in the tube preparation (1 μM) was shown to inhibit contractions induced by the nicotinic agonist, 1,1-dimethyl-4-phenyl-piperazine in tracheal strips.

6 These results show that methoctramine is able to demonstrate adequately the presence of autoinhibitory receptors functionally both *in vivo* and *in vitro* and confirms their pre-junctional location on pulmonary cholinergic nerve terminals and their classification as muscarinic M_2 subtypes. These results also indicate that while methoctramine is a potent muscarinic M_2 receptor antagonist, it does not possess the required selectivity to discriminate between cholinoceptor subtypes in preparations, such as the airways, where mixed populations of muscarinic and nicotinic cholinoceptors exist.

Keywords: Methoctramine; muscarinic and nicotinic cholinoceptors; guinea-pig airways

Introduction

The parasympathetic nerves supplying the lung and trachea provide the dominant neuronal control of airway smooth muscle tone. The events leading to nerve-induced contraction of airway smooth muscle involve the interaction of acetylcholine (ACh) at a number of different cholinoceptors (Barnes *et al.*, 1988). Transmission through the parasympathetic ganglia involves release of ACh from preganglionic nerve terminals onto nicotinic receptors on the cell bodies of the postganglionic nerve fibres (Hawkins & Paton, 1958). This is the main pathway for transmission through parasympathetic ganglia, but in some species, including man and rabbit, a role for facilitatory muscarinic M_1 receptors in parasympathetic ganglia is suggested (Bloom *et al.*, 1987; Lammers *et al.*, 1989). Activation of nicotinic receptors by ACh leads to the propagation of an action potential in the postganglionic nerve fibre and the release of ACh onto muscarinic M_3 receptors located on airway smooth muscle (Roffel *et al.*, 1988). Muscarinic receptors have also been identified on the terminals of pulmonary parasympathetic nerves where they have an autoinhibitory role. Activation of these receptors by the neurotransmitter inhibits the outflow of ACh from these postganglionic nerve terminals (Fryer & Maclagan, 1987; Kilbinger *et al.*, 1991) thereby controlling the degree of neuronally induced airway contraction. Autoinhibitory muscarinic receptors have been found in a number of species including guinea-pig, cat, dog, man and rat, (Blaber *et al.*, 1985; Ito & Yoshitomi, 1988; Minette & Barnes, 1988; Aas & Maclagan, 1990). Blockade of these receptors with the muscarinic receptor antagonist, gallamine, leads to a facilitation of neuronally-induced bronchoconstriction while having little or no effect on bronchoconstriction induced by exogenous ACh (Fryer & Maclagan, 1984).

The results obtained with gallamine suggested that the prejunctional receptors are of the M_2 subtype and this possibility

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has been further investigated in the present study by use of a novel potent and selective muscarinic M_2 receptor antagonist, methoctramine (Melchiorre *et al.*, 1987). This antagonist has been shown to have a high degree of selectivity for muscarinic M_2 receptors present in atria when compared to the muscarinic M_3 receptors found in tracheal smooth muscle (Giraldo *et al.*, 1988). Clearly such an antagonist provides a useful experimental tool for investigating the actions of the autoinhibitory M_2 receptors on pulmonary parasympathetic nerves, since both M_2 and M_3 receptors are present in airways on opposite sides of the neuroeffector junction and their activation exerts opposing effects. Methoctramine has also been reported to have antagonist properties at two other subtypes of cholinoceptors (Melchiorre *et al.*, 1987) which are found to be present in the airways, namely nicotinic and muscarinic M_1 receptors. Nicotinic receptors are found in parasympathetic ganglia (Hawkins & Paton, 1958) and facilitatory M_1 receptors have been demonstrated in rabbit and human airways (Bloom *et al.*, 1987; Lammers *et al.*, 1989).

The work described here investigates the actions of methoctramine *in vivo* and *in vitro*, on bronchoconstrictor responses elicited by stimulation of parasympathetic nerves innervating airway smooth muscle in the guinea-pig. Similar work with the M_2 antagonist, gallamine, has previously been performed in this laboratory (Fryer & Maclagan, 1987) and we wished to determine what advantages methoctramine has over gallamine. Ganglionic mechanisms were also studied *in vitro* by comparing responses elicited by pre- and postganglionic stimulation and revealed ganglionic nicotinic receptor antagonist properties of methoctramine. Abstracts of this work have been presented to the British Pharmacological Society (Watson *et al.*, 1989; 1990a) and the XIth International Congress of Pharmacology (Watson *et al.*, 1990b).

Methods

In vivo *preparation*

In vivo experiments were performed according to the protocol described by Fryer & Maclagan (1987). Blood pressure was recorded via a Statham P23 pressure transducer and expressed as the mean arterial blood pressure (BP) given by the equation: BP-Diastolic pressure + 1/3 Pulse pressure, where the pulse pressure is the difference between systolic and diastolic pressures. Bronchoconstriction was induced alternately by stimulation of the peripheral end of the cut right vagus nerve (30 Hz, 0.2 ms, 10–40 V, for 5 s every 180 s) and by intravenous administration of acetylcholine (5–10 $\mu g\,kg^{-1}$, 90 s after each vagal stimulation). Bronchoconstriction was recorded as an increase in pulmonary inflation pressure (Ppi) over the basal inflation pressure change produced by the pump, before and during cumulative i.v. administration of methoctramine.

In vitro *vagally-innervated tracheal tube preparation*

In vitro experiments using the vagally-innervated guinea-pig tracheal tube preparation (Blackman & McCaig, 1983), were performed according to the protocol described by Faulkner *et al.* (1986). Indomethacin (5 μM) and propranolol (1 μM) were present in the Krebs-Henseleit buffer solution (composition mM: NaCl 118.4, KCl 4.9, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, $CaCl_2 6H_2O$ 2.5, glucose 11.1) throughout all the experiments to eliminate prostaglandin-induced tone and noradrenergic nerve stimulation during transmural stimulation (TMS), respectively. A ganglion blocking concentration of hexamethonium (75 μM) was also present during TMS to ensure that responses recorded were due to stimulation of postganglionic nerve fibres. Contractions of the trachea were measured as an increase in intraluminal pressure (ILP), and were recorded on a polygraph Grass Model 7D.

After mounting in the organ bath, tissues were left to equilibrate for at least 60 min during which time the vagus nerves were stimulated regularly for 5 s at 40 s intervals (30 V; 30 Hz; 0.2 ms preganglionic stimulation (PGS)). Once stable responses were established after this period, cumulative concentration-response curves to ACh (0.1–100 μM) were performed during PGS, in the absence and presence of single concentrations of methoctramine (0.01–1 μM). Each tissue acted as its own control, thus enabling paired analysis of the results. In a second set of experiments, cumulative concentrations of methoctramine (0.01–10 μM) were given during either PGS or TMS, in the absence of exogenous ACh. In a further set of experiments, pirenzepine (1–300 nM) was applied to the preparation during PGS in a cumulative manner. Cumulative concentrations of ACh were added to the organ bath in the absence and then the presence of either pirenzepine or methoctramine, to determine postjunctional actions of both these antagonists.

In vitro *tracheal strip preparation*

Guinea-pigs were killed by cervical dislocation. The trachea was removed, cut open longitudinally along its ventral surface and divided into six sections, each containing 4–5 cartilaginous rings. The resultant tracheal strips were then suspended in 10 ml organ baths, at a resting tension of 2 g, which was found in preliminary experiments to produce optimal contractions to ACh. Isometric contractions of the tracheal strips were measured with Grass FT03 force displacement transducers and recorded on a polygraph Grass Model 7D. The organ baths contained Krebs Henseleit buffer solution maintained at 37°C and oxygenated with 95% O_2: 5% CO_2. Indomethacin (5 μM) was present in the Krebs solution to eliminate prostaglandin-induced tone.

After equlibration for 60 min, cumulative concentrations of ACh were added to the organ bath. The tissues were then washed 6–8 times over a further 60 min period, until a stable baseline was re-established. Reproducible, consecutive responses to the nicotinic agonist 1,1-dimethyl-4-phenyl-piperazine (DMPP) could not be obtained because of tachyphylaxis, so tissues were paired according to their responsiveness to ACh in order that one should act as the control while the other received methoctramine. Vehicle or a single concentration of methoctramine in the range 0.1–1.0 μM were administered to each pair of tissues, 20 min before the addition of cumulative concentrations of DMPP. In parallel experiments, the addition of 1 μM tetrodotoxin and 1 μM atropine to two separate strips confirmed the neuronal, cholinergic nature of the DMPP-induced contractions.

Drugs

The following drugs were used: acetylcholine bromide, histamine acid phosphate and atropine sulphate were obtained from BDH Ltd., (Dagenham, Essex). Hexamethonium bromide, indomethacin, succinylcholine, urethane ethyl carbamate, tetrodotoxin and 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP) were from Sigma Chemical Co. Ltd. (Poole, Dorset). Pirenzepine (Boots Chemicals Ltd., Nottingham) and propranolol hydrochloride (Imperial Chemical Industries Ltd,. Andover, Hants) were also used. Methoctramine was a generous gift from Dr C. Melchiorre, University of Camerino, Italy.

All drugs were made up fresh each day in 0.9% (w/v) saline, with the exception of DMPP which was made up in distilled water and of indomethacin which was made up in a buffer of composition (mM): KH_2PO_4 19.77 and Na_2HPO_4 118.34, adjusted to pH 7.8 with NaOH, and sonicated for 3 min. Ascorbic acid (1 μM) was added to the histamine solutions to prevent oxidation.

Statistical analysis of data

The Wilcoxon matched-pairs signed-ranks test was used to determine the level of significance of differences resulting from treatments within the same preparation and the Mann-Whitney U-test was used to determine the level of significance of differences between treatments in different preparations. All

results are expressed as mean values ± standard error of the mean (s.e.mean).

Results

Effect of methoctramine in vivo

Stimulation of the peripheral end of the cut right vagus nerve during the control period, produced an increase in pulmonary inflation pressure of 8 ± 1 mmH_2O, from a basal value of 100 ± 6 mmH_2O and a fall in heart rate of 205 ± 11 beats per min (b.p.m.), from a basal rate of 327 ± 13 b.p.m. A bronchoconstriction of similar magnitude to that induced by vagal nerve stimulation (6 ± 1 mmH_2O, from the same basal Ppi) could be produced by i.v. ACh and this was associated with a fall in heart rate of 170 ± 31 b.p.m. (from the same basal rate of 327 ± 13 b.p.m.). While both vagus nerve stimulation and i.v. ACh produced a fall in mean arterial blood pressure, the effect was only accurately measurable when induced by ACh (fall of 9 ± 2 mmHg, from a basal value of 36 ± 3 mmHg) since that induced by vagal nerve stimulation was smaller and shorter in duration.

Vagal nerve stimulation and i.v. ACh were given alternately at 90s intervals, while methoctramine was given in a cumulative manner by i.v. injections every 9 min. Preliminary experiments had shown that the peak effects of methoctramine in the lung were obtained within 6–9 min of i.v. injection and that recovery of control responses could be achieved within 15–20 min.

The effects of methoctramine on vagally- and ACh-induced bronchoconstriction and bradycardia are summarised in Figure 1. Methoctramine caused a dose-dependent inhibition of bradycardia induced by either vagal nerve stimulation (ED_{50}: 38 ± 5 nmol kg^{-1}) or i.v. ACh (ED_{50}: 38 ± 9 nmol kg^{-1}). In contrast, vagally-induced bronchoconstriction was facilitated by methoctramine despite some inhibition of ACh-induced bronchoconstriction. Inhibition of ACh-induced bronchoconstriction was statistically significant at doses of methoctramine of 120 nmol kg^{-1} and greater.

Effect of methoctramine on ACh-induced hypotension

ACh-induced hypotension was not significantly affected by methoctramine at doses up to 120 nmol kg^{-1}, but at the highest dose of 240 nmol kg^{-1} there was approximately a 30% inhibition, which was statistically significant (Figure 2).

Effect of methoctramine on the in vitro *innervated tracheal tube preparation*

In the absence of prostaglandin-induced tone the tracheal tube preparation had a low constant intraluminal pressure (ILP). Contractions of the tube induced by nerve stimulation or spasmogen added to the organ bath, were recorded as an increase in ILP. Exogenously applied ACh produced a slow contraction which was insensitive to tetrodotoxin (1 μM) but inhibited by atropine (1 μM), while stimulation of either pre- or postganglionic cholinergic nerves for 5 s caused rapid contractions both of which were blocked by tetrodotoxin and atropine.

Application of exogenous ACh, during nerve stimulation, caused a slow, concentration-dependent increase in basal tone of the trachea, while at the same time inhibited the contractions induced by PGS (Figure 3a). Similar tone changes induced by the spasmogen histamine (1–10 μM) did not cause inhibition of nerve-induced contractions. At concentrations of ACh up to 10 μM the sum of the slow increase in pressure caused by ACh plus the rapid increase in pressure caused by neuronally released ACh was much smaller than the pressure increase achieved with the maximum concentration of ACh used (0.3 mM). The reduction in PGS-induced contractions caused by ACh was antagonized by methoctramine (Figure 3b 0.01 μM and c 1 μM), producing a rightward shift in the concentration-effect curves for ACh. These concentrations of

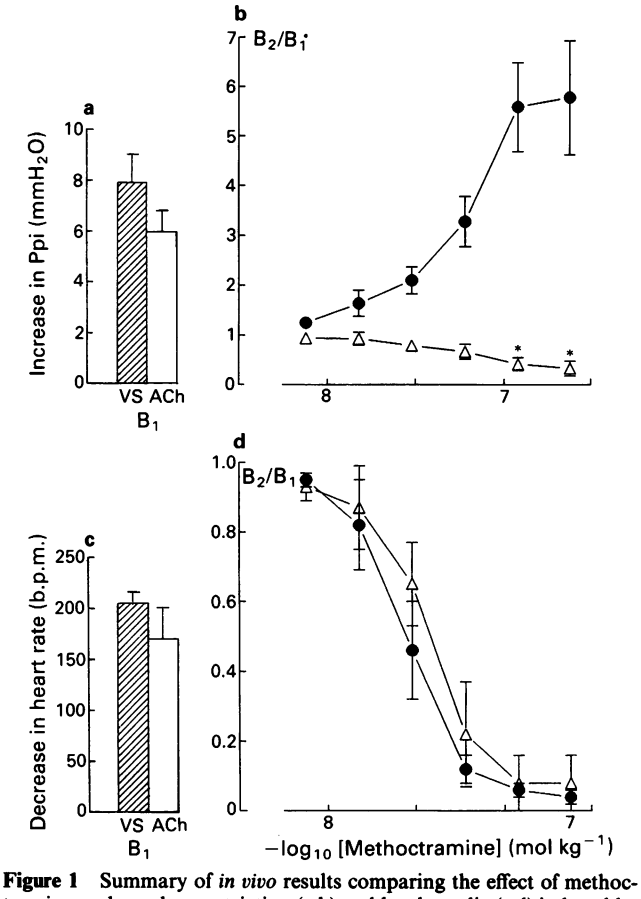


Figure 1 Summary of *in vivo* results comparing the effect of methoctramine on bronchoconstriction (a,b) and bradycardia (c,d) induced by either vagal nerve stimulation (VS: 30 Hz, 0.2 ms, 5 s, 10–40 V, solid circles and hatched columns) or by intravenous acetylcholine (ACh: 5–10 μg kg^{-1} open triangles and open columns). The control responses, before methoctramine (B_1), are shown (a) and (c) in absolute units. Results (b) and (d) are expressed as the ratio B_2/B_1 where B_2 is the response after methoctramine. All points are the mean with s.e.mean shown by vertical lines; $n = 8$; * $P < 0.05$ for inhibition of ACh-induced bronchoconstriction.

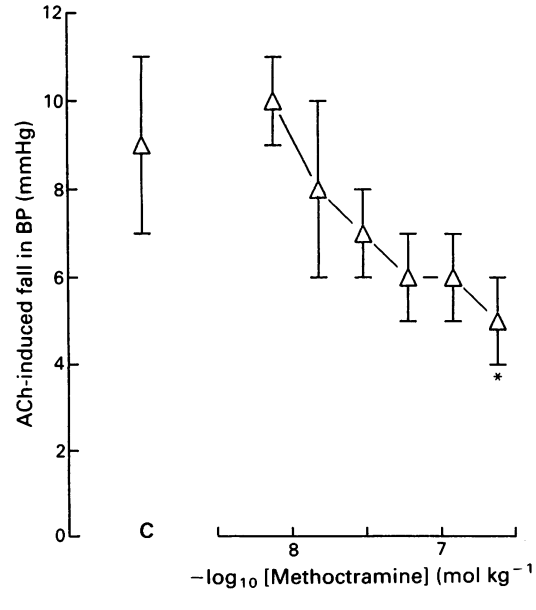


Figure 2 The effect of cumulative doses of methoctramine on the fall in mean arterial blood pressure (BP, mmHg) induced by intravenous acetylcholine (ACh, 5–10 μg kg^{-1}), in anaesthetized guinea-pigs. C indicates the mean control value before addition of methoctramine. All points are the mean with s.e.mean shown by vertical bars, $n = 8$; * $P < 0.005$, Wilcoxon-paired sign-ranked test compared with C.

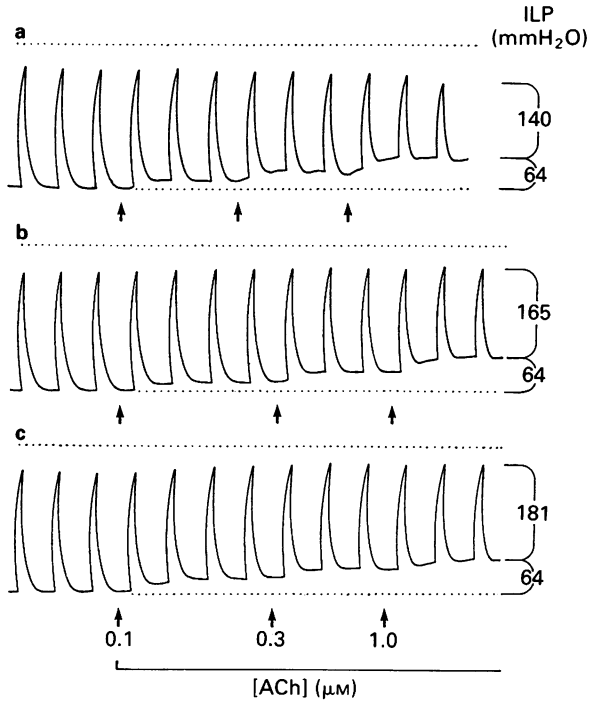


Figure 3 Contractions of the isolated tracheal tube elicited by preganglionic nerve stimulation (PGS: 30 V, 30 Hz, 0.2 ms, for 5 s every 40 s), recorded as an increase in intraluminal pressure (ILP) in mmH_2O. Acetylcholine (ACh) was added cumulatively to the organ bath during PGS, in the absence (a) and presence of methoctramine (0.01 μM (b) and 1 μM (c)) in the same preparation. The size of the stimulation-induced contraction (204 mmH_2O) before addition of methoctramine, was the same in (a), (b) and (c). The distance between the dotted lines on each panel, indicates the maximum contractile response (274 mmH_2O) attained to 0.1 mM ACh.

methoctramine had no effect on the contractions induced by exogenously applied ACh, though at the highest concentration of methoctramine used (10 μM) there was significant inhibition of the ACh concentration-effect curve (Figure 4). Table 1 summarises the results showing that methoctramine (0.01, 0.1 and 1 μM) caused a concentration-dependent antagonism of the inhibitory actions of ACh on PGS-induced contractions.

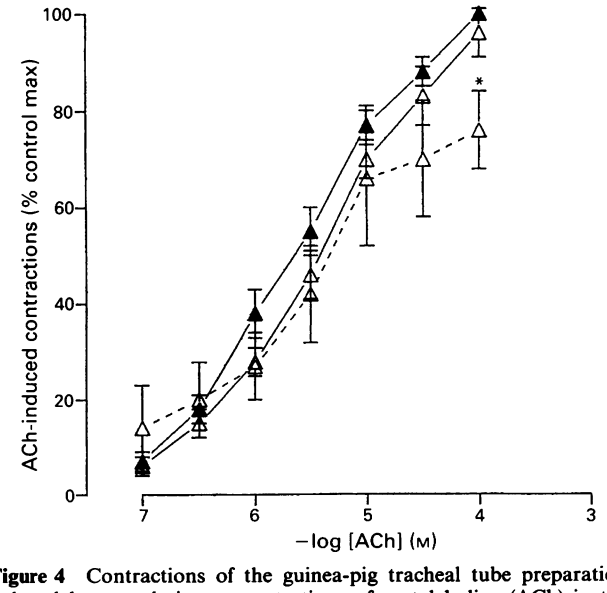


Figure 4 Contractions of the guinea-pig tracheal tube preparation induced by cumulative concentrations of acetylcholine (ACh) in the absence (▲—▲) and presence of methoctramine 1 μM (△—△) and 10 μM (△----△). Results are expressed as a % of the maximum contraction to ACh in the absence of methoctramine (control) and are the mean (s.e.mean shown by vertical lines) of at least 5 data points (* $P < 0.05$).

Table 1 Antagonism of acetylcholine (ACh)-induced inhibition of preganglionic stimulation-induced contractions of the guinea-pig isolated trachea, by methoctramine

	% *inhibition by ACh* (1 μM)		
Methoctramine (μM)	*Control* A	*+Methoctramine* B	*Difference* (A − B)
0.01	26 ± 8	16 ± 8	10 ± 1]
0.1	27 ± 6	10 ± 9	17 ± 5 *
1.0	30 ± 7	0 ± 8	30 ± 8]
	**		

Effect of methoctramine (0.01, 0.1 and 1 μM) on the inhibitory effect of ACh on contractions induced by PGS, compared at a concentration of ACh of 1 μM. This concentration of ACh produced an increase in tone which did not contribute to the inhibition observed to PGS. (Results are expressed as mean ± s.e.mean, $n = 6$. ** $P < 0.025$, according to Wilcoxon-paired sign-ranked test; * $P < 0.05$, according to a Mann-Whitney U test).

In contrast, when the effect of methoctramine (0.01–1 μM) was studied on contractions elicited by nerve stimulation, in the absence of exogenously applied ACh, facilitation of the rapid contractions was observed. This facilitation was the same using either PGS or TMS in the concentration-range of methoctramine 0.01–0.3 μM. An additional effect was seen at concentrations of methoctramine of 1 μM and greater, when a significant inhibition of PGS-induced contractions occurred when compared to TMS-induced contractions (Figure 5). At the highest concentration of methoctramine used (10 μM) both PGS and TMS-induced contractions were inhibited, as were contractions induced by exogenously applied ACh (Figures 4 and 5).

The effect of pirenzepine on the in vitro *innervated tracheal tube preparation*

The M_1 antagonist, pirenzepine in the concentration range 1–100 nM, had no effect on PGS-induced contractions (30 V; 30 Hz; 0.2 ms). At the highest concentration of pirenzepine used (300 nM) some degree of inhibition of both PGS-induced contractions and ACh-induced contractions was observed.

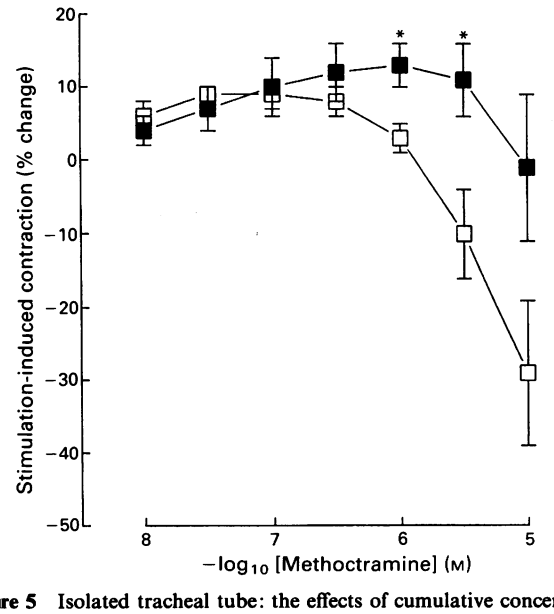


Figure 5 Isolated tracheal tube: the effects of cumulative concentrations of methoctramine on preganglionic stimulation (PGS)-induced contractions (□) and transmural stimulation (TMS)-induced contractions (■) are shown. Results are expressed as the percentage change in response from control values before addition of the antagonist. All points are the mean with s.e.mean shown by vertical lines, $n \geq 5$; * $P < 0.05$, between contractions induced by PGS compared to TMS, according to a Mann-Whitney U statistical test.

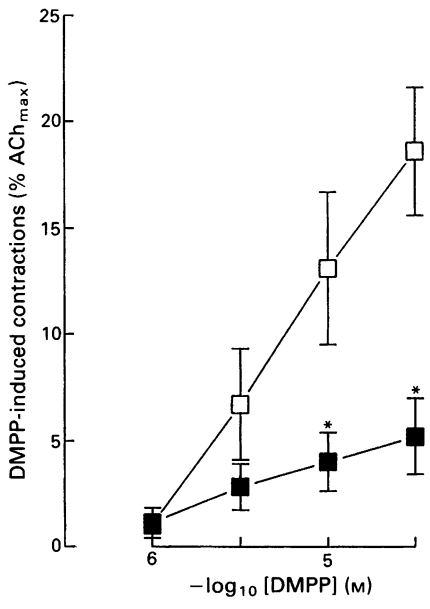


Figure 6 The effect of methoctramine (1 μM) on contractions of strips of guinea-pig trachea induced by the nicotinic cholinoceptor agonist, 1,1-dimethyl-4-phenyl-piperazine (DMPP). Contractions are expressed as a % of the maximum response to acetylcholine (ACh) (0.3 mM), since tissues were paired according to their responsiveness to ACh. Control contractions induced by DMPP in the absence of antagonist (□); response in the presence of 1 μM methoctramine (■). Results are the mean with s.e.mean shown by vertical lines, $n = 8$; * $P < 0.05$, according to a Mann-Whitney U test.

Effect of methoctramine on the in vitro *tracheal strip preparation*

In tracheal strip preparations, which had been paired according to their responsiveness to ACh, methoctramine (1 μM) caused an inhibition of contractions induced by cumulative concentrations of the nicotinic receptor agonist, DMPP (Figure 6). Tetrodotoxin 1 μM and atropine 1 μM completely inhibited the contractions induced by DMPP.

Discussion

The results presented in this paper, confirm the presence and location of the autoinhibitory muscarinic receptors on pulmonary parasympathetic nerves (Faulkner *et al.*, 1986) and their classification as muscarinic M_2 receptors (Fryer & Maclagan, 1987; Del Monte *et al.*, 1990; Doelman *et al.*, 1991; Kilbinger *et al.*, 1991). The results also demonstrate for the first time, that methoctramine is an antagonist for ganglionic nicotinic cholinceptors in the airways.

The *in vivo* data presented here with methoctramine show very similar results to those previously reported with the M_2 receptor antagonists, gallamine and pancuronium (Fryer & Maclagan, 1987). Unlike gallamine, which has a long lasting effect in guinea-pigs *in vivo*, methoctramine achieved peak pulmonary effects within 6 to 9 min of i.v. administration and responses returned to control levels within 15–20 min. There appears to be very little difference in the relative muscarinic M_2/M_3 receptor selectivities of these two compounds *in vivo*, therefore for *in vivo* work there seems little to choose from between these drugs apart from their differing time course of action.

Facilitation of stimulation-induced bronchoconstriction *in vivo*, occurred at all doses of methoctramine despite evidence from the data on ACh-induced bronchoconstriction, of significant postjunctional effects at doses of methoctramine of 120 nmol kg^{-1} and greater. An altenative measure of the potency of methoctramine for M_3 receptors is provided by its effect on ACh-induced hypotension, which is believed to be mediated via muscarinic receptors on the vascular endothelium with a similar profile to the M_3 subtype (Eglen *et al.*, 1988; McCormak *et al.*, 1988). Methoctramine (15–240 nmol kg^{-1}) produced a dose-related inhibition of the ACh-induced hypotension, but this did not reach statistical significance until the highest dose of methoctramine (240 nmol kg^{-1}). These data taken in conjunction with the effects of methoctramine on ACh-induced bronchoconstriction suggest that at best, *in vivo*, methoctramine in our hands is able to show about a 20 fold separation between M_2 and M_3 receptor antagonist activity. This is much less than the 100 fold separation previously reported by Melchiorre *et al.* (1987) and the reasons for this difference are unclear. There also appears to be some controversy in the literature as to the precise degree of selectivity of this drug. Studies by Eglen *et al.* (1988) on the effects of methoctramine on M_2 receptors on the atria and M_3 receptors on tracheal smooth muscle and blood vessel epithelium, indicate approximately a 60 fold difference in selectivity.

In vitro, experiments can more easily be used to determine the exact location of these inhibitory M_2 receptors than *in vivo* experiments, since stimulation of pulmonary parasympathetic nerves at both the pre- and postganglionic level can be achieved within the same preparation.

Application of exogenous ACh *in vitro*, to preparations during nerve stimulation, results in an inhibition of nerve-induced contractions via activation of the autoinhibitory M_2 receptors. However, since ACh is also an agonist for the postjunctional M_3 receptors on airway smooth muscle, this inhibition of stimulation-induced contractions is associated with an increase in tracheal tone, seen as an increase in the base line intraluminal pressure. At high concentrations of ACh ($\geq 10\,\mu$M) this increase in pressure in the tube reduces the contraction elicited by nerve stimulation because the tissue is unable to contract further. However, at concentrations of ACh less than 10 μM, inhibition of stimulation-induced contractions cannot be explained in this way and is probably due to the actions of ACh on the autoinhibitory M_2 receptors. Evidence for this comes from the fact that (i) at these concentrations of ACh ($< 10\,\mu$M), the sum of the slow increase in pressure caused by exogenously applied ACh plus the rapid increase in pressure caused by neurally released ACh, is much smaller than the pressure increase induced by the maximal concentration of ACh use (0.3 mM); (ii) the inhibitory effect of this concentration-range of ACh ($< 10\,\mu$M) on nerve-induced contractions can be antagonized by blockade of the autoinhibitory receptors by the M_2 receptor antagonist, methoctramine; and (iii) other spasmogens including the stable thromboxane analogue U46619 (Faulkner *et al.*, 1986) and histamine can produce similar increases in basal tone without inhibiting nerve-induced contractions.

The interpretation of results from these types of functional experiments, is complicated by the dual effect of ACh on pre- and postjunctional muscarinic receptors. At low concentrations of ACh these complications can be minimized and an assessment of M_2 receptor activity made by use of the selective M_2 receptor antagonist methoctramine in conjunction with ACh. However, accurate determination of a pA_2 value (Arunlakshana & Schild, 1959) for methoctramine on these autoinhibitory M_2 receptors must await the availability of a selective muscarinic M_2 receptor agonist.

Methoctramine facilitated contractions induced by both pre- and postganglionic nerve stimulation *in vitro*. This finding confirms the previous reports that the autoinhibitory M_2 receptors are located postganglionically on the prejunctional cholinergic nerve terminals (Faulkner *et al.*, 1986; Doelman *et al.*, 1991). A comparison of the effect of high concentrations of methoctramine ($\geq 1\,\mu$M), on contractions induced by these two methods of stimulation however, revealed an unexpected ganglionic blocking action of this drug. This ganglionic blocking action of methoctramine was not due to inhibition of facilitatory muscarinic M_1 receptors in the ganglia, since ganglionic blockade could not be reproduced using the muscarinic antagonist pirenzepine at concen-

trations known to block muscarinic M_1 receptors. Therefore, although M_1 receptors have been demonstrated to cause facilitation of pulmonary, ganglionic transmission in man and rabbit (Lammers *et al.*, 1989; Bloom *et al.*, 1987), they do not appear to be involved in ganglionic transmission in guinea-pig pulmonary cholinergic nerves.

The concentration of methoctramine, which in the vagally innervated tracheal tube preparation had been shown to have differential effects on contractions induced by stimulation of pre- or postganglionic nerves (1 μM), significantly antagonized contractions of tracheal strips induced by the nicotinic agonist, DMPP. This is not due to a postjunctional action of methoctramine, since contractions induced by exogenous ACh were not significantly altered by 1 μM methoctramine. Therefore, methoctramine shows significant antagonist activity at nicotinic receptors in pulmonary parasympathetic ganglia as well as its previously reported actions at nicotinic receptors in the frog rectus abdominis (Melchiorre *et al.*, 1987). The concentrations of methoctramine associated with this nicotinic receptor antagonist activity are in the micromolar range for both muscle and ganglionic nicotinic receptors.

While our *in vivo* results were unable to show the high degree of M_2/M_3 receptor selectivity previously reported for methoctramine by Melchiorre *et al.* (1987), these results indicate that despite this, methoctramine is at least as effective as gallamine in demonstrating autoinhibitory M_2 receptors on the pulmonary parasympathetic nerve *in vivo*. There appears to be little difference between the actions of these two drugs *in vivo*, apart from their different durations of action. Our *in vitro* results suggest that methoctramine is a potent muscarinic M_2 receptor antagonist with around a 100 fold separation between concentrations facilitating contractions induced by postganglionic nerve stimulation (0.01 μM) and those inhibiting contractions induced by ACh (10 μM). However, its usefulness may be limited by the fact that it also demonstrates significant ganglionic nicotinic receptor antagonist properties at micromolar concentrations. It is clear that like several other cholinoceptor antagonists, methoctramine has antagonist activity at nicotinic and muscarinic M_1 receptors in addition to its well-documented M_2 and M_3 antagonist properties. In preparations of airway smooth muscle, these other properties of methoctramine are important since nicotinic cholinoceptors are responsible for transmission through parasympathetic ganglia (Hawkins & Paton, 1958) and these ganglia are embedded within the airway walls. In addition, a modulatory role for facilitatory muscarinic M_1 receptors has been suggested in transmission in parasympathetic ganglia in man and rabbit (Bloom *et al.*, 1987; Lammers *et al.*, 1989) however, the present experiments were unable to demonstrate the presence of functional muscarinic M_1 receptors in guinea-pig parasympathetic ganglia.

Therefore while methoctramine is a potent muscarinic M_2 receptor antagonist both *in vivo* and *in vitro*, caution should be exercised when it is used in preparations with mixed cholinoceptor populations since methoctramine, in common with many other cholinoceptor antagonists, does not possess the required selectivity to discriminate effectively between muscarinic and nicotinic cholinoceptor subtypes under these circumstances.

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The effects of vesamicol on trains of endplate currents and on focally recorded nerve terminal currents at mammalian neuromuscular junctions

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1 The effects of vesamicol, an inhibitor of vesicular acetylcholine (ACh) storage, were studied on trains of endplate currents (e.p.cs) in the cut rat hemidiaphragm nerve-muscle preparation and on trains of focally recorded nerve terminal current waveforms in the mouse triangularis sterni nerve-muscle preparation.

2 In the rat, 0.1 and 1 μM (−)-vesamicol produced an enhancement of the rundown of e.p.c. amplitudes during trains of high frequency (50 Hz) nerve stimulation. However, 1 μM (+)-vesamicol had no effect on the rundown of e.p.c. amplitudes.

3 In the mouse, high concentrations of (−)-vesamicol (10–100 μM) produced a concentration- and stimulation-dependent decrease in the amplitude of the second negative-going deflection of focally recorded nerve terminal current waveforms.

4 At 1 mM, (−)-vesamicol produced a stimulation-independent decrease in the amplitude of the first negative-going deflection of the nerve terminal current waveforms, an increase in signal delay and evidence of nerve conduction failure. These all indicate a local anaesthetic-like block of nodal Na^+-channels.

5 In contrast to its effects on trains of e.p.cs, the effects of vesamicol on the nerve terminal current waveforms were not stereoselective, the (+)-isomer being equipotent with the (−)-isomer.

6 Low concentrations of the Na^+-channel blocking toxin, tetrodotoxin (15–60 nM), produced similar changes in the focally recorded nerve terminal current waveforms to those seen with vesamicol.

7 It is concluded that the stereoselective rundown of e.p.c. amplitudes produced by (−)-vesamicol is due to an effect, either direct or indirect, on ACh mobilization within motor nerve terminals. Furthermore, in mammalian species, the inhibitory effects of vesamicol on nodal Na^+-channels which are seen at high concentrations do not contribute to the principal neuromuscular effects of the compound.

Keywords: Rat hemidiaphragm; mouse triangularis sterni; neuromuscular junction; motor nerve ending; endplate currents; perineural waveform; acetylcholine mobilization; sodium currents

Introduction

Following the initial proposal (Marshall, 1970) and subsequent confirmation (Anderson *et al.*, 1983) that vesamicol (*trans*-2-[4-phenylpiperidino] cyclohexanol; AH5183) inhibits the transport of acetylcholine (ACh) into synaptic vesicles the compound has become widely used in mammalian preparations as a prejunctionally-active tool for studying the role of vesicular ACh in transmitter release (see Marshall & Parsons, 1987, for review). However, at high concentrations, vesamicol has been reported to exhibit a range of pharmacological actions in addition to the effects attributable to a block of ACh storage. These include postjunctional receptor and ion channel block (Enomoto, 1988); anticholinesterase activity (Van der Kloot, 1986); local anaesthetic activity (Marshall, 1970; Estrella *et al.*, 1987); twitch augmentation (Marshall, 1970; Estrella *et al.*, 1987); and effects at the sympathetic neuroeffector junction (Marshall, 1970; Wannan *et al.*, 1991). Many of these 'side-effects' do not exhibit the stereospecificity seen for the biochemical and pharmacological actions of the compound on synaptic vesicles and neuromuscular transmission. Hence, although the (−)-isomer of vesamicol is 20 times more potent at inhibiting the synaptic vesicle transport of ACh than the (+)-isomer (Bahr & Parsons, 1986), the two isomers are equipotent for the local anaesthetic and twitch augmentation effects (Estrella *et al.*, 1987) and for block of α_1-adrenoceptors (Wannan *et al.*, 1991).

A consequence of the non-cholinergic side-effects of vesamicol is the problem of unequivocally linking vesamicol-induced changes in neuromuscular transmission to an effect on the vesicular packaging of ACh. Thus, Girod *et al.* (1991) have shown that in the *Torpedo* electroplax the successive diminution of synaptic potential amplitudes during high frequency trains of motor nerve stimulation is a consequence of the ability of vesamicol to block Na^+-channels, and hence to prevent invasion of the nerve terminals by action potentials, rather than a result of its ability to inhibit vesicular ACh storage. In contrast, results from experiments in our own laboratory have been interpreted in terms of the enhanced rundown of endplate current (e.p.c.) amplitudes during high frequency trains of nerve stimulation in mammalian preparations being a reflection of an effect, direct or indirect, of vesamicol on transmitter mobilization (Prior *et al.*, 1989; Bowman *et al.*, 1990). However, it is also possible that the vesamicol-induced rundown in e.p.c. amplitudes could be due to postjunctional ion channel block as demonstrated for trimetaphan (Gibb & Marshall, 1984) or to an increased release during the first few e.p.cs of the train as a result of a block of nerve terminal K^+-channels similar to that seen with 4-aminopyridine (Lundh, 1978; Molgo *et al.*, 1979). Indeed, it has been suggested that the twitch augmentation effect of (+)- and (−)-vesamicol seen at a low rates of nerve stimulation (1 Hz) could be due, in part, to a block of nerve terminal K^+-channels (Estrella *et al.*, 1987).

To determine the role of the ion channel blocking activities of vesamicol in its neuromuscular effects at high frequencies of nerve stimulation at mammalian neuromuscular junctions, we have studied the concentration-dependence and stereoselectivity of vesamicol on trains of e.p.cs recorded from cut rat hemidiaphragm motor endplates and on perineurally recorded nerve terminal current waveforms recorded from motor nerve endings in the mouse triangularis nerve-muscle preparation.

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Methods

Endplate current trains

The cut rat hemidiaphragm preparation used has been described extensively elsewhere (Barstad & Lilliheil, 1968; Glavinovic, 1979; Gibb & Marshall, 1984). Briefly, hemidiaphragms were removed, along with 10–20 mm of their phrenic nerve supply, from male Sprague-Dawley rats (150–200 g) and pinned to the Sylgard base of a 5 ml tissue bath. Preparations were continually superfused (5–10 ml min^{-1}) at 32°C with Krebs-Hensleit solution of the following composition (mM): NaCl 118, KCl 5, $CaCl_2$ 2.5, $NaHCO_3$ 25, $MgSO_4$ 1 and glucose 11; gassed with 95% O_2:5% CO_2 to pH 7.4. The phrenic nerve was stimulated at 50 Hz via silver wire electrodes with fifty pulses of 50–100 μs duration and supramaximal voltage. Trains of e.p.cs were recorded from endplates voltage-clamped at −50 mV by a conventional two microelectrode voltage clamp recording technique (Axon Instruments, Axoclamp 2A). Intracellular microelectrodes were filled with either 3 M KCl (voltage recording, resistances 5–10 MΩ) or 0.6 M K_2SO_4 (current passing, resistances 2–5 MΩ).

In each fibre, following a minimum of 30 min rest with no nerve stimulation, four trains of e.p.cs, each separated by 20 s rest, were recorded in either the absence of vesamicol or in the presence of either 0.1 or 1 μM (−)-vesamicol or 1 μM (+)-vesamicol. For each train, e.p.c. rundown was calculated as the average loss of amplitude of the last ten e.p.c. in the train as a percentage of the amplitude of the first e.p.c. Values for each of the four separately recorded trains were averaged to give a mean value for each fibre studied.

Nerve terminal current waveforms

Experiments were performed on the left triangularis sterni nerve-muscle preparation (McArdle *et al.*, 1981) isolated from 20–25 g male Balb C mice. The entire dissection was performed under continuous superfusion with a physiological salt solution of the following composition (mM): NaCl 154, KCl 5, $MgCl_2$ 1.2, HEPES 5, $CaCl_2$ 2.5 and glucose 11; buffered to pH 7.4 and continually gassed with 100% O_2. Following isolation, preparations were mounted in a 5 ml tissue bath and superfused with physiological solution at a rate of 5–10 ml min^{-1} at 32°C. The intercostal nerves were stimulated via a suction electrode with pulses of 50–100 μs duration and supramaximal voltage. Muscle movement in response to nerve stimulation was abolished by adding 1 μM vecuronium bromide to the bathing medium. Presynaptic waveforms were recorded from the terminal region of intercostal nerves with glass microelectrodes placed inside the perineural sheath (Mallart, 1985). Extracellular microelectrodes were filled with 2 M NaCl and had resistances of 5–15 MΩ. Signals were monitored using a standard high impedance unity gain electrometer (World Precision Instruments, model 705).

For each perineural impalement, a single recording of 300 waveforms evoked at 50 Hz was made in the absence and in the presence of one of (−)-vesamicol (1, 10, 100, 1000 μM), (+)-vesamicol (100 μM) or tetrodotoxin (15, 30, 60 nM). Each concentration of vesamicol was perfused through the tissue bath for 5 min (5–10 ml min^{-1}) before making any recording. For experiments with tetrodotoxin, the preparations were exposed to each concentration of the toxin for 5, 10 and 15 min before recording a single train of 300 waveforms evoked at 50 Hz.

Analysis and statistics

Trains of e.p.cs and nerve terminal current waveforms were recorded (d.c. − 5 kHz) on FM tape for subsequent analysis. Signals were digitized at 25 kHz (Data Translation, DT2801A laboratory interface) and analysed with a suite of purpose-designed programmes (Dempster, 1988) running on an IBM-compatible microcomputer (Vanilla, SX-386).

All numerical data are expressed as mean ± s.e.mean of 3–9 impalements (mouse triangularis sterni), or 8–14 impalements (rat hemidiaphragm). For the nerve terminal current waveforms, each impalement was made in a different muscle preparation and for the endplate current trains no more than three fibres were sampled in any single muscle preparation. Statistical testing was performed with either a paired Student's t test (nerve terminal current waveforms), an unpaired Student's t test (endplate current trains) or, where appropriate, a one sample Student's t test on data normalized to a control value of 100%. In all statistical tests significance was set as $P < 0.05$.

Results

Effects of vesamicol on trains of endplate currents

In the absence of vesamicol, the amplitudes of successive e.p.cs in trains of currents diminished by around 30% until a plateau level was reached. In the presence of 0.1 or 1 μM (−)-vesamicol, the biochemically active isomer, there was a statistically significant increase of approximately 50% in the rundown in e.p.c. amplitudes during the train compared to that seen in the absence of the drug (Table 1). Neither concentration of (−)-vesamicol had any statistically significant effect on the amplitude or the decay time constant of the first e.p.c. in the train (Table 1). In contrast to the effects of (−)-vesamicol, 1 μM of (+)-vesamicol, the biochemically inactive isomer, had no significant effect on the rundown of e.p.c. amplitudes and, like the active isomer, had no effect on the amplitude and time course of the first e.p.c. of the train (Table 1).

Effects of vesamicol on nerve terminal current waveforms

In the absence of vesamicol, high frequency nerve stimulation (300 pulses at 50 Hz) produced a consistent and characteristic change in the shape of the nerve terminal current waveform (Figures 1 and 2). As stimulation progressed, the amplitude of the first negative-going deflection (the component of the waveform normally attributed to the inward nodal Na^+-current) gradually increased, so that by the 300th waveform, it was significantly increased to 108.8 ± 1.6% ($n = 9$) of its initial amplitude ($P < 0.05$, paired Student's t test). At the same time the amplitude of the second negative-going deflection (the component of the waveform normally attributed to the outward nerve terminal K^+-current) gradually decreased to 81.6 ± 2.0% ($n = 9$) of its initial value ($P < 0.05$, paired Student's t test).

At a concentration of 1 μM, (−)-vesamicol had no clearly discernible effect on the nerve terminal current waveforms. However, higher concentrations of the compound produced marked changes in the amplitude of both the first and second negative-going components of the waveforms (Figure 1). At concentrations of 100 μM and above, (−)-vesamicol produced a concentration-dependent decrease in the amplitude of the first negative-going component of the initial nerve terminal

Table 1 Effect of vesamicol on endplate current (e.p.c.) train parameters at 50 Hz

	n	$e.p.c._1$ (nA)	τ_1 (ms)	*e.p.c. rundown* (%)
Control	14	90 ± 13	0.519 ± 0.026	29.2 ± 3.5
(−)-Vesamicol 0.1 μM	12	77 ± 11	0.509 ± 0.045	44.7 ± 3.5*
(−)-Vesamicol 1 μM	8	82 ± 28	0.543 ± 0.053	46.1 ± 1.0*
(+)-Vesamicol 1 μM	10	76 ± 7	0.558 ± 0.024	30.8 ± 3.1

All data are mean ± s.e.mean averaged over the number of cells shown in n. $E.p.c._1$ and τ_1 are, respectively, the peak amplitude and time constant of decay of the first e.p.c. in the stimulation period. *Significantly different from control ($P < 0.05$, unpaired Student's t test).

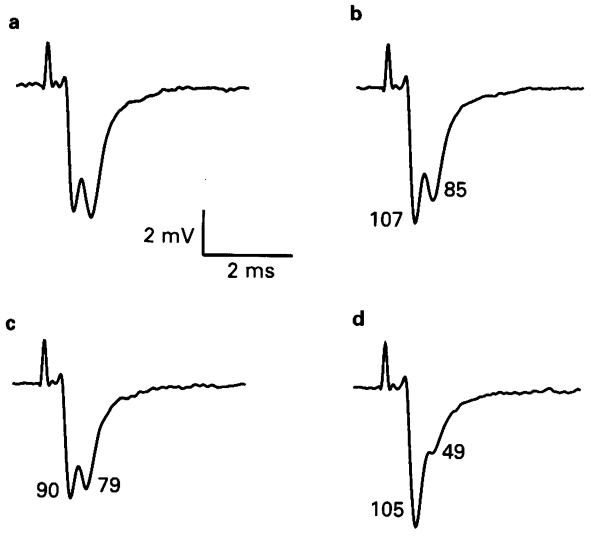


Figure 1 Representative examples of nerve terminal current waveforms recorded from a single impalement in the absence (a, b) and presence (c, d) of 100 μM (−)-vesamicol. Figure shows the first (a, c) and last (b, d) waveforms from a 6 s period of 50 Hz nerve stimulation. The numbers in (b), (c) and (d) give the amplitude of the two negative-going components of the waveform as a percentage of their amplitude in (a).

current waveform of the train of stimuli. Thus 100 μM (−)-vesamicol produced a slight, but not statistically significant, decrease in the amplitude of the first negative-going component of the initial waveform in the train of stimuli, which remained constant throughout the 6 s stimulation period (Figure 2a), while 1 mM (−)-vesamicol produced a statistically significant decrease in the amplitude of the first negative-going component of the initial waveform in the train of stimuli to 72.7 $\pm$ 5.8% of control ($n = 4$, $P < 0.05$, one sample Student's t test). The effects of (−)-vesamicol on the second negative-going component of the nerve terminal current waveforms were more pronounced than the effects on the first negative-going component and were seen at lower concentrations. Thus 10–100 μM (−)-vesamicol produced a concentration-dependent decrease in the amplitude of the second negative-going component of the initial waveform of the train of stimuli (Figure 2b). In addition, 100 μM (−)-vesamicol enhanced the stimulation-induced depression of the second negative-going component seen as stimulation progressed (Figure 2b).

None of the effects of vesamicol on nerve terminal current waveforms were stereoselective (Figure 3). Thus, as for 100 μM (−)-vesamicol, 100 μM (+)-vesamicol produced a small, statistically non-significant, decrease in the amplitude of the first negative-going component of all waveforms in the train of stimuli (Figure 4a) and a statistically significant decrease in the amplitude of the second negative-going component of the initial waveform (Figure 4b). Additionally, as for 100 μM (−)-vesamicol, the stimulation-induced decline in the amplitude of the second negative-going component of the waveform was enhanced by 100 μM (+)-vesamicol (Figure 4b). These effects of 100 μM (+)-vesamicol were of similar magnitude to those seen for 100 μM (−)-vesamicol.

In the presence of high concentrations of (−)-vesamicol (100 μM and 1 mM) there was a concentration-dependent, stimulation-induced, increase in signal delay, i.e. the time between the stimulus artefact and the start of the nerve terminal current waveform. The stimulation-induced increase in signal delay was rapid, being seen within the first 5–10 waveforms (Figure 5). Subsequent to the increase in signal delay, as stimulation progressed in the presence of the highest concentration of (−)-vesamicol (1 mM), the size of the nerve terminal current waveform fluctuated markedly and, occasionally, no

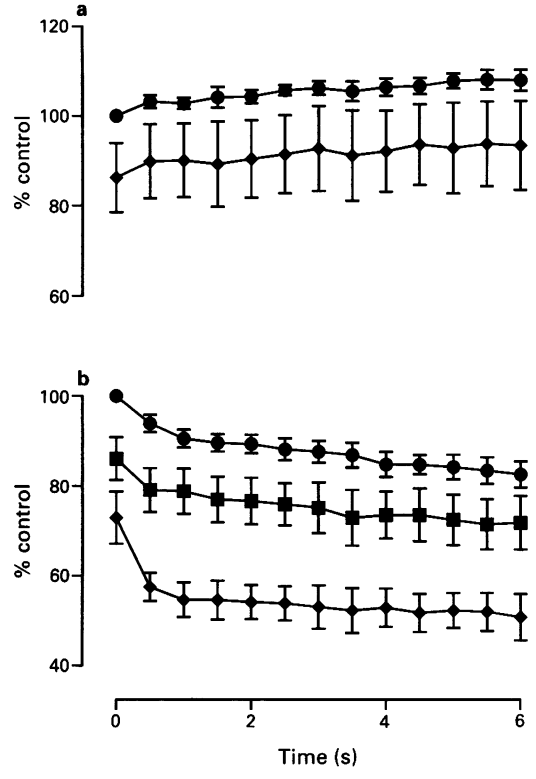


Figure 2 Effect of stimulation at 50 Hz on the amplitude of the first (a) and second (b) negative-going components of nerve terminal current waveforms recorded in the absence of (−)-vesamicol (●) and in the presence of 10 (■) and 100 μM (◆) (−)-vesamicol. For each impalement, individual amplitude values were expressed as a percentage of the amplitude of the appropriate negative-going component in the first control waveform. Each plotted point is the mean of data from the same six impalements; s.e.mean shown by vertical bars. (−)-Vesamicol, 10 μM, had no effect on the first negative-going component of the waveform and therefore data for this concentration has been omitted from (a) for clarity.

waveform was detected associated with a stimulus artefact. The variable waveform size, and occasional absence of a signal, suggest nerve conduction failure in some, or all, of the nerve fibres responsible for generating the signal.

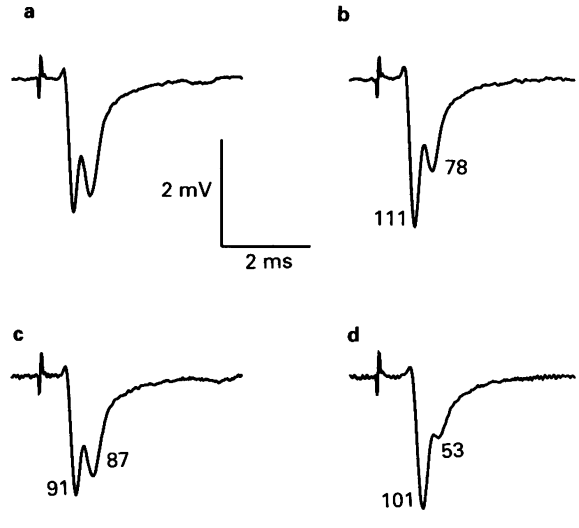


Figure 3 Representative examples of nerve terminal current waveforms recorded from a single impalement in the absence (a, b) and presence (c, d) of 100 μM (+)-vesamicol. Figure shows the first (a, c) and last (b, d) waveforms from a 6 s period of 50 Hz nerve stimulation. The numbers in (b), (c) and (d) give the amplitude of the two negative-going components of the waveform as a percentage of their amplitude in (a).

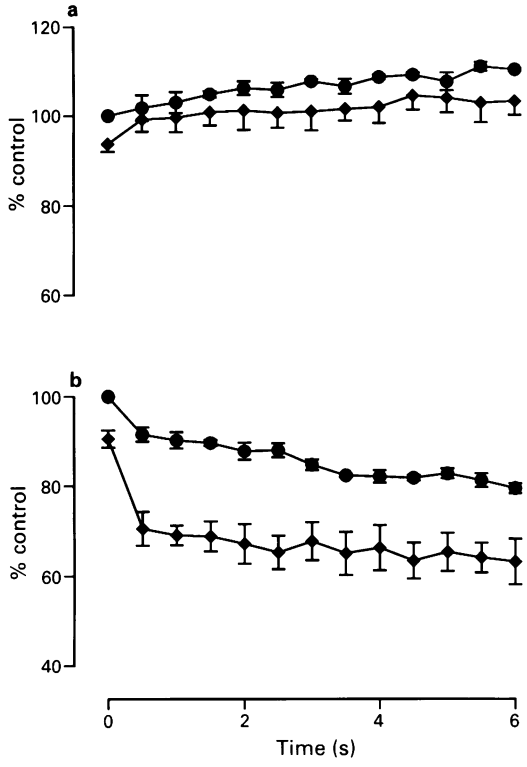


Figure 4 Effect of stimulation at 50 Hz on the amplitude of the first (a) and second (b) negative-going components of nerve terminal current waveforms recorded in the absence (●) and presence (◆) of 100 μM (+)-vesamicol. For each impalement, individual amplitude values were expressed as a percentage of the amplitude of the appropriate negative-going component in the first control waveform. Each plotted point is the mean of data from the same three impalements; s.e.mean shown by vertical bars.

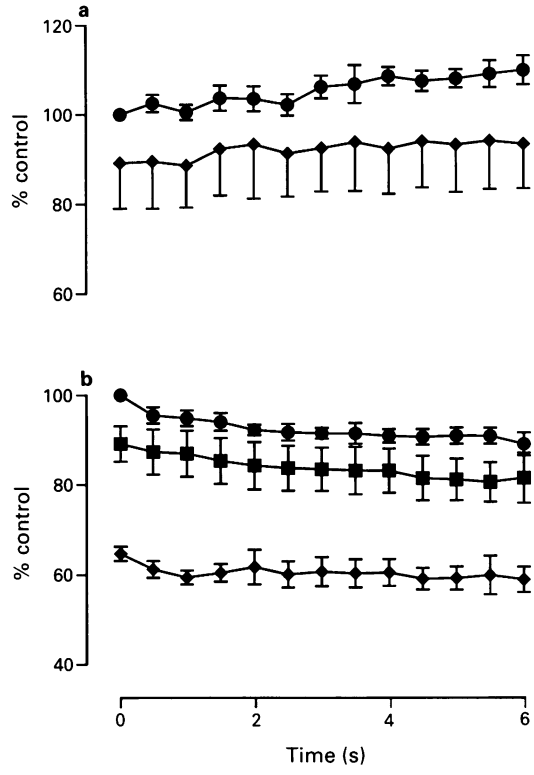


Figure 6 Effect of stimulation at 50 Hz on the amplitude of the first (a) and second (b) negative-going components of nerve terminal current waveforms recorded in the absence of tetrodotoxin (●) and following a 10 min incubation in 15 (■) or 30 nM (◆) of the toxin. For each impalement, individual amplitude values were expressed as a percentage of the amplitude of the appropriate negative-going component in the first control waveform. Each plotted point is the mean of data from the same three impalements; s.e.mean shown by vertical bars. Tetrodotoxin, 15 nM, had no effect on the first negative-going component of the waveform and therefore data for this concentration have been omitted from (a) for clarity.

Effects of tetrodotoxin on nerve terminal current waveforms

As 1 mM (−)-vesamicol was shown to produce marked effects on both negative-going components of the nerve terminal current waveforms, it is not clear whether its effects on the second negative-going component at lower concentrations are due to a block of nerve terminal K^+-channels or, alternatively, a consequence of an undetectable effect on the first negative-going component, i.e. the nodal Na^+-channels. Accordingly, we tested the effects of the specific Na^+-channel blocking toxin, tetrodotoxin (15–60 nM), under conditions identical to those used for vesamicol. The results obtained with tetrodotoxin were essentially similar to those seen for 10–100 μM (−)-vesamicol. Hence, tetrodotoxin produced a concentration-dependent depression of the second negative-going component of the nerve terminal current waveforms at concentrations that produced only a very small, non-significant, effect on the amplitude of the first negative-going component (Figure 6a). Unlike vesamicol, tetrodotoxin did not enhance the stimulation-induced depression in the amplitude of the second negative-going component of the nerve terminal current waveforms (Figure 6b).

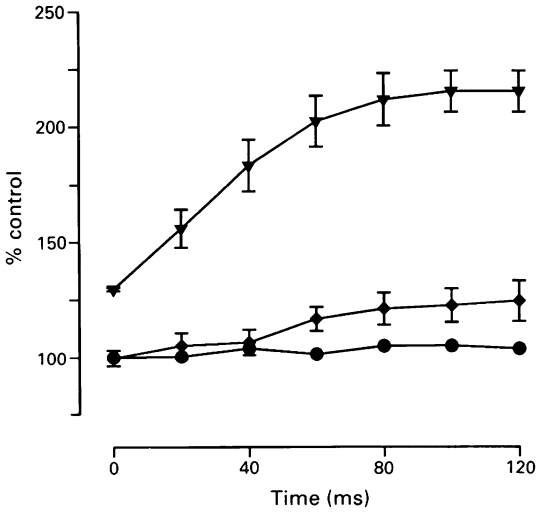


Figure 5 Effects of (−)-vesamicol on nerve terminal current waveform signal delay. Graph shows signal delay for first seven signals recorded at 50 Hz in the absence (●) and presence of 100 μM (◆) or 1 mM (▼) (−)-vesamicol. For each impalement, individual signal delay values were expressed as a percentage of the signal delay of the first control waveform. Each point is the mean of data from 4–8 different impalements; vertical bars show s.e.mean. Error bars for control points lie within the plotted symbols.

Discussion

As reported previously, vesamicol, an inhibitor of vesicular acetylcholine uptake, produced an enhanced rundown of evoked synaptic responses during high frequency repetitive stimulation (Suszkiw & Manalis, 1987; Prior *et al.*, 1989; Bowman *et al.*, 1990; Girod *et al.*, 1991). The compound also produced changes in prejunctional current waveforms that are similar to changes produced by tetrodotoxin and are consistent with the previously reported local anaesthetic activity of vesamicol (Marshall, 1970; Estrella *et al.*, 1987). Girod *et al.* (1991) have demonstrated in the *Torpedo* electroplax that the

effects of vesamicol on evoked synaptic responses can be demonstrated only at high concentrations of the drug and are not demonstrable when the synaptic responses are evoked by focal stimulation of release in tetrodotoxin-blocked preparations. They conclude that the effects of vesamicol in the *Torpedo* are a consequence of the local anaesthetic activity of the compound. Their experiments point to the difference between the actions of vesamicol on mammalian, reptilian and amphibian neuromuscular junctions on the one hand, and at the *Torpedo* electroplax on the other hand. Girod *et al.* (1991) suggest that the local anaesthetic activity of vesamicol should be taken into account when assessing the mechanism of action of the compound during high frequency trains of stimulation. As our own interpretation of the enhanced rundown of endplate responses during high frequency trains of stimuli in the presence of vesamicol and other drugs (Prior *et al.*, 1989; Bowman *et al.*, 1990) has been in terms of agents affecting transmitter mobilization from the depot to the readily releasable store, we have now re-assessed the actions of vesamicol by studying its concentration-dependent and stereospecific effects on the rundown of e.p.c. amplitudes and nerve terminal current waveforms. The effect of the biochemically-active isomer of vesamicol on e.p.c. rundown was observed at very low concentrations. Thus 0.1 μM (−)-vesamicol produced a virtual maximal effect on e.p.c. rundown. This concentration of (−)-vesamicol is close to the IC_{50} of 50 nM for the inhibitory effects of the agent on acetylcholine transport into isolated synaptic vesicles (Bahr & Parsons, 1986). Additionally, the biochemically inactive (+)-isomer of vesamicol was devoid of activity on e.p.c. rundown at a concentration of 1 μM. Thus, the effects of the vesamicol stereoisomers on the rundown of e.p.c. amplitudes during high frequency nerve stimulation are consistent with effects mediated through the same, or similar, mechanisms to those involved in inhibition of vesicular acetylcholine transport. However, the lack of an effect of vesamicol on the amplitude of the first e.p.c. of the train is in agreement with our previous observation that, at low rates of nerve stimulation, (−)-vesamicol does not affect evoked quantal release in the rat (Searl *et al.*, 1991) and suggests that the compound has no effect on the transmitter release mechanism *per se*. Furthermore, the lack of an effect of vesamicol on the time constant of decay of the first e.p.c. of the train suggests that at this concentration the compound does not affect the acetylcholine receptor-associated ion channel. This eliminates the possibility that the e.p.c. rundown is a consequence of a use-dependent endplate ion channel block.

In contrast to its effects on e.p.cs, the effects of vesamicol on nerve terminal current waveforms were observed at much higher concentrations than those used to demonstrate e.p.c. rundown. Thus, no effects on nerve terminal current waveforms were measurable at 1 μM (−)-vesamicol, which produced a maximal effect on e.p.c. rundown. The lowest concentration used at which effects on nerve terminal current waveforms were detected was 10 μM. This concentration produced a small, but significant, reduction of the second negative-going deflection of the waveform, but 1 mM was required to demonstrate a significant effect on the first negative-going deflection of the waveform, normally associated with the nodal Na^+-current. Additionally, unlike the effects on neuromuscular transmission, the effects of vesamicol, as reported previously for extracellularly recorded gross nerve action potentials (Estrella *et al.*, 1989) were not stereospecific. We thus conclude that the stereospecific enhanced rundown of e.p.c. amplitudes seen at low concentrations of vesamicol, in the rat hemidiaphragm, is unlikely to be related to the local anaesthetic activity of the compound.

Nevertheless, our results with vesamicol on the nerve terminal waveforms do provide more information on the local anaesthetic activity of vesamicol which may be applicable in species like *Torpedo* where the local anaesthetic activity of the compound is clearly of importance. Thus, the effect of 10 μM vesamicol was primarily on the second negative-going component of the nerve terminal waveform, i.e. the waveform normally associated with the nerve terminal K^+-current. On this basis, it could be argued that vesamicol has a specific effect on K^+-channels similar to that of the aminopyridines. However, a diminution of the second negative-going component of the waveforms produced by the specific K^+-channel blocker 3,4-diaminopyridine, similar in degree to that produced by 10 μM (−)-vesamicol, produces a marked increase in e.p.p. quantal content (Harvey & Rowan, 1990; Braga *et al.*, 1991). In a small number of experiments performed on the Mg^{2+}-paralysed, voltage-clamped (−55 mV) mouse triangularis sterni nerve-muscle preparation we observed no detectable effect of 10 μM (−)-vesamicol on e.p.c. quantal content measured from the mean e.p.c. amplitude (1 Hz) divided by the mean m.e.p.c. amplitude: control, 12.4 ± 2.9; 10 μM (−)-vesamicol, 10.4 ± 1.5 ($n = 6$, $P > 0.05$, paired Student's t test). This is in agreement with our previous observations in the snake (Searl *et al.*, 1990) and rat (Searl *et al.*, 1991) that (−)-vesamicol has no effect on e.p.c. quantal content when small numbers of quanta are being released. One noticeable difference between the effects of vesamicol and 3,4-diaminopyridine on the nerve terminal current waveforms is that, unlike vesamicol, even at very high concentrations, 3,4-diaminopyridine has no effect on the first negative-going component of the waveform (Braga *et al.*, 1991). However, low concentrations of the Na^+-channel selective toxin μ-conotoxin decrease the amplitude of both negative-going components of the nerve terminal current waveform (M.F.M. Braga, personal communication). Therefore, we investigated whether the effect of vesamicol on the second negative-going component of the waveform could be an effect secondary to a prime effect on the first negative-going component, the Na^+-current. We were able to demonstrate that tetrodotoxin, a specific Na^+-channel blocker, produced effects similar to those of vesamicol, i.e. a marked reduction in the amplitude of the second negative-going component of the waveform at concentrations producing only a slight, non-significant, reduction in the amplitude of the first negative-going component. The ability of low concentrations of Na^+-channel blockers selectively to affect the second negative-going component of the nerve terminal current waveforms presumably indicates that even a very small, undetectable, change in the nodal Na^+-current is sufficient to impair markedly the spread of the depolarizing wave into the nerve terminal and the subsequent activation of the nerve terminal voltage-dependent K^+-channels. Alternatively it is possible that low concentrations of Na^+-channel blockers selectively affect Na^+-channels in the nerve terminal membrane itself. The existence of such Na^+-channels in the nerve terminal membrane has been postulated (Konishi & Sears, 1984) and it is possible that these channels could play an important, as yet unidentified, role in determining the magnitude of the depolarization reaching the nerve ending. This could explain the large effects observed on the nerve terminal K^+-current in the absence of significant effects on the nodal Na^+-current.

In summary, the observed effects of vesamicol on nerve terminal current waveforms, plus the vesamicol-induced, stimulation-dependent, increase in signal delay time, leads us to suggest that it is a use-dependent block of the nodal Na^+-channels which underlies all of the observed effects of vesamicol on the nerve terminal current waveforms. The ability of vesamicol, but not tetrodotoxin, to enhance the stimulation-induced depression of the amplitude of the second negative-going component of the nerve terminal current waveforms presumably reflects differences in the use-dependence of the Na^+-channel block by the two agents. We therefore conclude that the use-dependent block of Na^+-channels at high concentrations is the primary cause of the local anaesthetic activity of both (−)- and (+)-vesamicol. Additionally, the results suggest that a block of acetylcholine mobilization, and not the local anaesthetic-like effect, underlies the stereospecific enhanced rundown of e.p.c. amplitudes seen at high frequencies of stimulation with low concentrations of (−)-vesamicol.

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The role of platelet-activating factor and peptidoleukotrienes in the vascular changes of rat passive anaphylaxis

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1 The role of platelet-activating factor (PAF) and peptidoleukotrienes as putative mediators of some of the vascular changes triggered by antigen was investigated in rats passively sensitized with monoclonal anti-DNP (2,4-dinitrophenyl) IgE.

2 Lethal anaphylaxis with respiratory distress, systemic hypotension, detachment of the intestinal mucosa, leukopenia and extravasation of protein-rich plasma was observed after antigen challenge of rats sensitized with partially purified monoclonal IgE at concentrations of 15 mg protein kg^{-1}.

3 Analysis of the peritoneal fluid obtained after i.v. challenge with DNP-BSA (bovine serum albumin) showed the presence of significant amounts of PAF (101 ± 8 pg/rat), whereas this mediator was undetectable in control animals. Leukotriene D_4 was the predominant peptidoleukotriene that could be recovered after antigen challenge, and showed an extremely high concentration (92 ± 15 ng/rat) as compared to PAF levels.

4 Extravasation of protein-rich plasma was observed shortly after challenge and reached a maximum at 30 min. Treatment of animals with i.v. PCA 4248 (1–2 mg kg^{-1}) and WEB 2086 (1 mg kg^{-1}), two chemically unrelated compounds which are antagonists of the PAF-receptor, produced a significant reduction of the extravasation of protein-rich plasma.

5 The same degree of protection could be afforded by MK-886, an inhibitor of leukotriene biosynthesis. Combined treatment with WEB 2086 and MK-886 provided greater inhibition of protein-rich plasma extravasation than either compound alone. PCA 4248 was also found to inhibit in a dose-dependent manner the systemic hypotension observed upon DNP-BSA challenge.

6 These data indicate that the lipid mediators PAF and peptidoleukotrienes are major effectors of the vascular disturbances observed in rat passive IgE-mediated anaphylaxis.

Keywords: Anaphylaxis; hypersensitivity; IgE; inflammatory mediators; leukotrienes; PAF; PAF antagonists; shock

Introduction

Various animal models have been employed as an approach to the study of human anaphylaxis. A major concern regarding the suitability of these models to characterize the network of inflammatory mediators involved, stems from the diverse classes of antibodies that can be elicited. For example, some studies on rat anaphylactoid syndromes produced by soluble immune complexes and IgG-aggregates have emphasized the role of several mediators released by mononuclear phagocytes as effectors of the extravasation of protein-rich plasma, systemic hypotension, release of lysosomal enzymes and interferences with haemostasis (Sánchez Crespo *et al.*, 1985; Doebber *et al.*, 1986; Fernández-Gallardo *et al.*, 1988). In addition, del Balzo *et al.* (1989) have emphasized the role of complement-derived anaphylatoxins in the amplification of cardiac anaphylaxis in the guinea-pig and another report has stressed the role of anaphylatoxins in the activation of platelets in the anaphylactoid syndrome elicited by soluble aggregates of IgG in the rat (Fernández-Gallardo *et al.*, 1989). This is in keeping with the results of Smedgård *et al.* (1989) who found the anaphylatoxin C5a to be the effector of platelet activation following endotoxin challenge in the rat, and with current views suggesting a major role in host defence for the generation of products by interaction of xenobiotics with biological fluids (Hellewell *et al.*, 1989).

On the other hand, classical views regarding the central role played by mast cells in the pathophysiology of anaphylaxis have been reinforced by a recent study carried out in genetically mast cell-deficient mice, which exhibited limited cardiopulmonary changes and no mortality after challenge with anti-IgE (Martin *et al.*, 1989). Since the description by Liu *et al.* (1980) of a hybridoma cell line that produces DNP-specific murine IgE, it has been possible to obtain large amounts of a single class of monoclonal antibody to be used in rats. This antibody permits a passive sensitization of circulating basophils and tissue mast cells that spares activation of the complement system, although a contribution of mononuclear phagocytes cannot be ruled out (Capron *et al.*, 1977; Rouzer *et al.*, 1982; Wei *et al.*, 1986). The purpose of this study was to determine the role of lipid mediators in the pathogenesis of vascular changes in IgE-mediated rat anaphylaxis.

Methods

In vivo *experimental design*

Male Sprague-Dawley rats of about 200 g body weight were used throughout the study. These animals were passively sensitized according to the method of Orange *et al.* (1977) by the i.p. administration of about 0.2 ml of anti-DNP (2,4-dinitrophenyl) IgE in a phosphate-buffered isotonic saline solution, pH 7.4. The solution injected contained, unless otherwise stated, about 3 mg of total protein as assessed by the method of Bradford (1976). Animals were used for experiments within a period of 24 h following sensitization, in view of previous reports showing a decline of maximal response thereafter (Leng *et al.*, 1988). Rats were anaesthetized by i.p. injection of Nembutal at a dose of 50 mg kg^{-1}, and tracheostomized with a polyethylene catheter (PE 250, Vigon, France). A femoral artery was cannulated with a polyethylene catheter (PE 50) for

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recording mean arterial pressure or for taking blood samples. A jugular vein was also cannulated (PE 10) and used for administration of drugs. Platelets and leukocytes were counted in blood samples of 44.5 μl anticoagulated with EDTA-K_2 using a Coulter counter model Plus I.

Assay of protein-rich plasma extravasation

Extravasation of protein-rich plasma was studied as described (Sánchez Crespo *et al.*, 1982). Briefly, rats received an i.v. infusion of ^{125}I-labelled bovine serum albumin (BSA) (about 200,000 c.p.m.) 10 min before DNP-BSA challenge. At different times thereafter, blood samples were taken to measure radioactivity, and extravasation was expressed as percentage reduction of the inital radioactivity per ml of blood. The spontaneous drop in the ^{125}I-labelled BSA count was regularly less than 9% during a 60 min period in control animals, and this value was subtracted from the drop in the count in the anaphylaxis experiments. The haematocrit value was obtained together with blood cell counts in the Coulter counter analysis.

Assay and characterization of PAF in peritoneal fluid

At 30 min after DNP-BSA challenge or at the time of lethal anaphylaxis, peritoneal fluid was obtained by lavage with 2 ml of isotonic saline solution. PAF was extracted by the procedure of Bligh & Dyer (1959), and processed as described (López Diez *et al.*, 1989). Briefly, after evaporation to dryness, the lipid extract was resuspended in the medium used as a mobile phase for straight-phase high performance liquid chromatography (h.p.l.c.). This was carried out in a Spheri-5 silica column using as a mobile phase 96% propan-2-ol/hexane (1/1, supplemented with 0.005% acetic acid) and 4% water which was linearly increased to 8% during a 15 min period (Blank & Snyder, 1983). The column was eluted for 60 min at 2 ml min^{-1}, and the fractions collected, evaporated to dryness, and resuspended in 100 μl of a N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES)-saline buffered medium supplemented with 0.25% BSA to carry out a bioassay on rabbit platelets according to the procedure described by Henson (1976). Briefly, rabbit platelet-rich plasma was obtained by low speed centrifugation and incubated with [^{3}H]-5-hydroxytryptamine ([^{3}H]-5HT) at a concentration of 1 μCi ml^{-1}. After two washes to remove plasma and non-incorporated [^{3}H]-5-HT, platelets were resuspended at a concentration of 10^8 ml^{-1}, and incubated with different concentrations of the h.p.l.c. fractions or a standard solution of PAF. The amount of PAF contained in the samples was corrected for recovery and expressed in pg as assessed from comparison with the activity of the standard. The limit of sensitivity of this assay was 5 pg.

Assay and characterization of peptidoleukotrienes

Collection of peritoneal fluid was carried out as described for PAF. The extraction procedure was a modification of that of Clancy & Hugli (1983). Briefly, each sample was adjusted to a total volume of 3 ml with saline solution, combined with 90 μl 5 M formic acid and 1.5 ml propan-2-ol, vortexed for 5 min at room temperature, and then mixed with 3.5 ml anhydrous ether. After further vortexing, the organic layer was taken, combined with 45 μl 10 M NH_4OH and the solvent removed under N_2. The extraction efficiency for LTC_4 was above 70%, as determined with a radiolabelled standard. Reverse-phase h.p.l.c. separation of the eicosanoids was carried out with a dual pump Kontron system model 420 (Kontron Instruments, Zurich, Switzerland), fitted with a Spheri-5 RP18 column (220 × 4.6 mm, 5 μm) and a 15 × 3.2 mm guard column, using as a system of solvents methanol/water/acetic acid (60/40/0.1, pH 5.4 [adjusted with 10% ammonium hydroxide] + 1 mM EDTA). The column was isocraticly eluted for 30 min, and then the strength of the mobile phase increased by a methanol gradient from 0% to 25% between 30 and 45 min, and from 25 to 35% between 50 and 70 min. Peaks of peptidoleukotrienes were detected by on line u.v. absorbance at 280 nm, and identified by comparison with authentic standards, analysis of u.v. absorbance spectra, and immunoreactivity with the antibody used for the radioimmunoassay (RIA). The quantities of leukotrienes were estimated by integration of the peak areas using a Spectra-Physics apparatus (San Jose, CA, U.S.A.).

Materials and drugs

Monoclonal anti-DNP IgE was obtained from an IgE-secreting hybridoma grown in ascites in BALB/c mice and was partially purified by ammonium sulphate precipitation followed by dialysis (Liu *et al.*, 1980). The final preparation of protein used to carry out passive sensitization contained about 7 mg ml^{-1} specific IgE, as indirectly inferred from a classical precipitation reaction using antigen and several dilutions of the antibody solution (Hudson & Hay, 1976). This estimated content of specific antibody in the sample was consistent with the data obtained from FPLC gel filtration chromatography of a 200 μl sample in a Superose-12 column (Pharmacia LKB, Uppsala, Sweden) and measurement of the protein present in the peak showing a molecular weight analogous to mouse IgE. Analysis by sodium dodecyl sulphate/polyacrylamide gel electrophoresis under reducing conditions, of the immunoprecipitate formed upon incubation of DNP-BSA with IgE anti-DNP showed the presence of two bands compatible with both the heavy and light chains of mouse IgE as described by Liu *et al.* (1980).

DNP-BSA containing 20 mol of DNP per mol of BSA was prepared according to the method of Eisen (1964), by using BSA from Sigma Chemical Company (St. Louis, MO, U.S.A.) and 2,4-dinitrobenzenesulphonic acid from Aldrich-Europe, Beerse, Belgium. PCA 4248 (2-[phenylthio]ethyl 5-methoxycarbonyl-2,4,6-trimethyl-1,4-dihydropyridine-3-carboxylate) was provided by ALTER Laboratories, Madrid, Spain (Ortega *et al.*, 1990). The solubilization of this product was carried out under protection of light exposure by mixing 10 mg of the compound with 20 μl dimethylsulphoxide (DMSO) under a stream of N_2, followed by the addition of 1 ml polyethyleneglycol-400 and 1 ml of distilled water. This solution could be diluted in saline solution for i.v. administration, as required, WEB 2086 (3-(4-(2-chlorophenyl)-9-methyl-6H-thieno-(3,2-f)(1,2,4)-triazolo-(4,3-a)(1,4)-diazepine-2-yl)-1-(4-morpholinyl)-1-propane), a kind gift from Dr H.O. Heuer, Boehringer Ingelheim, Ingelheim, F.R.G., was solubilized in a mixture of 0.1 M HCl and saline (1 : 20 v/v). MK-886 (3-[1-(4-chlorobenzyl)-3-t-butyl-thio-5-isopropylindol-2-yl]-2,2- dimethylpropanoic acid [Gillard *et al.*, 1989]), leukotriene B_4 (LTB_4), LTC_4, LTD_4 and LTE_4, used as standards for reverse-phase h.p.l.c. were a kind gift from Dr C.B. Pickett, Merck Frosst, Pointe Claire-Dorval, Quebec, Canada. [^{3}H]-hexadecyl-PAF, and reagents for RIA of LTC_4, LTD_4 and LTE_4, were purchased from Amersham International, U.K. MK-886 was solubilized in 0.1% methyl cellulose and given by mouth, at a dose of 10 mg kg^{-1}, 2 h before anaphylactic challenge, according to the manufacturer's instructions. PAF was obtained from Bachem Feinchemikalien AG, Bubendorf, Switzerland.

Statistical analyses

Data are expressed as the mean ± s.e.mean. For comparison of two groups of samples normally distributed, the Student's two-tailed t test was used to analyse differences for significance. For comparison of two groups of samples not normally distributed, the Mann-Whitney U test was used. Differences in the survival rate were evaluated by the χ^2-test with the Yates' correction. Statistical procedures were performed using a database and statistical package (Sigma, Horus Hardware Co., Madrid, Spain). A P value < 0.05 was considered significant.

Results

Effect of DNP-BSA challenge on systemic arterial pressure

Sensitized animals showed respiratory distress and a drop of mean arterial pressure after i.v. challenge with 0.7 mg kg^{-1} of DNP-BSA (Figure 1). This was not observed in either non-sensitized rats receiving DNP-BSA nor in those having received IgE heated for 1 h at 56°C, i.e. a treatment that destroys the biological effect of IgE by affecting the portion of molecule which interacts with cell receptors (Dorrington & Bennich, 1973). The hypotension occurred immediately after challenge, but the record frequently showed a modest elevation after the initial drop, and then a protracted decline which lagged until the death of the animal (Figure 1). The hypotension was dependent on the dose of antibody used for sensitization, and lethal anaphylaxis was observed in those animals that had received more than 15 mg protein kg^{-1} of anti-DNP IgE. Since this dose usually spared the death of the animal for up to 40 min after challenge, it was selected for experiments intended to show pharmacological modulation of the anaphylactic reaction. Under these conditions, arterial pressure dropped from 108 ± 5.0 to 49.4 ± 4.1 mmHg 2 min after antigen challenge (Table 1). This was followed by a further drop to 35.7 ± 8.3 mmHg during the next 30 min. Two out of 16 animals showed recovery to the basal level at 2 and 35 min after challenge, whereas the remainder showed a prolonged shock state until death occurred. Animals treated with PCA 4248 (1 mg kg^{-1}, i.v., 10 min before DNP-BSA challenge) showed a decline of arterial pressure from 110 ± 4.0 to 69.7 ± 9.5 mmHg, but in contrast to the drop observed in the rats not treated with the drug, this was not followed by secondary reduction during the ensuing minutes. Prechallenge arterial pressure levels were reached in most animals within 40 min, whereas only 2 out of 15 rats showed lethal anaphylaxis during the period of observation (Table 1).

Histological examination including transmission electron microscopy was carried out in specimens of small intestine from control and DNP-BSA challenged sensitized animals. Whereas few changes were observed in the peritoneal serosa, the mucosa showed prominent changes in the epithelial layer with wide areas detached. The lamina propria showed oedema and cellular infiltration with a prominent presence of eosinophils, which were identified by examination of their granules. The lumen of the vessels was occupied by red cells, which is most probably due to the occurrence of haemoconcentration (Figure 2).

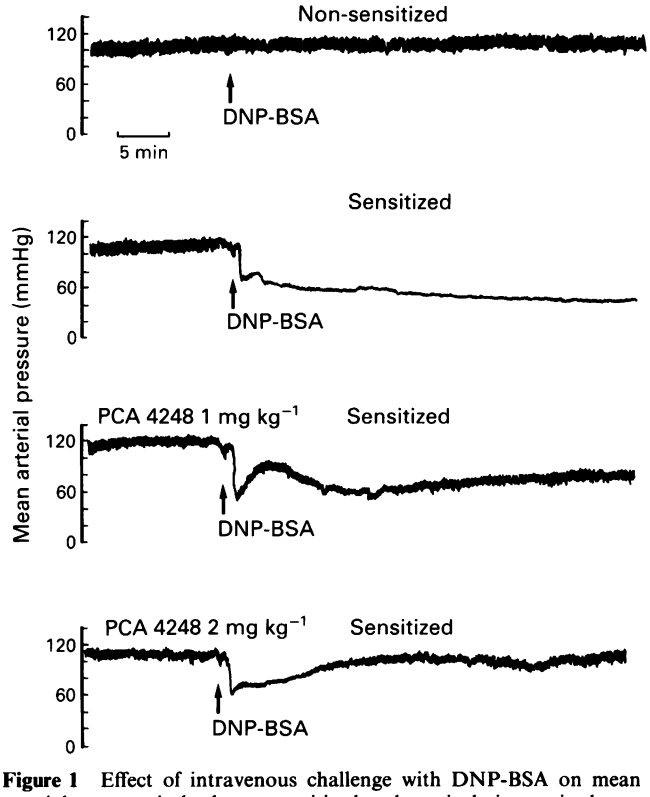


Figure 1 Effect of intravenous challenge with DNP-BSA on mean arterial pressure in both non-sensitized and passively immunized rats, and effect of a previous treatment with PCA 4248. Rats received 0.7 mg kg^{-1} DNP-BSA at the times indicated by the arrows. PCA 4248 was injected i.v. 10 min before DNP-BSA. These are typical recordings selected from 3 non-sensitized rats, and 15 or 16 animals in each group of sensitized animals.

Extravasation of protein-rich plasma

Antigen challenge of sensitized rats produced a time-dependent drop of the plasma ^{125}I-labelled BSA count. Figure 3 shows the temporal course of protein-rich plasma extravasation and the protection provided by PCA 4248 (1–2 mg kg^{-1}, i.v., 10 min before antigen challenge). Extravasation could be demonstrated as early as 2 min after challenge, and showed a decrease to 52 ± 12% of the initial blood-associated ^{125}I-labelled BSA count by 30 min. Rats treated with PCA 4248 at a dose of 1 mg kg^{-1}, showed a reduction of the ^{125}I-labelled BSA blood count to 76 ± 10% of the prechallenge level at 30 min, and to 80 ± 5% when PCA 4248 was administered at a dose of 2 mg kg^{-1}. This represents 58 ± 7% and 70 ± 4% inhibition, respectively, when expressed as a percentage of the response observed in vehicle-treated animals. The existence of extravasation of protein-rich plasma could also be demonstrated by the haematocrit value, which increased from 44 ± 1% to 50 ± 4, 30 min after challenge ($n = 8$), compared to 44 ± 3 and 43 ± 7 in a group of animals pretreated with PCA 4248 at the dose of 1 mg kg^{-1}. WEB 2086, a compound structurally unrelated to PCA 4248 but sharing the PAF-receptor antagonistic property (Casals-Stenzel *et al.*, 1987), was tested at the dose of 1 mg kg^{-1}, i.v., 10 min before DNP-BSA. As shown in Table 2, it was also found to blunt protein-rich plasma extravasation. MK-886 (10 mg kg^{-1}, orally, 2 h before DNP-BSA) produced a diminution of protein-rich plasma extravasation similar to that observed with both PAF-receptor antagonists. Combined administration of MK-886 and WEB 2086 showed a reduction of extravasation higher than that provided by treatment with either drug alone (Table 2).

The leukocyte count decreased following DNP-BSA challenge to reach 59 ± 15% of the prechallenge level 5 min thereafter (Figure 4). This was followed by a recovery to

Table 1 Effect of PCA 4248 on mean arterial pressure and survival rate during passive anaphylaxis

	Mean arterial pressure				
PCA 4248	*Prechallenge*	*At 2 min*	*At 30 min*	*% survival*	n
Vehicle	108 ± 5.0	49.42 ± 4.1	35.71 ± 8.3	12.5	16
1 mg kg^{-1}	110 ± 4.0	69.66 ± 9.5	103 ± 10.0*	86.6*	15
2 mg kg^{-1}	112 ± 5.5	56.32 ± 4.6	112 ± 5.5*	81.2*	16

Rats were treated as described in the legend to Figure 1 and in Methods. Mean arterial pressure was continuously recorded for up to 40 min, when most animals pretreated with vehicle died. Mean arterial pressure values registered 2 and 30 min after DNP-BSA challenge were taken to construct this table. Survival rate was recorded during a period of observation of 40 min. Data represent mean ± s.e.mean.
* Indicates $P < 0.001$ as compared to vehicle-treated animals (χ^2 with the Yates' correction).

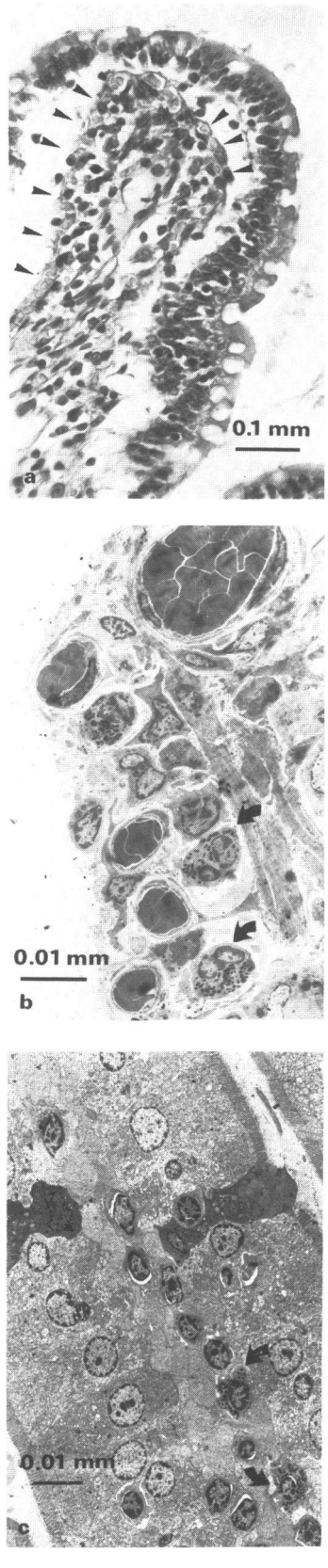


Figure 2 Micrographs of specimens of small intestine from rats sensitized with anti-DNP IgE and challenged with 0.7 mg of DNP-BSA. The sample was obtained 30 min after antigen challenge, fixed by immersion, and processed for both light microscopy and transmission electron microscopy: (a) shows detachment of the epithelium from the lamina propria (arrowheads) of an intestinal villus. Electron micrograph (b) shows an area of intestinal mucosa denuded of the epithelial layer, infiltration of eosinophils (arrows), and occupation of the vascular lumen by red blood cells. Electron micrograph (c) shows the detached epithelium of a villus with infiltrating eosinophils (arrows) in the central area.

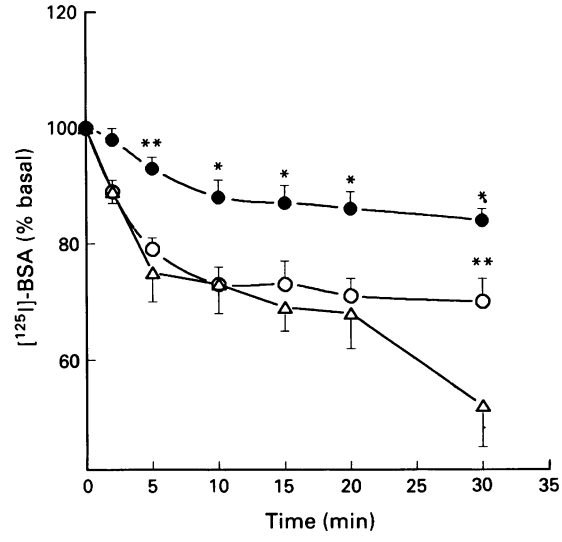


Figure 3 Time course of the effect of PCA 4248 on the extravasation of ^{125}I-labelled BSA during passive anaphylaxis. PCA 4248 or vehicle were administered i.v. to IgE-sensitized rats 10 min before i.v. challenge with DNP-BSA (0.7 mg kg^{-1}). At the times indicated, 100 μl blood samples were taken via a femoral catheter to measure [^{125}I]-BSA. Results are expressed as percentage of the basal [^{125}I]-BSA count rate. Vehicle-treated rats (△); rats treated with PCA 4248 at the dose of 1 mg kg^{-1} (○) and 2 mg kg^{-1} (●). Data represent mean of 7 to 11 animals in each group; s.e.mean shown by vertical bars. * Indicates $P < 0.05$; ** $P < 0.01$.

80 ± 20% by 30 min. The platelet count showed variations of less than 14% throughout the experiments, which suggests the absence of a true thrombocytopenia linked to platelet activation and organ sequestration.

Presence of PAF in peritoneal fluid in IgE-sensitized rats

The volume of fluid collected from the peritoneum of DNP-BSA challenged rats was 3.1 ± 0.2 ml ($n = 22$, range 2.7–4.2 ml) and the recovery of protein was 8.2 ± 0.8 mg (range 5.3–10.9 mg). Ten control rats contained 0.8 ± 0.1 ml of peritoneal fluid and 1.9 ± 0.3 mg of protein. The number of peritoneal cells was 9.3 ± 2.6 × 10^6 ml^{-1} (range 6.4 × 10^6–1.3 × 10^7 cells ml^{-1}) and did not significantly differ from the number obtained from normal controls (9.0 ± 2.4 cells ml^{-1}). IgE-sensitized animals at the time of lethal anaphylaxis or killed 30 min after antigen challenge, were found to contain

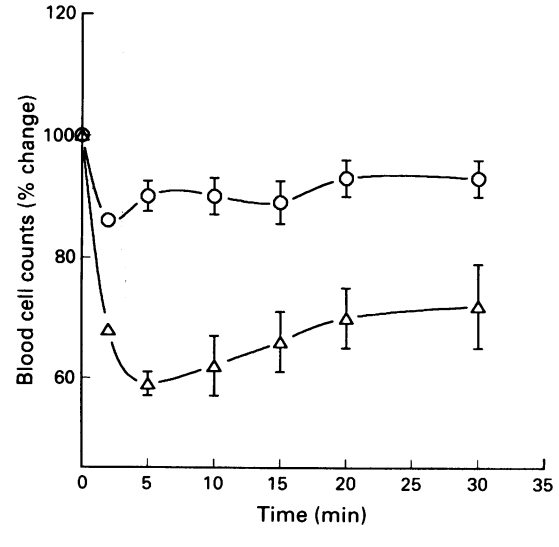


Figure 4 Variations of leukocyte and platelet counts during IgE-mediated anaphylaxis. Blood samples were taken to assess platelet (○) and leukocyte counts (△). Data represent mean of 3 to 6 animals; s.e.mean shown by vertical bars.

Table 2 Inhibition by WEB 2086 and MK-886 of protein-rich plasma extravasation in rat passive anaphylaxis

Treatment	*Dose*	*Extravasation (% inhibition)*	P
Vehicle	—	0	
WEB 2086	1 mg kg^{-1}	47.5 ± 5.2	<0.01*
MK-886	10 mg kg^{-1}	53.7 ± 4.4	<0.01*
MK-886 + WEB 2086	10 mg kg^{-1} + 1 mg kg^{-1}	70.7 ± 4.4	<0.05**

WEB 2086 was administered i.v., 10 min before DNP-BSA challenge. MK-886 was given orally 2 h before challenge. The inhibition of [^{125}I]-BSA extravasation was studied at 30 min after DNP-BSA challenge, and is expressed as percentage inhibition of the response observed in vehicle-treated animals. Data represent mean ± s.e.mean. * As compared to vehicle-treated animals. ** As compared to rats treated with either MK-886 or WEB 2086 alone.

PAF in the peritoneal fluid (Table 3), whereas only 1 out of 10 control animals showed 19 pg PAF (13 pg mg^{-1} protein), and the remaining 9 animals showed values below the detection limit of 5 pg. The amounts of PAF assayed in a group of rats undergoing i.p. challenge with the same dose of DNP-BSA were slightly lower than those detected in animals undergoing i.v. challenge (Table 3). Characterization of the lipid product obtained from the peritoneum as authentic PAF was based on its time of retention of 21 min in straight-phase h.p.l.c., i.e. analogous to that of a [^{3}H]-hexadecyl-PAF standard, as well as the loss of its platelet-activating properties upon treatment with phospholipase A_2 from *Naja naja* venom.

Detection of peptidoleukotrienes in peritoneal fluid

Reverse-phase h.p.l.c. analysis of peritoneal fluid obtained from passively immunized rats at times similar to those mentioned for PAF recovery, showed the presence of several major peaks displaying absorbance at 280 nm, an u.v. absorbance spectrum compatible with leukotrienes, and reactivity with an antibody used for the combined RIA of LTC_4, LTD_4, and LTE_4. The identity of these compounds was further confirmed by comparison of their times of retention with those showed by standards of LTC_4 (16.4 ± 0.2 min), LTD_4 (35.1 ± 0.1 min), LTB_4 (38.3 ± 0.1 min), and LTE_4 (39.4 ± 0.2 min). LTD_4 was the predominant leukotriene in antigen-challenged rats, whereas little LTE_4 was detected. LTC_4 was only found in animals sensitized with 37 mg protein kg^{-1} of the anti-DNP IgE preparation, which probably indicates that LTC_4 accumulates because of enzymatic overload at the gamma-glutamyl transpeptidase step due to massive release of leukotrienes. In experiments with rats sensitized with 15 mg protein kg^{-1} of anti-DNP IgE, the amount of LTD_4 that could be detected in the peritoneal fluid was 92 ± 15 ng/rat (n = 8), whereas non-sensitized rats contained less than 5.0 ± 0.3 ng/rat (n = 15). Rats pretreated with MK-886 prior to DNP-BSA challenge showed a 73% inhibition of LTD_4 production, and those treated with PCA 4248 showed a generation of LTD_4 similar to that of vehicle treated animals (Table 4). The amount of LTD_4 measured in a group of 6 passively sensitized rats challenged with i.p. DNP-BSA was 68 ± 12 ng/animal (Table 4), i.e. slightly lower than the quantities detected in animals undergoing i.v. challenge. The pattern of leukotriene distribution was not modified when i.p. L-cysteine, which inhibits the conversion of LTD_4 to LTE_4 was omitted (Sok *et al.*, 1980). A problem observed during the development of these experiments was the identification of a major peak that eluted at a retention time of 10–12 min, and which was present in all of the samples including those from non-sensitized animals as well as those from animals pretreated with MK-886. This peak was tentatively characterized as a sulphoxide on the basis of its u.v. spectrum, since it showed a maximal absorbance at 285 nm. In addition, it had a retention time analogous to that of a leukotriene sulphoxide prepared by treatment of LTC_4 with an excess of H_2O_2 at room temperature (Foster *et al.*, 1988).

Discussion

This study was intended to define the role of lipid mediators in rat IgE-mediated passive anaphylaxis. Some stress was placed on PAF, a phospholipid mediator characterized as 1-O-hexadecyl/octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (Demopoulos *et al.*, 1979; Blank *et al.*, 1979; Benveniste *et al.*, 1979), the receptor for which in guinea-pig lung has recently been cloned and characterized as a member of the superfamily of G protein-coupled receptors (Honda *et al.*, 1991). The role of PAF in anaphylaxis has been established in the rabbit (Pinckard *et al.*, 1979), guinea-pig (Darius *et al.*, 1986), mouse (Terashita *et al.*, 1987) and sheep (Tomioka *et al.*, 1989). However, there is only one report on the role of PAF in the shock elicited in rats, and this refers to animals immunized to produce IgG (Damas, 1989). Our data confirm previous studies as to the occurrence of systemic hypotension, transient leukopenia and extravasation (Sanyal & West, 1958; Ross *et al.*, 1986; Leng *et al.*, 1989), and show that the lesions observed in the small intestine are similar, if not identical, to the ischaemic necrosis described in rats following administration of either PAF, combinations of PAF and endotoxin

Table 3 Quantitation of PAF in peritoneal fluid from sensitized rats challenged with DNP-BSA

Pretreatment	*Challenge*	*PAF* (pg/animal)	*PAF* (pg mg^{-1} protein)	n
Vehicle	i.v.	101 ± 8	13 ± 3	8
PCA 4248	i.v.	172 ± 19	20 ± 2	4
Vehicle	i.p.	88 ± 12	9 ± 1	10

Peritoneal fluid was obtained at the time of death or at 30 min after either i.v. or i.p. challenge with DNP-BSA. Protein content was assayed by the method of Bradford. The limit of sensitivity of PAF assay was 5 pg. Data represent mean ± s.e.mean.

Table 4 Quantitation of leukotriene D_4 (LTD_4) in peritoneal fluid from DNP-challenged rats

Pretreatment	*Challenge*	*LTD_4* (ng/animal)	*LTD_4* (ng mg^{-1} protein)	n
Vehicle	i.v.	92 ± 15	11 ± 3	8
PCA 4248	i.v.	101 ± 26	12 ± 2	6
MK-886	i.v.	25 ± 6	3 ± 1	9
Vehicle	i.p.	68 ± 12	7 ± 3	6

PCA 4248 was administered i.v., 10 min before DNP-BSA at a dose of 1 mg kg^{-1}. MK-886 was given orally at a dose of 10 mg kg^{-1} 2 h before DNP-BSA. DNP-BSA was given either i.v. or i. p., as indicated.

(González-Crussi & Hsueh, 1983; Hsueh *et al.*, 1986), or tumour necrosis factor (Sun & Hsueh, 1988). The evidence in favour of PAF as a mediator of passive anaphylaxis provided in this study are its appearance in the peritoneal exudate of antigen-challenged rats, and the beneficial effect of two selective PAF-receptor antagonist on the evolution of the shock. Furthermore, the infusion of PAF into experimental animals causes a number of pathophysiological changes similar to those observed during systemic anaphylaxis (McManus *et al.*, 1980; Sánchez Crespo *et al.*, 1982), including bowel necrosis (Hsueh *et al.*, 1986).

Extravasation of protein-rich plasma is a prominent event in anaphylaxis, but its mediation has not been fully ascertained. In a report by Leng *et al.* (1988) the LTD_4-receptor antagonist L-649,923 and the 5-lipoxygenase inhibitor ONO-LP-049 showed no effect on skin extravasation, a moderate effect on peritoneal extravasation, and no effect on systemic hypotension. Our data indicate that either an inhibitor of leukotriene biosynthesis or a PAF-receptor antagonist may blunt protein-rich plasma extravasation, whereas the combined intereference with both families of mediators enhances the effect of either drug alone. We have not detected a reduction of platelet count following DNP-BSA challenge. This is at variance with the anaphylactoid syndrome produced by soluble aggregates of IgG, and might be due to the lack of complement activation and anaphylatoxin formation in IgE-mediated anaphylaxis.

PAF was detected in the peritoneal exudate of sensitized rats at concentrations lower than those of LTD_4, but much higher than could be expected from a mere contamination of samples with blood, as previous studies (Caramelo *et al.*, 1984; Chang *et al.*, 1987) have reported a mean content of 2.8 pg PAF per mg protein in rat blood, i.e. 4.6 to 7 fold lower than the concentration found here. This finding was rather unexpected in view of the prominent effect of the two PAF-receptor antagonists on the course of rat passive anaphylaxis. The observation is also at variance with studies on human whole blood, where the formation of PAF and leukotrienes in response to ionophore A23187 challenge reaches similar levels (Fradin *et al.*, 1989) as if both familes of lipid mediators originated from a common 1-O-alkyl-2-araquidonyl-*sn*-glycero-3-phosphocholine precursor by the action of a phospholipase A_2 to yield lyso-PAF and nonesterified archidonate.

The predominance of leukotrienes observed in the present study possibly stems from the nature of the cell types involved. Thus, the activation of submucosal mast cells, which do not generate PAF, but produce peptidoleukotrienes (Mencía-Huerta & Benveniste, 1979), might be a major explanation for this finding. Alternative explanations could be an ineffective recovery of PAF due to intracellular retention (Lynch & Henson, 1986), a formation of PAF by mononuclear phagocytes not located in the peritoneal cavity (Mencía-Huerta & Benveniste, 1979; Iñarrea *et al.*, 1983), or a predominant production of PAF-analogues of the 1-acyl subclass for which the current PAF-bioassays show limited sensitivity (Sturk *et al.*, 1989; Triggiani *et al.*, 1990).

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X-irradiation attenuates relaxant responses in the rabbit ear artery

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1 The relaxant actions of acetylcholine, substance P and calcitonin gene-related peptide, and the levels of neuropeptide Y and calcitonin gene-related peptide were assessed in the rabbit central ear artery 1, 4 and 6 weeks after a single dose of 45 Gy X-irradiation, a dose similar to that used clinically in intraoperative radiotherapy.

2 Relaxant responses induced by acetylcholine and substance P (both endothelium-dependent) and calcitonin gene-related peptide (endothelium-independent) were reduced, and endogenous neuropeptide Y and calcitonin gene-related peptide levels were unaffected after X-irradiation.

3 The mechanism(s) by which a single dose of 45 Gy X-irradiation may selectively damage relaxant, but not direct, contractile responses of the smooth muscle (as we have shown previously) of the rabbit central ear artery are discussed.

Keywords: Rabbit ear artery; intraoperative radiotherapy; vasodilatation

Introduction

There is now considerable evidence for dual control of vascular tone by perivascular nerves and endothelial cells (for review, see Lincoln & Burnstock, 1990). Neuropeptide Y (NPY)-like immunoreactivity has been localized in sympathetic perivascular nerves of the rabbit central ear artery (REA), and exogenous NPY is known to have neuromodulatory effects on the sympathetic transmission in this preparation (Wong-Dusting & Rand, 1988; Saville *et al.*, 1990). The REA also has a sensory-motor innervation with perivascular nerves containing substance P- (SP) and calcitonin gene-related peptide-(CGRP)-like immunoreactivity (Morris & Bevan, 1986; Maynard *et al.*, 1990). SP and CGRP induce vasodilatation on preconstricted REA preparations in an endothelium-dependent (Bolton & Clapp, 1986) and endothelium-independent (Maynard *et al.*, 1990) manner, respectively.

Much has been reported on the effects of radiation on the structure but not on the functioning of the blood vessels (for reviews see Law, 1981; Fajardo & Berthrong, 1988; Reinhold *et al.*, 1990). Recent evidence has suggested that endothelial cell function (see Fajardo, 1989) and vascular smooth muscle (Hopewell *et al.*, 1989) may be affected by irradiation. In a previous publication, we showed that a single dose of 45 Gy X-irradiation, similar to that used in intraoperative radiotherapy (Abe *et al.*, 1980; Kinsella & Sindelar, 1989; Le Couteur *et al.*, 1989), significantly affected the sympathetic transmission but not the direct contractile action of noradrenaline (NA) on the REA (Stewart-Lee *et al.*, 1991).

Since our previous findings indicated that there is X-irradiation-induced perivascular nerve damage, in this study we have examined the effects of a single dose of 45 Gy X-irradiation on the content of NPY and CGRP in the perivascular nerves, and on the endothelium-dependent (acetylcholine (ACh) and SP) and endothelium-independent (CGRP) relaxant actions on preconstricted REA preparations.

Methods

X-irradiation procedure

Details of the X-irradiation experimental procedure have been described previously (Stewart-Lee *et al.*, 1991). Briefly, both ears of New Zealand White rabbits (4–6 months; 2–3.5 kg) were locally irradiated with a single dose of X-rays (45 Gy, 215 kV, 12.5 mA). There were 6 to 9 animals in each of the age-matched control and X-irradiated animal groups. The latter were examined at 1, 4 and 6 weeks after X-irradiation.

Peptide assays

NPY and CGRP assays were performed on mid-segments of the REA which were measured, weighed and the peptides extracted. NPY and CGRP levels were quantitated by an inhibition enzyme-linked immunosorbent assay as described earlier (Belai *et al.*, 1988).

Pharmacology

Proximal segments of the REA were mounted under isometric conditions and optimal resting conditions of 1.0 g for at least 1 h, and used for pharmacological investigations as previously described (Stewart-Lee *et al.*, 1991). Cumulative concentration-response curves were established for NA (0.1–100 μM) and were unaffected after 45 Gy X-irradiation (Stewart-Lee *et al.*, 1991).

To study relaxant responses to ACh, SP and CGRP, REA segments were preconstricted with NA (3–10 μM) which produced approximately 70% of the maximal NA contraction (Stewart-Lee *et al.*, 1991). As ACh does not cause desensitization of receptors, it was added cumulatively (0.1–100 μM) to the organ bath, whereas SP (0.1 μM) and CGRP (0.01 μM) were added as single, predetermined concentrations (Illes & van Falkenhausen, 1986; Maynard *et al.*, 1990) and washed out once a maximal relaxant response was established.

Drugs and peptides

NA and ACh were obtained from the Sigma Chemical Company, Poole, U.K. SP, CGRP, NPY and anti-CGRP were purchased from Cambridge Research Biochemicals, Cambridge, U.K., and anti-NPY was obtained from Immunodiagnostics Ltd., U.K.

Expression of results and statistical methods

Relaxant responses are all expressed as a percentage change of the control response (taken as 100%). pD_2 values for ACh were calculated from the mean of the $-\log EC_{50}$ [ACh], i.e. the concentration of ACh which produced 50% of the maximal relaxant response. Slope values (Table 2) were obtained by use of a Pharmacologic Calculation System based on the *Manual of Pharmacologic Calculations with Computer*

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Table 1 Tissue content of neuropeptide Y (NPY) and calcitonin gene-related peptide (CGRP) in the rabbit central ear artery from control segments and preparations 6 weeks after a single dose of 45 Gy X-irradiation

	NPY (pmol cm^{-1})	*CGRP* (pmol cm^{-1})
Control segments	0.65 ± 0.10 (6)	0.13 ± 0.03 (6)
Irradiated segments	0.59 ± 0.11 (6)	0.14 ± 0.01 (6)

Values are mean ± s.e.mean. (*n*) indicates the number of preparations.

Programs (Tallarida & Murray, 1981). Results are expressed as the mean ± standard error of the mean (s.e.mean), and statistical significance was calculated by use of Student's unpaired *t* test. A probability level of $P \leqslant 0.05$ was considered significant.

Results

Peptide assays

Immunoassays were performed on control and 6 weeks post-irradiated REA preparations. There was no significant change in the amount of either NPY or CGRP in the REA 6 weeks after 45 Gy X-irradiation in comparison with control preparations (Table 1).

Pharmacology

In preconstricted control preparations, ACh produced concentration-dependent relaxant responses. The maximum relaxant effect of ACh measured in control preparations (80.9 ± 3.7% relaxation of NA-induced preconstriction) was significantly reduced 1, 4 and 6 weeks after 45 Gy irradiation (Table 2). The diminution of the relaxant response increased with time after irradiation, being greatest after 6 weeks. The pD_2 value measured in segments from control animals, however, was not significantly different up to 6 weeks after 45 Gy X-irradiation (Table 2).

Both SP (0.1 μM) and CGRP (0.01 μM) produced relaxant responses in preconstricted REA segments from control animals (27.4 ± 4.4% and 57.6 ± 5.4% relaxation of NA-induced preconstriction, respectively) which were significantly reduced after 45 Gy X-irradiation (Figure 1a,b).

Table 2 Statistical analysis of the effect of acetylcholine (ACh) on isolated transverse ring preparations of the rabbit ear artery as influenced by X-irradiation treatment: age-matched control preparations are compared with those 1, 4 and 6 weeks after a single dose of 45 Gy X-radiation

Time after irradiation (weeks)	*Maximum relaxation to acetylcholine*	*pD_2 value*	*Slope*
Control (7)	100.0 ± 4.6	5.9 ± 0.2	57.1 ± 4.6
1 (6)	50.5 ± 10.3***	6.2 ± 0.1^{NS}	34.6 ± 7.0*
4 (6)	42.6 ± 5.1***	6.0 ± 0.2^{NS}	31.2 ± 5.0**
6 (6)	36.9 ± 7.0***	5.8 ± 0.1^{NS}	21.9 ± 3.3***

All values are given as mean ± s.e.mean and *n* (in parentheses) is the number of observations. Asterisks denote significant differences (* $P \leqslant 0.05$; ** $P \leqslant 0.01$; *** $P \leqslant 0.001$); NS denotes no significant differences between control and irradiated preparations. pD_2 values were calculated as the −log molar concentration of ACh producing 50% of the maximal response. Maximal relaxation to ACh is expressed as a percentage of the control response (taken as 100%).

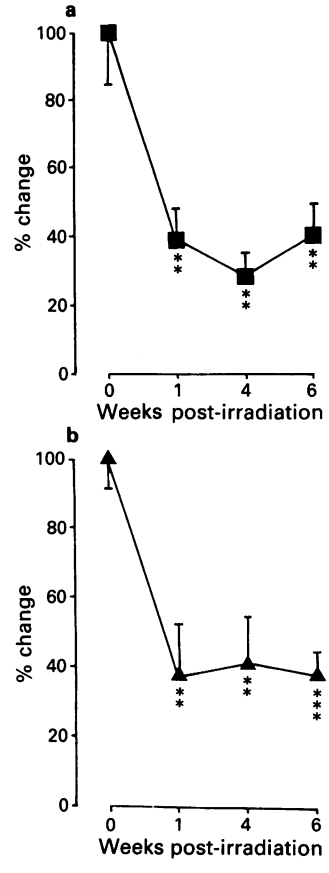


Figure 1 Graphs illustrating the percentage change of relaxations mediated by substance P (0.1 μM) (a) and calcitonin gene-related peptide (CGRP, 0.01 μM) (b) on preconstricted preparations (noradrenaline, 3–10 μM) of the rabbit isolated central ear artery. Preparations were taken from age-matched control animals and animals 1, 4 and 6 weeks after a single dose of 45 Gy X-irradiation. Symbols represent means and vertical bars denote s.e.mean (** $P \leqslant 0.01$; *** $P \leqslant 0.001$).

Discussion

A single dose of 45 Gy X-irradiation reduced the levels of NA (Stewart-Lee *et al.*, 1991), but not NPY (this study) in the REA. This finding suggests that X-irradiation may have a differential effect on these two vasoactive substances, which are thought to be colocalised in the same sympathetic nerve terminals in the REA (Saville *et al.*, 1990). Alternatively, 45 Gy X-irradiation may damage sympathetic fibres that do not contain NPY. NA is stored in both small and large dense-cored vesicles (Miserez *et al.*, 1988), whereas NPY, which was unaffected 6 weeks after 45 Gy X-irradiation, is stored exclusively in large dense-cored vesicles (Wharton & Gulbenkian, 1987). Therefore, it is possible that the small dense-cored, NA-containing vesicles are selectively radiation-sensitive. This possibility is consistent with the observation that endogenous levels of CGRP were unaffected, since this polypeptide is also stored in large dense-cored vesicles (Wharton & Gulbenkian, 1987) in sensory-motor nerves of the REA (Maynard *et al.*, 1990).

The finding that after 45 Gy X-irradiation, the endothelium-dependent relaxant responses to ACh (Owen & Bevan, 1985; Maynard *et al.*, 1990) and SP (Bolton & Clapp, 1986) in the REA were progressively attenuated with time may be a reflection of the structural changes to the endothelium caused by this treatment (Kristek *et al.*, 1990). However, since the relaxant responses to CGRP, which acts independently of the endothelium in the REA (Maynard *et al.*, 1990), were also reduced to a similar degree, X-irradiation may have induced a general impairment of the relaxant capacity of the REA smooth muscle. It should be noted that there was no effect on

NA-induced contractions after 45 Gy X-irradiation (Stewart-Lee *et al.*, 1991). This suggests that the vasodilator mechanism(s) may be more radiation-sensitive than the vasoconstrictor mechanism(s) of the REA smooth muscle. It is possible that the selective damage may be at the level of the transduction mechanisms associated with vasodilatation, but not involved in vasoconstriction, e.g. calcium sequestration (Kuriyama, 1981).

Our previous studies have shown that perivascular nerves, endothelial cells and smooth muscle cells (Kristek *et al.*, 1990), and sympathetic transmission (Stewart-Lee *et al.*, 1991) are all adversely affected by a single dose of 45 Gy X-irradiation. The present paper reveals that even though levels of endogenous CGRP in perivascular nerves are unaffected after 45 Gy X-irradiation, the relaxant action of this polypeptide is impaired.

Since there has been a revival in the use of intraoperative radiotherapy in clinical practice (Goldson, 1981; Abe & Takahashi, 1981; Suit *et al.*, 1985; Hoopes *et al.*, 1987; Gillette *et al.*, 1988), it is important to improve our knowledge regarding the effect of using single massive doses of radiation on or near blood vessels. Further experiments are necessary to elucidate the mechanisms underlying these changes, which could severely disrupt the normal local control of vascular tone and blood flow after X-irradiation.

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One way cross tolerance between cromakalim and salbutamol in the uterus of the rat *in vivo*

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1 Cross tolerance between the potassium (K^+) channel opener, cromakalim and the β_2-adrenoceptor agonist, salbutamol, was investigated in the uterus of the non-pregnant rat *in vivo*. Uterine sensitivity to salbutamol was similar in both vehicle-treated and cromakalim-tolerant rats. In salbutamol-tolerant rats, uterine responses to cromakalim were markedly decreased compared with saline-infused rats, such that maximum inhibition of uterine contractions was less than 40%.

2 Propranolol treatment and salbutamol tolerance each produced similar reductions in sensitivity of the uterus to salbutamol of approximately 10 fold. The same dose of propranolol did not influence uterine sensitivity to cromakalim, which suggests that the relaxant action of cromakalim is not due to a direct or indirect activation of uterine β_2-adrenoceptors.

3 Salbutamol produced a marked (11.7 fold) increase in uterine adenosine 3':5'-cyclic monophosphate (cyclic AMP) concentrations measured *ex vivo*, which was completely inhibited by propranolol pretreatment, but was unaffected by glibenclamide pretreatment. Cromakalim did not increase uterine cyclic AMP concentrations, suggesting that stimulation of adenylate cyclase is not significant in the uterine relaxant action of cromakalim.

4 The lack of propranolol antagonism of cromakalim and of cromakalim-induced changes in uterine cyclic AMP concentrations suggests that the cross tolerance observed between salbutamol and cromakalim may be at the level of K^+-channels.

Keywords: Cromakalim; salbutamol; propranolol; cyclic AMP; uterus; K^+-channels

Introduction

It has been suggested that cromakalim, a relaxant of smooth muscle, acts by opening plasma membrane K^+-channels (Cook, 1988; Hamilton & Weston, 1989; Winquist *et al.*, 1989; Newgreen *et al.*, 1990). Glibenclamide, a selective blocker of ATP-dependent K^+-channels, produces competitive-like antagonism of cromakalim in a number of smooth muscles including uterus (Buckingham *et al.*, 1989; Cavero *et al.*, 1989; Eltze, 1989; Wilson, 1989; Winquist *et al.*, 1989; Piper *et al.*, 1990). *In vivo*, a single bolus dose of cromakalim produces prolonged inhibition of uterine contractions (Downing *et al.*, 1989); however, repeated bolus injections of cromakalim do not maintain inhibition of uterine activity. Development of tolerance during repeated administration of cromakalim results in a 25 fold desensitization of the uterus to the drug. The mechanism by which tolerance develops is not known.

β_2-Adrenoceptor agonists such as salbutamol are also relaxants of smooth muscle and act by increasing membrane adenylate cyclase activity leading to increased intracellular concentrations of adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Johansson & Andersson, 1980; 1981; Harden, 1983). It has been suggested that β_2-adrenoceptor agonists also open K^+-channels inducing membrane hyperpolarization and this contributes to their mechanism of smooth muscle relaxation (Diamond & Marshall, 1969; Kroeger & Marshall, 1973; Yamaguchi *et al.*, 1988; Sadoshima *et al.*, 1988; Kume *et al.*, 1989; Longmore *et al.*, 1991). Uterine tolerance to salbutamol has also been observed *in vivo* (Abel & Hollingsworth, 1986). Development of tolerance to β_2-adrenoceptor agonists has been shown to be due to internalization and loss of membrane receptors and uncoupling of the membrane-receptor complex from the adenylate cyclase complex with consequent loss of production of second messenger cyclic AMP (Harden, 1983).

Despite the differences in mechanisms of action of the two relaxants, preliminary studies indicated that cross tolerance between cromakalim and salbutamol occurs in the uterus. The aim of this study was to characterize the phenomenon of cross tolerance between cromakalim and salbutamol in the uterus of the rat *in vivo*. It is possible that an indirect stimulation of uterine cyclic AMP concentrations by cromakalim may occur *in vivo* upon reflex release of adrenaline into the circulation after cromakalim-induced hypotension. The possible involvement of β_2-adrenoceptor stimulation and cyclic AMP production in cromakalim-induced uterine relaxation and cross tolerance with salbutamol was investigated by use of the β_2-adrenoceptor antagonist, propranolol, and by measuring uterine cyclic AMP concentrations *ex vivo* after cromakalim treatment. Preliminary findings have been presented to the British Pharmacological Society (Downing & Hollingsworth, 1991).

Methods

Female rats, 200–250 g, were purchased from Charles River, U.K.

Surgical techniques

Rats were anaesthetized with tribromoethanol (240 mg kg^{-1}, i.p.), bilaterally ovariectomized and the right jugular vein cannulated. A small latex balloon was placed in one uterine horn. Morphine sulphate (5 mg kg^{-1}, s.c.) was given postoperatively to all rats. Animals were allowed 24 h for recovery, then continuous recording of pressure cycles produced by uterine contractions was started in conscious rats (Downing & Sherwood, 1985). Contractions were quantified as the integral of the area under the pressure curve. The rats were allocated to one of the following groups.

In vivo *recordings*

(I) *Cromakalim/salbutamol cross tolerance* Uterine responses to bolus i.v. doses of cromakalim (0.05–0.25 mg kg^{-1}) or salbutamol (20–400 μg kg^{-1}) were recorded for 60 min and expressed as percentage inhibition of the integral of uterine

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contractions recorded for the 60 min immediately before each bolus dose. Three hours elapsed between each bolus dose to allow the drug to clear. Three hours after the last bolus dose, the rats were given an i.v. infusion of saline ($0.15\,ml\,h^{-1}$) or salbutamol ($120\,\mu g\,kg^{-1}\,h^{-1}$) for 24 h or 3 i.v. injections of vehicle (14% ethanol/saline, $1\,ml\,kg^{-1}$) or cromakalim ($1\,mg\,kg^{-1}$) 8 h apart. After tolerance induction/vehicle treatment, uterine responses to cromakalim or salbutamol were determined as before, such that the following combinations were examined: (a) Uterine sensitivity to cromakalim before and after salbutamol tolerance (13 rats)/vehicle infusion (10 rats). (b) Uterine sensitivity to salbutamol before and after cromakalim tolerance (10 rats)/vehicle injections (10 rats). Pre- and post-tolerance log ID_{50} values were estimated for each rat by probit analysis. Log ID_{50} values were compared statistically by analysis of variance followed by Student's t test.

(II) *Influence of propranolol on uterine sensitivity to cromakalim and salbutamol* Uterine responses to bolus i.v. doses of cromakalim or salbutamol were determined as described before. Rats were then given an i.v. infusion of saline ($0.15\,ml\,h^{-1}$) for 2 h, salbutamol ($120\,\mu g\,kg^{-1}\,h^{-1}$) for 24 h or propranolol ($400\,\mu g\,kg^{-1}\,h^{-1}$) for 2 h. After infusion, uterine responses to cromakalim or salbutamol were determined as before such that the following combinations were examined: (a) Uterine sensitivity to salbutamol before and after induction of salbutamol tolerance (12 rats). (b) Uterine sensitivity to salbutamol before and after propranolol (9 rats)/saline (10 rats) infusion. (c) Uterine sensitivity to cromakalim before and after propranolol (10 rats)/saline (10 rats) infusion.

Pre- and post-infusion log ID_{50} values were estimated for each rat and compared statistically by analysis of variance followed by Student's t test.

Biochemical studies

Rats were anaesthetized with tribromoethanol and a cannula placed in the right jugular vein. Anaesthesia was maintained throughout the experiments. Rats were then allocated to one of the following groups:

(III) *Time course of cromakalim-induced changes in cyclic AMP concentrations* Rats were given bolus i.v. doses of cromakalim ($0.25\,mg\,kg^{-1}$) or vehicle (14% ethanol/saline, $1\,ml\,kg^{-1}$). Rats were killed at 1, 2, 5, 10, 20, 40 or 60 min after bolus dose ($n = 4$–8 rats per time point), the uterus removed as rapidly as possible and frozen in liquid nitrogen. The uterus was homogenized in 5% w/v trichloroacetic acid, then the homogenate centrifuged at 1200 g and the pellet used for protein determination by the Lowry-Folin method (Lowry *et al.*, 1951). The supernatant was shaken with 4×4 volumes of water-saturated diethyl ether to remove trichloroacetic acid, the ether layer being discarded. Aliquots of the extract (25 and 50 μl) were assayed for cyclic AMP with tritiated cyclic AMP radioimmunoassay kits (Amersham (H^3) cAMP assay system).

(IV) *Influence of propranolol on cromakalim or salbutamol-induced changes in cyclic AMP concentrations* Rats were anaesthetized and jugular veins cannulated as described before. The rats were then given i.v. bolus doses of propranolol ($400\,\mu g\,kg^{-1}$) or saline ($1\,ml\,kg^{-1}$) followed 5 min later by cromakalim ($0.25\,mg\,kg^{-1}$), salbutamol ($100\,\mu g\,kg^{-1}$) or saline ($1\,ml\,kg^{-1}$) ($n = 4$–6 rats). Rats were killed at 2 min after the second bolus dose, the uterus removed, frozen, homogenized and extracted and assayed for cyclic AMP as described before.

(V) *Influence of glibenclamide on cromakalim or salbutamol-induced changes in cyclic AMP concentrations* Rats were anaesthetized and jugular veins cannulated as described before. The rats were then given an i.v. infusion of glibenclamide ($20\,mg\,kg^{-1}$ in 0.02 N sodium hydroxide in 4% glucose) followed 15 min later by bolus i.v. doses of cromakalim ($0.25\,mg\,kg^{-1}$), salbutamol ($100\,\mu g\,kg^{-1}$) or saline ($1\,ml\,kg^{-1}$) ($n = 4$–6 rats). At 2 min after bolus dose, the animals were killed and the uterus removed, frozen, homogenized and extracted and assayed for cyclic AMP as described previously.

Cyclic AMP radioimmunoassay

Cyclic AMP concentrations in extracts of uterine tissue were assayed over the range 0.25–16 pM. Coefficient of variation within assay was 4.1% ($n = 4$) and for several assays over 6 months was 4.5% ($n = 12$). Recovery of cyclic AMP from uterine tissue during extraction was 62.5 ± 2.5%. Values of cyclic AMP concentrations are uncorrected for procedural losses.

Drugs and analysis of results

Cromakalim was obtained from SmithKline Beecham, salbutamol from Glaxo and glibenclamide from Hoechst. Propranolol was purchased from Sigma Chemical Co., (Poole, U.K.), tribromoethanol from Fluka Chemicals (Glossop, U.K.) and morphine sulphate from Evans (Dunstable, U.K.). Values are quoted as means ± s.e.mean. The significance of difference between means was calculated by analysis of variance followed by Student's t test.

Results

(I) *Cromakalim/salbutamol cross tolerance*

Uterine sensitivity to salbutamol increased in both vehicle-treated and cromakalim-tolerant rats and to similar extents, 4.6 fold and 5.5 fold respectively (pre-vehicle log ID_{50}, 1.94 ± 0.16, post-vehicle log ID_{50}, 1.28 ± 0.10, $P < 0.01$, pre-cromakalim log ID_{50}, 2.18 ± 0.18, post-cromakalim log ID_{50}, $1.44 \pm 0.14\,\mu g\,kg^{-1}$, $P < 0.01$) (Figure 1). Cromakalim tolerance, therefore, had no effect on uterine sensitivity to salbutamol. Uterine sensitivity to cromakalim showed an 8.7 fold increase in saline-infused rats (pre-saline log ID_{50}, -0.76 ± 0.10, post-saline log ID_{50}, $-1.70 \pm 0.09\,mg\,kg^{-1}$, $P < 0.001$). However, uterine responses to cromakalim were markedly decreased in salbutamol-tolerant rats and maximum inhibition of uterine contractions was less than 40% (Figure 2).

(II) *Influence of propranolol on uterine sensitivity to cromakalim or salbutamol* Salbutamol infusion resulted in a decrease in uterine sensitivity to salbutamol of 14.1 fold (pre-tolerance log ID_{50}, 1.69 ± 0.19, post-tolerance log ID_{50}, $2.84 \pm 0.21\,\mu g\,kg^{-1}$, $P < 0.001$) (Figure 3). Propranolol infusion resulted in a similar reduction in uterine sensitivity to salbutamol of 11.2 fold (pre-saline log ID_{50}, 1.73 ± 0.14, post-saline log ID_{50}, 1.69 ± 0.19; pre-propranolol log ID_{50}, 1.87 ± 0.11, post-propranolol log ID_{50}, $2.92 \pm 0.10\,\mu g\,kg^{-1}$, $P < 0.001$). Propranolol did not influence uterine responses to cromakalim. Uterine sensitivity to cromakalim increased in both saline- and propranolol-infused rats (pre-saline log ID_{50}, -0.69 ± 0.10, post-saline log ID_{50}, -1.12 ± 0.16; pre-propranolol log ID_{50}, -0.81 ± 0.09, post-propranolol log ID_{50}, $-1.15 \pm 0.10\,mg\,kg^{-1}$). Uterine sensitivity to cromakalim after propranolol infusion was not significantly different from that observed after saline infusion (Figure 4).

(III) *Time course of cromakalim-induced changes in cyclic AMP concentrations*

Concentrations of cyclic AMP in uterine tissue at various times after cromakalim bolus are given in Table 1. The changes in uterine cyclic AMP concentrations after cromakalim treatment were not significantly different from those after vehicle treatment throughout the 60 min period.

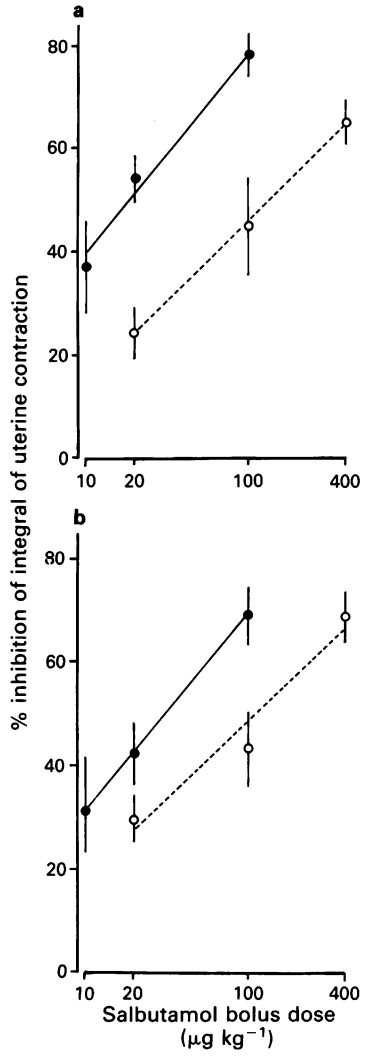


Figure 1 Effect of salbutamol on uterine contractions (a) before (○) and after (●) treatment with 14% ethanol/saline vehicle (3 × 1 ml kg^{-1}, i.v., 8 h apart, 10 rats) and (b) before (○) and after (●) treatment with cromakalim (3 × 1 mg kg^{-1}, i.v., 8 h apart, 10 rats). Ordinate scale: % inhibition of integral of uterine contractions. Abscissa scale: salbutamol bolus dose (μg kg^{-1}) log scale.

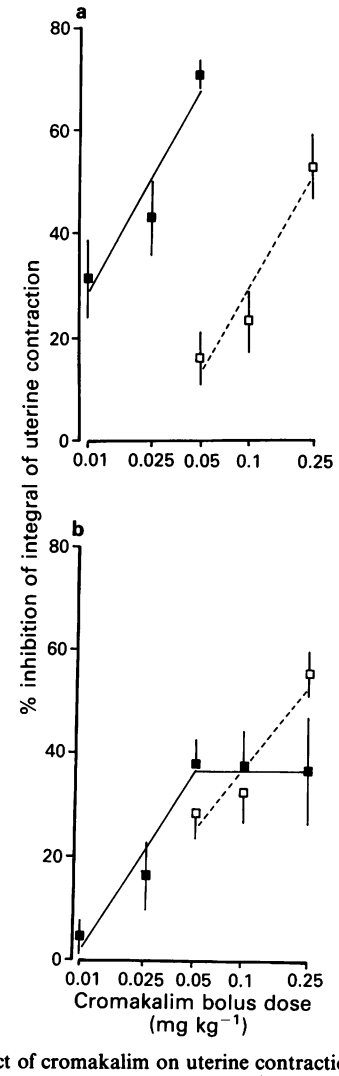


Figure 2 Effect of cromakalim on uterine contractions (a) before (□) and after (■) infusion of saline (0.15 ml h^{-1} i.v. for 24 h, 10 rats) and (b) before (□) and after (■) infusion of salbutamol (120 μg kg^{-1} h^{-1} i.v., for 24 h, 13 rats). Ordinate scale: % inhibition of integral of uterine contractions. Abscissa scale: cromakalim bolus dose, (mg kg^{-1}), log scale.

(IV) *Influence of propranolol on cromakalim or salbutamol-induced changes in cyclic AMP concentrations*

Propranolol plus saline treatment had no effect on uterine cyclic AMP concentrations (Figure 5). Salbutamol produced a marked increase in uterine cyclic AMP concentrations at

Table 1 Time course of changes in uterine cyclic AMP concentrations after cromakalim or vehicle treatment

	Cyclic AMP concentrations (pmol mg^{-1} protein)	
Time (min)	*Vehicle*	*Cromakalim* (0.25 mg kg^{-1})
1	9.61 ± 0.67 (6)	11.32 ± 1.55 (6)
2	8.71 ± 1.09 (8)	11.63 ± 2.60 (8)
5	8.60 ± 1.84 (6)	10.53 ± 3.66 (7)
10	7.53 ± 2.24 (6)	8.92 ± 1.73 (6)
20	9.18 ± 1.29 (5)	7.91 ± 1.07 (4)
40	11.14 ± 3.33 (4)	6.87 ± 1.00 (5)
60	6.95 ± 0.46 (4)	7.47 ± 0.92 (4)

Values are means ± s.e.mean and numbers of rats are given in parentheses. There were no significant differences with treatment.

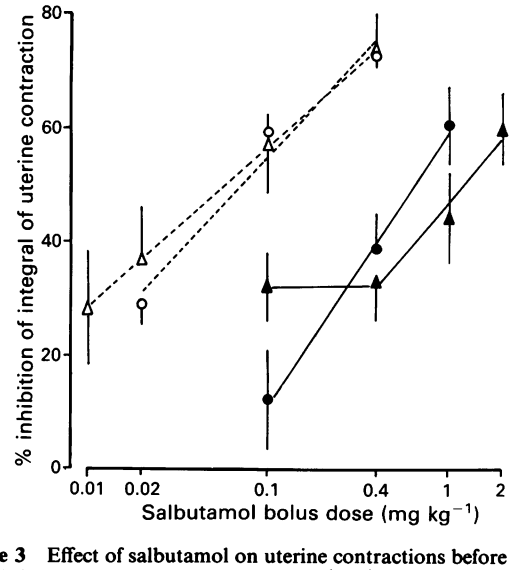


Figure 3 Effect of salbutamol on uterine contractions before (○) and after infusion of salbutamol (120 μg kg^{-1} h^{-1} for 24 h, 12 rats, ●), propranolol (400 μg kg^{-1} h^{-1} for 2 h, 9 rats, ▲) or saline (0.15 ml h^{-1} for 2 h, 10 rats, △). Ordinate scale: % inhibition of integral of uterine contraction. Abscissa scale: salbutamol bolus dose (mg kg^{-1}) log scale.

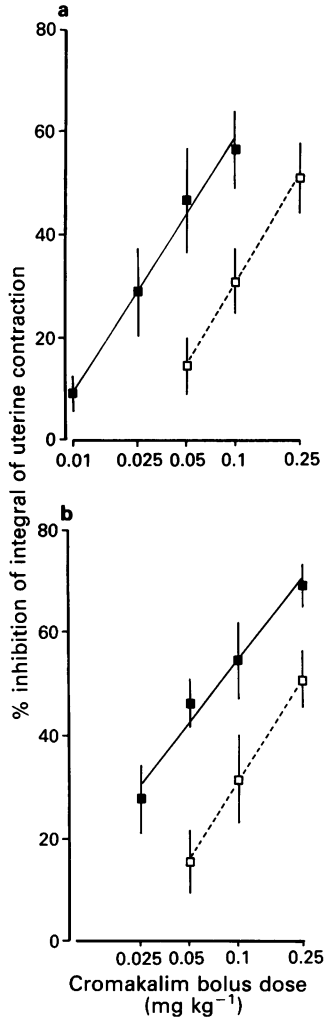


Figure 4 Effect of cromakalim on uterine contractions (a) before (□) and after (■) infusion of saline (0.15 ml h^{-1} i.v. for 2 h, 10 rats) and (b) before (□) and after (■) infusion of propranolol (400 $\mu g\,kg^{-1}\,h^{-1}$ i.v. for 2 h, 10 rats). Ordinate scale: % inhibition of integral of uterine contractions. Abscissa scale: cromakalin bolus dose (mg kg^{-1}) log scale.

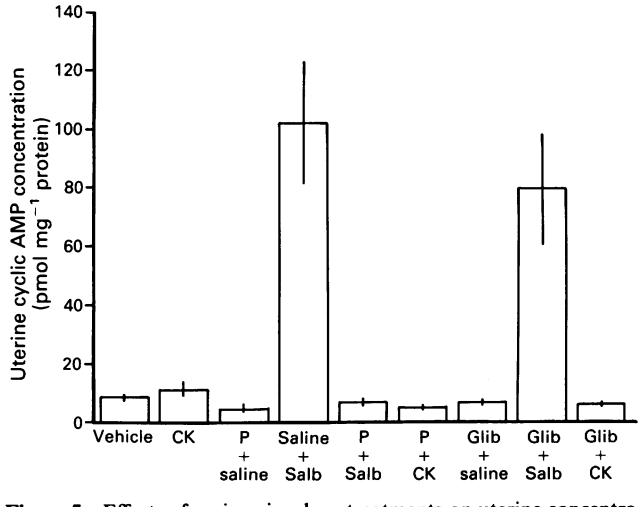


Figure 5 Effects of various i.v. drug treatments on uterine concentrations of cyclic AMP measured *ex vivo*. Ordinate scale: cyclic AMP concentrations (pmol mg^{-1} protein). Abscissa scale: drug treatment. Vehicle = 14% ethanol/saline. CK = cromakalim (0.25 mg kg^{-1}). P = propranolol (400 $\mu g\,kg^{-1}$). Saline = 1 ml kg^{-1}. Salb = salbutamol (100 $\mu g\,kg^{-1}$). Glib = glibenclamide (20 mg kg^{-1}). Values are means of n = 4–8 rats; vertical lines show s.e.mean.

2 min after bolus dose ($P < 0.001$) which was inhibited by prior propranolol treatment. Cyclic AMP concentrations in uterine extracts from propranolol plus cromakalim-treated rats were similar to those from animals treated with vehicle for propranolol.

(V) *Influence of glibenclamide on cromakalim or salbutamol-induced changes in cyclic AMP concentrations*

Glibenclamide plus saline treatment had no effect on uterine cyclic AMP concentrations (Figure 5). Glibenclamide did not significantly reduce salbutamol-induced increase in uterine cyclic AMP concentrations. Uterine cyclic AMP concentrations in glibenclamide plus cromakalim-treated rats were similar to those observed in vehicle-treated rats.

Discussion

In vehicle-treated rats, an increase in uterine sensitivity to salbutamol and cromakalim was observed. For salbutamol, the increase in uterine sensitivity of approximately 5 fold, is apparent by 48 h after surgical balloon implantation, whereas for cromakalim, uterine sensitivity appears to increase progressively from 30 h after surgery. The apparent change in sensitivity may, in part, reflect a decline in uterine prostaglandin production, which would have been stimulated during balloon insertion and would have stimulated uterine contractions. It may be that 24 h recovery between surgery and uterine sensitivity testing is insufficient. However, integrals of uterine contractions recorded at 48 h after surgery (65.2 ± 3.3 units per h) are slightly greater than those recorded at 24 h after surgery (53.9 ± 2.7, n = 23 rats). Therefore, the change in uterine sensitivity to the drugs is not due solely to the *in vivo* recording technique. The increase in uterine sensitivity to salbutamol may also be, in part, a result of ovariectomy. Oestrogen has been shown to reduce β_2-adrenoceptor-mediated cyclic AMP production in rabbit uterus (Riemer *et al.*, 1988). Uterine sensitivity to β_2-adrenoceptor agonists may increase progressively during the period of declining oestrogen influence. In contrast to salbutamol, uterine sensitivity to cromakalim is increased by oestradiol treatment (Downing & Hollingsworth, unpublished), and therefore, declining oestrogen influence after ovariectomy is unlikely to be a cause of the marked increase in sensitivity to cromakalim with time. The mechanism of the increase in uterine sensitivity to cromakalim is not known. No increase in sensitivity is observed *in vitro* at 4 h after initial cromakalim concentration-effect testing, and therefore this phenomenon is unlikely to be cromakalim-induced (Piper *et al.*, 1991). In addition, no increase in cardiovascular sensitivity to cromakalim has been observed (Downing & Hollingsworth, 1989) suggesting that this phenomenon is specific to the uterus.

Cross tolerance between cromakalim and salbutamol was observed in the uterus of the rat, but in one direction only. Uterine sensitivity to salbutamol was unaffected by development of tolerance to cromakalim. However, uterine responses to cromakalim were markedly depressed by development of tolerance to salbutamol.

The possibility that cromakalim relaxes uterine smooth muscle indirectly *in vivo* by reflex release of adrenaline resulting from cromakalim-induced hypotension, thus activating uterine β_2-adrenoceptors, was tested by use of propranolol. Propranolol produces blockade of uterine β_2-adrenoceptors and antagonizes adrenaline released in response to cromakalim-induced fall in blood pressure. At doses which were effective at reducing salbutamol-induced inhibition of uterine contractions and stimulation of uterine cyclic AMP concentrations, propranolol had no effect on uterine sensitivity to cromakalim. Furthermore, a large bolus dose of cromakalim (0.25 mg kg^{-1}, i.v.) did not increase

uterine cyclic AMP concentrations. Although in most isolated tissues cromakalim does not change cyclic AMP concentrations (Newgreen *et al.*, 1990), cromakalim has been shown to increase cyclic AMP concentrations in taenia coli (McHarg *et al.*, 1989). It may be that significant losses of cyclic AMP occurred due to the action of endogenous phosphodiesterases before the tissue was frozen; however, marked increases in uterine cyclic AMP concentrations in response to salbutamol were observed with the same methodology, suggesting that cromakalim-induced changes in cyclic AMP would have been observed, had they occurred. It therefore appears unlikely that cromakalim acts directly or indirectly on β_2-adrenoceptors in the uterus.

The lack of antagonism of cromakalim by propranolol and the absence of changes in uterine cyclic AMP concentrations after cromakalim bolus suggest that the desensitization to cromakalim observed in salbutamol-tolerant rats is not due to loss of β_2-adrenoceptors or uncoupling of adenylate cyclase. Potassium channel opening may be involved in the mechanism of action of β_2-adrenoceptor agonists (Diamond & Marshall, 1969; Kroeger & Marshall, 1973; Izumi & Kishikawa, 1982; Yamaguchi *et al.*, 1988). Kume *et al.* (1989) have demonstrated that isoprenaline and cyclic AMP activate calcium-dependent K^+-channels in rabbit isolated tracheal myocytes. Cromakalim has also been reported to activate calcium-dependent K^+-channels in arterial vascular smooth muscle (Kusano *et al.*, 1987; Gelband *et al.*, 1989; Post *et al.*, 1989). The activity of calcium-activated K^+-channels is modulated by a number of regulatory factors, including ATP (Groschner *et al.*, 1991), cyclic AMP-dependent phosphorylation (Sadoshima *et al.*, 1988; Kume *et al.*, 1989) at a site closely associated with the channel (Ewald *et al.*, 1985), and guanosine 5'-monophosphate (Williams *et al.*, 1988). These factors may in turn be modulated by salbutamol and/or cromakalim and may also represent a site at which tolerance develops. The K^+-channel itself may also become tolerant to salbutamol or cromakalim. Although glibenclamide does not antagonize salbutamol as an inhibitor of uterine contractions (Downing & Hollingsworth, 1990), nor influence salbutamol-induced changes in cyclic AMP concentrations, ATP-dependent K^+-channel opening may be a minor component of β_2-adrenoceptor agonist action (Longmore *et al.*, 1991). Blockade or desensitization of potassium channels by cromakalim may be overridden by the cyclic AMP-mediated inhibition of uterine contractions and thus uterine sensitivity to salbutamol would not be reduced in cromakalim-tolerant rats. Desensitization of potassium channels in addition to loss of β_2-receptors may occur during development of tolerance to salbutamol and may result in cross tolerance with cromakalim.

An alternative explanation for cross tolerance between salbutamol and cromakalim in one direction only is that cromakalim tolerance may be due to loss of cromakalim binding sites only. Such a mechanism would be unlikely to influence uterine sensitivity to salbutamol. Cromakalim may not induce tolerance of putative second messenger systems or of K^+-channels. Development of tolerance to salbutamol, however, may include loss of salbutamol binding sites, uncoupling of second messenger systems and desensitization of K^+-channels. In this case, uterine sensitivity to cromakalim would be reduced in salbutamol-tolerant rats.

In conclusion, cross tolerance between salbutamol and cromakalim has been observed in one direction; uterine sensitivity to cromakalim was reduced in salbutamol-tolerant rats, but sensitivity to salbutamol was unchanged in cromakalim-tolerant rats. Propranolol treatment did not influence uterine sensitivity to cromakalim, nor were significant changes in uterine cyclic AMP concentrations observed after cromakalim treatment. Cross desensitization between cromakalim and salbutamol may be due to tolerance at the level of uterine K^+-channels.

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Endothelin-1-induced [^{3}H]-inositol phosphate accumulation in rat trachea

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1 The effects of endothelin-1 (ET-1) and of the muscarinic cholinoceptor agonist, carbachol, on [^{3}H]-inositol phosphate ([^{3}H]-InsP) accumulation and smooth muscle contraction were determined in rat isolated tracheal tissue.

2 ET-1 (1 μM) and carbachol (10 μM) induced significant accumulation of [^{3}H]-InsPs in *myo*-[2-^{3}H]-inositol-loaded rat tracheal segments. Several components of the tracheal wall including the airway smooth muscle band, the cartilaginous region and the intercartilaginous region generated significant levels of [^{3}H]-InsPs in response to ET-1 and carbachol. Following stimulation with ET-1, a greater proportion of tracheal [^{3}H]-InsPs were generated in the intercartilaginous region (49%) than in either the airway smooth muscle band (25%) or cartilaginous region (26%). However, when the respective weights of these regions is taken into account, ET-1-induced accumulation of [^{3}H]-InsPs was greatest in the airway smooth muscle band. The tracheal epithelium did not appear to generate [^{3}H]-InsPs in response to ET-1 or modulate either basal or ET-1-induced accumulation of [^{3}H]-InsPs in rat tracheal segments.

3 In the rat tracheal smooth muscle band, ET-1 caused a time- and concentration-dependent accumulation of [^{3}H]-InsPs. Concentrations of ET-1 as low as 10 nM produced significant accumulation of [^{3}H]-InsPs (1.23 $\pm$ 0.10 fold increase above basal levels of 295 $\pm$ 2 d.p.m. mg^{-1} wet wt., $n = 3$ experiments). At 10 μM, the highest concentration used, ET-1 produced similar levels of [^{3}H]-InsP accumulation (7.03 $\pm$ 0.55 fold above basal levels, $n = 5$) to that produced by a maximally effective concentration of carbachol (10 mM; 7.97 $\pm$ 0.31 fold increase above basal levels, $n = 4$). ET-1-induced accumulation of [^{3}H]-InsPs was not significantly affected by indomethacin (5 μM), nordihydroguaiaretic acid (NDGA, 10 μM), WEB 2086 (10 μM) or phosphoramidon (10 μM).

4 ET-1 also produced concentration-dependent contractions of epithelium-denuded rat tracheal ring preparations. The mean concentration of ET-1 producing 50% of the maximum contractile response to carbachol (EC_{50}) was 31 nM (95% confidence limits, 20–49 nM, $n = 12$). The presence of an intact tracheal epithelium, indomethacin (5 μM), WEB 2086 (10 μM) and phosphoramidon (10 μM) had no significant effect on the mean EC_{50} for ET-1-induced contraction ($n = 5$). In contrast, NDGA (10 μM) inhibited ET-1-induced contractions (4.0 fold increase in mean EC_{50}, $P < 0.001$, $n = 5$). However, this effect of NDGA did not appear to be related to inhibition of leukotriene synthesis via lipoxygenase since the leukotriene antagonist SKF 104353 did not affect ET-1-induced contractions ($n = 5$) and moreover, leukotriene C_4 and leukotriene D_4 did not contract rat isolated tracheal smooth muscle preparations ($n = 4$).

5 The threshold concentrations of ET-1 that produced increases in smooth muscle contraction and [^{3}H]-InsPs accumulation were similar, although the EC_{50} for [^{3}H]-InsP accumulation was 2.9 fold greater than that for smooth muscle contraction. For carbachol, the EC_{50} for [^{3}H]-InsP accumulation (mean EC_{50} = 5.0 μM, 1.2–21 μM, $n = 4$) was 25 fold greater than that for smooth muscle contraction (mean EC_{50} = 0.20 μM, 0.17–0.24 μM, $n = 12$).

6 It seems likely that ET-1 has a direct effect on InsP generation in rat tracheal smooth muscle and that this is largely responsible for the spasmogenic actions of this peptide.

Keywords: Endothelin-1; inositol phosphates; rat tracheal smooth muscle

Introduction

The 21-amino acid peptide endothelin-1 (ET-1) was first isolated from porcine aortic endothelial cells by Yanagisawa and coworkers (1988). In addition to producing potent vasoconstriction, ET-1 has been shown to produce numerous other actions including a positive inotropic effect in atria (Ishikawa *et al.*, 1988) and release of atrial natriuretic peptide (Fukuda *et al.*, 1988).

Relatively little is known of the airway effects of ET-1, although recent evidence suggests that ET-1 is synthesized within the lung and may exert an influence on its function. *In situ* hybridization and immunohistochemical studies indicate that ET-1 is synthesized and released from airway epithelial cells (MacCumber *et al.*, 1989; Black *et al.*, 1989; Rozengurt *et al.*, 1990). However, a growing body of data suggests that airway smooth muscle may be a primary target for this peptide. For example, quantitative autoradiographic studies of human and animal central airways have demonstrated high concentrations of specific binding sites for ET-1 in airway smooth muscle (Turner *et al.*, 1989; Henry *et al.*, 1990). *In vitro* functional studies suggest that stimulation of such sites produces a long-lasting and slowly reversible contraction of airway smooth muscle (Uchida *et al.*, 1988; Turner *et al.*, 1989; Henry *et al.*, 1990; Hemsen *et al.*, 1990; McKay *et al.*, 1991). Furthermore, ET-1 produces a significant bronchoconstrictor response in anaesthetized guinea-pigs *in vivo* (Payne & Whittle, 1988; Lagente *et al.*, 1989; Macquin-Mavier *et al.*, 1989; Pons *et al.*, 1991).

The mechanism(s) through which ET-1 produces contraction of airway smooth muscle are not clear. In some studies, a dependence on extracellular calcium was reported (Turner *et al.*, 1989; Advenier *et al.*, 1990), although others have shown ET-1-induced contraction in the absence of extracellular calcium (Hay, 1990; McKay *et al.*, 1991). In addition, calcium-channel blocking drugs, such as nicardipine have been reported to inhibit ET-1-induced contractions of guinea-pig and human airway smooth muscle preparations (Uchida *et al.*,

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1988; Maggi et al., 1989; Advenier et al., 1990; Hay, 1990), but not those in rat trachea (Turner et al., 1989). Thus, ET-1-induced contraction of airway smooth muscle in some species may involve an action on receptor-operated calcium-channels. In contrast, recent studies in vascular smooth muscle have demonstrated that ET-1-induced contraction may be mediated via the generation of inositol (1,4,5) trisphosphate (Ins(1,4,5)P_3) which is linked to the mobilization of intracellular calcium stores (Marsden et al., 1989; Xuan et al., 1989; Pang et al., 1989). This possibility has only recently been investigated with respect to ET-1 in airway smooth muscle (Hay, 1990), although this pathway has previously been demonstrated for some other spasmogens of airway smooth muscle including histamine, muscarinic cholinoceptor agonists, tachykinins and leukotrienes (Grandordy et al., 1986; 1988; Madison & Brown, 1988; Mong et al., 1988). In addition to the direct effects of ET-1, several recent studies have shown that ET-1 may release secondary mediators the actions of which may indirectly and variably contribute to the overall spasmogenic effects of ET-1 (Payne & Whittle, 1988; Macquin-Mavier et al., 1989; Battistini et al., 1990a,b).

In the current study, the relationship between ET-1-induced contraction of airway smooth muscle and [^{3}H]-inositol phosphate accumulation was determined in rat isolated tracheal preparations and compared to those effects induced by the muscarinic cholinoceptor agonist, carbachol. Furthermore, we examined the effects of several receptor antagonists and enzyme inhibitors to determine whether other secondary mediators contributed to these ET-1-induced effects.

Methods

Tissue preparation

Male Wistar rats (8–10 weeks) were stunned and killed by cervical dislocation and exsanguination. The trachea was rapidly excised, cleaned of adhering connective tissue and the tracheal epithelium deliberately removed by rubbing with a moist cotton wool swab (Goldie et al., 1986). To determine the extent of [^{3}H]-InsP accumulation in the entire tracheal wall, the trachea was cut into ring segments approximately 2–3 mm long.

We also compared the ability of various structures within the tracheal wall to generate [^{3}H]-InsPs; the airway smooth muscle, the epithelium, the cartilaginous region and intercartilaginous region. In these experiments, the trachea was not initially cut into segments. The airway smooth muscle band, which lies between the cartilage horns along the posterior membrane of the trachea, was dissected from the trachea with the aid of an optical magnifier (2-times magnification). The smooth muscle band was then cut transversely to generate 4 segments of similar length. The remainder of the trachea, which contains the alternating cartilaginous and intercartilaginous regions was stretched longitudinally and pinned flat. Although these two regions could be easily distinguished visually, it was not possible to dissect readily the intercartilaginous tissue from between neighbouring cartilage rings and to form a preparation comprising only intercartilaginous tissue. Therefore, to determine the relative contributions of the cartilaginous and intercartilaginous regions to the generation of [^{3}H]-InsPs, the trachea was cut at intervals, close to the cartilage, to provide two preparations (P1 and P2) which contained different proportions of the two regions. Both P1 and P2 had 2 cartilaginous regions; however, P1 contained a single intercartilaginous region whereas P2 contained three. The relative contributions of the cartilaginous region (x) and the intercartilaginous region (y) to the accumulation of InsPs was then determined by solving equations (1) and (2) simultaneously:

$$2x + y = \text{InsPs(P1)} \quad (1)$$

$$2x + 3y = \text{InsPs(P2)} \quad (2)$$

where InsPs(P1) and InsPs(P2) are the amounts of [^{3}H]-InsPs accumulated from P1 and P2, respectively. The ability of tracheal epithelium to generate [^{3}H]-InsPs was assessed indirectly by comparing tracheal segments that had an intact epithelium with those in which the epithelium had been mechanically removed, as described above.

Assay of [^{3}H]-inositol phosphate

For each experiment, preparations from 8–10 rats were pooled and randomly divided into 20 groups. Preparations were preincubated in 5 ml of Krebs-Henseleit solution (KH) for 30 min at 37°C and then incubated with *myo*-[2-^{3}H]-inositol (5 μCi) in 1 ml of carbogen-aerated KH for 3 h at 37°C with gentle shaking. Previous work on airway smooth muscle suggests that the presence of agonists such as carbachol during this loading period stimulates the incorporation of [^{3}H]-inositol into a hormone-sensitive phosphoinositide pool, allowing steady-state conditions to prevail during subsequent periods of agonist stimulation (Chilvers et al., 1989). In the current study, addition of 1 μM carbachol for the final 30 min of the loading period produced no noticeable increase in the subsequent accumulation of [^{3}H]-InsPs produced by ET-1. This suggests that the 3 h loading period used was sufficient to enable steady-state conditions to be achieved.

After loading with *myo*-[^{3}H]-inositol, preparations were washed twice with 5 ml of KH for 15 min and then washed a third time for a further 15 min. When atropine, indomethacin, nordihydroguaiaretic acid (NDGA), WEB 2086 or phosphoramidon were used, each was added after the third wash period (30 min before exposure to an agonist) and were in contact with tracheal tissue for the remainder of the experiment. After washing, segments were incubated for 15 min with 1 ml KH containing 5 mM LiCl to inhibit the breakdown of inositol monophosphate to inositol and thus enhance the subsequent accumulation of InsPs (Berridge et al., 1982). Tracheal segments were then stimulated for 15 min by addition of 20 μl of ET-1 or other agonists and the incubation terminated by the addition of 1.5 ml chloroform: methanol (1:2, v/v) with vigorous shaking. After standing for 15 min, chloroform (0.5 ml) and water (0.5 ml) were added sequentially to the samples to separate it into an aqueous and a lipid phase. The entire upper methanol/water phase was applied to an anion-exchange chromatography column (1 ml of Dowex AG1-X8 in formate form) and [^{3}H]-InsPs fractionated by washing the column with buffers of increasing ionic strength. Inositol was eluted with 10 ml of water and glycerophosphoinositol was eluted with 15 ml of buffer consisting of 5 mM sodium tetraborate and 60 mM sodium formate (Buffer I).

In some experiments with isolated airway smooth muscle segments, [^{3}H]-inositol monophosphates ([^{3}H]-InsP_1s), [^{3}H]-inositol bisphosphates ([^{3}H]-InsP_2s) and [^{3}H]-inositol trisphosphates ([^{3}H]-InsP_3s) were eluted stepwise with 15 ml of Buffer II (5 mM sodium tetraborate and 150 mM sodium formate), 10 ml of Buffer III (0.1 M formic acid and 0.3 M ammonium formate) and 10 ml of Buffer IV (0.1 M formic acid and 0.75 M ammonium formate) respectively. The [^{3}H]-InsP_3 fraction was not separated further and may therefore have contained a significant amount of the biologically less active [^{3}H]-Ins(1,3,4)P_3 isomer (Chilvers et al., 1990), as well as tetrakis- and higher polyphosphates. Otherwise, Buffer IV was added immediately after Buffer 1 to elute [^{3}H]-InsP_1, [^{3}H]-InsP_2 and [^{3}H]-InsP_3 in a single 10 ml fraction. Three 1 ml aliquots of this eluted fraction was mixed with 10 ml of scintillant (5.8 g l^{-1} 2,5-diphenyloxazol (PPO) in Triton X100: toluene, 1:2) and radioactivity counted in a Tri-Carb liquid scintillation counter (Packard, Model 1500). Preliminary experiments revealed that total [^{3}H]-InsP accumulation ([^{3}H]-InsP_1 plus [^{3}H]-InsP_2 plus [^{3}H]-InsP_3) estimated by the first elution method (sum of the radioactivities recovered from Buffers II, III and IV) was similar to the estimate obtained by the second elution method (radioactivity in Buffer

IV). Total [^{3}H]-InsP accumulation was expressed as d.p.m. mg^{-1} wet wt. tracheal tissue or as d.p.m./trachea, as indicated.

Functional studies

Rat trachea was isolated as described above. Four tracheal ring preparations (2 mm long segments) were obtained from each trachea and unless otherwise stated, denuded of epithelium (Goldie *et al.*, 1986). These preparations were suspended under a resting tension of 0.5 g and placed in siliconized organ baths containing 3 ml of KH at 37°C, bubbled continuously with 5% CO_2 in O_2. Changes in isometric tension in tracheal smooth muscle were measured with a Model 7D Polygraph via FTO3 force-displacement transducers (Grass Instruments). Tracheal rings were allowed to equilibrate for 45 min before exposure to a single dose of carbachol (0.3 μM) to assess airway smooth muscle viability (100%). Preparations were washed for 15 min. In the current study, we determined the influence of several receptor antagonists (WEB2086 (10 μM), SKF 104353 (3 μM)) and enzyme inhibitors (phosphoramidon (10 μM), indomethacin (5 μM), NDGA (10 μM)) on carbachol- and ET-1-induced contractions. Preparations were exposed for 30 min to a selected antagonist/inhibitor or solvent (control) and then to cumulatively administered concentrations of carbachol (0.01–10 μM). Tracheal segments were washed and rested for 15 min and then re-exposed for 30 min to the antagonist/inhibitor or solvent, as before. A concentration-effect curve to ET-1 (1–300 nM) was then obtained. Contractile responses to carbachol and ET-1 are expressed as a percentage of the maximum response obtained with 10 μM carbachol (C_{max}). For each experiment, EC_{50} values (concentration of spasmogen producing 50% C_{max}) were derived directly from the concentration-effect curves by interpolation. Geometric mean EC_{50} values with associated 95% confidence limits were obtained from n different animals.

Statistical analyses

Unless otherwise stated, data are presented as mean ± s.e.mean of the indicated number of experiments (n) and differences between treatments assessed by analysis of variance followed by a modified t-statistic (Wallenstein *et al.*, 1980). P values less than 0.05 were considered to be statistically significant.

Drugs

Drugs used were: *myo*-[2-^{3}H]-inositol (17.8 Ci $mmol^{-1}$, Amersham), endothelin-1 (Auspep, Melbourne, Australia), carbamylcholine chloride, indomethacin, nordihydroguaiaretic acid, phosphoramidon, histamine diphosphate (Sigma Chemical Company, St. Louis, USA), WEB 2086 (3-[1,1-dimethylethyl]hexahydro-1,4,7b-trihydroxy-α-methyl-9H-1,7a(epoxymethanol) - 1H,6aH - cyclapenta[c]furo[2.3 - b]furo[3',2':3,4] cyclopenta[1,2-d]furan-5,9,12[4H]-trione, (Boehringer, Ingelheim), leukotriene C_4, leukotriene D_4, SKF 104353 (SmithKline Beecham Pharmaceuticals, King of Prussia, U.S.A.). Indomethacin was dissolved in 0.1 M Na_2CO_3 and nordihydroguaiaretic acid in ethanol. Stock solutions of endothelin-1 (50 μM) were stored in 0.1 μM acetic acid. All other drugs were dissolved and diluted in 0.9% saline. The composition of the KH solution was (in mM): NaCl 117, KCl 5.36, $NaHCO_3$ 25.0, KH_2PO_4 1.03, $MgSO_4 \cdot 7H_2O$ 0.57, $CaCl_2 \cdot 2H_2O$ 2.5 and glucose 11.1.

Results

Accumulation of [^{3}H]-InsPs in rat tracheal segments

Exposure of *myo*-[2-^{3}H]-inositol-loaded rat tracheal segments to ET-1 caused a significant accumulation of [^{3}H]-InsPs. For example, stimulation with 1 μM ET-1 for 15 min produced a 4.3 ± 0.4-fold increase (n = 3 experiments) in total [^{3}H]-InsP accumulation above basal levels (184 ± 12 d.p.m. mg^{-1} wet wt). This was significantly greater than the level of [^{3}H]-InsPs accumulated in response to 1 mM carbachol (3.3 ± 0.2-fold increase; n = 4; $P < 0.05$). Following stimulation with 1 μM ET-1, the relative proportions of [^{3}H]-InsPs recovered from elution buffers II, III and IV, which subsequently elute [^{3}H]-$InsP_1$, [^{3}H]-$InsP_2$ and [^{3}H]-$InsP_3$ (plus higher polyphosphates) respectively, was 61 ± 1%:32 ± 2%:7 ± 1% (n = 3). These data indicate that approximately 93% of the total accumulated [^{3}H]-InsPs were the major metabolic products of [^{3}H]-$InsP_3$ degradation, namely [^{3}H]-$InsP_1$ and [^{3}H]-$InsP_2$.

Accumulation of [^{3}H]-inositol phosphates in various tracheal wall structures

ET-1 (1 μM) and carbachol (1 mM) induced significant accumulation of [^{3}H]-InsPs in the airway smooth muscle band, the cartilaginous region and the intercartilaginous region of the rat trachea (Figure 1). Approximately half of the [^{3}H]-InsPs accumulated in response to 1 μM ET-1 were generated within the intercartilaginous region of the tracheal wall (Figure 1a). In contrast, the greatest proportion of [^{3}H]-InsPs accumulated in response to 1 mM carbachol were generated within the airway smooth muscle band. When the respective weights of the regions are accounted for (average weight in mg/trachea; smooth muscle, 4.3; intercartilaginous region 13.6 and cartilaginous region, 16.5), the greatest level of [^{3}H]-InsP accumulation produced in response to either ET-1 or carbachol was observed in the airway smooth muscle band (Figure 1b). For example, ET-1-induced accumulation of [^{3}H]-InsPs in the airway smooth muscle band was 3.5 and 1.6 fold greater than that observed in the cartilaginous and intercartilaginous regions, respectively. Similarly, carbachol-induced accumulation of [^{3}H]-InsPs was at least 5.5 fold higher in the airway

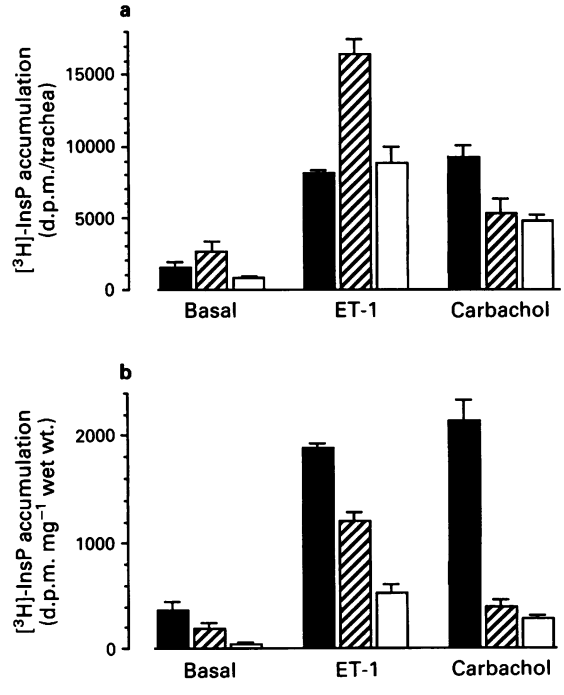


Figure 1 Accumulation of [^{3}H]-inositol phosphates ([^{3}H]-InsPs) in different regions of the rat isolated, epithelium-denuded trachea under basal conditions or following stimulation with 1 μM endothelin-1 (ET-1) or 1 mM carbachol; airway smooth muscle band (solid columns), intercartilaginous region (hatched columns) and cartilaginous region (open columns). Accumulation of [^{3}H]-InsPs has been expressed in terms of (a) d.p.m. per trachea and (b) d.p.m. mg^{-1} wet wt. region. Data were obtained from three experiments using 12 animals.

smooth muscle band than in the other regions (Figure 1b). Removal of the tracheal epithelium had no significant effect on [^{3}H]-InsP accumulation either under basal conditions or following stimulation with 0.1 μM ET-1 (3.66 $\pm$ 0.19 fold increase above basal accumulation in epithelium denuded segments versus 3.60 $\pm$ 0.18 fold increase in epithelium-intact segments, n = 3).

Accumulation of [^{3}H]-inositol phosphates in the airway smooth muscle band

ET-1-induced accumulation of total [^{3}H]-InsPs within the tracheal airway smooth muscle band was time- and concentration dependent (Figure 2 and 3a). ET-1 (1 μM) produced significant accumulation of total [^{3}H]-InsPs within 5 min and the rate of accumulation appeared linear for at least 15 min thereafter. Hence, in all subsequent experiments a 15 min stimulation period was used. Significant [^{3}H]-InsP accumulation was produced with ET-1 concentrations as low as 10 nM

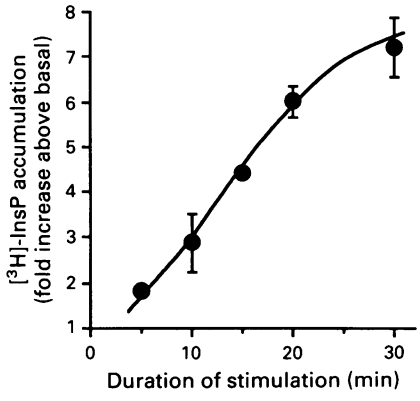


Figure 2 Accumulation of [^{3}H]-inositol phosphates ([^{3}H]-InsPs), above basal levels, produced by increasing periods of stimulation of rat isolated, epithelium-denuded tracheal smooth muscle segments with 1 μM (●) endothelin-1 (n = 3 experiments). Basal levels of [^{3}H]-InsP accumulation did not change significantly during the various periods of incubation (mean basal levels = 174 $\pm$ 9 d.p.m. mg^{-1} wet wt.).

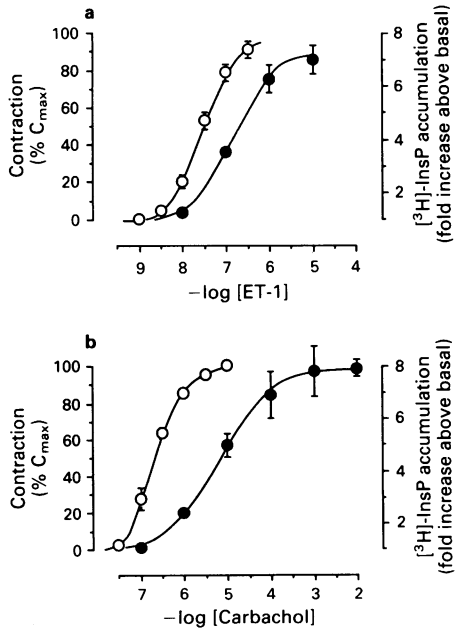


Figure 3 Mean concentration-effect curves for (a) endothelin-1 (ET-1) and (b) carbachol showing agonist-induced changes in isometric tension (○, C_{max} = response to 10 μM carbachol, n = 12 preparations from 12 animals) and [^{3}H]-inositol phosphate ([^{3}H]-InsP) accumulation (●, n = 4–6 experiments) in rat isolated, epithelium-denuded tracheal smooth muscle preparations.

(1.23 $\pm$ 0.10 fold increase above basal levels of 295 $\pm$ 2 d.p.m. mg^{-1} wet wt, n = 3 experiments) and there was a tendency for [^{3}H]-InsP accumulation to plateau after exposure to 10 μM ET-1, the highest concentration used. The mean increase in [^{3}H]-InsP accumulation above basal levels induced with 10 μM ET-1 (7.03 $\pm$ 0.55 fold above basal levels, n = 5, Figure 3a) was not significantly different from the mean maximum increase produced in the presence of the cholinoceptor agonist, carbachol (10 mM, 7.97 $\pm$ 0.31, n = 4; Figure 3b). The EC_{50} concentration of ET-1-induced accumulation of [^{3}H]-InsPs was 90 nM (95% confidence limits, 47–170 nM, n = 5).

ET-1-induced (0.1 μM) accumulation of [^{3}H]-InsPs in the airway smooth muscle band was not significantly affected by indomethacin (5 μM), NDGA (10 μM), WEB 2086 (10 μM) or phosphoramidon (10 μM) (Figure 4). Similarly, the accumulation of [^{3}H]-InsPs in response to 0.1 μM ET-1 (3.02 $\pm$ 0.19 fold above basal levels, n = 6) was not affected by 0.1 μM atropine (3.17 $\pm$ 0.25 fold above basal levels, n = 3), whereas accumulation of [^{3}H]-InsPs produced in response to 10 μM carbachol was prevented by atropine (0.84 $\pm$ 0.04 fold above basal levels, n = 3). At the concentrations used, none of these inhibitors alone had any significant effect on basal [^{3}H]-InsP accumulation in unstimulated preparations.

Exposure of *myo*-[2-^{3}H]-inositol-loaded rat tracheal smooth muscle preparations to the membrane depolarizing spasmogen, KCl (60 mM) produced a small, but not statistically significant, increase in accumulation of [^{3}H]-InsPs above basal levels (1.36 $\pm$ 0.16 fold, n = 5). At a concentration of 100 μM, histamine produced a small accumulation of [^{3}H]-InsPs above basal levels (1.46 $\pm$ 0.14 fold, n = 5, $P < 0.05$). This represents only 7.6% of the increase obtained with 10 μM ET-1.

Carbachol- and endothelin-1-induced contractions in rat tracheal segments

Carbachol produced concentration-dependent contraction of epithelium-denuded rat isolated tracheal preparations (mean EC_{50} value, 0.20 μM; 95% confidence limits, 0.17–0.24; n = 12; Figure 3b). Carbachol-induced contractions were not significantly affected by the presence of an intact epithelium (mean EC_{50} value, 0.21 μM (0.14–0.31), n = 5) or by any of the antagonists/inhibitors tested (Table 1).

ET-1 caused potent concentration-dependent contractions of epithelium-denuded rat tracheal smooth muscle preparations (Figure 3a). Significant contractions to ET-1 were observed with concentrations as low as 10 nM. At the highest concentration of ET-1 used (0.3 μM), the concentration-effect curve had not plateaued but the increase in tone was 91.1 $\pm$ 2.2% C_{max} (n = 12). The mean concentration of ET-1 producing 50% C_{max} was 31 nM (95% confidence limits, 20–49 nM). A similar profile was observed in epithelium-intact

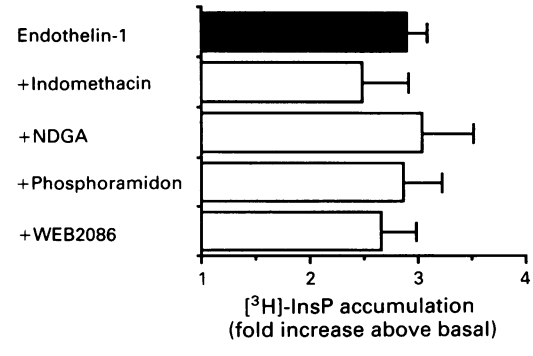


Figure 4 Influence of indomethacin (5 μM), nordihydroguaiaretic acid (NDGA, 10 μM), phosphoramidon (10 μM) and WEB 2086 (10 μM) on [^{3}H]-inositol phosphate ([^{3}H]-InsP) accumulation produced in rat isolated epithelium-denuded tracheal smooth muscle preparations following stimulation with endothelin-1 (0.1 μM). Shown are the mean responses (columns) from 3–4 experiments; horizontal bars show s.e.mean.

Table 1 Contractile potencies of carbachol and endothelin-1 (ET-1) in epithelium-denuded rat isolated tracheal preparations determined in the absence and presence of receptor antagonists and enzyme inhibitors

Antagonist/Inhibitor	n	*Mean concentration producing 50% C_{max}* (95% confidence limits) *Carbachol* (μM)	*ET-1* (nM)
None (control)	12	0.20 (0.17–0.24)	31 (20–49)
Indomethacin (5 μM)	5	0.14 (0.08–0.26)	31 (15–62)
NDGA (10 μM)	5	0.23 (0.14–0.38)	123** (57–265)
SKF 104353 (3 μM)	5	0.21 (0.16–0.28)	38 (23–63)
WEB 2086 (10 μM)	5	0.27 (0.17–0.43)	32 (12–85)
Phosphoramidon (10 μM)	5	0.26 (0.18–0.38)	33 (26–43)

** Significantly different from control values, $P < 0.001$.
n, number of animals.
For abbreviations, see text.

preparations (0.3 μM ET-1 produced 88.2 ± 3.6% C_{max} and the mean EC_{50} value was 38 nM (21–67), $n = 5$). ET-1-induced contractions of epithelium-denuded rat isolated tracheal smooth muscle preparations were not influenced by indomethacin, phosphoramidon or WEB 2086 (Table 1). In contrast, 10 μM NDGA produced a 4.0 fold decrease in the potency of ET-1 ($P < 0.001$, $n = 5$). This was apparently not due to the inhibition of leukotriene synthesis or effect since leukotriene C_4 and D_4 did not contract rat isolated tracheal smooth muscle preparations ($n = 4$) and SKF 104353 was without effect on ET-1-induced contractions (Table 1).

The threshold concentrations of ET-1 producing contraction and accumulation of total [^{3}H]-InsPs in airway smooth muscle were similar (Figure 3), although the mean EC_{50} value for total [^{3}H]-InsP accumulation was 2.9 fold higher than that for smooth muscle contraction. For carbachol, the mean EC_{50} value for [^{3}H]-InsP accumulation (5.0 μM) was 25 fold greater than that for airway smooth muscle contraction (0.20 μM).

Discussion

The current study revealed that several regions within the tracheal wall, including airway smooth muscle, the cartilaginous region and the intercartilaginous tissue, generate InsPs in response to ET-1 and carbachol. Of these regions, the airway smooth muscle band was clearly the most responsive (on a wet weight basis) to the [^{3}H]-InsP generating actions of both ET-1 and carbachol. However, it is also noteworthy that the region generating the greatest amounts of [^{3}H]-InsPs in response to ET-1 was the intercartilaginous region of the tracheal wall. The particular cell type(s) within the intercartilaginous region that generated InsPs in response to ET-1 cannot be determined from the current study. However, in view of the recent reports that the tissue between adjacent cartilage rings contains a dense, highly ordered vascular network (McDonald, 1988) and that blood vessels may generate InsPs in response to ET-1 (Marsden *et al.*, 1989; Xuan *et al.*, 1989), it is possible that ET-1-induced accumulation of InsPs in this region includes a vascular component. Confirmation of this may require additional autoradiographic studies (Hwang *et al.*, 1990). Similarly, the presence of blood vessels such as terminal arterioles, which traverse the cartilage, may have contributed to the smaller, but significant amount of [^{3}H]-InsPs generated within the cartilaginous region following stimulation with ET-1. The contribution of the tracheal cartilage ring to ET-1-induced generation of tracheal [^{3}H]-InsPs is likely to be minor in view of the relatively small overall contribution of the cartilaginous region to the observed effect and recent autoradiographic evidence that tracheal cartilage has only a very sparse population of ET-1 binding sites (Henry *et al.*, 1990). Although airway epithelial cells have been shown to generate [^{3}H]-InsPs when stimulated with an appropriate agonist (McCann *et al.*, 1989; Bainbridge *et al.*, 1989), in the present study mechanical removal of rat tracheal epithelium had no significant effect on the levels of ET-1-induced accumulation of [^{3}H]-InsPs. Furthermore, quantitative autoradiographic studies in airway preparations from a number of animal species including the rat, revealed that the density of specific ET-1 binding sites, presumably necessary for ET-1-induced [^{3}H]-InsP generation, was very low over the epithelium when compared with the numbers present in the tracheal smooth muscle band (Henry *et al.*, 1990).

Exposure of [^{3}H]-inositol-loaded rat tracheal smooth muscle tissue to ET-1 produced a time- and concentration-dependent accumulation of [^{3}H]-InsPs. In addition, ET-1-induced [^{3}H]-InsP accumulation and contraction in rat tracheal airway smooth muscle occurred at similar threshold concentrations. Furthermore, ET-1 and carbachol produced similar maximal increases in both [^{3}H]-InsP accumulation and smooth muscle contraction. In isolation, these results do not necessarily imply a causal relationship between ET-1-induced [^{3}H]-InsP accumulation and contraction. However, recent studies indicate that phosphatidylinositol(4,5)bisphosphate is the major and possibly exclusive phosphoinositide hydrolysed by hormone-sensitive phospholipase C in airway smooth muscle (Takuwa *et al.*, 1986; Chilvers & Nahorski, 1990). Thus, the accumulation of these [^{3}H]-InsPs provides an indirect index of phosphatidylinositol(4,5)biphosphate hydrolysis and may quantitatively reflect the turnover of the intracellular calcium-mobilizer, Ins(1,4,5)P_3. These events have been closely linked to contraction (Hashimoto *et al.*, 1985; Twort & van Breemen, 1989) and suggest that ET-1-induced contraction of airway smooth muscle may be mediated via the generation of Ins(1,4,5)P_3 and mobilization of intracellular calcium. Our findings are in accord with those of Hay (1990) who found that ET-1-induced contraction of guinea-pig isolated tracheal smooth muscle also involved the stimulation of phosphoinositide turnover and utilization of intracellular calcium. However, mechanisms in addition to phosphoinositide metabolism may be involved because several recent studies have suggested a role for extracellular calcium (Turner *et al.*, 1989; Advenier *et al.*, 1990) and dihydropyridine-sensitive calcium channels (Uchida *et al.*, 1988; Maggi *et al.*, 1989; Advenier *et al.*, 1990) in ET-1-induced contraction of airway smooth muscle.

Although ET-1-induced contraction and [^{3}H]-InsP accumulation in rat tracheal airway smooth muscle occurred at similar threshold concentrations, the concentration-effect curve for contraction lay slightly (2.9 fold) to the left of the curve describing [^{3}H]-InsP accumulation. A close

concentration-effect relationship for these two phenomena in airway smooth muscle has recently been observed for ET-1, histamine and leukotriene D_4 (Weichman *et al.*, 1982, Grandordy & Barnes, 1987; Mong *et al.*, 1988; Hay, 1990). In contrast, the concentration-effect curve describing carbachol-induced [^{3}H]-InsP accumulation in rat tracheal smooth muscle was displaced markedly (25 fold) to the right of the curve describing contractile responses to this agonist. These results agree with the previously reported effects of muscarinic cholinoceptor agonists on [^{3}H]-InsP accumulation in airway smooth muscle (Grandordy & Barnes, 1987; Meurs *et al.*, 1988; van Amsterdam *et al.*, 1989). Such data have been interpreted as indicating a marked capacity for InsP generation and accumulation in response to muscarinic agonists far beyond that which is required to activate contraction. Alternatively, cholinoceptor agonists such as carbachol may have invoked other transduction systems (Katsuyama *et al.*, 1990) not stimulated by ET-1.

Our results with ET-1 are consistent with studies showing that phosphoinositide metabolism plays a primary role in the pharmacomechanical coupling of receptor-mediated airway smooth muscle contraction for a number of other spasmogens including muscarinic cholinoceptor agonists, histamine, 5-hydroxytryptamine (5-HT), bradykinin, tachykinins and leukotrienes (Grandordy *et al.*, 1986, 1988; Grandordy & Barnes, 1987; Lemoine *et al.*, 1988; Chilvers *et al.*, 1989; Mong *et al.*, 1988). In contrast, KCl failed to alter significantly [^{3}H]-InsP accumulation above basal levels in rat tracheal smooth muscle. These data agree with previous reports that the calcium-mobilizing and spasmogenic actions of KCl in airway smooth muscle occur via activation of voltage-dependent calcium channels and influx of extracellular calcium (Kotlikoff, 1988) rather than via generation of InsPs and release of calcium from intracellular stores (Baron *et al.*, 1984; Grandordy *et al.*, 1986). Histamine (100 μM) produced only a small increase in [^{3}H]-InsP accumulation above basal levels and this is consistent with its weak spasmogenic activity in rat isolated tracheal smooth muscle (Lulich & Paterson, 1980).

In vivo studies of anaesthetized guinea-pigs have demonstrated that the bronchoconstriction observed following intravenous administration of ET-1 is due, at least in part, to the actions of cyclo-oxygenase products such as thromboxane A_2 and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Payne & Whittle, 1988; Lagente *et al.*, 1989). Furthermore, Battistini and coworkers (1990a) have suggested that ET-1-induced contractions in guinea-pig isolated airways are mediated through the release of thromboxane A_2. However, in the present study in rat isolated trachea, neither ET-1-induced accumulation of [^{3}H]-InsPs, nor smooth muscle contraction were significantly modulated by the cyclo-oxygenase inhibitor, indomethacin. This is in accord with *in vitro* studies of isolated airway smooth muscle preparations from rat (Henry *et al.*, 1990) and man (Advenier *et al.*, 1990; McKay *et al.*, 1991) showing that indomethacin had no inhibitory influence on ET-1-induced contractions. Similarly, Pons *et al.* (1991) report that the bronchoconstriction produced following aerosol administration of ET-1 to guinea-pig isolated lungs occurred through an indomethacin-insensitive process. Whereas ET-1-induced effects were indomethacin-insensitive, the combined cyclo-oxygenase/lipoxygenase inhibitor NDGA produced significant inhibition of ET-1-induced contractions without altering ET-1-induced accumulation of InsPs. Although these results suggest that ET-1-induced contraction may be partly mediated via lipoxygenase products such as the leukotrienes, this explanation is unlikely since the leukotriene antagonist SKF 104353 did not affect ET-1-induced contractions and leukotrienes C_4 and D_4 did not contract rat tracheal smooth muscle. At present, the mechanism through which NDGA inhibits ET-1-induced contraction is not clear although this seems to be independent of effects on InsP accumulation.

Although a recent study has indicated that platelet-activating factor may mediate ET-1-induced contraction in guinea-pig airways (Battistini *et al.*, 1990b), the inability of a platelet-activating factor receptor antagonist, WEB 2086, to affect either ET-1-induced contraction or InsP accumulation in the current study argues against this mechanism operating in rat airways. Overall, our studies utilizing selected receptor antagonists and enzyme inhibitors indicate that both the InsP accumulating and tension generating effects of ET-1 observed in rat isolated tracheal smooth muscle appear to be the result of a direct action on smooth muscle rather than due to the actions of secondary spasmogenic mediators such as prostaglandins, leukotrienes, platelet-activating factor, histamine or thromboxane.

In this study we have established that ET-1 induced significant accumulation of [^{3}H]-InsPs in rat tracheal segments. It seems likely that the generation of [^{3}H]-InsPs is due to a direct effect of ET-1 via specific receptors on the tracheal smooth muscle and is responsible, at least in part, for ET-1-induced contraction in this preparation.

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Subtype classification of the presynaptic α-adrenoceptors which regulate [^{3}H]-noradrenaline secretion in guinea-pig isolated urethra

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1 The following experiments were carried out to investigate the presence and type of functional presynaptic receptors in adrenergic nerves of the guinea-pig urethra.

2 The urethra from male guinea-pigs was incubated with [^{3}H]-noradrenaline and superfused with Tyrode solution *in vitro*. The fractional secretion of [^{3}H]-noradrenaline evoked by 300 electrical pulses was measured.

3 The [^{3}H]-noradrenaline secretion was positively frequency-dependent, yielding a half-maximal secretion at 8 ± 5 Hz. Stimulation was usually applied at 5 Hz.

4 The [^{3}H]-noradrenaline secretion was not altered by noradrenaline (1 or 100 μM), norephedrine (1 μM), isoprenaline (0.1 μM), 5-hydroxytryptamine (10 μM), oxotremorine (10 μM), adenosine (0.2 mM), propranolol (1 μM), atropine (1 μM) or 8-phenyltheophylline (10 μM).

5 The [^{3}H]-noradrenaline secretion was enhanced by clonidine (3 μM), chlorpromazine (10 μM), metitepine (1 μM), 4-aminopyridine (0.5 mM), tetraethylammonium (2 mM), 3-isobutyl-1-methylxanthine (4 mM), 8-bromo cyclic AMP (1 mM) and forskolin (25 μM).

6 The α-adrenoceptor antagonists rauwolscine, yohimbine, phentolamine, prazosin and AR-C 239 maximally enhanced the [^{3}H]-noradrenaline secretion to about 300% of control. The partial α-adrenoceptor agonist oxymetazoline maximally enhanced the secretion to about 200% of control. The order of apparent EC_{50} values was rauwolscine < yohimine < phentolamine < oxymetazoline < prazosin < AR-C 239.

7 The enhancing effects of yohimbine (1 μM) with tetraethylammonium (2 mM), 8-bromo cyclic AMP (1 mM), or forskolin (25 μM) were additive, but not those of yohimbine (1 μM) with prazosin (10 μM), 4-aminopyridine (0.5 mM), or 3-isobutyl-1-methylxanthine (4 mM).

8 These results suggest that the [^{3}H]-noradrenaline secretion in the guinea-pig urethra is regulated by presynaptic α_{2A}-adrenoceptors which may, in a cyclic AMP-independent manner, be coupled to a 4-aminopyridine-sensitive potassium channel. The secretion is not influenced by compounds acting at β-adrenoceptors, muscarinic cholinoceptors or adenosine receptors.

Keywords: AR-C 239; 4-aminopyridine; clonidine; noradrenaline secretion; oxymetazoline; prazosin; presynaptic adrenoceptor; rauwolscine; urethra; yohimbine

Introduction

Activation of smooth muscle α-adrenoceptors is important for keeping the urethral canal closed in the maintenance of urinary continence (Wein *et al.*, 1987). However, agonist action on presynaptic α-adrenoceptors reduces the electrically-evoked noradrenaline secretion from sympathetic nerve endings in several tissues (Langer, 1981; Starke, 1987; Starke *et al.*, 1989; Stjärne, 1989), including rabbit and human urethra (Mattiasson *et al.*, 1984). Therefore, pre- and postsynaptic actions of noradrenaline may under some circumstances be counteractive.

α-Adrenoceptors can be classified by use of the selective α_1-adenoceptor antagonist prazosin and the selective α_2-adrenoceptor antagonist yohimbine. A further division into α_2-adrenoceptor subtypes has been suggested based on pharmacological data (Nahorski *et al.*, 1985; Bylund, 1985; 1988; Bylund *et al.*, 1988) and supported by gene sequencing work (Kobilka *et al.*, 1987; Regan *et al.*, 1988; Lorenz *et al.*, 1990; for a review, see Harrison *et al.*, 1991). Receptors with higher affinity for oxymetazoline than for AR-C 239 and prazosin are defined as α_{2A}-adrenoceptors, whereas the reverse order of affinity characterizes α_{2B}-adrenoceptors (Petrash & Bylund, 1986; Bylund *et al.*, 1988).

Presynaptic α-adrenoceptors are mostly of the α_2-adrenoceptor variety (cf. Langer, 1981). Subclassification of functional presynaptic α_2-adrenoceptors has only recently been attempted (Connaughton & Docherty, 1990; Shen *et al.*, 1990; Gobbi *et al.*, 1990; Limberger, *et al.*, 1991) and indicate the presence of presynaptic α_{2A}- and α_{2B}-adrenoceptor subtypes in different tissues. The present experiments were carried out to investigate the presynaptic modulation, and by use of selective antagonists the subtype of α-adrenoceptors involved, in the secretion of [^{3}H]-noradrenaline in the guinea-pig urethra *in vitro*.

Methods

Male guinea-pigs (200–450 g) were used in these experiments. The urinary bladder was tied at the base and cut, and the urethra was removed *en bloc*. A piece of the urethra immediately adjacent to the bladder and proximal to the cavernous part, including the preprostatic, prostatic and membranous parts, was dissected free and tied at both ends. The preparation was placed in Tyrode solution containing (mM) NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.5, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, D-glucose 5.6, ascorbate 0.114 and aerated with 5% CO_2 in O_2 to give pH 7.4.

The method employed is essentially the same as that used for studies of [^{3}H]-noradrenaline secretion in the guinea-pig vas deferens (Alberts *et al.*, 1981) and in the guinea-pig ileum myenteric plexus (Alberts & Stjärne, 1982; Alberts *et al.*, 1985). The urethra was preincubated with R-(−)-[7-^{3}H]-noradrenaline (10 μCi ml^{-1}, 0.7–0.77 μM; the stock solution was 13.0–14.2 Ci mmol^{-1}, Du Pont de Nemours New England

Nuclear, Boston, MA, U.S.A.) and unlabelled R-(−)-noradrenaline (1.23–1.3 μM, to give a final noradrenaline concentration of 2 μM) in Tyrode solution for 60 min at 37°C. When the incubation was terminated, desipramine (0.6 μM) and normetanephrine (10 μM) were added to the Tyrode solution in order to inhibit reuptake of [^{3}H]-noradrenaline.

The urethra was mounted in an organ bath under a passive isometric tension of 10 mN and superfused with Tyrode solution at a rate of 1 ml min^{-1} and allowed to rest for 1 h.

During the experiment the superfusate was collected continuously and divided into fractions for each 6 min period. Electrical stimulation was delivered via two platinum ring electrodes mounted 4 cm apart with trains of 300 pulses (0.5 ms) at 1–10 Hz with a Grass S44 stimulator set to yield an electrical field of approximately 25 V cm^{-1} between the electrodes (143 mA).

Electrical stimulation caused enhancement of the efflux of ^{3}H in excess of the spontaneous release. This evoked secretion was expressed as the fractional secretion of the total amount of ^{3}H contained in the tissue at the start of the stimulation period. Therefore, the tissue content of ^{3}H was extracted in 0.4 M perchloric acid and measured after the experiment. Under control conditions the fractional secretion at 5 Hz was 0.5–1.5% per stimulation period.

During each experiment four successive stimulation periods (S_1–S_4) were applied to the preparation. The minimum interval at which stimulation periods were started was 24 min. The chemical compound to be tested was included in the superfusion medium 18 min before the third stimulation. Effects of chemical compounds were always compared to controls obtained in the same experiments. The results were calculated as ratios of the secretory response in the presence of the compound divided by that in the absence of the compound. In consequence, the standard error (s.e.mean) of every ratio reflects the s.e.mean of the numerator and the s.e.mean of the denominator (Dahlberg, 1940). Data are expressed as mean ± s.e.mean throughout. When chemicals were not added to the medium the ratio $(S_3 + S_4)/(S_1 + S_2)$ was 108 ± 9% ($n = 11 + 12$; where n of $S_3 + S_4 = 11$ and n of $S_1 + S_2 = 12$, and $P > 0.05$ from 100%). In the Results section, however, n denotes only the particular number of stimulation periods done in the presence of the compound tested (i.e., stimulation periods $S_3 + S_4$) from which the measured or calculated values are obtained.

The fractional secretion of [^{3}H]-noradrenaline evoked by 300 pulses at 5 Hz was depressed by the omission of calcium from the Tyrode solution to 3 ± 2% ($n = 6$, $P < 0.001$) and by tetrodotoxin (0.3 μM) to 2 ± 1% ($n = 6$, $P < 0.001$) of control and was therefore considered to be of neuronal origin.

The curves describing the frequency-dependence as well as the concentration-response for some of the chemical compounds were hyperbolic. These curves were characterized by an equation containing two constants, $y = P_1x/(P_2 + x)$, where y is the evoked fractional secretion of [^{3}H]-noradrenaline relative to control, x is the stimulation frequency or concentration of the compound, P_1 is the maximal effect asymptotically approached at 'infinitely' high stimulation frequency or concentration of the compound, i.e. the 'maximal secretion' or the 'maximal enhancement', and P_2 is the frequency or concentration yielding half of the maximal effect, 'Freq$_{50}$' or 'EC$_{50}$', respectively. The constants were calculated by use of an iterative non-linear regression computer programme (Nonlin84, 1987 edition, Statistical Consultants, Edgewood, KY, U.S.A. or NLIN, version 6.04, SAS Institute Inc., Cary, NC, U.S.A.; cf. Alberts & Ögren, 1988). The graph shown in Figure 1 is drawn using the fitted constants.

When observed, the influence of a chemical compound on the spontaneous release of [^{3}H]-noradrenaline was calculated from the ratio of fraction number 13 (from the start of the experiment) and fraction number 9 (6 min per fraction). Compounds were added to the medium during fraction number 11.

Statistical evaluation of the results was done with Student's t test or approximate Student's t test (cf. Daniel, 1991).

The following chemical compounds were used in the study: adenosine, atropine sulphate, 8-bromoadenosine 3':5'-cyclic monophosphate sodium salt (8-Br cyclic AMP), chlorpromazine hydrochloride, forskolin, 3-isobutyl-1-methylxanthine, (−)-isoprenaline (+)-bitartrate, (−)-noradrenaline bitartrate, norephedrine hydrochloride, DL-normetanephrine hydrochloride, oxymetazoline hydrochloride, 8-phenyltheophylline, (±)-propranolol hydrochloride, 5-hydroxytryptamine hydrochloride, tetrodotoxin, yohimbine hydrochloride (all from Sigma, St. Louis, MO, U.S.A.), 4-aminopyridine, tetraethylammonium chloride (Merck-Schuchardt, Hohenbrunn bei München, Germany), AR-C 239 Cl (2-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl-4,4-dimethyl-1,3(2H,4H)-iso quinolinedione), clonidine hydrochloride (Boehringer Ingelheim, Ingelheim, Germany), desipramine hydrochloride, phentolamine methane sulphonate (Ciba-Geigy, Basel, Switzerland), methiothepine maleate (metitepine) (Ro 8-6837; Roche, Basel, Switzerland), oxotremorine sesquifumarate (Aldrich, Steinheim/Albuch, Germany), prazosin hydrochloride (Pfizer, Brussels, Belgium), and rauwolscine hydrochloride (Research Biochemicals Inc., Natick, MA, U.S.A.). All other chemicals were of analytical grade.

Forskolin and metitepine were dissolved in ethanol and diluted in Tyrode solution. A stock solution of 8-phenyl) - 1 - piperazinyl]ethyl - 4,4 - dimethyl - 1,3(2H,4H) - iso - containing 0.2 M sodium hydroxide and diluted in Tyrode solution. A stock solution of prazosin was made in methanol and diluted one thousand-fold in Tyrode solution. A stock solution of AR-C 239 (50 mM) was made in ethanol (50%) and diluted one thousand fold in Tyrode solution. All other compounds were dissolved in double distilled water or Tyrode solution.

Results

Effect on the spontaneous release of [^{3}H]-noradrenaline

Although the underlying mechanisms were not analysed, it was found that high concentrations of prazosin enhanced the spontaneous release of [^{3}H]-noradrenaline in a concentration-dependent manner. For instance, when the effect of prazosin (7.9 μM) was tested, the basal efflux of [^{3}H]-noradrenaline was 6624 ± 785 d.p.m. ml^{-1} ($n = 6$). The enhancement of the spontaneous release in the presence of prazosin (7.9, 10, 12.6, 30 and 100 μM) was to 150 ± 26% ($n = 6$, $P < 0.05$), 142 ± 17% ($n = 12$, $P < 0.01$), 167 ± 19% ($n = 18$, $P < 0.001$), 222 ± 46% ($n = 6$, $P < 0.01$) and 495 ± 74% ($n = 3$, $P < 0.01$) of control, respectively. This finding is in accordance with observations in adrenergic nerves in e.g. the rat hippocampus (Frankhuyzen & Mulder, 1982).

At a high concentration noradrenaline (0.1 mM) enhanced the spontaneous release of [^{3}H]-noradrenaline to 206 ± 14% ($n = 3$, $P < 0.01$) of control.

Effects on the [^{3}H]-noradrenaline secretion evoked by electrical stimulation

The secretion of [^{3}H]-noradrenaline was strongly dependent on the frequency of stimulation (Figure 1). When the total number of electrical pulses delivered per stimulation period was kept constant at 300, an increase in the frequency from 1 Hz to 10 Hz enhanced the evoked fractional secretion of [^{3}H]-noradrenaline to 681 ± 221% of that at 1 Hz. The 'maximal secretion' calculated is 1.57 ± 0.50% ($n = 24$) of the ^{3}H content of the tissue per stimulation period, and the frequency yielding half of the 'maximal secretion' (Freq$_{50}$) calculated is 8 ± 5 Hz. In order to use as low a stimulation frequency as possible, but still evoke readily measurable amounts of transmitter, stimulation was at 5 Hz in all of the

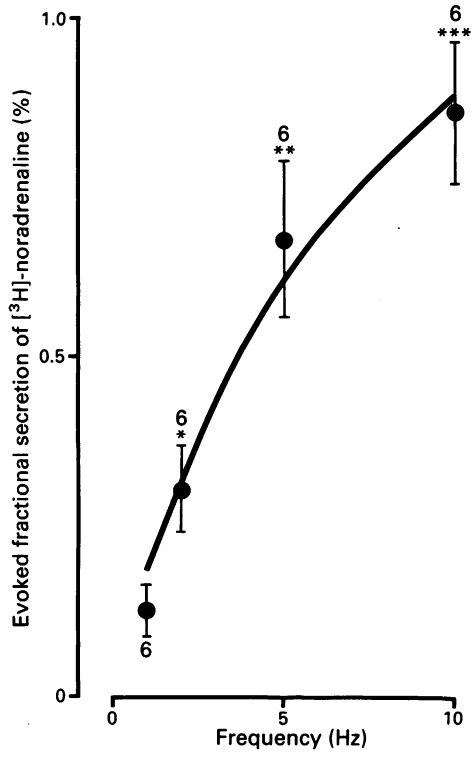


Figure 1 Effect of the frequency of stimulation on the fractional secretion of [^{3}H]-noradrenaline in the guinea-pig urethra. Stimulation was with 300 pulses at 1–10 Hz (0.5 ms). An equal number of stimulations was made in an ascending and a descending order of frequencies. The statistical difference from the secretion at 1 Hz is: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The number of stimulations carried out is shown.

following experiments (except some of those shown in Tables 1 and 2).

First, the effect of receptor agonists was tested. As shown in Table 1, all agonists used were ineffective under the present experimental conditions, despite the fact that a very low stimulation frequency (1 Hz) was also used.

Second, the effect of antagonists was tested (Table 2). While the β-adrenoceptor, muscarinic and adenosine receptor antagonists were ineffective, the α-adrenoceptor antagonists and the potassium channel blockers enhanced the [^{3}H]-noradrenaline secretion. Also, the inhibitor of cyclic nucleotide

Table 1 Lack of effect of receptor agonists on the evoked fractional secretion of [^{3}H]-noradrenaline in the guinea-pig urethra

	Relative to control (%)	n
Stimulation at 5 Hz:		
Noradrenaline (1 μM)	82 ± 20	4
Noradrenaline (0.1 mM)	90 ± 24	5
Norephedrine (1 μM)	116 ± 15	6
Isoprenaline (0.1 μM)	125 ± 19	6
5-Hydroxytryptamine (10 μM)	85 ± 12	6
Oxotremorine (10 μM)	84 ± 10	6
Adenosine (0.2 mM)	98 ± 11	6
Stimulation at 1 Hz:		
Noradrenaline (1 μM)	92 ± 12	6

Stimulation was with 300 pulses per stimulation period. The statistical difference (P) from the control level (100%) in the absence of compounds is not significant in any case: $P > 0.05$. The number of stimulations (n) made in the presence of each compound is shown.

Table 2 Effect of receptor agonists/antagonists, potassium channel blockers and compounds affecting intraneuronal cyclic nucleotide concentrations on the [^{3}H]-noradrenaline secretion in the guinea-pig urethra

	Relative to control (%)	P	n
Stimulation at 5 Hz:			
Clonidine (3 μM)	148 ± 17	**	12
Propranolol (1 μM)	100 ± 10	NS	6
Chlorpromazine (10 μM)	157 ± 16	**	4
Metitepine (1 μM)	184 ± 24	***	12
Atropine (1 μM)	107 ± 21	NS	12
8-Phenyltheophylline (10 μM)	125 ± 14	NS	6
4-Aminopyridine (0.5 mM)	376 ± 32	***	6
Tetraethylammonium (2 mM)	224 ± 50	**	6
3-Isobutyl-1-methylxanthine (4 mM)	143 ± 18	*	12
8-Bromo cyclic AMP (1 mM)	238 ± 17	***	6
Forskolin (25 μM)	199 ± 34	***	5
Stimulation at 1 Hz:			
Yohimbine (1 μM)	307 ± 56	***	6

Stimulation was with 300 pulses per stimulation period. The statistical difference (P) from the control level (100%) in the absence of compounds is: NS, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The number of stimulations (n) made in the presence of each compound is shown.

phosphodiesterase 3-isobutyl-1-methylxanthine, the 8-bromo analogue of cyclic AMP, and the activator of adenylate cyclase forskolin, enhanced the [^{3}H]-noradrenaline secretion.

The α-adrenoceptor antagonists rauwolscine, yohimbine, phentolamine, prazosin and AR-C 239 all enhanced the [^{3}H]-noradrenaline secretion to a similar extent, i.e. the 'maximal enhancement' was about 300% of control (Table 3). The 'maximal enhancement' value for the partial agonist oxymetazoline was, naturally, lower than that for the genuine antagonists.

The apparent EC_{50} values for yohimbine and prazosin were significantly ($P < 0.05$) different. The apparent EC_{50} value of oxymetazoline was also significantly different from those of rauwolscine, yohimbine and AR-C 239 (Table 3).

In the presence of noradrenaline (0.1 mM) the enhancing effect of yohimbine (1 μM) was abolished (Table 4).

In a separate series of experiments, yohimbine (1 μM) was added simultaneously to the medium with one of several compounds in order to investigate possible additive effects on the [^{3}H]-noradrenaline secretion (Table 4). The effects of yohimbine with prazosin, 4-aminopyridine, or 3-isobutyl-1-methylxanthine were not additive. In contrast, the effects of yohimbine with tetraethylammonium, 8-bromo cyclic AMP, or forskolin were additive.

Discussion

The secretion of noradrenaline is positively related to the frequency of stimulation in most tissues (for references, see Alberts & Stjärne, 1982). The present results show that in the guinea-pig urethra, the noradrenaline secretion is also strongly frequency-dependent (Figure 1). In tissues where the secretion is frequency-dependent the effect of presynaptic receptor ligands is largest at low frequencies of stimulation (cf. Starke *et al.*, 1989; Alberts & Ögren, 1988). Therefore, the effects of all chemical compounds were tested at frequencies lower than that yielding half of the 'maximal secretion'.

Several categories of compounds at high or optimal concentrations were without effect on the [^{3}H]-noradrenaline secretion in the guinea-pig urethra (Tables 1 and 2). Isoprenaline, at a concentration that is optimal in enhancing the [^{3}H]-noradrenaline secretion in the guinea-pig ileum myenteric plexus

Table 3 Constants describing the concentration-response curves for α-adrenoceptor antagonists enhancing the [^{3}H]-noradrenaline secretion in the guinea-pig urethra

	Maximal enhancement (% of control)	P	EC_{50} (μM)	P	n
Rauwolscine	326 ± 30	***	0.016 ± 0.0064	*	27
Yohimbine	293 ± 11	***	0.10 ± 0.025	*	90
Phentolamine	361 ± 41	***	0.92 ± 0.44	NS	34
Oxymetazoline	176 ± 12		2.03 ± 0.82		159
Prazosin	380 ± 66	**	7.35 ± 3.14	NS	153
AR-C 239	364 ± 30	***	11.94 ± 4.77	*	17

The constants were calculated by a non-linear regression programme (cf. Methods). Stimulation was at 5 Hz with 300 pulses per stimulation period. The control secretion in the absence of compounds is 100%.
The statistical difference (*P*) from the values obtained for oxymetazoline is: NS, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The number of stimulations (*n*) made in the presence of each compound is shown. The concentration-range tested was rauwolscine (3–100 nM), yohimbine (0.03–3 μM), phentolamine (0.3–10 μM), oxymetazoline (1–10 μM), prazosin (1–10 μM) and AR-C 239 (3–100 μM).

(Alberts *et al.*, 1985), and propranolol were ineffective. Similarly, adenosine, which does not affect the [^{3}H]-noradrenaline secretion in the myenteric plexus (Alberts *et al.*, 1985), or 8-phenyltheophylline, oxotremorine and atropine, which do influence the [^{3}H]-noradrenaline secretion in the myenteric plexus (cf. Alberts & Stjärne, 1982), were found not to alter the secretion in the urethra. These results suggest that the [^{3}H]-noradrenaline secretion in the guinea-pig urethra is not regulated by compounds acting at β-adrenoceptors, muscarinic cholinoceptors or adenosine receptors.

None of the exogenous agonists noradrenaline, norephedrine or 5-hydroxytryptamine altered the [^{3}H]-noradrenaline secretion (Table 1). This may be due to activation of presynaptic α-adrenoceptors by saturating concentrations of endogenous noradrenaline secreted by the electrical stimulation.

In contrast, the α-adrenoceptor antagonists had a marked effect. Moreover, the enhancing effect of yohimbine was counteracted by noradrenaline (Table 4). The finding that the values of 'maximal enhancement' for the α_1- and α_2-adrenoceptor antagonists prazosin and yohimbine were not significantly different ($P > 0.05$), and that the enhancements of both antagonists at high concentrations were not additive (Table 4), suggests the involvement of a single receptor population. The apparent EC_{50} values of yohimbine and prazosin, however, were significantly different, suggesting that the [^{3}H]-noradrenaline secretion in the guinea-pig urethra is regulated by α_2-adrenoceptors.

The two imidazolines, clonidine (Medgett *et al.*, 1978) and oxymetazoline (Sanders *et al.*, 1975), are partial α-adrenoceptor agonists. Of these two, oxymetazoline has been found to have the lower intrinsic activity at presynaptic autoreceptors (Frankhuyzen & Mulder, 1982). Accordingly, in the presence of the full agonist, endogenous noradrenaline, both clonidine and oxymetazoline enhanced the [^{3}H]-noradrenaline secretion (Tables 2 and 3) (cf. Starke *et al.*, 1989; Alberts, 1989a).

The classification into α_{2A}- and α_{2B}-adrenoceptor subtypes is based on the order of affinities of oxymetazoline, AR-C 239 and prazosin (cf. the Introduction). The fact that oxymetazoline is a partial agonist, rendered it useful for comparison with the α-adrenoceptor antagonists in this functional study. The calculated apparent EC_{50} value of oxymetazoline was significantly lower than that of AR-C 239, and significantly higher than that of rauwolscine and yohimbine (Table 3). The rank order of EC_{50} values rauwolscine < yohimbine < phentolamine < oxymetazoline < prazosin < AR-C 239 is therefore characteristic of α_{2A}-adrenoceptors (Bylund *et al.*, 1988). If, in contrast, the EC_{50} value for oxymetazoline had been higher than that for AR-C 239 and prazosin, the receptors could not have been α_{2A}-adrenoceptors. Thus, the present results suggest that the [^{3}H]-noradrenaline secretion in the guinea-pig urethra is regulated by presynaptic α_{2A}-adrenoceptors.

The enhancement of the [^{3}H]-noradrenaline secretion caused by metitepine (Table 2) is probably also due to α-adrenoceptor blockade (cf. Schlicker & Göthert, 1981).

Chlorpromazine is a ligand at α-adrenoceptors (cf. Bylund *et al.*, 1988). The concentration-response curve (3–100 μM, not shown) was bell-shaped with the peak at chlorpromazine (10 μM) (Table 2).

The potassium channel blockers, 4-aminopyridine (Alberts *et al.*, 1981, cf. also Alberts & Ögren, 1988) and tetraethylammonium (Alberts *et al.*, 1981, cf. also Albert & Ögren, 1989), enhanced the [^{3}H]-noradrenaline secretion in the urethra (Table 2). While the effects of yohimbine and tetraethylammonium were additive, those of yohimbine and 4-aminopyridine were not (Table 4). Therefore, the α_{2A}-adrenoceptor-mediated effect may be coupled to a potassium channel sensitive to 4-aminopyridine but not to tetraethylammonium (cf. Rudy, 1988).

The compounds that act upon the cyclic AMP system, 3-isobutyl-1-methylxanthine, 8-Br cyclic AMP and forskolin, all enhanced the [^{3}H]-noradrenaline secretion (Table 2) in harmony with results from adrenergic nerves in, e.g., the

Table 4 Combined effect of compounds added to the medium simultaneously with yohimbine on the [^{3}H]-noradrenaline secretion in the guinea-pig urethra

	Relative to control (%)	P	n
Yohimbine (1 μM) + noradrenaline (0.1 mM)	108 ± 13	**	4
Yohimbine (1 μM) + prazosin (10 μM)	307 ± 47	NS	6
Yohimbine (1 μM) + 4-aminopyridine (0.5 mM)	363 ± 47	NS	12
Yohimbine (1 μM) + tetraethylammonium (2 mM)	548 ± 88	***	10
Yohimbine (1 μM) + 3-isobutyl-1-methylxanthine (4 mM)	323 ± 33	NS	6
Yohimbine (1 μM) + 8-bromo cyclic AMP (1 mM)	509 ± 22	***	6
Yohimbine (1 μM) + forskolin (25 μM)	430 ± 72	*	6

Stimulation was at 5 Hz with 300 pulses per stimulation period (control level = 100%). The statistical difference (*P*) from the level (283 ± 24%, $n = 20$) in the presence of yohimbine (1 μM) alone is: NS, $P > 0.05$, * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$. The number of stimulations (*n*) made in the presence of the compounds is shown.

guinea-pig vas deferens (Stjärne *et al.*, 1979) and the guinea-pig ileum myenteric plexus (Alberts *et al.*, 1985; for cholinergic nerves see Alberts & Ögren, 1988; Alberts, 1989b). The enhancing effects of yohimbine and 3-isobutyl-1-methylxanthine were not additive in the urethra (Table 4), in contrast to the effects of yohimbine and 3-isobutyl-1-methylxanthine in the guinea-pig ileum myenteric plexus (Alberts *et al.*, 1985). However, even a high concentration of 3-isobutyl-1-methylxanthine (4 mM, Table 2) only slightly enhanced the [^{3}H]-noradrenaline secretion in the urethra. Furthermore, the effects of yohimbine and 8-Br cyclic AMP, or forskolin, were additive (Table 4). Taken together therefore, the present results suggest that cyclic AMP is not the second messenger of the presynaptic α-adrenoceptor effect in the adrenergic nerves of the guinea-pig urethra.

In conclusion, the present results suggest that the [^{3}H]-noradrenaline secretion in the guinea-pig urethra is modulated by presynaptic α_{2A}-adrenoceptors but not influenced by compounds acting at β-adrenoceptors, muscarinic cholinoceptors or adenosine receptors. The α_{2A}-adrenoceptors may, in a cyclic AMP-independent manner, be coupled to a potassium channel sensitive to 4-aminopyridine.

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Interaction of the central analgesic, tramadol, with the uptake and release of 5-hydroxytryptamine in the rat brain *in vitro*

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1 Tramadol is a centrally acting analgesic with low opioid receptor affinity and therefore presumably other mechanisms of analgesic action. Tramadol inhibits noradrenaline uptake but since 5-hydroxytryptamine (5-HT) is also involved in the modulation of pain perception, we tested the effects of tramadol on 5-HT uptake and release *in vitro*.

2 Tramadol inhibited the uptake of [^{3}H]-5-HT into purified rat frontal cortex synaptosomes with an IC_{50} of 3.1 μM. The (+)-enantiomer was about four times more potent than the (−)-enantiomer; the main metabolite of tramadol, O-desmethyltramadol, was about ten times less potent.

3 Rat frontal cortex slices were preincubated with [^{3}H]-5-HT, then superfused and stimulated electrically. Tramadol facilitated the basal outflow of [^{3}H]-5-HT, at concentrations greater than 1 μM, while the uptake inhibitor 5-nitroquipazine enhanced both basal and stimulation-evoked overflow. Effects of the (+)-enantiomer were more potent than either the racemate, the (−)-enantiomer or the principal metabolite.

4 The effects of tramadol on the basal outflow of [^{3}H]-5-HT were almost completely abolished when the superfusion medium contained a high concentration of the selective 5-HT uptake blocker, 6-nitroquipazine.

5 The results provide evidence for an interaction of tramadol with the neuronal 5-HT transporter. An intact uptake system is necessary for the enhancement of extraneuronal 5-HT concentrations by tramadol indicating an intraneuronal site of action.

Keywords: Tramadol; 6-nitroquipazine; 5-HT uptake; 5-HT release; frontal cortex slices; frontal cortex synaptosomes

Introduction

Pain perception is modulated by a variety of neurotransmitters including opioids, noradrenaline and 5-hydroxytryptamine (5-HT) (Yaksh, 1988). The centrally acting analgesic, tramadol, (1**RS**;2**RS**)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride, has a low affinity for opioid receptors in the micromolar range (Hennies *et al.*, 1988) which may not adequately explain its analgesic and antinociceptive potency which is only five to ten times lower than that of morphine (Friderichs *et al.*, 1978). We have recently shown that noradrenaline uptake blockade may be a further mechanism contributing to its antinociceptive effects (Driessen *et al.*, 1990). Since 5-hydroxytryptaminergic pathways are particularly important in pain modulation (Besson & Chaouch, 1987) we investigated whether tramadol may also interact with this neurotransmitter system.

Methods

General

Male Sprague Dawley rats weighing 160–380 g were killed by decapitation and the brain was quickly removed and chilled. Frontal cortices were dissected according to Glowinski & Iversen (1966). Slices from frontal cerebral cortex (0.4 mm thick, 5 mm diameter) were prepared as described and characterized previously (Reimann *et al.*, 1981).

Accumulation of [^{3}H]-5-hydroxytryptamine in synaptosomes

Cortices were homogenized in ice-cold 0.32 M sucrose (1.5 ml per 100 mg tissue) in a glass homogenizer with a loosely fitting teflon pestle by 10 full up and down strokes at 800 revolutions per min. The homogenate was filtered through Thomapor gauze, 100 μm mesh, and centrifuged at 1000 *g* for 15 min at 4°C. Further subfractionation was performed according to Dodd *et al.* (1981) by discontinuous gradient centrifugation. The supernatant was layered on 4.5 ml 1.2 M sucrose and centrifuged at 190000 *g* for 25 min. Particles at the gradient interface were collected in a volume of about 2.5 ml and diluted with ice-cold 0.32 M sucrose to a final volume of 8.0 ml. This suspension was layered on 4.5 ml 0.8 M sucrose and centrifuged again for 25 min at 190000 *g*. The resulting synaptosomal pellet was gently resuspended in 4 ml ice-cold incubation medium per 100 mg original tissue weight by use of a teflon/glass homogenizer.

The incubation medium was as follows (mM): NaCl 119, KCl 3.9, $CaCl_2$ 0.51, $MgSO_4$ 0.65, Na_2HPO_4 15.6, NaH_2PO_4 3.4, glucose 10, ascorbic acid 0.57. Pargyline, 10 μM, was added for inhibition of monoamine oxidase. The pH was adjusted to 7.4 by addition of NaOH.

The synaptosomes were preincubated for 5 min at 37°C in the absence or presence of the drugs to be tested. The uptake was started by addition of 10 μl [^{3}H]-5-HT yielding a concentration of 0.1 μM. The final incubation volume was 1 ml, and the protein concentration, determined according to Lowry *et al.* (1951), was 90–193 μg ml^{-1}. The incubation lasted for 30 s if not stated otherwise and was stopped by addition of 6 ml ice-cold medium, immediately followed by vacuum filtration through nitrocellulose membranes (pore seize 0.65 μm; Sartorius) presoaked in incubation medium. For determination of radioactivity, the wet filters were solubilised in 2 ml ethylene glycol monoethyl ether. Radioactivity was counted after addition of 10 ml Ready Safe (Beckman). Values were corrected for the accumulation of radioactivity in synaptosomes incubated at 0°C. All assays were performed in triplicate.

Outflow of tritium from slices after preincubation with [^{3}H]-5-hydroxytryptamine

Cortical slices were preincubated with 0.1 μM [^{3}H]-5-HT at 37°C for 30 min, then transferred to glass superfusion chambers equipped with platinum electrodes and superfused with

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medium at a rate of 1 ml min^{-1} for 105 min at 37°C. Starting 50 min after the onset of superfusion 5 min samples of the perfusate were collected. The slices were stimulated electrically for two periods of 2 min each, after 60 and 90 min of superfusion (S_1; S_2). Rectangular pulses of 2 ms duration, 24 mA current strength and a frequency of 3 Hz were delivered from a stimulator constructed by the Biomedical Technics Department of Grünenthal.

At the end of the experiment, the slices were solubilised in 0.5 ml Soluene 350 (Packard). The radioactivity in superfusates and slices was measured by liquid scintillation spectrometry after addition of Ready Gel (Beckman) and Ready Safe (Beckman), respectively. The incubation and superfusion medium contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, ascorbic acid 0.57, disodium EDTA 0.03; the medium was saturated with 5% CO_2 in O_2. The pH was adjusted to 7.4 by the addition of NaOH. Pargyline, 10 μM, was added to the medium throughout the superfusion. Drugs were added 15 min before S_2 or were present throughout the superfusion as indicated.

Calculations and statistics

The kinetic parameters of synaptosomal uptake, K_m and V_{max}, were estimated by adaptation of the data to the Michaelis-Menten hyperbola, and IC_{50}s by adaptation to the IB_1 model of the Top-fit programme package (Thomae) on a Wang 2236-D type computer.

The fractional rate of tritium outflow in release experiments was calculated by dividing the tritium content in the superfusate by the calculated tritium content in the slice at the start of the respective collection period. The stimulation-evoked overflow of tritium was calculated by subtraction of the estimated basal outflow and was expressed as a percentage of the tissue tritium content at the start of stimulation. For further evaluation of basal tritium efflux, ratios were calculated between the fractional rate of outflow observed in the 5 min period before S_2 (pre-S_2) over that observed in the 5 min period before S_1 (pre-S_1).

Means ± s.e.mean are given throughout. Differences between means were tested for significance by the Mann-Whitney test.

Compounds

5-[1,2-^{3}H(N)]-hydroxytryptamine-creatinine-sulphate, specific activity 21.0, 25.4 and 29.6 Ci $mmol^{-1}$ (New England Nuclear); pargyline HCl (Serva); morphine HCl (E. Merck); tramadol HCl, (−)- and (+)-enantiomer of tramadol, O-desmethyltramadol HCl (Grünenthal). 6-Nitroquipazine maleate was a kind gift of Duphar Pharma, and zimeldine dihydrochloride monohydrate of Astra Chemicals. All drugs were dissolved in water.

Results

Effects on the accumulation of [^{3}H]-5-hydroxytryptamine in synaptosomes

Preliminary experiments indicated that the rapid initial rate of tritium accumulation in frontal cortex synaptosomes lasted only for 30 s. This time period was therefore chosen for subsequent experiments. Kinetic analysis showed an apparent K_m of 0.051 ± 0.005 μM, and V_{max} was 5.4 ± 0.2 pmol $30\,s^{-1}\,mg^{-1}$ protein ($n = 4$ with 7 concentrations per experiment).

Synaptosomes were incubated with 0.1 μM [^{3}H]-5-HT either in the presence or absence of drugs. Accumulation of tritium under control conditions (no drug present) was 4.2 ± 0.1 pmol [^{3}H]-5-HT equivalent mg^{-1} protein ($n = 36$). The accumulation of radioactivity was most potently inhibited by 6-nitroquipazine, followed by zimeldine and tramadol (Figure 1). Calculated IC_{50} values are given in Table 1. The principal metabolite of tramadol, O-desmethyltramadol, was less active by about one order of magnitude (Figure 1; Table 1). Some stereoselectivity of the uptake inhibition by tramadol was observed since the (+)-enantiomer is about four times more potent than the (−)-form (Table 1). Inhibition of uptake by the test drugs was not complete. Maximum inhibition at 100 μM amounted to 85.6 ± 1.5% with 6-nitroquipazine, 88.2 ± 1.8% with zimeldine, 80.2 ± 2.4% with tramadol, 86.9 ± 2.2% with its (+)-enantiomer, 76.9 ± 1.1% with its (−)-enantiomer and 65.4 ± 1.9% with its metabolite O-desmethyltramadol. For comparison, morphine was practically devoid of 5-HT uptake inhibition; its maximum effect was 12.8 ± 3.1% inhibition at 100 μM ($n = 3$) (data not shown).

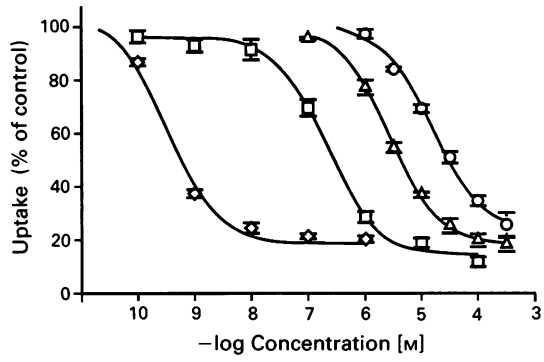


Figure 1 Effects of various drugs on the accumulation of tritium in frontal cortex synaptosomes incubated with 0.1 μM [^{3}H]-5-hydroxytryptamine. The accumulation in the presence of nitroquipazine (◇), zimeldine (□), tramadol (△) and O-desmethyltramadol (○) is expressed as a percentage of control. Each data point represents the mean of 6 experiments; s.e.mean shown by vertical bars.

Effects on tritium outflow from slices preincubated with [^{3}H]-5-hydroxytryptamine

Slices from the frontal cortex were preincubated with [^{3}H]-5-HT then superfused with medium containing 10 μM pargyline and stimulated electrically for two periods (S_1, S_2). When no drug was present, stimulation at S_1 resulted in an overflow of 1.2 ± 0.02% of the tritium in the tissue ($n = 97$) and was enhanced to 4.9 ± 0.1% in the presence of 1 μM 6-nitroquipazine ($n = 23$).

Tramadol, when added 15 min before S_2, enhanced the basal outflow without major effects on the stimulation-evoked overflow (Figure 2). The basal outflow was evaluated in the fractions preceding the stimulation periods; 10 μM tramadol enhanced the overflow by about 100% (Table 2). Basal outflow was facilitated more potently by the (+)-enantiomer

Table 1 Effect of drugs on the accumulation of tritium in frontal cortex synaptosomes incubated with 0.1 μM [^{3}H]-5-hydroxytryptamine

Drug	*IC_{50} (μM)*
6-Nitroquipazine	0.00038 ± 0.00002
Zimeldine	0.23 ± 0.03
Tramadol	3.1 ± 0.4
Tramadol (+)-enantiomer	2.1 ± 0.2
Tramadol (−)-enantiomer	8.6 ± 0.6
O-desmethyltramadol	24.2 ± 2.3

In each experiment, at least six drug concentrations were used. Means ± s.e.mean of $n = 6$ individual experiments.

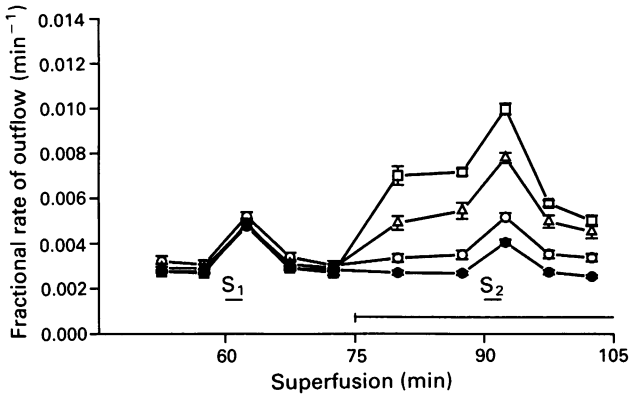


Figure 2 Effect of tramadol on the outflow of tritium from brain cortical slices preincubated with 0.1 μM [^{3}H]-5-hydroxytryptamine. After preincubation, slices were superfused with medium containing 10 μM pargyline and were stimulated electrically for two periods (S_1/S_2). Tramadol was added 15 min before S_2 as indicated by the bar. (●) Controls without tramadol; (○) tramadol 1 μM; (△) tramadol 10 μM; (□) tramadol 100 μM. Each data point represents the mean of 5–6 experiments; s.e.mean shown by vertical bars.

as compared to the (−)-enantiomer, and the metabolite was considerably less potent (Table 2). An evaluation of the stimulation-evoked overflow was not possible since the enhancement of the basal outflow precludes an exact estimation of the basal outflow for the subtraction from the total overflow.

Addition of the 5-HT uptake inhibitor 6-nitroquipazine, 1 μM, to the superfusion medium results in an attenuation of the effects of tramadol (Figure 3; Table 2). Residual effects were only observed at 100 μM. Under these conditions, a small but significant inhibition by tramadol of the stimulation-evoked overflow was observed. The ratio S_2/S_1 dropped to 0.85 ± 0.01 at 10 μM (n = 6) and 0.68 ± 0.02 at 100 μM tramadol (n = 5) as compared to 0.96 ± 0.01 (n = 6) under control conditions (absence of tramadol).

6-Nitroquipazine, added at S_2, also enhanced the basal outflow at the concentrations tested (Figure 4); the facilitation was almost 60% (Table 2). But it also enhanced the stimulation-evoked overflow, as can be seen from the graph. Effects were even more pronounced on the stimulation-evoked as compared to the basal overflow and differed therefore from the effects of tramadol (cf. Figure 2).

Discussion and conclusions

Neuronal amine uptake is a primary mechanism for the termination of neurotransmitter action and therefore drugs may interfere with the uptake mechanism to modulate neurotransmission. To determine the influence of a drug on the uptake of a specific neurotransmitter, some precautions have

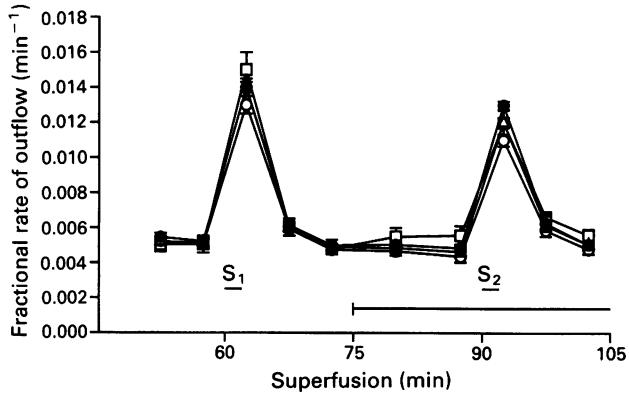


Figure 3 Effect of tramadol on the outflow of tritium from brain cortical slices preincubated with 0.1 μM [^{3}H]-5-hydroxytryptamine. After preincubation, slices were superfused with medium containing 10 μM pargyline and 1 μM nitroquipazine and were then stimulated electrically for two periods (S_1, S_2). Tramadol was added 15 min before S_2 as indicated by the bar. (●) Controls without tramadol; (○) tramadol 1 μM; (△) tramadol 10 μM; (□) tramadol 100 μM. Each data point represents the mean of 5-6 experiments; s.e.mean shown by vertical bars.

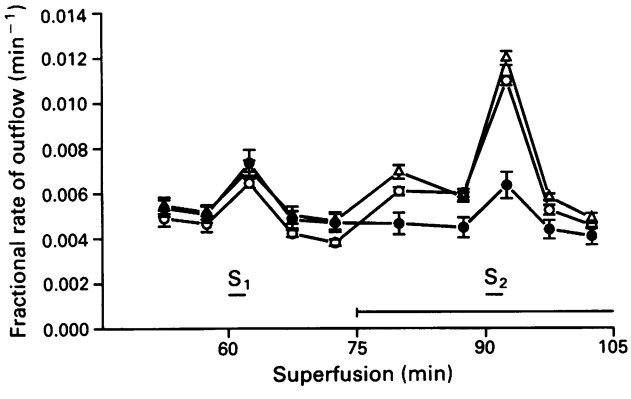


Figure 4 Effect of nitroquipazine on the overflow of tritium from brain cortical slices preincubated with 0.1 μM [^{3}H]-5-hydroxytryptamine. After preincubation, slices were superfused with medium containing 10 μM pargyline and were stimulated electrically for two periods (S_1, S_2). Nitroquipazine was added 15 min before S_2 as indicated by the bar. (●) Controls without nitroquipazine; (○) nitroquipazine 1 μM; (△) nitroquipazine 10 μM. Each data point represents the mean of 6 experiments; s.e.mean shown by vertical bars.

Table 2 Effects of the investigated drugs on the basal outflow of tritium from rat cerebral cortex slices preincubated with [^{3}H]-5-hydroxytryptamine

Drug added at S_2	*Drug added throughout superfusion*	*Basal outflow (pre-S_2/pre-S_1) at a drug concentration of* 0	1 μM	10 μM	100 μM
Tramadol	—	0.97 ± 0.02 (6)	1.14 ± 0.03 (6)*	1.88 ± 0.04 (6)*	2.67 ± 0.04 (5)*
Tramadol	6-Nitroquipazine 1 μM	0.91 ± 0.01 (6)	0.90 ± 0.03 (6)	0.95 ± 0.03 (6)	1.19 ± 0.06 (5)*
Tramadol (+)-enantiomer	—	0.89 ± 0.01 (8)	1.27 ± 0.16 (4)*	1.53 ± 0.08 (4)*	2.21 ± 0.12 (4)*
Tramadol (−)-enantiomer	—	0.89 ± 0.01 (8)	0.96 ± 0.01 (4)*	1.35 ± 0.08 (4)*	2.22 ± 0.15 (4)*
O-desmethyl-tramadol	—	0.82 ± 0.02 (4)	0.83 ± 0.01 (4)	1.05 ± 0.03 (4)*	1.71 ± 0.02 (4)*
6-Nitroquipazine	—	0.87 ± 0.03 (6)	1.37 ± 0.02 (6)*	1.14 ± 0.03 (6)*	

After preincubation, slices were superfused with medium containing pargyline and, when indicated, 6-nitroquipazine. They were stimulated electrically for two periods (S_1/S_2). Drugs given at S_2 were added to the medium 15 min before the onset of stimulation. Fractional rates of tritium outflow were determined in the fractions preceding the stimulation periods (pre-S_1, pre-S_2). Significant differences from corresponding controls (concentration of drug added at S_2 = 0): * $P < 0.05$. Means ± s.e.mean of number of experiments in parentheses.

to be taken. In order to approach true uptake, i.e. unidirectional transmitter flow, only the initial linear rate of uptake should be measured (Graefe, 1976). This precondition was met by the short incubation time of 30 s in the present study. The uptake of [^{3}H]-5-HT into the rat frontal cortex synaptosomes followed Michaelis-Menten-kinetics and the K_m-value, indicative for high affinity, agrees with that of other investigators (Richelson & Pfenning, 1984). 5-HT may also accumulate in catecholaminergic nerve terminals (Iversen, 1974) but with a much lower affinity for the carrier (K_m 8 μM). Therefore we can assume that, under the present conditions, most of the radiolabelled transmitter has been taken up by 5-hydroxytryptaminergic terminals. Purification of the synaptosomes by gradient centrifugation was performed in order to minimize uptake in or attachment to other cell organelles.

Nitroquipazine was a very potent 5-HT uptake blocker in the present study. As with zimeldine, inhibition of total uptake of exogenous 5-HT was monophasic, albeit not complete, indicative of a highly selective interaction with the uptake mechanism for 5-HT (Ross *et al.*, 1976; Vaatstra *et al.*, 1981). Inhibition by zimeldine of 5-HT uptake was comparable to previously published data (Ross & Renyi, 1977; Richelson & Pfenning, 1984) while the efficacy of nitroquipazine was about two orders of magnitude higher than that reported by Vaatstra *et al.* (1981) who, however, studied uptake in whole brain homogenates, which may explain the apparent discrepancy. Nevertheless, the potency of uptake inhibition by nitroquipazine in our experiments is in good agreement with data from binding studies with [^{3}H]-6-nitroquipazine (K_i 0.12 nM) recently published by Hashimoto & Goromaru (1990).

Tramadol blocked the uptake of 5-HT in a similar manner to nitroquipazine and zimeldine. Its potency was only about one order of magnitude less than that of zimeldine and contrasts with a previous study which reported the blockade of 5-HT uptake with considerably lower potency (Hennies *et al.*, 1982), differences which may be due to the use of whole brain synaptosomes. The investigation of the enantiomers of tramadol showed evidence of a stereoselective interaction with a four fold higher potency in favour of the (+)-enantiomer. Investigations on the interaction with the 5-HT transporter often yield small stereoselective effects although some results are in favour of a clearcut stereoselective uptake mechanism (Smith, 1986). An unexpected finding is that the loss of the O-methyl group results in a strong reduction of activity; this small alteration of the molecule did not affect the interaction with the noradrenaline transporter (unpublished observations).

Apparent neurotransmitter uptake inhibition in experiments measuring accumulation of transmitter into synaptosomes may be the result of either true blockade of uptake sites or induction of neurotransmitter release (Baumann & Maitre, 1976; Maxwell *et al.*, 1976). To avoid misinterpretation of the uptake inhibition observed, we tested tramadol and nitroquipazine in a concomitant investigation of 5-HT release from slices of the same brain region.

Overflow of tritium from cortical slices preincubated with [^{3}H]-5-HT is likely to represent authentic 5-HT when monoamine oxidase is inhibited by pargyline. The model is often used for the study of the presynaptic regulation of 5-HT release (Cerrito & Raiteri, 1979; Göthert & Weinheimer, 1979; Göthert, 1980; Baumann & Waldmeier, 1981), and using this system, facilitation of [^{3}H]-5-HT overflow can be observed with uptake inhibitors which prevent the reuptake of previously released transmitter.

6-Nitroquipazine is a potent and highly selective inhibitor of neuronal 5-HT uptake (Vaatstra *et al.*, 1981; Hashimoto & Goromori, 1990). Its facilitating effect on the stimulation-evoked overflow has been described (Göthert *et al.*, 1983; Classen *et al.*, 1984) and was also observed in the present investigation. Tramadol, which was demonstrated to be an uptake inhibitor in the synaptosomal uptake experiments, showed a concentration-dependent enhancement of the basal overflow in brain slice release experiments. This effect cannot be due to interference with autoreceptors, since they are known not to modulate the basal transmitter outflow (Göthert, 1980; Galzin *et al.*, 1985) and since affinity for 5-HT receptor sites has not been shown for tramadol (Vaught *et al.*, 1991). The effect is, however, dependent on a functional uptake site since it was almost completely abolished in the presence of 6-nitroquipazine. Summarising the results from uptake and release studies, it appears that tramadol competes with 5-HT for the 5-HT carrier to enter the nerve terminal where it subsequently induces the stimulus-independent release of 5-HT. A similar mechanism has been claimed for the actions of the indirect 5-HT-mimetic, fenfluramine (Fuxe *et al.*, 1975). The release studies confirm the stereoselectivity of the effects of tramadol found in the uptake experiments, and the loss of activity of the metabolite O-desmethyltramadol was also observed. The small inhibition of the stimulation-evoked overflow in the presence of 6-nitroquipazine may be due to enhancement by tramadol of extraneuronal noradrenaline (Driessen *et al.*, 1990) and not to an interaction with 5-HT receptors since it lacks affinity for 5-HT receptor sites (Vaught *et al.*, 1991).

5-Hydroxytryptamine pathways are part of the spinoreticulospinal system which plays an important role in the modulation of pain transmission at the spinal level (Roberts, 1984; Besson & Chaouch, 1987). Intrathecal injection of 5-HT inhibits the activity of dorsal horn neurones and causes antinociception (Yaksh & Wilson, 1979; Schmauss *et al.*, 1983; Solomon & Gebhart, 1988). It has been postulated that enhancement of 5-hydroxytryptaminergic neurotransmission by 5-HT uptake inhibitors may explain the analgesic efficacy of these drugs (Yaksh & Wilson, 1979; Keilstein *et al.*, 1988). The effects of tramadol on 5-HT uptake occur at serum concentrations which are achieved with analgesic doses in the mouse tail-flick test, 0.8 μM at the threshold and 10.6 μM at the maximum effective dose (Friderichs & Becker, 1991). In addition, four fold higher concentrations were observed in the CNS as compared to plasma levels when [^{11}C]-tramadol concentrations were measured by positron emission in men and mice (G. Stoecklin, KFA Jülich, personal communication). Further support for a participation of the 5-hydroxytryptaminergic component of tramadol is derived from the antagonism of the 5-HT$_2$ receptor blocking drug, ritanserin, to the antinociceptive effect of intrathecally injected tramadol in rats (Driessen *et al.*, 1991). These observations provide evidence that reinforcement of 5-HT neurotransmission by tramadol may be of relevance to its *in vivo* antinociceptive effects.

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Responses to vasodilator drugs on pulmonary artery preparations from pulmonary hypertensive rats

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1 Relaxant responses to six vasodilator drugs, with different mechanisms of action, were examined on noradrenaline (0.1 μM)-contracted ring preparations of pulmonary artery and aorta taken from rats with pulmonary hypertension induced by monocrotaline or chronic hypoxia.

2 On pulmonary artery preparations from monocrotaline-treated rats, compared with controls, (a) the maximum relaxation to pinacidil and cromakalim was significantly increased, but their potency (negative log EC_{50}) was unchanged, (b) the potencies of nitroprusside and sodium nitrite were significantly reduced (10 fold and 3 fold respectively), but there was no change in the maxima, (c) for nicorandil there was an increase in maximum relaxation and a decrease in potency (3 fold), and (d) for atriopeptin II there was no change in potency or maximum.

3 The increase in maximum relaxation for pinacidil and the decrease in potency for nitroprusside were also demonstrated in pulmonary artery preparations from rats with chronic hypoxic pulmonary hypertension. The other four drugs were not examined in preparations from hypoxic rats.

4 In both models of pulmonary hypertension, no change in maximum response or potency was seen on aortic preparations for any of the vasodilator drugs.

5 In control preparations, none of the drugs was more potent on pulmonary artery than on aorta (i.e. they were not pulmonary-selective). In preparations from pulmonary hypertensive rats, pinacidil was selective for pulmonary artery, in contrast to nitroprusside which was selective for aorta.

6 It is concluded that the development of pulmonary hypertension in rats is accompanied by changes in the responsiveness of the pulmonary arteries to some vasodilator drugs; whether or not these changes occur depends on the mechanism of action of the vasodilator drug, but they are independent of the method of inducing pulmonary hypertension.

7 It is postulated that the reduction in potency seen for nitroprusside, sodium nitrite and nicorandil may be due to desensitization of soluble guanylate cyclase in pulmonary vascular smooth muscle in pulmonary hypertension.

Keywords: Vasodilator drugs; pulmonary hypertension; pulmonary artery; monocrotaline treatment; chronic hypoxia; potassium channel opening drugs; nitrovasodilators

Introduction

Pulmonary blood vessels from rats with experimentally-induced pulmonary hypertension differ structurally and functionally from normal pulmonary vessels. The structural changes which are associated with the development of pulmonary hypertension include intimal thickening, increased collagen production, medial hypertrophy and muscularization of non-muscular arterioles (Hislop & Reid, 1974; Rabinovitch *et al.*, 1979; Tozzi *et al.*, 1989). The accompanying changes in the functional properties of these vessels are reflected in their responsiveness to vasoactive agents. There are several studies in which contractile responses to vasoconstrictor agents have been reported to increase or decrease in pulmonary arteries from pulmonary hypertensive rats compared with controls (Hilliker & Roth, 1985; Altiere *et al.*, 1986; Langleben *et al.*, 1988; Wanstall & O'Donnell, 1990a). It is relevant to know whether or not there are also changes in the responsiveness of these vessels to vasodilator drugs, since vasodilator therapy may be useful in the treatment of patients with some types of pulmonary hypertension (Rubin, 1987).

Thus the primary aims of the present study were to determine to what extent the development of pulmonary hypertension in rats is accompanied by changes in the responsiveness of pulmonary arteries to vasodilator drugs, and if the changes in responsiveness are related to the mechanism of action of the vasodilator drugs and/or are dependent on the means of inducing pulmonary hypertension.

Vasodilator drugs which have previously been studied in pulmonary arteries taken from rats with pulmonary hypertension induced by monocrotaline include felodipine (calcium entry blocking drug), acetylcholine (ACh) and isoprenaline. When compared with data in control vessels, the vasorelaxant effects of felodipine were increased (Wanstall *et al.*, 1991) whereas those of ACh and isoprenaline were reduced (Altiere *et al.*, 1986). In the present study, pulmonary hypertension was induced in rats either by monocrotaline pretreatment or by exposure to chronic hypoxia, and vasodilator drugs with mechanisms that differ from those of felodipine, ACh and isoprenaline were selected for study. These are (a) pinacidil and cromakalim, which act by opening potassium channels (Weir & Weston, 1986; Southerton *et al.*, 1988), (b) nitroprusside and sodium nitrite, which act via cytosolic (soluble) guanylate cyclase (Ignarro & Kadowidtz, 1985), (c) nicorandil, which acts by both the above mechanisms (Kreye *et al.*, 1991; Kukovetz *et al.*, 1991) and (d) atriopeptin II, which acts on membrane (particulate) guanylate cyclase (Tremblay & Hamet, 1989).

A secondary aim of the study was to determine whether or not any changes in responsiveness seen in pulmonary artery were also seen in a non-pulmonary vessel, so data were obtained for the aorta as well as the pulmonary artery. From these data, the relationship of the potency of each of the drugs on the two vessel types (i.e. selectivity between pulmonary artery and aorta) was obtained.

Preliminary accounts of some of the results have been presented in abstract form (Wanstall & O'Donnell, 1990b,c).

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Methods

Pretreatment of animals

Male Wistar rats, 6–7 weeks old at the start of treatment, were used. Some of the rats were treated with a single s.c. injection of monocrotaline (105 mg kg^{-1}) or saline (1 ml kg^{-1}) 4 weeks before the experiment (Wanstall & O'Donnell, 1990a). Other rats were exposed to chronic hypoxia by housing them in chambers containing 10–11% oxygen for a period of 2 weeks before the experiment. Control rats were housed in room air (21% oxygen, normoxia) for the same period of time. The hypoxic chamber was continuously flushed with a mixture of nitrogen and compressed air, at a flow rate of 1.7 l min^{-1}. The ratio of nitrogen to air was adjusted so that the oxygen component of the gas in the chamber was 10–11% (measured with a Datex Normocap Gas Monitor). Trays of soda lime were included in the chamber to maintain the CO_2 content of the gas in the chamber below 0.5%. The rats were removed from the chamber for a maximum of 15 min each day, when the chamber was cleaned, and food and water were replenished.

The weights of the rats (in g) were: (a) at the time of treatment: saline-treated 225 ± 10.1 (n = 14); monocrotaline-treated 232 ± 6.4 (14); normoxia 209 ± 2.9 (4); hypoxia 215 ± 9.4 (4); (b) at the time of the experiment: saline 375 ± 12.3; monocrotaline 253 ± 15.8; normoxia 310 ± 7.4; hypoxia 266 ± 10.8.

On the day of the experiment, rats were anaesthetized with pentobarbitone (60 mg kg^{-1}, i.p.), were artificially ventilated via a tracheal cannula, and the pulmonary artery pressure (PAP) was recorded via a hypodermic needle inserted into the pulmonary artery through the right ventricle (Wanstall & O'Donnell, 1990a). A blood sample was taken for measurement of haematocrit. After removal of the pulmonary artery and aorta, the heart and lungs were removed and weighed for the determination of the ratio of the weight of right ventricle to the weight of left ventricle plus septum (RV/LV + S), and the ratio of lung wet to dry weight, as described previously (Wanstall & O'Donnell, 1990a). Increases in mean PAP, RV/(LV + S), lung wet/dry weight and haematocrit were taken as indications of pulmonary hypertension, right ventricular hypertrophy, lung oedema and polycythemia, respectively.

Blood vessel preparations

Single ring preparations (3 mm in length, endothelium not removed) of main pulmonary artery and ventral aorta were set up in physiological salt solution (PSS), at 37°C, around 2 stainless steel wires in a vertical organ bath. Force in the circular muscle was recorded isometrically with a Statham Universal Transducer (UC3 + UL5) attached to a micrometer (Mitutoyo, Tokyo, Japan), as described previously (Wanstall & O'Donnell, 1988). The composition of the PSS (in mM) was: NaCl 118, KCl 5.9, $CaCl_2$ 1.5, $MgSO_4$ 0.72, $NaHCO_3$ 25, glucose 11.7 and ascorbic acid 1.14 (95% O_2/5% CO_2; pH 7.4).

Aortic preparations were set up under a resting force of 10 mN (Wanstall & O'Donnell, 1988). Pulmonary artery preparations were set up under resting forces of 10 mN (preparations from control rats) or 20 mN (preparations from monocrotaline-treated or hypoxic pulmonary hypertensive rats). These different resting forces were selected, as in previous studies (Wanstall & O'Donnell, 1990a; Wanstall *et al.*, 1991), to reflect the different *in vivo* pulmonary artery pressures in pulmonary normotensive and hypertensive rats.

At the completion of the experiment, the distance between the two horizontal wires (with the preparation under the selected resting force, see above), was measured with the micrometer holding the transducer, and the wet weight of the preparation was determined. The cross-sectional area of the preparation, in the plane perpendicular to the direction of the applied force, was then calculated as described by Wanstall & O'Donnell (1988), from the formula:

$$\text{cross-sectional area} = w(hd)^{-1}$$

where h = distance between the wires plus the diameters of the two wires (mm), w = wet weight (mg) and d = density = 1.06 mg mm^{-3}. Since all the preparations were the same length (3 mm, see above), differences in cross-sectional area between vessels reflected differences in vessel wall thickness. Thus an increase in cross-sectional area indicated the development of vascular hypertrophy.

Experimental protocols

Preliminary procedure After an equilibration period of 1 h, the preparations were contracted with noradrenaline (0.1 μM). When the contraction was stable, acetylcholine (ACh, 1 μM) was added, a relaxant response indicating the presence of endothelium. After washing with PSS, the preparations were then contracted with potassium-depolarizing PSS (in which 80 mM NaCl was replaced with 80 mM KCl).

Spasmolytic effects of vasodilator drugs After the above preliminary procedure, the preparations were contracted with noradrenaline (0.1 μM, approximate EC_{80}–EC_{90}). This concentration of noradrenaline gave contractions that were 75–80% (pulmonary artery) and 95–100% (aorta) of the contraction to potassium depolarizing PSS. When the contraction was stable, a cumulative concentration response (relaxation) curve to a vasodilator drug was obtained. On most preparations this procedure was then repeated with a second vasodilator drug. The order in which different vasodilators was examined was randomized in different preparations. Each of the vasodilator drugs tested could be washed off the tissue and the noradrenaline contraction repeated.

In preliminary experiments on pulmonary artery preparations from untreated rats it was shown that, for nitroprusside and pinacidil, responses were not different in preparations set up at resting forces of 10 mN or 20 mN. Thus it was concluded that differences in resting force alone would not affect responsiveness of the preparations to the vasodilator drugs.

Analysis of data

Responses to the vasodilator drugs were expressed as 'percentage reversal' of the noradrenaline-induced contraction, i.e. 100% reversal = reversal back to the baseline before the addition of noradrenaline. For each vasodilator drug, potency was expressed as the negative log EC_{50} (where EC_{50} is the concentration producing 50% of the maximum relaxation to the particular vasodilator drug), and selectivity for pulmonary artery compared with aorta was calculated from: antilog [mean neg log EC_{50} (pulmonary artery) − mean neg log EC_{50} (aorta)]. A value greater than 1 indicates selectivity for pulmonary artery and a value less than 1 selectivity for aorta.

Drugs and solutions

Acetylcholine chloride (ACh, Sigma): atriopeptin II (Sigma); cromakalim (BRL 34915, gift from Beecham Laboratories): monocrotaline (crotaline, Sigma): nicorandil (gift from Rhone-Poulenc); (−)-noradrenaline acid tartrate (Sigma); pinacidil (racemic; gift from Leo Pharmaceuticals); sodium nitrite (B.D.H. Chemicals); sodium nitroprusside (Sigma).

Solutions of monocrotaline (105 mg ml^{-1}) were prepared as the hydrochloride as follows: 500 mg of monocrotaline was dissolved in 1.5 ml 1 M HCl, 1 ml of deionized water was added, the pH was adjusted to 7.0 with 1 M NaOH, and deionized water was added to give a final volume of 4.76 ml. Solutions of other drugs were prepared as follows: ACh (10 mM), atriopeptin II (10 μM), nicorandil (10 mM), sodium nitrite (1 M)

and sodium nitroprusside (10 mM) in deionized water; noradrenaline (10 mM) and pinacidil (10 mM), in 10 mM HCl; cromakalim (10 mM) in 70% ethanol. Dilutions were made in PSS and kept on ice during the course of an experiment. The solvent for cromakalim, in amounts corresponding to the concentrations of cromakalim used, had no effect on the blood vessel preparations.

Statistical analyses

Mean values are quoted together with their standard errors (s.e.mean). The significance of differences between mean values was assessed by Student's t test (neg log EC_{50} values) or by Mann-Whitney U-test (percentage values).

Results

Monocrotaline-treated rats

Effects of monocrotaline treatment on rats Rats pretreated with monocrotaline had pulmonary hypertension, right ventricular hypertrophy and lung oedema, when compared with corresponding control rats (saline, Table 1). They did not have polycythemia. Pulmonary arteries from monocrotaline-treated rats had thicker walls than those from control rats. This was seen as an increase in the cross-sectional areas of the preparations, as defined in the Methods (Table 1). No increase in vessel wall thickness was seen for aortic preparations (Table 1).

Relaxant responses of pulmonary artery and aorta to vasodilator drugs Pinacidil, cromakalim, nicorandil, nitroprusside, sodium nitrite and atriopeptin II all relaxed noradrenaline-contracted preparations of pulmonary artery and aorta. Cromakalim was the only drug that did not cause complete (100%) reversal of the contractions (Figures 1 and 2).

In pulmonary artery preparations from monocrotaline-treated rats the maximum relaxations to pinacidil, cromakalim and nicorandil were significantly greater than in controls; for pinacidil and nicorandil these were greater than 100%, indicating that there was inherent tone in the preparations (Figure 1). The potencies (negative log EC_{50} values) of pinacidil and cromakalim were no different from controls, but that of nicorandil was significantly reduced (3 fold; Table 2). The potencies of nitroprusside and sodium nitrite were significantly less than in controls (10 fold and 3 fold respectively; Table 2) but there was no significant change in the maximum relaxations to these two drugs (Figure 1). The responses to atriopeptin II did not differ from those in controls with respect to either maximum or potency (Table 2, Figure 1).

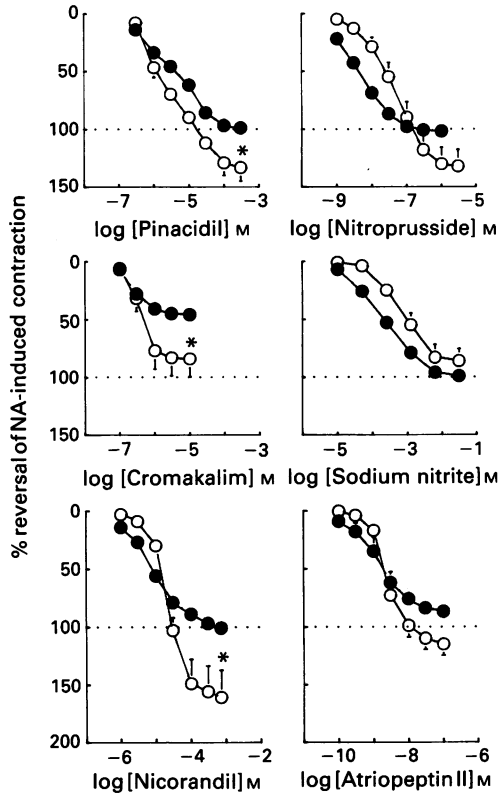


Figure 1 Pulmonary artery preparations: mean concentration-response (relaxation) curves to pinacidil, cromakalim, nicorandil, nitroprusside, sodium nitrite and atriopeptin II on noradrenaline (0.1 μM)-contracted preparations from rats treated with saline (●) or monocrotaline (○). Points are mean values, and the s.e.means are shown by the vertical bars except when smaller than the size of the symbols. Asterisks indicate significant differences in maximum relaxation between preparations from saline- and monocrotaline-treated rats (Mann-Whitney U test). For the number of experiments for each drug refer to Table 2. The contractions to noradrenaline (0.1 μM) were (in mN): saline 12.3 ± 0.48 (n = 26); monocrotaline 12.7 ± 0.83 (n = 26).

On aortic preparations from monocrotaline-treated rats, relaxant responses did not differ significantly from corresponding data in control rats for any of the vasodilator drugs except nicorandil, which showed a significant, but small (2 fold), decrease in potency (Figure 2; Table 2).

Pulmonary artery: aorta selectivity In preparations from saline-treated control rats, all the vasodilator drugs, with the exception of nitroprusside, were equipotent on pulmonary

Table 1 Effects of treatment of rats with monocrotaline or exposure of rats to hypoxia

	Saline[a] (n = 14)	*MCT*[b] (n = 14)	*Normoxia*[c] (n = 4)	*Hypoxia*[d] (n = 4)
Mean PAP (mmHg)	17 ± 2.4	26 ± 2.6*	17 ± 2.6	29 ± 4.2*
RV/(LV + S)	0.32 ± 0.005	0.67 ± 0.033***	0.31 ± 0.013	0.72 ± 0.055***
Lung wet/dry weight	4.5 ± 0.11	5.1 ± 0.08***	4.6 ± 0.06	4.7 ± 0.01
Haematocrit (%)	46 ± 0.7	50 ± 2.3	42 ± 1.4	54 ± 0.6***
Cross-sectional area of preparations (mm^2)				
Pulmonary artery	0.71 ± 0.059	1.22 ± 0.035***	0.72 ± 0.074	1.17 ± 0.096***
Aorta	1.19 ± 0.018	1.13 ± 0.046	1.16 ± 0.070	1.12 ± 0.074

Results shown are mean values ± s.e.

[a] Rats received saline (1 ml kg^{-1}, s.c.) 4 weeks previously.

[b] Rats received monocrotaline (MCT, 105 mg kg^{-1}, s.c.) 4 weeks previously.

[c] Rats housed in room air for 2 weeks.

[d] Rats housed in an environment containing 10% O_2 for 2 weeks.

Asterisks indicate value is significantly higher than the corresponding value in control rats: * $0.05 > P > 0.01$; *** $P < 0.001$ (Student's t test).

PAP: pulmonary artery pressure; RV/(LV + S): weight of right ventricle/weight of left ventricle plus septum.

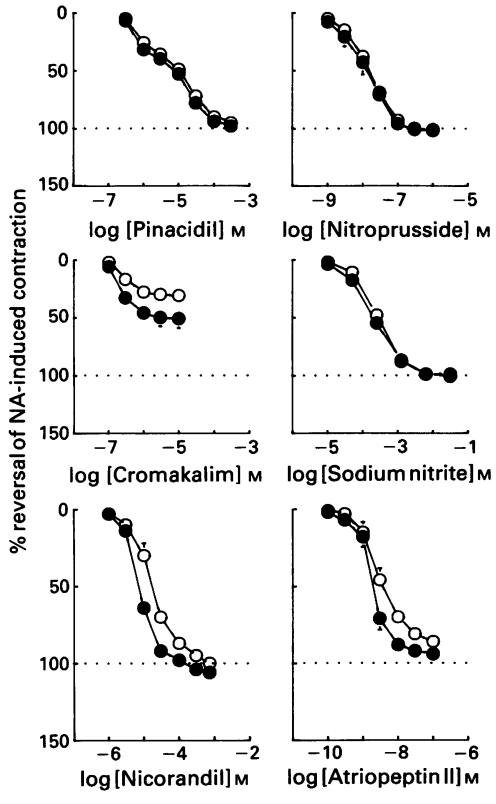


Figure 2 Aortic preparations: mean concentration-response (relaxation) curves to pinacidil, cromakalim, nicorandil, nitroprusside, sodium nitrite and atriopeptin II on noradrenaline (0.1 μM)-contracted preparations from rats treated with saline (●) or monocrotaline (○). Points are mean values, and the s.e.means are shown by the vertical bars except when smaller than the size of the symbols. For the numbers of experiments for each drug refer to Table 2. The contractions to noradrenaline (0.1 μM) were (in mN): saline 16.1 ± 0.60 (n = 25); monocrotaline 11.3 ± 0.72 (n = 25).

artery and aorta, i.e. did not show pulmonary artery: aorta selectivity (Table 2). Nitroprusside was slightly selective for pulmonary artery.

In preparations from monocrotaline-treated rats pinacidil was selective for the pulmonary artery, whilst nitroprusside and sodium nitrite were selective for the aorta (Table 2).

Rats exposed to chronic hypoxia

Effects of chronic hypoxia on rats Rats exposed to hypoxia for 2 weeks, like those treated with monocrotaline, had pulmonary hypertension and right ventricular hypertrophy (Table 1); the pulmonary arteries from these rats had thicker walls than those from control rats (refer cross-sectional areas, Table 1). Unlike monocrotaline-treated rats, hypoxic rats did not have lung oedema but they did have polycythemia (Table 1).

Relaxant responses of pulmonary artery and aorta to vasodilator drugs In this series of experiments, data were obtained only for pinacidil and nitroprusside. These two drugs were chosen because they each exhibited one of the changes in responsiveness seen in the pulmonary artery preparations from monocrotaline-treated rats, viz. an increase in maximum relaxation (pinacidil), and a decrease in potency (nitroprusside) (see above).

In pulmonary arteries from chronically hypoxic rats, the maximum relaxation to pinacidil was greater (Figure 3), and the potency of nitroprusside was less (7 fold; Table 3), than in preparations from normoxic control rats. Thus the changes in responsiveness were the same as those seen in monocrotaline-treated rats for those two drugs. Chronic hypoxia had no effect on responses to either drug on aorta (Figure 3, Table 3).

Pulmonary artery: aorta selectivity In preparations from normoxic rats, neither pinacidil nor nitroprusside showed pulmonary artery:aorta selectivity, but in preparations from chronically hypoxic rats, pinacidil was selective for the pulmonary artery and nitroprusside was selective for the aorta (Table 3).

Discussion

In this study six vasodilator drugs with different mechanisms of action were examined on pulmonary artery preparations taken from rats with two types of experimentally-induced pulmonary hypertension. The responsiveness to vasodilator drugs was assessed in terms of their potency and maximum relaxation, and the data obtained in preparations from pulmonary hypertensive rats were compared with data from pulmonary normotensive control rats. The results of the study have shown that (a) the development of pulmonary hypertension in rats is accompanied by changes in the responsiveness of pulmonary arteries to some, but not all, of the vasodilators

Table 2 Potency (negative log EC_{50}) and selectivity (pulmonary artery: aorta) of vasodilator drugs on blood vessel preparations from rats treated with saline or monocrotaline (MCT)

	$-\log EC_{50}$				*Selectivity*[c] (pulmonary artery: aorta)	
	Pulmonary artery		*Aorta*			
	Saline[a]	*MCT*[b]	*Saline*	*MCT*	*Saline*	*MCT*
Pinacidil	5.40 ± 0.07 (4)	5.67 ± 0.16 (4)	5.19 ± 0.13 (4)	5.07 ± 0.04 (4)	1.6	4.0†
Cromakalim	6.62 ± 0.06 (5)	6.45 ± 0.08 (5)	6.65 ± 0.05 (5)	6.54 ± 0.09 (5)	0.9	0.8
Nicorandil	5.12 ± 0.03 (5)	4.68 ± 0.08** (5)	5.12 ± 0.02 (4)	4.79 ± 0.08* (4)	1.0	0.8
Nitroprusside	8.36 ± 0.04 (4)	7.37 ± 0.11*** (4)	7.90 ± 0.18 (4)	7.83 ± 0.09 (4)	2.9†	0.3‡
Sodium Nitrite	3.69 ± 0.12 (4)	3.19 ± 0.11* (4)	3.71 ± 0.07 (4)	3.57 ± 0.06 (4)	1.0	0.4‡
Atriopeptin II	8.99 ± 0.18 (4)	8.67 ± 0.02 (4)	8.76 ± 0.06 (4)	8.55 ± 0.13 (4)	1.7	1.3

[a] Rats received saline (1 ml kg^{-1}, s.c.) 4 weeks previously.
[b] Rats received monocrotaline (MCT 105 mg kg^{-1}, s.c.) 4 weeks previously.
[c] Selectivity = antilog [mean $-\log EC_{50}$ pulmonary artery − mean $-\log EC_{50}$ aorta].
* Value significantly lower than corresponding value in control rats: * $0.05 > P > 0.01$; ** $0.01 > P > 0.001$; *** $P < 0.001$.
† Selective for pulmonary artery i.e. $-\log EC_{50}$ on pulmonary artery significantly greater than that on aorta: $0.05 > P > 0.01$.
‡ Selective for aorta i.e. $-\log EC_{50}$ on pulmonary artery significantly less than that on aorta: $0.05 > P > 0.01$.

Table 3 Potency ($-\log EC_{50}$) and selectivity (pulmonary artery: aorta) of pinacidil and nitroprusside on blood vessel preparations from rats exposed to room air (normoxia) or hypoxia

	$-\log EC_{50}$				*Selectivity*[c] (pulmonary artery: aorta)	
	Pulmonary artery		*Aorta*			
	Normoxia[a]	*Hypoxia*[b]	*Normoxia*	*Hypoxia*	*Normoxia*	*Hypoxia*
Pinacidil	5.68 ± 0.09 (4)	5.93 ± 0.11 (4)	5.70 ± 0.13 (4)	5.32 ± 0.19 (4)	1.0	4.1†
Nitroprusside	8.12 ± 0.08 (4)	7.30 ± 0.07*** (4)	8.16 ± 0.15 (4)	8.07 ± 0.23 (4)	0.9	0.2‡

Results shown are mean ± s.e. Number of observations is in parentheses.
[a] Rats housed in room air for 2 weeks.
[b] Rats housed in an environment containing 10% O_2 for 2 weeks.
[c] Selectivity = antilog [mean $-\log EC_{50}$ pulmonary artery − mean $-\log EC_{50}$ aorta].
*** Value significantly lower than corresponding value in control rats $P < 0.001$ (Student's t test).
† Selective for pulmonary artery i.e. $-\log EC_{50}$ on pulmonary artery significantly greater than that on aorta: $0.05 > P > 0.01$.
‡ Selective for aorta i.e. $-\log EC_{50}$ on pulmonary artery significantly less than that on aorta $0.05 > P > 0.01$.

studied, and (b) the changes in responsiveness are dependent on the mechanism of action of the vasodilator drug but are independent of the means of inducing pulmonary hypertension.

The changes in responsiveness that were associated with pulmonary hypertension in the present study were of two types, i.e. an increase in maximum relaxation or a decrease in potency. The former was seen for the two drugs examined that act by opening potassium channels, pinacidil and cromakalim (Weir & Weston, 1986; Southerton *et al.*, 1988), and the latter for the drugs which act via activation of soluble guanylate cyclase, nitroprusside and sodium nitrite (Ignarro & Kadowidtz, 1985). Nicorandil, a drug that opens potassium channels and activates soluble guanylate cyclase (Kreye *et al.*, 1991; Kukovetz *et al.*, 1991), showed both an increase in maximum relaxation and a decrease in potency. In contrast, for atriopeptin II, which causes vasodilatation by activation of particulate guanylate cyclase (Tremblay & Hamet, 1989), i.e. does not act by either of the above mechanisms, the responses were unaffected. These findings indicate that whether or not

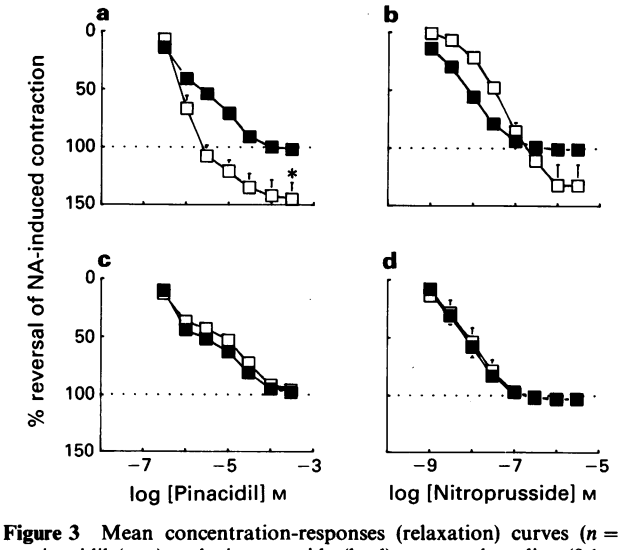


Figure 3 Mean concentration-responses (relaxation) curves ($n = 4$) to pinacidil (a, c) and nitroprusside (b, d) on noradrenaline (0.1 μM)-contracted preparations of pulmonary artery (a, b) and aorta (c, d) from normoxic control rats (■) and chronic hypoxic rats (□). Points are mean values, and the s.e.means are shown by the vertical bars except when smaller than the symbols. The asterisk indicates a significant difference in maximum relaxation between preparations fron normoxic and hypoxic rats (Mann-Whitney U test). The contractions to noradrenaline were (in mN): pulmonary artery: normoxia 15.3 ± 0.90 ($n = 8$); hypoxia 16.9 ± 1.89 ($n = 8$); aorta: normoxia 11.8 ± 0.64 ($n = 8$); hypoxia 12.4 ± 1.03 ($n = 8$).

(and in what manner) pulmonary hypertension affects the responsiveness of pulmonary arteries to vasodilator drugs, depends on the mechanism whereby each drug causes vasodilatation.

It is likely that the above changes in responsiveness are linked specifically to the development of pulmonary hypertension for two reasons. Firstly responses to the same vasodilator drugs in a vessel from outside the pulmonary circulation, the aorta, were no different in preparations from pulmonary hypertensive and normotensive rats. Secondly it was shown that the increase in maximum relaxation for pinacidil and the decrease in potency for nitroprusside occurred in pulmonary arteries from pulmonary hypertensive rats, irrespective of the method used to induce pulmonary hypertension (monocrotaline treatment or chronic hypoxia). The two methods of inducing pulmonary hypertension differ in as much as monocrotaline treatment causes lung injury and lung oedema whereas chronic hypoxia causes hypoxic pulmonary vasoconstriction (Reeves & Herget, 1984). However, both treatments (monocrotaline and chronic hypoxia) caused raised pulmonary artery pressures and pulmonary vascular hypertrophy. Thus, the increased relaxation of isolated pulmonary arteries to the potassium channel opening drugs and the decreased potency on these preparations of the nitrovasodilator drugs could either be associated with the hypertrophy of the pulmonary arteries and/or be an indirect result of the raised pulmonary artery pressure. Since endothelial cell damage can occur in pulmonary hypertension (Rosenberg & Rabinovitch, 1988), it is also possible that the changes in vasodilator responsiveness seen in the present study might reflect dysfunction of the endothelium. However this is unlikely since pulmonary vessels from both monocrotaline-treated and hypoxic rats were shown to have a functional endothelium, as indicated by a relaxant response to ACh, and on rat pulmonary artery, responses to neither pinacidil nor nitroprusside are modified by removal of the endothelium (Wanstall & Kay, unpublished observations).

The observations that there was some inherent tone in the pulmonary artery preparations from pulmonary hypertensive rats, but not in those from normotensive control rats, confirms findings from a previous study on pulmonary arteries from monocrotaline-treated rats (Wanstall *et al.*, 1991). It could be argued that the maximum relaxation produced by the potassium channel opening drugs appeared greater in pulmonary arteries from pulmonary hypertensive rats simply because of the inherent tone in these vessels. However if this were the only explanation for the increase in maximum relaxation produced by these drugs, an increase in maximum would also have been seen for each of the other vasodilator drugs studied, and this was not the case. Thus it is necessary to consider alternative (or additional) explanations for the increase in maximum relaxation for the potassium channel opening drugs.

In a previous study (Wanstall *et al.*, 1991), we suggested that the involvement of voltage operated calcium channels (VOCs) in contractions to noradrenaline might be greater in pulmonary arteries from pulmonary hypertensive rats than in controls, and that this might explain the observed increase in the inhibition of these contractions by felodipine, a drug that blocks calcium influx through VOCs. A similar explanation could be applied to the increased relaxation seen in the present study for the potassium channel opening drugs, which also inhibit calcium influx through VOCs, albeit indirectly by causing hyperpolarization (Cook, 1988). Alternatively, in pulmonary arteries from pulmonary hypertensive rats, the potassium channels may be closer to the open state than in control arteries. The potassium channels on which these drugs have been proposed to act are ATP-dependent (Standen *et al.*, 1989), i.e. are normally inhibited by intracellular ATP. Intracellular levels of ATP may be depleted in the pulmonary arteries from the hypertensive rats, as a result of a reduction in oxidative phosphorylation (Voelkel, 1986). It has been suggested that, under these conditions, the potassium channels may be in a less inhibited state and, thus, more readily opened by potassium channel opening drugs (Duty & Weston, 1991).

Possible explanations for the decrease in potency of the nitrovasodilator drugs include decreased relaxation of the vascular smooth muscle in response to guanosine 3′:5′-cyclic monophosphate (cyclic GMP, the second messenger responsible for the action of the drugs), or desensitization of soluble guanylate cyclase (the enzyme on which the drugs act). The first of these possible explanations is unlikely because there was no decrease in the potency of atriopeptin II, a drug which also acts via cyclic GMP, albeit via particulate rather than soluble guanylate cyclase (Tremblay & Hamet, 1989). However the second explanation is tenable.

Desensitization of soluble guanylate cyclase could occur if there were an increase in the production of endothelium-derived relaxing factor (EDRF) from the pulmonary vascular endothelium. It has been suggested that EDRF may be an important physiological modulator of hypoxic pulmonary vasoconstriction (Brashers *et al.*, 1988; Liu *et al.*, 1991). Furthermore it has been shown in isolated blood vessels that hypoxia can stimulate the release of EDRF *in vitro* (Pohl & Busse, 1989). Thus it is possible that under *in vivo* hypoxic conditions, which occur in chronically hypoxic rats, and which are likely to occur in monocrotaline-treated rats due to ventilatory dysfunction (Lai *et al.*, 1991), EDRF production from the pulmonary endothelial cells may be increased. It is now accepted that one EDRF is nitric oxide, or a closely related precursor (Palmer *et al.*, 1987). Sustained exposure of isolated blood vessels to nitric oxide has been shown to cause a reduction in the responses of the vessels to other drugs which activate guanylate cyclase (Henry *et al.*, 1989), and incubation of guanylate cyclase with nitric oxide *in vitro* has been reported to result in a reduction in the responsiveness of the enzyme to subsequent exposure to nitric oxide (Braughler, 1983), i.e. there is evidence for nitric oxide-induced desensitization of the enzyme. Thus it is plausible that, in pulmonary hypertension, there may be an increase in EDRF (nitric oxide) production from the pulmonary vascular endothelium which then leads to desensitization of guanylate cyclase in the pulmonary vascular smooth muscle, and results in a reduction in the potency of nitrovasodilator drugs.

Not all types of pulmonary hypertension are necessarily suitable for treatment with vasodilator drugs (Reeves *et al.*, 1986; Rubin, 1987). Vasodilator therapy may be valuable in patients with primary pulmonary hypertension (Pavlevsky & Fishman, 1985; Reeves *et al.*, 1986) and in the treatment of post-operative pulmonary hypertensive crises following cardiac surgery, but the value of vasodilators in secondary pulmonary hypertension is controversial (Reeves *et al.*, 1986; Adnot *et al.*, 1987). However, in any patient in which vasodilator drug therapy is appropriate, selectivity of the drugs for pulmonary as opposed to systemic blood vessels is obviously desirable. None of the drugs included in the present study was selective for pulmonary artery compared with aorta when examined on preparations from control rats. In contrast, on preparations from pulmonary hypertensive rats, pinacidil was significantly selective for pulmonary artery, while nitroprusside showed selectivity for aorta. Thus a drug which is non-selective when studied on vessels from normal rats may nevertheless show selectivity under conditions of pulmonary hypertension. This finding emphasizes the importance of assessing the selectivity of potential pulmonary vasodilator drugs in vessels taken from pulmonary hypertensive rats rather than in normal healthy vessels.

In conclusion, this study has shown that, in pulmonary hypertension, functional changes in the pulmonary vasculature occur not only for vasoconstrictor spasmogens (as is well documented in various previous studies, see Introduction), but also for vasodilator drugs. The influence of pulmonary hypertension on the responsiveness of pulmonary arteries to vasodilator drugs varies depending on the mechanism of action of the drug, there being an increase in maximum relaxation to poassium channel opening drugs and a decrease in the potency of nitrovasodilator drugs. The increased responsiveness to the potassium channel opening drugs in arteries from pulmonary hypertensive rats and the pulmonary:aorta selectivity of pinacidil suggest that potassium channel opening drugs may be worth investigating as pulmonary vasodilators in those pulmonary hypertensive patients in which vasodilator therapy is indicated.

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Role of the adrenal medulla in the metabolic and pressor actions of 8-OH-DPAT

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1 Intravenous administration of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, 150 $\mu g\,kg^{-1}$) into conscious sham-operated rats caused significant increases in basal glycaemia with minor changes in basal insulinaemia. Glucose-stimulated (intravenous glucose tolerance test) plasma insulin levels were significantly inhibited in 8-OH-DPAT-treated sham-operated animals. These metabolic changes were associated with significant and sustained falls in blood pressure (BP) and heart rate (HR) preceded by transient (< 5 min) increases only in BP.

2 In adrenodemedullated animals, 8-OH-DPAT failed to cause an initial vasoconstriction, hyperglycaemia, or inhibition of glucose-stimulated plasma insulin despite eliciting falls in BP and HR that were comparable to those observed in sham-operated animals.

3 Noradrenaline, adrenaline and dopamine levels in the adrenal tissue were reduced by about 95% in adrenodemedullated rats as compared to sham-operated rats. A functionally intact adrenal cortex was indicated by the presence of corticosterone in the plasma of both adrenodemedullated and sham-operated rats.

4 The present data demonstrate that 8-OH-DPAT mediates an initial increase in BP and changes in metabolic parameters via intact adrenal medulla and may thus be consequential to the release of adrenaline, whereas the sustained cardiovascular effects of 8-OH-DPAT are not.

Keywords: Adrenal medulla; catecholamines; insulin; glucose; 8-OH-DPAT; conscious Sprague-Dawley rats; blood pressure

Introduction

5-Hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) receptor agonists such as 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and flesinoxan decrease blood pressure (BP) and heart rate (HR) in many species through an interaction with central 5-HT$_{1A}$-receptors (Fozard *et al.*, 1987; Ramage & Fozard, 1987; Doods *et al.*, 1988; for review see Mir & Fozard, 1990). These actions are associated with an increase in vagal tone and sympathoinhibition (Ramage & Fozard, 1987; Ramage *et al.*, 1988).

8-OH-DPAT has been shown also to act on the endocrine system. For example, 8-OH-DPAT, through activation of 5-HT$_{1A}$-receptors, decreases plasma insulin and increases basal plasma glucose levels in several rat strains, via a central mechanism of action (Chaouloff & Jeanrenaud, 1987, 1988; Bouhelal *et al.*, 1990; Laude *et al.*, 1990).

5-HT$_{1A}$-receptor agonists enhance plasma levels of corticosterone (Koenig *et al.*, 1987; Aulakh *et al.*, 1988; Chaouloff *et al.*, 1990c; Bouhelal & Mir, 1990) and catecholamines (Badgy *et al.*, 1989; Chaouloff *et al.*, 1990a; Laude *et al.*, 1990). We have shown previously that despite the fact that the effects of 8-OH-DPAT on glucose and insulin are centrally mediated, they are dependent upon the adrenal glands being intact (Bouhelal & Mir, 1990). Similarly, the initial short-lasting pressor effect of 8-OH-DPAT (Gradin *et al.*, 1985; Fozard *et al.*, 1987; Bagdy *et al.*, 1989) is also dependent on intact adrenal glands (Bouhelal & Mir, 1990). Both parts of the adrenal gland (cortex and medulla) can participate in glucose metabolism, through their respective secretory products (corticosteroids and catecholamines). The objective of the present study was to investigate the importance of the medulla versus the cortex of the adrenal gland in the mediation of the metabolic (hyperglycaemia and inhibition of insulin release during a glucose tolerance test) and pressor effects of 8-OH-DPAT by using adrenodemedullated and sham-operated conscious Sprague-Dawley rats.

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Methods

Animals

Adrenodemedullated and sham-operated Sprague-Dawley rats (300–380 g) were supplied by IFFA-CREDO (Les Oncins, France). They had free access to food and were given a solution of 1% saccharose and 0.9% NaCl to drink during the course of the study. Animals were maintained on a constant 12 h light cycle. Animals were used about 2 to 3 weeks after adrenodemedullation. Successful adrenodemedullation was assessed by measuring the adrenal catecholamines at the end of the experiments.

Intravenous glucose tolerance test (IVGTT) and cardiovascular recordings in conscious animals

Experiments were conducted with minor modification of the procedures described in detail previously (Bouhelal *et al.*, 1990). Briefly, catheters were implanted under sodium hexobarbitone (NA-Evipan) anaesthesia (160 $mg\,kg^{-1}$ i.p.) in the femoral artery and vein. Cannulae were passed subcutaneously and exteriorized at the back of the neck. At the end of surgery cannulae were filled with heparinized saline to avoid blood clotting. Experiments were performed at least three days after surgery. BP and HR were recorded continuously from the arterial cannula as described in detail previously (Fozard *et al.*, 1987). After a stabilization period of about 1 h an arterial blood sample (200 μl) was taken to establish basal glucose and insulin values. Subsequently saline (1 $ml\,kg^{-1}$ i.v.) or 8-OH-DPAT (150 $\mu g\,kg^{-1}$ i.v.) was injected via the venous cannula and another blood sample taken 15 min later to determine the acute effects of saline or 8-OH-DPAT on plasma glucose and insulin concentrations. The dose of 150 $\mu g\,kg^{-1}$ i.v. of 8-OH-DPAT has been previously characterized in detail for the metabolic and cardiovascular effects in rats (Chaouloff & Jeanrenaud, 1987, 1988; Bouhelal *et al.*, 1990; Bouhelal & Mir, 1990); therefore, this dose was used in the present study. Glucose (0.5 $g\,kg^{-1}$ in 0.5 ml water) was

then injected i.v. and further blood samples withdrawn after 1, 3, 6, 12, 24, and 48 min. The arterial cannula was rinsed with heparinized saline following each blood sampling and care was taken not to dilute the sample with the residual saline in the cannula. Preliminary experiments showed that glucose loading did not affect the cardiovascular changes due to 8-OH-DPAT administration.

Plasma glucose, insulin and corticosterone measurements

Blood samples were collected in Eppendorf tubes through the arterial cannula and kept at 4°C before centrifugation (8800 *g* for 3 min). Aliquots (10 μl) of the plasma were used to determine glucose concentrations by use of an automatic glucose analyser (Ektachem, Kodak). The remainder of the plasma was stored at −20°C for subsequent insulin and corticosterone assay. Insulin was determined, by use of the radioimmunoassays described by Hales & Randle (1963). Corticosterone was determined by a modification of a radioimmunoassay described by Abraham (1977). Briefly, 20 μl of plasma were diluted 500 fold with a phosphate buffer containing 0.2% gelatin. Diluted samples (500 μl) and standards were heated at 98°C for 10 min in order to denature corticosterone-binding rat plasma proteins. After the samples had cooled to room temperature, [^{3}H]-corticosterone (100 μl, about 10,000 c.p.m.) and anti-corticosterone antibody (100 μl) were successively added. The antibody concentration was selected to produce 50% of maximal binding. After an incubation at 4°C for 2 h, separation of free corticosterone from antibody bound corticosterone was achieved by use of 200 μl of coated charcoal suspension (0.6%). Following centrifugation (2500 r.p.m. for 15 min), supernatants containing bound corticosterone were collected and counted in a β-counter.

Adrenal tissue catecholamine measurements

At the end of the experiment, adrenal glands were removed and stored at −180°C and subsequently, tissue adrenaline, noradrenaline and dopamine concentrations were measured by reverse phase high performance liquid chromatography and electrochemical detection according to the method of Kempf & Mandel (1981).

Drugs and solutions

(±)-8-OH-DPAT hydrogen bromide (Research Biochemicals Inc.) was dissolved in 0.9% (w/v) NaCl for *in vivo* experiments. Human [^{125}I]-insulin (Amersham International) and anti-insulin antiserum raised in guinea-pigs were from Medipro (Basel, Switzerland). Anticorticosterone antibody (Radioassay Systems Laboratories, Inc., Carson, Ca, U.S.A.) and tritiated corticosterone were from New England Nuclear (Regensdorf, Switzerland).

Calculations and statistics

Data have been expressed as means ± s.e.mean. Statistical analysis of data was performed by use of unpaired Student's *t* test and were considered significant at the $P < 0.05$ level. Positive incremental areas under the curve for plasma insulin and glucose concentration versus the time (AUC) was determined using the trapezoidal rule (Le Floch *et al.*, 1990).

Results

Conscious sham-adrenodemedullated rats

In sham-operated animals, 8-OH-DPAT (150 μg kg^{-1} i.v.) significantly increased basal plasma glucose concentrations after 15 min (Figure 1a), without a change in basal plasma insulin (Figure 1b). Following an intravenous injection of glucose (IVGTT) to 8-OH-DPAT-treated animals, the stimulated

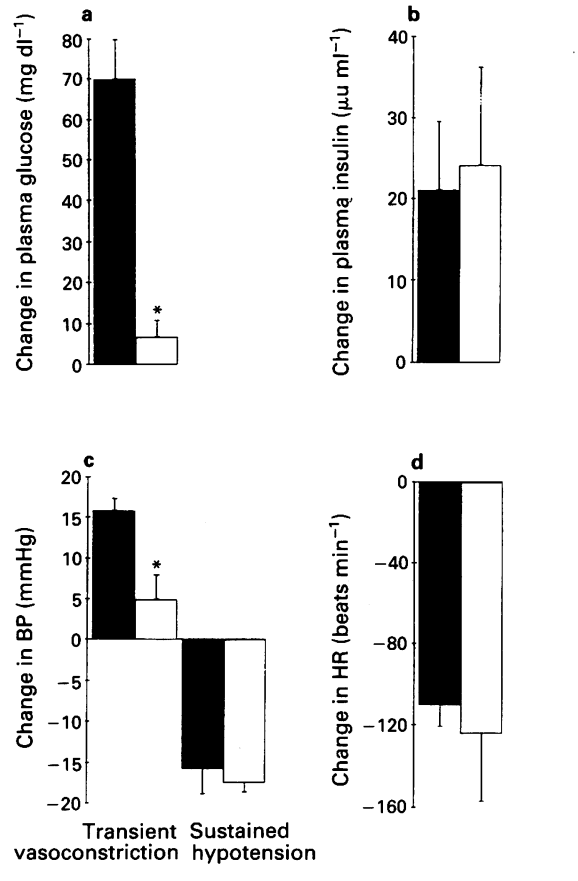


Figure 1 Effects of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) on plasma glucose, insulin, blood pressure (BP) and heart rate (HR) in conscious sham-operated and adrenodemedullated Sprague-Dawley rats. Basal plasma glucose and insulin concentrations were determined before 8-OH-DPAT (150 μg kg^{-1}, i.v.) injections and 15 min later. (a) Maximal changes in basal plasma glucose after 8-OH-DPAT. Basal plasma glucose levels were 137 ± 4 and 137 ± 9 mg dl^{-1}, $n = 6$, respectively in sham-operated and adrenodemedullated animals. (b) Maximal changes in basal plasma insulin after 8-OH-DPAT. Basal plasma insulin levels were 49 ± 5 and 41 ± 5 μu ml^{-1} respectively for sham-operated and adrenodemedullated animals, $n = 6$. (c) Maximal changes in BP during the transient vasoconstriction and the sustained hypotension phases. Baseline values were respectively 109 ± 2 mmHg, $n = 8$ and 101 ± 4 mmHg, $n = 6$ for sham-operated and adrenodemedullated animals. (d) Maximal changes in HR following 8-OH-DPAT administration. Baseline values were 417 ± 14 and 433 ± 9 respectively for sham-operated and adrenodemedullated animals. Results are represented as mean with s.e.mean of changes shown by vertical bars. Solid columns: sham-operated animals; open columns: adrenodemedullated animals. For further experimental details, see text. *$P < 0.05$, value significantly different from the corresponding control value.

plasma insulin levels were reduced in association with an increase in plasma glucose levels. When both the first 15 min of 8-OH-DPAT-induced hyperglycaemia and the IVGTT were taken into account (0–63 min), a significant decrease in AUC for plasma insulin was found in the 8-OH-DPAT-treated group whereas the plasma glucose was increased by 41%, although the increase did not reach statistical significance due to the variation (Table 1). As expected, 8-OH-DPAT at the same dose produced sustained falls in both BP and HR which were preceded by transient (< 5 min) increases in BP (Figure 1c and d). Control saline injections did not produce any change of the above parameters (data not shown).

Conscious adrenodemedullated rats

In contrast to sham-operated animals, 8-OH-DPAT did not modify plasma levels of glucose or insulin either under basal

Table 1 Effect of 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) on areas under the curve (AUC) for basal and intravenous glucose tolerance test (IVGTT)-stimulated plasma glucose and insulin concentrations versus time

	AUC glucose		*AUC insulin*	
	Control	*8-OH-DPAT*	*Control*	*8-OH-DPAT*
Sham-operated (0–63 min)	3012 ± 388	5082 ± 1035	3626 ± 525	2310 ± 198*
Adrenodemedullated (0–63 min)	1510 ± 151	2004 ± 190	4023 ± 804	4250 ± 555

Values are expressed in $\mu u\,ml^{-1}$ and $mg\,dl^{-1}$ respectively for plasma insulin and glucose. * $P < 0.05$, value significantly different from the corresponding control value. Areas under the curve (AUC) were calculated by use of the trapezoidal rule. Values determined for 63 min take into account both the effect on the basal levels during the first 15 min and the effects on the intravenous glucose tolerance test (IVGTT) during the 48 min period.

conditions or during the IVGTT in adrenodemedullated rats (Figure 1a; Table 1). 8-OH-DPAT produced similar falls in both BP and HR as compared to sham-operated animals (Figure 1c and d) that were, however, not preceded by the initial pressor response observed in sham-operated animals.

Assessment of adrenodemedullation

Adrenaline, noradrenaline and dopamine levels were respectively 426 ± 23, 87 ± 7 and 12 ± 1 $\mu g\,g^{-1}$ of tissue in sham-operated animals and significantly reduced at 14 ± 11, 3 ± 3 and 1 ± 1 $\mu g\,g^{-1}$ of tissue in adrenodemedullated animals. There were no significant differences between animals receiving 8-OH-DPAT or saline in either group (data not illustrated). Basal plasma corticosterone concentrations in sham-operated animals were significantly ($P < 0.05$) greater 57.7 ± 5.1 $\mu g\,dl^{-1}$, $n = 11$ than in adrenodemedullated animals 33.6 ± 3.5 $\mu g\,dl^{-1}$, $n = 10$. No statistically significant increases in corticosterone concentrations were observed 15 and 27 min after 8-OH-DPAT administration in either group of animals (data not illustrated).

Discussion

The major conclusion that emerges from this study is that the hyperglycaemic response of 8-OH-DPAT, its inhibitory action on glucose-stimulated insulin release and the transient vasoconstrictor effect are mediated by the medulla of the adrenal gland. The extent of adrenodemedullation was assessed by the determination of catecholamines in the adrenal gland tissue. A 95% reduction in noradrenaline, adrenaline and dopamine indicates nearly a complete adrenodemedullation and suggests a role for the medullary catecholamines in the metabolic and vasoconstrictor effects of 8-OH-DPAT. The fact that 8-OH-DPAT (Bagdy *et al.*, 1989; Chaouloff *et al.*, 1990a) increases plasma adrenaline and that selective α_2- and β_2-adrenoceptor antagonists (Chaouloff & Jeanrenaud, 1987; Chaouloff *et al.*, 1990a) suppress 8-OH-DPAT-mediated hyperglycaemia supports the involvement of adrenal catecholamines in the metabolic effects of 8-OH-DPAT. Moreover, the fact that the plasma glucose levels are significantly increased in sham-operated rats following 8-OH-DPAT administration, without significant increase in corticosterone, suggests that the hyperglycaemia is not a consequence of corticosterone release in the present study. The adrenaline-releasing effects of 8-OH-DPAT are antagonized by the mixed 5-HT_{1A}-β-adrenoceptor antagonist, (−)-pindolol, but not by selective β_1- or β_2-adrenoceptor antagonists (Chaouloff *et al.*, 1990a). Moreover, adrenaline release and hyperglycaemia is induced by several other selective 5-HT_{1A}-receptor agonists such as buspirone, ipsapirone (Chaouloff *et al.*, 1990b) and flesinoxan (Mir *et al.*, unpublished observations), thus implicating a key role for the 5-HT_{1A}-receptors in the regulation of adrenaline release and glucose metabolism.

8-OH-DPAT has been shown to increase plasma levels of corticosterone in the rat (Koenig *et al.*, 1987; Aulakh *et al.*, 1988; Chaouloff *et al.*, 1990c), which can be considered as another potential contributory factor to the 8-OH-DPAT-induced hyperglycaemia. However, the nature and extent of the involvement of corticosterone in this respect is not clearly established (Chaouloff *et al.*, 1990c). In the present study no significant stimulation of corticosterone levels was observed 15 min following 8-OH-DPAT administration. This lack of stimulation appears unlikely to be due to a non-functional cortex since in both adrenodemedullated and sham-operated animals corticosterone production was present.

With regard to the cardiovascular effects of 8-OH-DPAT, the transient vasoconstriction in response to 8-OH-DPAT, preceding the falls in BP and HR is abolished in adrenodemedullated animals. This finding, confirms a previous observation in adrenalectomized animals (Bouhelal & Mir, 1990), and together with the observations that 8-OH-DPAT increases plasma catecholamine levels (Bagdy *et al.*, 1989; Chaouloff *et al.*, 1990a) and that the pressor response is attenuated by prazosin (Gradin *et al.*, 1985), supports the involvement of catecholamines in the pressor response to 8-OH-DPAT. 8-OH-DPAT also produces a small transient pressor response in pithed rats (Fozard *et al.*, 1987; Bouhelal *et al.*, 1990). This action is unlikely to be due to catecholamine release, since Chaouloff *et al.* (1990d) have shown that penthobarbitone anaesthesia completely suppresses the adrenaline release which normally occurs in conscious animals. Rather, a direct vascular action in response to a bolus intravenous injection is a possible mechanism. The fact that 8-OH-DPAT produces similar hypotensive and bradycardic effects in sham and adrenodemedullated rats confirms previous observations in adrenalectomized rats (Bouhelal & Mir, 1990), and lends further support to the difference in mechanisms in the hypotensive and bradycardic effects *vis-a-vis* the vasoconstrictor and metabolic effects. Paradoxically, the metabolic changes (hyperglycaemia and hypoinsulinaemia) would generally be expected to arise from sympathoexcitation and/or a decrease in vagal tone (Ahren *et al.*, 1986), which is the opposite of what has been established to be the basis of the 8-OH-DPAT effects on the cardiovascular system (see Introduction). Thus it appears that 8-OH-DPAT exerts a differential action on the autonomic nervous system with respect to its metabolic and major cardiovascular effects (hypotension and bradycardia). In Figure 2, a tentative model to explain the mechanisms of 5-HT_{1A}-mediated metabolic and cardiovascular effects is illustrated. 5-HT and 8-OH-DPAT stimulate the release of corticotrophin-releasing factor (CRF) (Jones *et al.*, 1976; Calogero *et al.*, 1989). In addition to its well known ACTH-releasing activity, CRF is also known to stimulate directly sympathetic tone to the adrenal medulla via a central site of action (for review see Fisher, 1989), manifested by increases in efferent adrenal nerve activity, plasma adrenaline concentrations and an increase in BP (Brown & Fisher, 1983; Brown *et al.*, 1985). Hyperglycaemia has also been observed after intracerebroventricular injections of CRF in the rat and the dog (Brown *et al.*, 1982). The recent observations that ganglionic nicotine receptor blockade with hexamethonium decreases the 8-OH-DPAT-induced increases in plasma adrenaline and

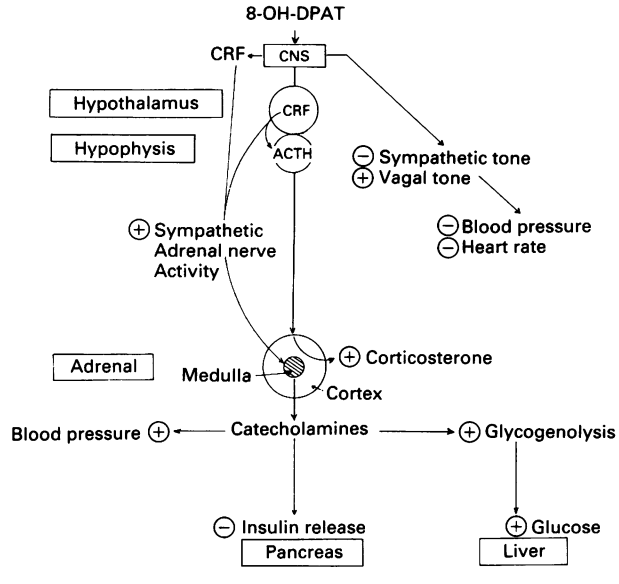


Figure 2 Tentative model for 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) - receptor-mediated metabolic and cardiovascular effects in the rat. CRF: corticotropin-releasing factor; ACTH: adreno-corticotropic hormone; −: decrease; +: increase; CNS: central nervous system. For further details see Discussion.

glucose (Chaouloff *et al.*, 1990c) would also be consistent with a centrally mediated sympatho-adrenal stimulation. Therefore, the possibility that 8-OH-DPAT-mediated catecholamine release, which would result in hyperglycaemia and hypoinsulinaemia, is triggered by CRF deserves further attention.

Taken together, these data strongly suggest that 8-OH-DPAT exerts its metabolic and transient pressor effect primarily via an action on the adrenal medulla. The data further confirm the differences in the mechanisms underlying the hypotensive and bradycardic actions of 8-OH-DPAT *vis-a-vis* the effects on glucose homeostasis. The major cardiovascular effects (hypotension and bradycardia) of 5-HT$_{1A}$ agonists may thus be regarded as being mediated purely by neuronal pathways whereas the metabolic effects involve mixed neuronal and humoral mechanisms (see Figure 2).

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Differences in intrinsic efficacy of benzodiazepines are reflected in their concentration-EEG effect relationship

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1 The relevance of EEG effect parameters as a measure of the central nervous system effects of benzodiazepines was evaluated. The concentration-EEG effect relationships of the benzodiazepine agonist midazolam, partial agonist bretazenil, antagonist flumazenil and inverse agonist Ro 19-4603 were quantified and compared with the intrinsic efficacy and affinity of these compounds at the γ-aminobutyric acid (GABA)-benzodiazepine receptor complex.

2 The pharmacokinetics and pharmacodynamics of the compounds were determined after a single intravenous bolus administration of $5\,mg\,kg^{-1}$ midazolam, $2.5\,mg\,kg^{-1}$ bretazenil, $10\,mg\,kg^{-1}$ flumazenil or $2.5\,mg\,kg^{-1}$ Ro 19-4603 to male Wistar derived rats. In a separate experiment the distribution between blood, cerebrospinal fluid and brain concentrations of these compounds was determined. A sensitive assay was developed to measure bretazenil and Ro 19-4603 concentrations in small samples of biological fluids.

3 The benzodiazepine-induced changes in amplitudes in the 11.5–30 Hz frequency band, as determined by aperiodic analysis, was used as EEG effect measure. Concentration-EEG effect relationships were derived by a pharmacokinetic-pharmacodynamic modelling procedure and in the case of midazolam, bretazenil and Ro 19-4603 successfully quantified by the sigmoidal E_{max} model. Large differences in maximal effect of midazolam ($E_{max} = 73 \pm 2\,\mu Vs^{-1}$), bretazenil ($E_{max} = 19 \pm 1\,\mu Vs^{-1}$) and Ro 19-4603 ($E_{max} = -6.5 \pm 0.4\,\mu Vs^{-1}$) were observed, reflecting their differences in intrinsic efficacy. A close correlation was found between the EC_{50} values based on free drug concentration and receptor affinity as determined by displacement of [^{3}H]-flumazenil in a washed brain homogenate at 37°C. In the concentration range of receptor saturation flumazenil did not produce any changes in the EEG effect measure.

4 The study demonstrated that the change in amplitudes in the 11.5–30 Hz frequency band of the EEG is a relevant measure of the pharmacological effect intensity of benzodiazepines, because it seems to reflect their affinity and intrinsic efficacy at the central GABA-benzodiazepine receptor complex.

Keywords: Pharmacokinetics; pharmacodynamics; modelling; benzodiazepines; EEG; efficacy; partial agonist; antagonist; inverse agonist

Introduction

Knowledge of the relationships that can describe and predict the time course of drug action *in vivo* is considered to be essential for the optimization of drug therapy (Levy, 1983; Holford & Sheiner, 1982). Despite the advances in analytical methodologies to measure drug and metabolite concentrations, the lack of appropriate, objective measures of pharmacological effect intensity has limited the characterization of these relationships, especially for drugs which act on the central nervous system (CNS) (Dingemanse *et al.*, 1988a). Recently, quantitative EEG effect parameters have been shown to provide continuous, objective, reproducible and sensitive measures of the central nervous system effects of benzodiazepines, allowing the characterization of *in vivo* concentration-effect relationships of these drugs (Koopmans *et al.*, 1988; Greenblatt *et al.*, 1989; Breimer *et al.*, 1990; Mandema *et al.*, 1991b,c). However, the relevance of the EEG parameters with respect to the pharmacological effects of benzodiazepines in terms of potency and intrinsic efficacy remains to be established.

The mechanism of action of benzodiazepines is well known and involves their specific interaction with the γ-aminobutyric acid (GABA)-benzodiazepine receptor complex and their ability to enhance GABA-induced inhibitory action (Haefely *et al.*, 1985; Haefely, 1989). During the past few years, compounds have been discovered that cover the whole spectrum of intrinsic efficacy ranging from the traditional full agonists to inverse agonists (Braestrup *et al.*, 1983; Gardner, 1988; Haefely, 1988; 1989). This continuum includes partial (inverse) agonists, which produce only a part of (inverse) agonistic activity and antagonists, which selectively antagonize the effects of both (partial) agonists and inverse agonists without producing any intrinsic effects by themselves. Therefore, it is essential to evaluate for validity reasons whether EEG effect parameters reflect the interaction of benzodiazepines with and their intrinsic efficacy at the GABA-benzodiazepine receptor complex. In a previous study a close correlation between the potency of different benzodiazepine agonists with respect to their EEG effects, their receptor affinity and anticonvulsant effects as determined by the pentylenetetrazol (PTZ)-induced seizure threshold model was found (Mandema *et al.*, 1991b). The purpose of the present investigation was to quantify the concentration-EEG effect relationship of a benzodiazepine agonist midazolam, partial agonist bretazenil (Ro 16-6028), antagonist flumazenil and inverse agonist Ro 19-4603 (Figure 1) and to determine whether their differences in intrinsic efficacy are adequately reflected in pharmacodynamic parameters derived from EEG effect measures.

Methods

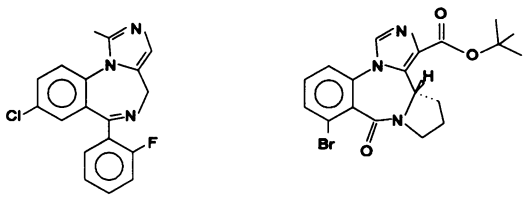
Animals

Male Wistar derived rats (225–275 g) were used throughout the study. The animals were housed individually in plastic cages with a normal 12 h light-dark cycle and fed on a commercially available diet (Standard Laboratory Rat, Mouse and Hamster Diets, RMH-TM, Hope Farms, Woerden, The Netherlands) and water *ad libitum*. From the night before experimentation the animals were deprived of food but had free access to water.

Pharmacokinetic-pharmacodynamic experiments

For the measurement of EEG signals, seven cortical electrodes were chronically implanted into the skull of the animals one week before the kinetic-dynamic experiments as previously

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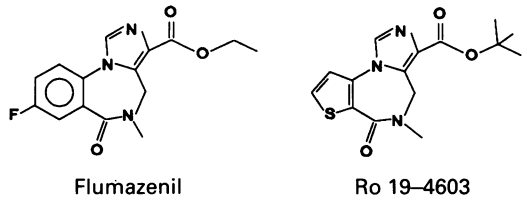


Figure 1 Chemical structures of the benzodiazepine agonist midazolam, partial agonist bretazenil (Ro 16-6028), antagonist flumazenil and inverse agonist Ro 19-4603.

described (Mandema & Danhof, 1990). Indwelling cannulae were implanted into the right jugular vein (for drug administration) and right femoral artery (for sample collection) under light ether anaesthesia, one day before the experiment. Dimethylacetamide (DMA) was used to make intravenous administration of the water insoluble benzodiazepines possible (Dingemanse *et al.*, 1988b). Drugs were dissolved at a concentration to give a dose volume of 100 μl of DMA. Rats were randomly assigned to four treatment groups that each received 2.5 mg kg^{-1} bretazenil, 10 mg kg^{-1} flumazenil, 2.5 mg kg^{-1} Ro 19-4603 or 100 μl DMA (placebo) intravenously, administered in 2 min by an infusion pump. The pharmacokinetic and pharmacodynamic data of midazolam were the same as found in a previous study (Mandema *et al.*, 1991b). Arterial blood samples (100–200 μl) were taken at intervals and immediately diluted with 0.5 ml of ice-cold 0.42% sodium fluoride solution in water and kept on ice to inhibit esterase activity. The diluted blood samples were stored at −35°C until analyzed for drug concentrations.

During the course of the experiment the animals had to walk in a slowly rotating drum (10 r.p.h.) to control the level of vigilance and prevent the animals from falling asleep spontaneously. EEG recordings were started 15 min before drug administration for baseline determination and continuously monitored by use of a system 50000 EEG recorder (Van Gogh BV, Amsterdam, The Netherlands). Following injection of the drug, the EEG recordings were continued until the signals had returned to pre-administration values. Two EEG leads, fronto-central and central-occipital on the left hemisphere, were subjected to on-line aperiodic analysis for quantification (Gregory & Pettus, 1986). The benzodiazepine induced change from baseline in the amplitudes (μVs^{-1}) in the 11.5–30 Hz (beta) frequency band of the fronto-central lead were calculated and used as a measure of drug effect (Mandema *et al.*, 1991b,c). The effect data points were calculated by averaging at least 60 s of consecutive EEG data. More frequent measurements were taken at the time of rapid change in drug concentration, e.g. immediately after administration of the bolus dose.

Distribution between blood and CNS

The distribution of flumazenil and midazolam between blood, CSF and brain was determined after administration of a cocktail of 10 mg kg^{-1} midazolam and 5 mg kg^{-1} flumazenil to a group of 7 animals weighing 200–250 g. CSF (by a puncture of a cisterna magna), blood (by an aortic puncture) and brain tissue were collected at 30 min after drug administration. Samples of 100 μl of blood were taken and treated as described earlier for midazolam and flumazenil blood concentration determination. The remainder of the blood was centrifuged and plasma was removed to determine midazolam plasma concentrations (100 μl sample). The brains were excised from the crania and one hemisphere was weighed and gently homogenized in 3 ml of ice-cold 0.42% sodium fluoride solution in water and rapidly stored at −35°C until analysis. Samples of 200 μl of brain homogenate were used to determine midazolam and flumazenil brain concentrations. The distribution between blood, CSF and brain of Ro 16-6028 and Ro 19-4603 was determined accordingly after administration of a cocktail of 5 mg kg^{-1} Ro 16-6028 and 5 mg kg^{-1} Ro 19-4603 to a group of 10 rats weighing 225–275 g. As protein binding of several benzodiazepines has been shown to be independent of concentration over a wide concentration range (0.01–10 mg l^{-1}) (Moschitto & Greenblatt, 1983), no interaction was expected with respect to the distribution to the CNS when the drugs were administered together.

Drug analysis

The blood, CSF and brain concentrations of flumazenil and midazolam were determined by a specific high pressure liquid chromatographic assay (h.p.l.c.) using u.v. detection, as previously described (Mandema *et al.*, 1991a,c). A sensitive assay was developed to measure the concentrations of bretazenil and Ro 19-4603 in small blood samples by h.p.l.c. and u.v. detection. A solution of 20 ng diazepam (internal standard) in 20 μl methanol was added with NaF solution to the diluted blood samples. Subsequently the samples were further diluted with 0.5 ml 0.1 N sodium hydroxide and extracted with 5 ml dichloromethane-pentane (1:1) for 30 s on a vortex mixer. After centrifugation the organic layer was separated and evaporated to dryness under reduced pressure. The residue was reconstituted with 200 μl mobile phase of which 100 μl was injected into the chromatographic system. The chromatographic system consisted of a M-45 solvent delivery pump, a WISP 710B automatic injector, a 10 cm μBondapack C18 column and a Lambda-Max model 418 LC spectrophotometer (all of Waters), which was set at 235 nm for bretazenil and 250 nm for Ro 19-4603. The mobile phase for bretazenil consisted of a mixture of 0.01 M phosphate buffer, pH = 5.0, and methanol in a ratio 35/65, with a flow rate of 1.0 ml min^{-1}. The mobile phase for Ro 19-4603 consisted of a mixture of 0.01 M phosphate buffer, pH = 5.0, and methanol in a ratio 40/60, with a flow rate of 0.8 ml min^{-1}. The within and between day coefficients of variation were less than 6% for Ro 19-4603 and less than 8% for bretazenil in the concentration range studied. The detection limit was about 5 ng ml^{-1} for both drugs, when a 200 μl blood sample was used. The brain and CSF samples were treated in similar manner to the blood samples.

Bretazenil and Ro 19-4603 were found to be stable in fresh rat blood *in vitro* kept at 37°C for 4 h.

Receptor binding

Binding affinities to the central GABA benzodiazepine receptor complex were determined based on displacement of [^{3}H]-flumazenil (New England Nuclear-757, specific activity 78.0 Ci mmol^{-1}) in a washed brain homogenate at 37°C as described previously (Mandema *et al.*, 1991b). Briefly, brains were obtained from drug-naive rats kept under similar conditions as described above. Total brains minus brain stems were gently homogenized in 10 vol of ice-cold, 'enriched' Tris buffer, containing 50 mM Tris, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH = 7.4). The homogenate was centrifuged at 48,000 *g* for 20 min and the supernatant was discarded. The pellet was resuspended in 10 vol of the 'enriched' Tris buffer. The procedure was repeated four times after which the homogenate was rapidly frozen and stored at −70°C. Brain homogenate aliquots (250 μl), to which GABA was added at a concentration of 33 μM and containing a protein concentration of 1.5 mg ml^{-1}, were incubated at 37°C with

approximately 6.4 ng ml^{-1} [^{3}H]-flumazenil and various concentrations of the displacing ligands. Alcohol, necessary to dissolve the benzodiazepines, was fixed at a concentration of 0.3% (Hollander-Jansen *et al.*, 1989). After equilibrium was reached (5 min) the samples were filtered through a presoaked glass-fibre filter (Whatman GF/B) under mild suction (Millipore 1225 Sampling Manifold). The filter was immediately washed twice with 5 ml of ice-cold, 'enriched' Tris buffer after which radioactivity was measured. Specific binding was assessed by subtracting the non-specific binding, obtained in the presence of 10 μg ml^{-1} flumazenil, from the total binding.

The receptor binding characteristics (K_d and B_{max}) of the radioligand [^{3}H]-flumazenil were determined in a saturation experiment under similar conditions as described above in the concentration range of 0 to 200 ng ml^{-1}. Free radioligand concentrations were calculated by subtracting the concentration of totally bound [^{3}H]-flumazenil at equilibrium from the concentration of [^{3}H]-flumazenil added to the incubation mixture. In both the displacement and saturation experiments binding was determined in triplicate at each concentration.

Data analysis

The pharmacokinetics of the different benzodiazepines were quantified for each individual rat. The plasma concentration time profiles were described by a poly-exponential equation:

$$C(t) = \sum_{i=1}^{n} A_i(e^{-\alpha_i t}) \quad (1)$$

where C(t) is the plasma concentration at time t and A_i and α_i are respectively the coefficients and exponents of the equation. Different exponential models were investigated and the most suitable model was chosen according to the Akaike information criterion (Akaike, 1974; Yamaoka *et al.*, 1978). Basic pharmacokinetic parameters such as clearance (Cl), volume of distribution at steady-state (Vdss) and mean residence time (MRT) were calculated from the coefficients and exponents of the fitted functions by standard methods (Gibaldi & Perrier, 1982).

The sigmoidal E_{max} model was used to describe the relationship between drug concentration and EEG effect (Holford & Sheiner, 1982):

$$E(C) = \frac{E_{max} \cdot C^N}{EC_{50}{}^N + C^N} \quad (2)$$

where E(C) is the observed effect (change from baseline) at concentration C, E_{max} is the maximal effect, EC_{50} is the concentration at half maximal effect and N is a constant expressing the shape of the concentration-effect relationship. The pharmacokinetic model was used to generate the concentrations of the drug at the times of effect measurement.

The receptor binding characteristics of [^{3}H]-flumazenil as determined in the saturation experiment have already been presented in a previous study (Mandema *et al.*, 1991b). IC_{50} values of the benzodiazepines were determined by fitting the following equation to the displacement data:

$$B = \frac{B_o \cdot IC_{50}}{IC_{50} + C_d} \quad (3)$$

In which B_o is the specific binding of the radioligand with no displacer present and C_d is the concentration of displacer added. K_i values were derived from the IC_{50} values according to the Cheng-Prusoff equation:

$$K_i = \frac{IC_{50}}{1 + \frac{L}{K_d}} \quad (4)$$

in which L is the concentration of radioligand used in the displacement studies. The equations were fitted to the data by use of the non-linear least squares regression program Siphar (Simed SA, Creteil, France). Statistical analysis was conducted by a one-way analysis of variance. A significance level of 5% was selected.

Results

Pharmacokinetics

The time course of drug concentrations after intravenous administration of the four benzodiazepines is shown in Figure 2. The solid lines represent the best fits of the pharmacokinetic model to the pooled data of all animals within one treatment group. For all four drugs, a bi-exponential equation (equation 1) was found to best characterize the data.

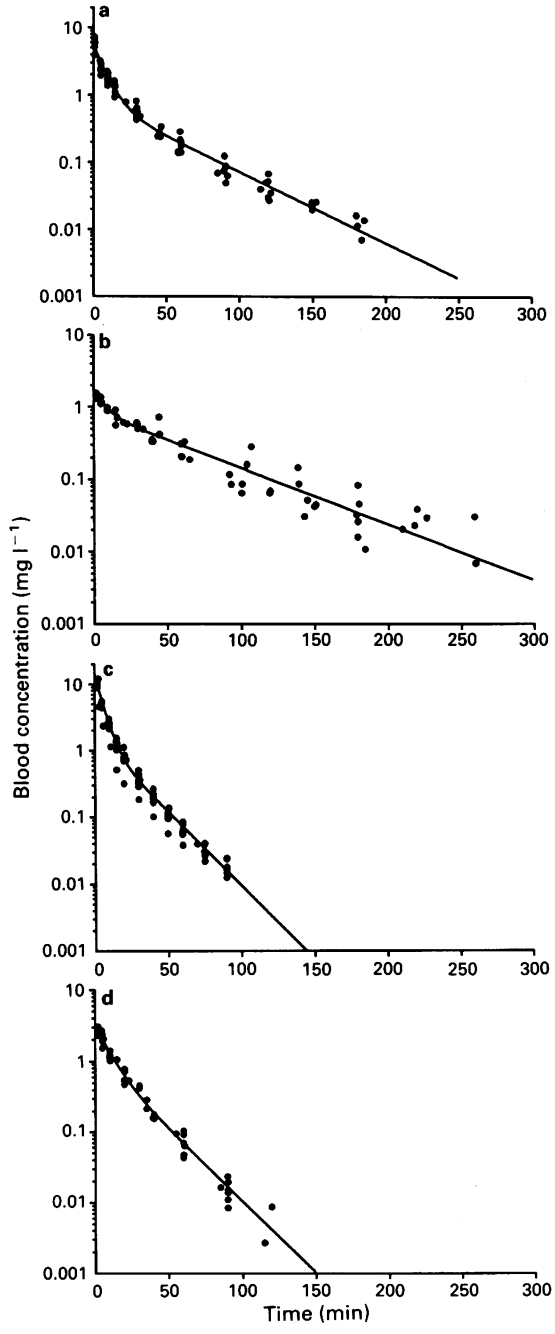


Figure 2 Blood concentration (●) versus time profiles of all individual animals, which had received an intravenous bolus administration of (a) 5 mg kg^{-1} midazolam, (b) 2.5 mg kg^{-1} bretazenil, (c) 10 mg kg^{-1} flumazenil or (d) 2.5 mg kg^{-1} Ro 19-4603. The solid lines represent the best fits of the pharmacokinetic model to the data.

Table 1 Pharmacokinetic parameter estimates of the four benzodiazepines

	Midazolam	*Bretazenil*	*Flumazenil*	*Ro 19-4603*
Number of animals	7	7	8	7
Cl (ml $min^{-1} kg^{-1}$)	92 ± 4	47 ± 3	114 ± 13	65 ± 6
Vdss (l kg^{-1})	2.2 ± 0.1	2.2 ± 0.1	1.1 ± 0.1	0.98 ± 0.09
MRT (min)	24 ± 1	48 ± 4	9.7 ± 0.2	15.0 ± 0.8

Cl, clearance; Vdss, volume of distribution at steady-state; MRT, mean residence time. The pharmacokinetic parameters are based on blood concentrations. The values are presented as means ± s.e.mean. Significant differences between the four drugs were found for Cl, Vdss and MRT (ANOVA, $P < 0.05$).

The pharmacokinetic parameters of each drug were calculated for the individual animals and are summarized in Table 1. Midazolam concentrations were originally measured in plasma (Mandema *et al.*, 1991b). The plasma to blood concentration ratio of midazolam was 1.23 ± 0.05 ($n = 7$), which was used to calculate the pharmacokinetic parameters of midazolam based on blood concentrations shown in Table 1. The pharmacokinetic parameters showed significant differences between the four drugs. The clearance value ranged from 114 ml $min^{-1} kg^{-1}$ for flumazenil to 47 ml $min^{-1} kg^{-1}$ for bretazenil and the mean residence time ranged from 9.7 min for flumazenil to 48 min for bretazenil.

The parameters describing the distribution of the four benzodiazepines between blood and the CNS in steady-state are shown in Table 2. Large differences were found between the CSF to blood concentration-ratio for the four drugs, with midazolam exhibiting the lowest value of 0.050 and flumazenil the highest value of 0.526. The differences in blood-brain distribution were much less pronounced.

Pharmacodynamics

The administration of the solvent DMA produced a small reduction in the EEG effect measure (amplitudes in the 11.5–30 Hz frequency band). However, it was large enough to distort the relatively small effects of the partial agonist, antagonist and inverse agonist. Therefore, the EEG effect versus time profiles of all individual animals were corrected for the averaged vehicle effect response. The corrected changes in EEG effect measure as a function of time for the four benzodiazepines are shown in Figure 3. This figure shows the averaged changes in EEG effect of all rats within one treatment group. For clarity the s.e.mean values are omitted. Figure 3 clearly shows the large differences in intrinsic efficacy of the four compounds. Midazolam produced the largest increase in EEG effect, bretazenil produced a much smaller increase, whereas Ro 19-4603 produced a small but significant decrease in the amplitudes in the 11.5–30 Hz frequency band. Flumazenil produced an initial increase in the EEG effect measure, which only lasted for 3 min, after which time no significant differences from the baseline EEG values were observed for the remainder of the registration period. No hysteresis was observed between plasma/blood concentrations and EEG effects for all four drugs and the values were directly correlated to each other. Figure 4 shows the averaged concentration-EEG effect relationship of the four drugs. The pharmacokinetic fits shown in Figure 2 were used to calculate the drug concentrations at the time points of effect measurement. The concentration-EEG effect relationships of midazolam, bretazenil and Ro 19-4603 were successfully quantified by the sigmoidal E_{max} model, as is shown by the solid lines in Figure 4. Flumazenil only produced a statistically significant increase in the EEG effect parameter at concentrations above 8 mg ml^{-1}. The peak effect after administration of flumazenil was 26 ± 3 μVs^{-1} at a concentration of 13 mg l^{-1}. In the concentration range of 0.1 mg l^{-1} to 1 mg l^{-1} the averaged effect was 0.7 ± 0.3 μVs^{-1} and not significantly different from baseline.

The concentration-EEG effect relationship of midazolam and bretazenil could be adequately quantified in individual animals and the estimated pharmacodynamic parameters are summarized in Table 3. The concentration-EEG effect relationship of the inverse agonist could not be adequately quantified by the sigmoidal E_{max} model in the individual animals. The pharmacodynamic parameters of Ro 19-4603 shown in Table 3 were estimated by fitting the sigmoidal E_{max} model to the averaged concentration-effect data and represent the parameter estimates of the fit and the estimates of the standard error of the parameter estimates. Since the pharmacodynamic parameters of the drugs were obtained in a different manner a statistical comparison with ANOVA was not possible and multiple t tests were used assuming the estimate of s.e.mean to be a population value. The maximal effects of midazolam, bretazenil and Ro 19-4603 showed significant differences. The EC_{50} values, based on total blood concentrations and CSF concentrations of bretazenil and Ro 19-4603,

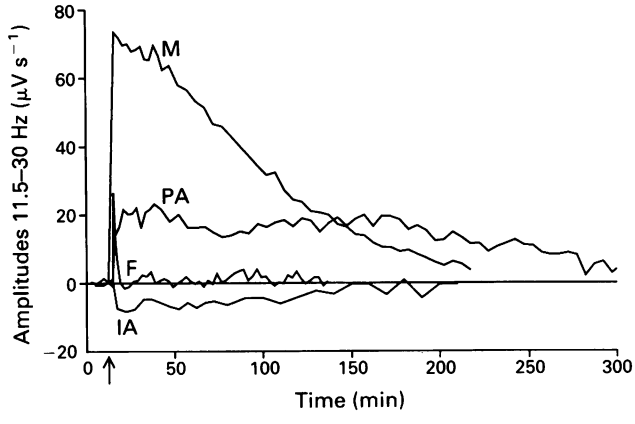


Figure 3 Averaged EEG effect (change in amplitudes in the 11.5–30 Hz frequency band) versus time profiles of all individual animals, which had received an intravenous bolus administration, marked by the arrow, of 5 mg kg^{-1} midazolam (M), 2.5 mg kg^{-1} bretazenil (PA), 10 mg kg^{-1} flumazenil (F) or 2.5 mg kg^{-1} Ro 19-4603 (IA).

Table 2 Distribution parameters of the four benzodiazepines between blood and the central nervous system

	Midazolam	*Bretazenil*	*Flumazenil*	*Ro 19-4603*
Number of animals	7	10	7	10
CSF/blood	0.050 ± 0.005	0.115 ± 0.006	0.526 ± 0.045	0.279 ± 0.008
Brain/blood (ml g^{-1})	2.10 ± 0.06	0.82 ± 0.02	0.51 ± 0.02	0.56 ± 0.01

The values are presented as means ± s.e.mean. Significant differences between the four drugs were found for the CSF to blood and brain to blood concentration-ratios (ANOVA, $P < 0.05$).

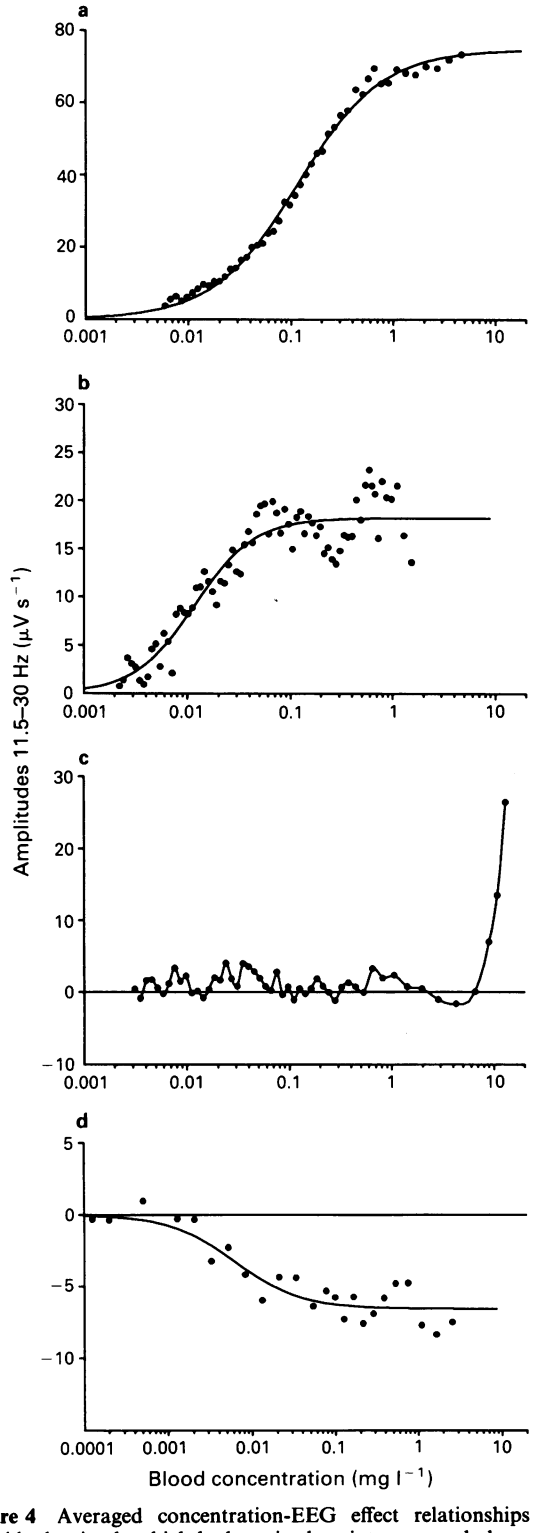


Figure 4 Averaged concentration-EEG effect relationships of all individual animals which had received an intravenous bolus administration of (a) 5 mg kg^{-1} midazolam, (b) 2.5 mg kg^{-1} bretazenil, (c) 10 mg kg^{-1} flumazenil or (d) 2.5 mg kg^{-1} Ro 19-4603. The sigmoidal E_{max} model was fitted to the concentration-effect data of midazolam, bretazenil and Ro 19-4603. Note the different scales on the y-axis.

were not statistically significantly different from each other. However, the values were significantly lower than the corresponding value for midazolam.

The receptor binding parameters of [^{3}H]-flumazenil as determined in the saturation experiment at 37°C were K_d = 7.1 ± 1.5 ng ml^{-1} and B_{max} = 0.545 ± 0.025 ng mg^{-1} protein (Mandema *et al.*, 1991b). For all benzodiazepines evaluated in the displacement studies the addition of a slope factor N in equation 3 did not improve the 'goodness of fit', indicating the

Table 3 Pharmacodynamic parameter estimates of the benzodiazepines

	Midazolam	*Bretazenil*	*Ro 19-4603*
Number of animals	7	7	7
E_{max} (μVs^{-1})	73 ± 2	19 ± 1	−6.5 ± 0.4
N	1.5 ± 0.2	2.0 ± 0.3	1.1 ± 0.4
EC_{50} (ng ml^{-1})	85 ± 8	12 ± 3	6.2 ± 1.9
$EC_{50,CSF}$ (ng ml^{-1})	4.3 ± 0.8	1.4 ± 0.4	1.7 ± 0.5
K_i[a] (ng ml^{-1})	4.9 ± 0.5	1.2 ± 0.2	1.5 ± 0.5

E_{max}, maximal effect; N, sigmoidicity of concentration-effect relationship; EC_{50}, blood concentration producing half-maximal effect; $EC_{50,CSF}$, CSF concentration producing half-maximal effect; K_i, concentration producing 50% receptor occupancy. The values are presented as means ± s.e.mean.
[a] The K_i values were determined based on displacement of [^{3}H]-flumazenil *in vitro* at 37°C.

presence of only one binding site. This assumption is the basis of the Cheng-Prusoff equation used to derive the K_i values from the IC_{50} values. The validity of the used models is further exemplified by the fact that the K_i value (6.8 ± 1.3 ng ml^{-1}) of flumazenil determined in the displacement studies was identical to the K_d value calculated from the saturation data. In Table 3 the receptor affinity, presented by their K_i values, is given for the three compounds exhibiting an intrinsic effect. For all three benzodiazepines the CSF concentration required to produce 50% of the maximal EEG effect is in the same range as the K_i values.

Discussion

The characterization of relevant relationships between the pharmacokinetics and pharmacodynamics of benzodiazepines requires the availability of appropriate measures of drug effects. Recently, quantitative EEG measures were shown to characterize successfully the concentration-EEG effect relationships of various benzodiazepine agonists (Koopmans *et al.*, 1988; Greenblatt *et al.*, 1989; Breimer *et al.*, 1990; Mandema *et al.*, 1991b,c). The EEG effect measures appear to fulfil many of the characteristics of ideal pharmacodynamic measures, being continuous, objective, sensitive and reproducible (Dingemanse *et al.*, 1988a). However, the pharmacological relevance of EEG parameters with respect to the various CNS effects of benzodiazepines remains to be established. The anxiolytic, anticonvulsant, muscle relaxant and sedative-hypnotic actions of benzodiazepines result from the interaction of these compounds with the GABA-benzodiazepine receptor complex and their ability to modulate GABA-induced inhibitory actions (Möhler & Okada, 1978; Haefely *et al.*, 1985; Haefely, 1989). The modulation of GABAergic activity by benzodiazepines is quite unique because of the fact that compounds have been discovered that cover the whole spectrum of intrinsic efficacy ranging from full agonists, enhancing GABAergic activity, to inverse agonists, which reduce the effects of GABA. This continuum also includes partial agonists, antagonists and partial inverse agonists (Braestrup *et al.*, 1983; Gardner, 1988; Haefely, 1988; 1989). In the present investigation the concentration-EEG effect relationships of four benzodiazepine ligands with large differences in their intrinsic efficacy at the GABA-benzodiazepine receptor complex were characterized.

The benzodiazepines studied were selected on the basis of their known differences in intrinsic efficacy. Midazolam is an extensively studied benzodiazepine ligand, which exhibits the pharmacological profile of a full agonist (Pieri *et al.*, 1981). Flumazenil was shown to antagonize selectively the central effects of benzodiazepines without possessing any intrinsic effects of its own (Hunkeler *et al.*, 1981; Polc *et al.*, 1981; Bonetti *et al.*, 1982). The partial agonistic activity of bretazenil was shown in a variety of tests (Haefely, 1984; Martin *et al.*,

1988; Potier *et al.*, 1988; Haefely, 1988; Yakushiji *et al.*, 1989). Interestingly, this drug was found to possess potent anxiolytic and anticonvulsant properties, but much less sedative and motor impairment activity (Martin *et al.*, 1988; Haefely, 1988; Haefely *et al.*, 1990). Ro 19-4603 was found to be a potent inverse agonist exhibiting a specific high affinity binding to the central benzodiazepine receptor comparable to that of bretazenil and was shown to possess intrinsic anxiogenic and proconvulsant properties (Pieri, 1988; Belzung *et al.*, 1990).

The benzodiazepine-induced changes in amplitudes in the 11.5–30 Hz (beta) frequency band were used as EEG effect measure. In a previous study a close correlation was found between the free drug concentrations of four benzodiazepine agonists required to produce 50% of their maximal EEG effect and the concentrations required to produce 50% receptor occupancy, as determind in an *in vitro* displacement study at 37°C (Mandema *et al.*, 1991b). In the present study it was clearly shown that differences in intrinsic efficacy of the benzodiazepines are also adequately reflected in the EEG effect parameter (Figures 3 and 4; Table 3). The concentration-EEG effect relationships of midazolam, bretazenil and Ro 19-4603 were successfully quantified by the sigmoidal E_{max} model and to our knowledge this is the first study that established these relationships for a partial agonist and inverse agonist *in vivo*. The concentration-EEG effect relationship of the inverse agonist could not be adequately quantified by the sigmoidal E_{max} model in the individual animals. This is probably due to the relatively small effects of this drug with respect to the normal fluctuations in the EEG effect measure independent of drug concentrations. The pharmacodynamic parameters of midazolam and bretazenil derived from the averaged concentration-EEG effect relationships did not differ from the parameters presented in Table 3. The averaged maximal effect observed for the partial agonist bretazenil was $19\,\mu V\,s^{-1}$ and substantially less than the maximal effect of midazolam (Table 3). Studies on the relative intrinsic efficacy of bretazenil with respect to full agonists are relatively scarce. However, the maximal increase of the convulsant threshold dose of pentylenetetrazol by bretazenil is about one quarter of that achieved with the full agonist diazepam (Haefely, 1990), which is similar to the ratio found in this study between the maximal effects of bretazenil and midazolam. In a previous study a close agreement between the pharmacodynamic parameters (EC_{50} and relative E_{max}) obtained in the EEG model and pentylenetetrazol convulsant threshold model was also found (Mandema *et al.*, 1991b). The inverse agonist Ro 19-4603 produced a decrease in the EEG effect measure with a maximal effect of $-6.5\,\mu V\,s^{-1}$. This decrease is relatively small, but significantly different from baseline. That only a small decrease was produced by Ro 19-4603 may be caused by the fact that the EEG effects were measured under controlled vigilance conditions. Under these conditions, the additional vigilance enhancing effects of the inverse agonist may only be small. This is in accordance with the observations made by Ongini *et al.* (1983), who showed that the inverse agonist FG 7142 increased the state of alertness and elicited EEG changes (decrease in beta power) in cats especially when given during periods of drowsiness.

The EC_{50} values should be corrected for differences in protein binding in order to be able to compare the relative potencies of these drugs (Mandema *et al.*, 1991b). Plasma protein binding of flumazenil cannot be determined due to the instability of this compound in plasma at 37°C (Mandema *et al.*, 1991a). As an alternative, CSF concentrations were used as representative for the free drug concentration. Since benzodiazepines were shown to equilibrate rapidly between blood and CSF (Arendt *et al.*, 1983), the ratios determined 30 min after the intravenous administration may be considered similar to the steady-state values. The CSF to plasma ratio of midazolam was 0.041 ± 0.007 and similar to the fraction unbound in plasma of 0.037 ± 0.005 as determined in a previous study (Mandema *et al.*, 1991b), indicating that the CSF concentrations are more or less equal to the free drug concentrations in plasma. Large differences were found between the CSF to blood ratio of the four drugs. The EC_{50} values of midazolam, bretazenil and Ro 19-4603 based on CSF concentrations are in the same order of magnitude as the concentrations required to produce 50% receptor occupancy (K_i) and the same rank order of potency was found based upon the two measures (Table 3). No close correlation seemed to exist when total brain concentrations were used to correct the EC_{50} values.

Flumazenil produced a significant increase in the EEG effect measure at relatively high concentrations ($>8\,mg\,l^{-1}$). Since the original reports that flumazenil had no intrinsic efficacy (Hunkeler *et al.*, 1981; Polc *et al.*, 1981; Bonetti *et al.*, 1982), various studies have appeared in the literature claiming that flumazenil might possess some partial agonist and even inverse agonist properties (for reviews see File & Pellow, 1986; Brodgen & Goa, 1988), of which the partial agonist properties were mostly observed after administration of high doses. However, considering the high affinity of flumazenil for the central benzodiazepine receptor ($K_i = 6.8 \pm 1.3\,ng\,ml^{-1}$) and high CSF to plasma ratio, it is unlikely that the EEG effects produced by this drug are the result of the interaction of this drug with this receptor. In view of the results obtained for the other three compounds, any intrinsic effects of flumazenil due to interaction with the high affinity benzodiazepine receptor must occur in the concentration range when receptor saturation takes place. However, in the blood concentration range of 100 to $1000\,ng\,ml^{-1}$ (approximately 90 to 99% receptor occupation) the average effect of flumazenil was $0.7 \pm 0.3\,\mu V\,s^{-1}$ and not significantly different from zero. The mechanism of the increase produced by flumazenil is thus unclear. However, benzodiazepines have been shown to interact with several other neurotransmitter systems, especially at high doses (Morgan *et al.*, 1983; Haefely *et al.*, 1985).

Large differences between the pharmacokinetic parameters were observed (Table 1). Flumazenil was rapidly eliminated from the rat body with a mean residence time of only 9.7 min due to a large clearance value and a small volume of distribution. The mean residence time of Ro 19-4603 was somewhat longer due to the lower clearance value of this drug. The disappearance of bretazenil from the rat body was considerably slower with a mean residence time of 48 min, which can be explained by the lower clearance value and larger volume of distribution found for this drug when compared to the other four compounds (Table 1). Flumazenil was found to be highly instable in fresh rat blood kept *in vitro*, probably due to aspecific esterases present in rat blood (Mandema *et al.*, 1991a). However, bretazenil and Ro 19-4603 were found to be stable when kept in fresh rat blood *in vitro* at 37°C for 4 h, which is most likely due to the change of the ethyl-ester group of flumazenil to a t-butyl-ester group. This lower sensitivity to esterases may also explain the lower clearance values found for these drugs, although little is known about the metabolic pathway of these three compounds in rats.

In conclusion, the concentration-EEG effect relationships of the benzodiazepine agonist midazolam, partial agonist bretazenil, antagonist flumazenil and inverse agonist Ro 19-4603 were quantified. The differences in intrinsic efficacy of these compounds were reflected in the EEG effect parameter (change in amplitudes in the 11.5–30 Hz (beta) frequency band). Furthermore, a close correlation was found between the free drug EC_{50} values and receptor affinity. These results suggest that the change in amplitudes in the 11.5–30 (beta) Hz frequency band is a realistic measure of the CNS effects of benzodiazepines, reflecting their interaction with and intrinsic efficacy at the central GABA-benzodiazepine receptor complex.

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Endogenous nitric oxide and sensory neuropeptides interact in the modulation of the rat gastric microcirculation

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1 The effects of depletion of sensory neuropeptides from primary afferent neurones by capsaicin pretreatment, on the changes in resting gastric mucosal blood flow following administration of inhibitors of nitric oxide biosynthesis have been investigated in the pentobarbitone-anaesthetized rat.

2 Bolus administration of the NO-synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; 0.8–12.5 mg kg^{-1} i.v.), induced a dose-dependent increase in systemic arterial blood pressure (BP) and a reduction in resting mucosal blood flow, as determined by laser Doppler flowmetry.

3 Concurrent administration of L-arginine (300 mg kg^{-1} i.v.) attenuated the effects of L-NAME (6.25 mg kg^{-1}) on resting mucosal blood flow and BP. The enantiomer, D-NAME (50 mg kg^{-1} i.v.), which does not inhibit NO biosynthesis, had no effect on either parameter.

4 The fall in mucosal blood flow induced by submaximal doses of L-NAME (0.8–3.2 mg kg^{-1}) was substantially augmented in rats pretreated 2 weeks earlier with capsaicin.

5 The fall in resting mucosal blood flow induced by the less potent NO-synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA; 1.6–25 mg kg^{-1} i.v.) was likewise significantly augmented in capsaicin-pretreated rats.

6 Pretreatment (15 min) with indomethacin (5 mg kg^{-1} i.v.) did not augment further the microvascular actions of L-NAME or L-NMMA in capsaicin-pretreated rats, suggesting the lack of interaction of endogenous prostanoids with these other mediators in regulating local blood flow. The effects of L-NAME on BP were not altered by capsaicin and indomethacin administration.

7 These findings indicate that endogenous sensory neuropeptides and NO can interact in the regulation of the gastric microcirculation.

Keywords: Nitric oxide; sensory neuropeptides; gastric mucosal blood flow; primary afferent neurones; capsaicin; N^G-nitro-L-arginine methyl ester

Introduction

The endothelium-derived vasodilator, nitric oxide (Palmer *et al.*, 1987; Khan & Furchgott, 1987; Ignarro *et al.*, 1987), formed from L-arginine (Palmer *et al.*, 1988a,b), plays an important role in the regulation of the resting gastric mucosal microcirculation (Pique *et al.*, 1989). Furthermore, endogenous nitric oxide (NO) interacts with sensory neuropeptides released from primary afferent neurones, in the modulation of the gastric mucosal integrity (Whittle *et al.*, 1990). Thus, in rats pretreated with capsaicin two weeks before the study to deplete sensory neuropeptides, acute administration of the inhibitor of NO biosynthesis N^G-monomethyl-L-arginine (L-NMMA; Palmer *et al.*, 1988b; Rees *et al.*, 1989) induced extensive necrotic damage to the gastric mucosa (Whittle *et al.*, 1990).

Since the gastric microcirculation has a crucial role in the maintainance of mucosal integrity, the actions and interactions of local vasoactive mediators in the microvasculature are of great significance. Hence, in the present study the interactions between endogenous NO and sensory neuropeptides in the regulation of gastric mucosal blood, measured by laser Doppler flowmetry, have now been explored. Thus, the effects of N^G-nitro-L-arginine as the methyl ester (L-NAME), a potent inhibitor of NO biosynthesis (Palacious *et al.*, 1989; Ishii *et al.*, 1990; Moore *et al.*, 1990; Mülsch & Busse, 1990; Rees *et al.*, 1990), on gastric mucosal blood flow have now been evaluated in both control and capsaicin-pretreated rats and compared to those of L-NMMA. In addition, by using indomethacin to inhibit mucosal cyclo-oxygenase (Whittle & Vane, 1987), the interaction of endogenous prostanoids with these local mediators in the gastric microcirculation has also been investigated.

Methods

Measurement of gastric mucosal blood flow

Male Wistar rat (230–260 g body weight) were deprived of food but not water for 18–20 h before the experiment. Animals were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.) and the stomach exposed by a midline incision. A small bore (8.5 mm o.d.) plastic cannula was then inserted via a small incision in the forestomach and tied in place, to allow free access to the gastric lumen. Gastric blood flow was recorded continuously with a laser Doppler blood flow monitor (Model MBF3D, Moor Instruments Ltd, Devon) as described before (Tepperman & Whittle, 1991).

The principle of laser Doppler flowmetry for assessment of gastric blood flow has been described previously in detail (Kiel *et al.*, 1985; Holm-Rutili & Berglindh, 1988). A stainless steel laser optic probe (1.65 mm o.d.; Moor Instruments) was inserted into the gastric lumen via the plastic cannula and was allowed to rest gently on the gastric corpus mucosa. Changes in laser Doppler flow (LDF) were assessed in response to intravenous bolus injection (1 ml kg^{-1}) of isotonic saline or the compound under investigation. Average LDF values were determined for the 3 min control period just before the administration of L-NAME. Similarly the average LDF was calculated for a 3 min period, 10–15 min after L-NAME (0.8–12.5 mg kg^{-1} i.v.) or in some further experiments, L-NMMA (1.6–25 mg kg^{-1} i.v.), when values of LDF had stabilized. In some experiments, indomethacin (5 mg kg^{-1} i.v.) in a dose inhibiting mucosal prostanoid biosynthesis by >90% (Whittle *et al.*, 1990) was injected 15 min before the administration of L-NAME, or L-NMMA.

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The mean systemic arterial blood pressure (BP) was measured from a cannula inserted into a carotid artery and connected to a pressure transducer (Bell & Howell, Ashford, U.K.) and a chart recorder (Grass model 7D polygraph). Changes in BP from resting levels are shown as ΔBP (mmHg).

Capsaicin-pretreatment

Adult rats (190–210 g) were treated with increasing doses of capsaicin for three consecutive days (20, 30 and 50 mg kg^{-1}, s.c.) to deplete neuropeptides in primary afferent neurones as described previously (Esplugues *et al.*, 1989). All capsaicin injections were made under halothane anaesthesia and, to counteract any respiratory impairment associated with administration of capsaicin, the rats were pretreated with terbutaline (0.1 mg kg^{-1}, i.m.) and aminophylline (10 mg kg^{-1}, i.m.) before capsaicin injection. The animals were used 2 weeks after completion of the capsaicin treatment. Control animals received a similar regimen of treatment with the vehicle alone.

Materials

N^{G}-nitro-L-arginine methyl ester (Sigma Chemical Co., Poole, U.K.) and its D-enantiomer (Bachem AG, Bubendorf, Switzerland) was dissolved in isotonic saline immediately before use. L-Arginine hydrochloride (Sigma Chemical Co) was dissolved freshly in isotonic saline. N^{G}-monomethyl-L-arginine acetate, synthesized in the Department of Medicinal Chemistry, Wellcome Research Laboratories by Dr H. Hodson, was freshly dissolved in isotonic saline before use.

Capsaicin (Fluka Chemic AG, Buchs, Switzerland) was prepared in a 50 mg ml^{-1} solution containing absolute ethanol, Tween 80 and isotonic saline (10:10:80 v/v/v). Terbutaline sulphate was obtained in an injectable form from Astra Pharmaceuticals, Kings Langley and aminophylline (Sigma Chemical Co.) was dissolved in isotonic saline. Indomethacin (Sigma Chemical Co.) was dissolved in sodium bicarbonate solution (5% v/v) and diluted 1:4 with distilled water to obtain an isotonic solution for intravenous administration.

Statistical analysis

All data are expressed as mean ± s.e.mean. Comparisons between groups were made by Student's *t* test for paired or unpaired data as appropriate, where *P* values of less than 0.05 were taken as significant.

Results

Effect of L-NAME on gastric mucosal blood flow

In the control series of studies, bolus intravenous injection of isotonic saline had no significant action on resting LDF ($n = 4$). Bolus intravenous administration of L-NAME (0.8–12.5 mg kg^{-1}) induced a dose-dependent and significant fall in resting LDF, reaching plateau levels within 15 min, with a maximal reduction of 49 ± 2% of basal ($n = 7$) being achieved with the highest dose (Figure 1). In contrast, the enantiomer D-NAME, in doses up to 50 mg kg^{-1} i.v. had no significant effect on LDF, as shown in Figure 2.

The effects of L-NAME (6.25 mg kg^{-1} i.v.) on LDF were inhibited (by 94 ± 5%, $n = 5$; $P < 0.001$) by pretreatment (5 min) with L-arginine (300 mg kg^{-1} i.v.). This dose of L-arginine itself had no significant effect on resting LDF (Figure 2).

Effects of capsaicin pretreatment

The effects of the threshold dose of L-NAME (0.8 mg kg^{-1} i.v.) on resting LDF were significantly augmented in rats that had been pretreated with capsaicin two weeks earlier, as shown in Figure 1. Furthermore, the actions of L-NAME (1.6 and 3.2 mg kg^{-1} i.v.) on LDF were likewise substantially enhanced

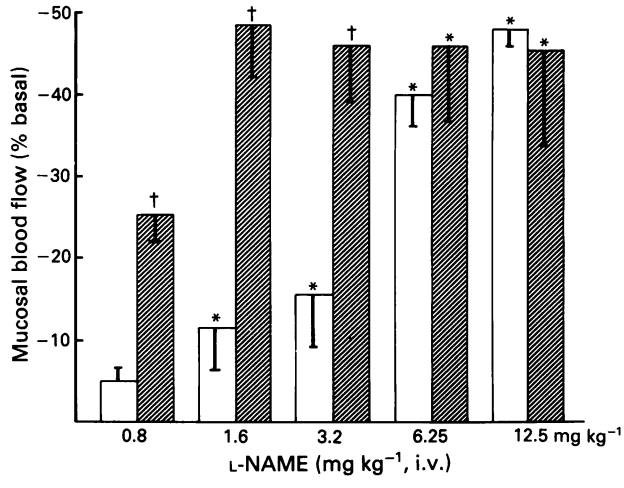


Figure 1 Effect of capsaicin-pretreatment, 2 weeks before the study, on the actions of N^{G}-nitro-L-arginine methyl ester (L-NAME, 0.8–12.5 mg kg^{-1} i.v.) on resting laser Doppler flow (LDF) as an index of gastric mucosal blood flow in the anaesthetized rat. Results, shown as the fall in LDF (% of basal) following L-NAME in control (open columns) and capsaicin-pretreated (hatched columns) rats, are the mean of 5–7 experiments for each, vertical bars show s.e.mean. Significant difference from resting LDF is shown as $*P < 0.05$ and from effects in control rats as $\dagger P < 0.05$.

(Figure 1). The effects of near-maximal doses of L-NAME (6.25–12.5 mg kg^{-1}) were not, however, significantly potentiated in capsaicin-pretreated rats (Figure 1).

In further studies, the effects seen with L-NAME in capsaicin-pretreated rats, were compared to those of L-NMMA. The reduction in resting LDF by a threshold dose of L-NMMA (1.6 mg kg^{-1} i.v.) in control rats was significantly greater in capsaicin-pretreated rats (−0.4 ± 0.2% and −34 ± 7% of basal respectively, $n = 5$ for each; $P < 0.01$). Likewise, the reduction in resting LDF by L-NMMA (3.2 mg kg^{-1} i.v.) was significantly augmented by capsaicin pretreatment (Figure 3).

Effects on systemic arterial blood pressure

Intravenous administration of L-NAME (0.8–12.5 mg kg^{-1}) induced dose-dependent increases in resting BP (resting value,

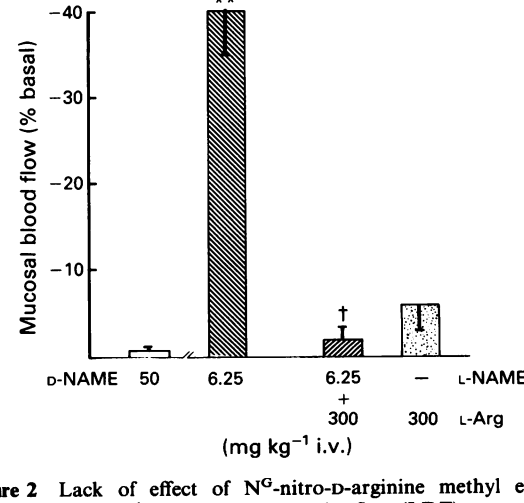


Figure 2 Lack of effect of N^{G}-nitro-D-arginine methyl ester (D-NAME, 50 mg kg^{-1} i.v.) on laser Doppler flow (LDF) as an index of gastric mucosal blood flow and the reversal of the effects of L-NAME (6.25 mg kg^{-1} i.v.) by prior (5 min) administration of L-arginine (L-Arg, 300 mg kg^{-1} i.v.) in a dose itself not affecting resting LDF. Results, shown as change in LDF (% of basal), are the mean of 5–7 experiments in each group, vertical bars show s.e.mean. Significant difference from resting values of LDF are shown as $**P < 0.01$ and inhibition of the effects of L-NAME as $\dagger P < 0.01$.

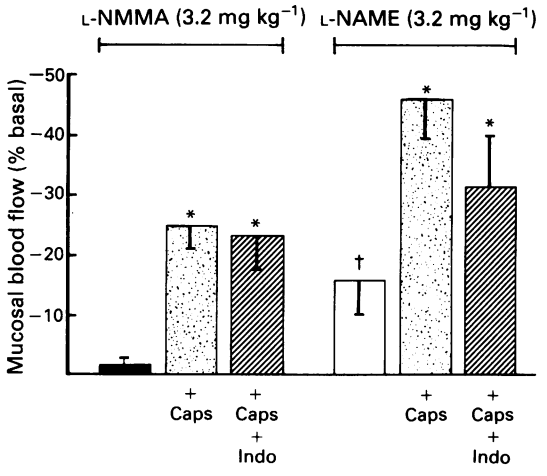


Figure 3 Effect of pretreatment (15 min) with indomethacin (Indo, 5 mg kg^{-1} i.v.), on the fall in laser Doppler flow (LDF), as an index of mucosal blood flow, induced by N^G-nitro-L-arginine methyl ester (L-NAME, 3.2 mg kg^{-1} i.v.) and N^G-monomethyl-L-arginine (L-NMMA, 3.2 mg kg^{-1} i.v.) in capsaicin-pretreated rats. Results, shown as change in LDF (% of basal) are the mean of 5–7 experiments in each group, vertical bars show s.e.mean. Significant difference from resting values is shown by $\dagger P < 0.05$ and significant increase by capsaicin administration is shown by $*P < 0.05$; there was no further significant change induced by indomethacin.

110 ± 5 mmHg) as shown in Table 1, reaching plateau values within 10 min, and which were well maintained for at least 60 min. The hypertensive effects of L-NAME (6.25 mg kg^{-1} i.v.) were substantially attenuated (from Δ33 ± 4 to Δ12 ± 2 mmHg, $n = 5$; $P < 0.05$) by prior administration (5 min) of L-arginine (300 mg kg^{-1} i.v.). This dose of L-arginine itself had no significant effect on BP (Δ3 ± 1 mmHg, $n = 4$). Intravenous administration of D-NAME (50 mg kg^{-1}) had no significant effect on BP (Δ1 ± 1 mmHg, $n = 3$).

Capsaicin-pretreatment had no significant action on the value of resting BP (115 ± 5 mmHg, $n = 8$). Furthermore, capsaicin-pretreatment had no significant effect on the hypertensive actions of L-NAME (0.8–12.5 mg kg^{-1} i.v.), as shown in Table 1.

Effects of indomethacin

In a further series of studies, pretreatment (15 min) with indomethacin (5 mg kg^{-1} i.v.) had no significant effect on the fall in LDF induced by L-NAME (3.2 mg kg^{-1}) or L-NMMA (3.2 mg kg^{-1}) in capsaicin-pretreated rats (Figure 3). Furthermore, the fall in LDF induced by a near-maximal dose L-NAME following capsaicin pretreatment (6.25 mg kg^{-1}) was not significantly altered by administration of indomethacin (−40 ± 4 and −38 ± 9% of basal respectively, $n = 5$ for each). Likewise, the augmented fall in LDF observed with a higher dose of L-NMMA (25 mg kg^{-1} i.v.) induced by capsaicin pretreatment (from −25 ± 8% to −43 ± 6% of basal, $n = 5$, $P < 0.01$) was not significantly increased by indomethacin administration (−36 ± 10% of basal, $n = 4$). Indomethacin alone had no significant effect on resting LDF (−4 ± 2% of basal; $n = 5$).

Indomethacin administration had no significant effect on the hypertensive actions of L-NAME (0.8–12.5 mg kg^{-1}) in capsaicin-pretreated rats (Table 1).

Discussion

The present finding that the potent NO-synthase inhibitor, L-NAME substantially reduces resting laser Doppler flow (LDF) in the rat gastric mucosa, confirms a role of endogenous NO in the regulation of gastric mucosal blood flow. Furthermore, evidence has now been obtained for an interaction of NO with endogenous sensory neuropeptides from capsaicin-sensitive afferent neurones, in the modulation of the gastric microcirculation.

In previous studies, a reduction in resting gastric blood flow has been demonstrated following bolus intravenous administration of L-NMMA, assessed by both hydrogen gas clearance and radiolabelled microsphere techniques in the rat (Pique *et al.*, 1989; 1991; Pizcueta *et al.*, 1991), as also confirmed in the present study by use of laser Doppler flowmetry. Other workers, however, have failed to demonstrate a fall in resting LDF in the rat gastric mucosa following administration of L-NMMA or L-NAME, although this may reflect the overall dose given by infusion (Walder *et al.*, 1990). A reduction in pentagastrin-stimulated hyperaemia by L-NMMA and L-NAME was observed in that study (Walder *et al.*, 1990), as also seen with hydrogen gas clearance techniques following bolus administration of L-NMMA in doses having no direct action on acid secretion (Pique *et al.*, 1991). It was also demonstrated that mucosal blood flow under resting conditions was more resistant to reduction by L-NMMA than the elevated mucosal blood flow during secretory stimulation, which may reflect the relative contribution of NO to the local vascular tone under these different conditions (Walder *et al.*, 1990; Pique *et al.*, 1991).

Capsaicin pretreatment two weeks before the study, to deplete sensory neuropeptides from primary afferent neurones (Sternini *et al.*, 1987; Holzer, 1988; 1991; Green & Dockray, 1988), substantially augmented the effects of L-NAME on mucosal blood flow. This was particularly apparent with the threshold and lower doses of L-NAME, where the fall in mucosal blood flow was increased to an extent comparable to those obtained with maximal doses in non-capsaicin pretreated rats. The reduction in LDF induced by L-NMMA, which was less potent than L-NAME, was likewise augmented in capsaicin-pretreated rats. The response to the maximal dose of L-NAME was not enhanced further by capsaicin pretreatment, which probably reflects the maximal degree of indirect vasoconstriction that could be achieved in this microvascular bed under resting conditions. It could also possibly indicate that the NO synthase inhibitors and capsaicin pretreatment are

Table 1 Effect of capsaicin pretreatment and indomethacin on the hypertensive actions of N^G-nitro-L-arginine methyl ester (L-NAME)

	ΔBP (mmHg)		
L-NAME (mg kg^{-1})	*L-NAME*	*L-NAME + Caps*	*L-NAME + Caps + Indo*
0.8	9 ± 2*	7 ± 2*	5 ± 2*
1.6	13 ± 3*	11 ± 3*	11 ± 4*
3.2	18 ± 5*	18 ± 5*	18 ± 5*
6.25	33 ± 4**	31 ± 5**	33 ± 9*
12.5	43 ± 5**	41 ± 7**	42 ± 9**

Capsaicin (Caps) was administered 2 weeks before the study, and indomethacin (Indo, 5 mg kg^{-1} i.v.) was administered 15 min before L-NAME (0.8–12.5 mg kg^{-1} i.v.) in the anaesthetized rat. Results, shown as the increase in systemic arterial blood pressure (ΔBP; mmHg) are the mean ± s.e.mean of 5–7 experiments for each dose level. Statistically significant increase in BP from resting values is shown as $*P < 0.05$, $**P < 0.01$; there was no significant effect of capsaicin pretreatment alone or with indomethacin administration on the responses to L-NAME.

acting through a final common mechanism that reduces NO release. Thus, sensory neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) are considered as endothelium-dependent vasodilators (Furchgott, 1983; Brain *et al.*, 1985), whose systemic vasodepressor actions may be attenuated by NO synthase inhibition (Whittle *et al.*, 1989; Whittle, 1990). Inhibition of NO formation would therefore be expected to reduce their local microvascular actions in the mucosa. However, capsaicin administration would act more profoundly to eliminate the local actions of these neuropeptides, while the release of NO is likely to be under a complex regulation by several local mediators.

The effects of capsaicin pretreatment itself on resting mucosal blood flow could not be evaluated in the present investigation because of the relative, rather than absolute values of blood flow changes determined by LDF measurement. However, previous studies using hydrogen gas clearance have suggested that depletion of sensory neuropeptides by capsaicin does not influence the absolute levels of resting mucosal blood flow (Pique *et al.*, 1990). Although CGRP, the predominant sensory neuropeptide located in rat gastric tissue, can induce vasodilatation in the mucosa (Holzer & Guth, 1991) and can attenuate the injurious actions of the vasoconstrictor endothelin-1 (Whittle & Lopez-Belmonte, 1991), such sensory neuropeptides alone do not appear to play a major role in the modulation of the resting microvascular blood flow. Thus, the augmented reduction of LDF would not appear to simply reflect the summation of the removal of the influence of these two types of mediators on resting vasodilator tone, and suggests that such a microvascular role for the sensory neuropeptides under basal conditions may only operate under conditions of reduced NO availability.

The reduction in resting LDF induced by submaximal doses of L-NAME was reversed by pretreatment with L-arginine, confirming that its vasoconstrictor actions in the microcirculation were as a consequence of the inhibition of NO biosynthesis from this precursor amino acid. The enantiomer D-NAME did not affect LDF, while as also observed by others, bolus administration of L-NAME but not D-NAME induced a dose-dependent increase in rat systemic BP, which was attenuated by the administration of L-arginine (Gardiner *et al.*, 1990; Rees *et al.*, 1990). In contrast to its effects in the gastric microcirculation, the hypertensive actions of L-NAME were not significantly altered in capsaicin-pretreated rats, indicating that the interactions between endogenous NO and sensory neuropeptides in the gastric microcirculation are not generalized effects in all vascular territories. It will be of interest, therefore, to explore in detail such interactions with NO in those vascular beds in which sensory neuropeptides are thought to play a significant role in modulating blood flow.

An interaction between NO and sensory neuropeptides in the regulation of gastric mucosal integrity has previously been observed, with L-NMMA inducing acute mucosal injury in capsaicin-pretreated rats (Whittle *et al.*, 1990). Since microvascular blood flow is a critical determinant of mucosal integrity, it is possible that the augmented changes in mucosal blood flow induced by administration of NO inhibitors following capsaicin-pretreatment, as observed in the present study, contributes to such damage. Indomethacin pretreatment substantially augmented this mucosal injury in capsaicin-pretreated rats (Whittle *et al.*, 1990), yet in the present study had no effect on the enhanced changes in LDF induced by L-NMMA or L-NAME. These findings could suggest that endogenous prostanoids do not interact with NO and sensory neuropeptides in the modulation of resting mucosal blood flow, yet do influence the tissue susceptibility to local ischaemia. Such actions may reflect protective actions of prostanoids, possibly prostacyclin, on the microvascular endothelium. Since there is evidence that NO can reduce gastro-intestinal vascular damage in endotoxic shock (Hutcheson *et al.*, 1990; Boughton-Smith *et al.*, 1990), it is feasible that NO and the sensory neuropeptides can also interact to promote microvascular integrity in the gastric mucosa under physiological conditions, by mechanisms additional to an enhancement of local blood flow.

The rat gastric mucosa possess high levels of the constitutive NO-synthase (Whittle *et al.*, 1991), but whether this calcium- and NADPH-dependent enzymic activity is solely located in the microvascular endothelium awaits studies on its mucosal cell distribution. The capsaicin-sensitive primary afferent neurones that store and release sensory neuropeptides have been demonstrated in close proximity to the submucosal microvasculature (Ekblad *et al.*, 1985), the vessels that regulate mucosal blood flow, and thus are appropriately located to influence the microcirculation. It is also pertinent that using immunohistochemical techniques, NO synthase has been localized in neuronal cell bodies and nerve fibres within the myenteric plexus of the rat duodenum (Bredt *et al.*, 1990). Furthermore, neuronally-derived NO has been proposed as a mediator of the non-cholinergic non-adrenergic relaxation on the rat stomach musculature following electrical stimulation (Li & Rand, 1990; Boeckxstaens *et al.*, 1991) and the reflex relaxation of guinea-pig stomach following distension (Desai *et al.*, 1991). It is therefore possible that the interactions between endogenous sensory neuropeptides and NO observed in the present study in the gastric mucosa may involve actions on local neuromodulator processes that regulate the mucosal microvasculature.

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Interference of cetirizine with the late eosinophil accumulation induced by either PAF or compound 48/80

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1 The effect of topical or systemic treatment with the histamine H_1-receptor antagonist, cetirizine, on the rat pleural eosinophil accumulation induced by PAF or compound 48/80 was investigated. The number of pleural resident eosinophils increased 6 h after the intrathoracic (i.t.) injection of PAF (1 μg/cavity), peaked within 24 h and persisted significantly augmented for at least 96 h. Compound 48/80 (25 μg/cavity) also produced a long lasting pleural eosinophilia but this was first noted only 24 h after stimulation.

2 Intraperitoneal pretreatment with cetirizine inhibited eosinophilia induced by either PAF (ED_{50} = 19 mg kg^{-1}) or compound 48/80 (ED_{50} = 14 mg kg^{-1}) whereas meclizine, another histamine H_1-receptor antagonist, was inactive.

3 Administered locally, cetirizine (5 and 15 μg/cavity) also dose-dependently inhibited both PAF- and compound 48/80-induced eosinophilia, providing evidence that its inhibitory effect is not due to any action upon circulating eosinophils. Consistent with this result, incubation of isolated peritoneal eosinophils with cetirizine failed to modify *in vitro* eosinophil migration caused by PAF.

4 Since the late eosinophilia induced by PAF may depend on the synthesis of a transferable protein mediator, cetirizine was administered to donor or recipient rats in order to determine whether it interferes with the generation or with the expression of this protein. We showed that only the treatment of recipient rats abolished the transfer of the eosinophilotactic activity, indicating that cetirizine does not modify the generation but inhibits the expression of this activity.

5 Our findings indicate that cetirizine is able to inhibit eosinophil accumulation by acting locally. The mechanism is neither related to its recognized ability to antagonize histamine H_1-receptors nor to a direct action upon circulating eosinophils.

Keywords: Cetirizine; PAF; compound 48/80; eosinophil recruitment; rat pleurisy

Introduction

Cetirizine {2-(4-[(4-chlorophenyl)phenylmethyl]-1-piperazinyl)ethoxy} acetic acid dihydrochloride is a novel histamine H_1-receptor antagonist which abrogates tissue eosinophil accumulation induced by either allergen (Fadel *et al.*, 1987; Michel *et al.*, 1988; Charlesworth *et al.*, 1989), compound 48/80 (Fadel *et al.*, 1987) or platelet activating factor (PAF) (Fadel *et al.*, 1990). Cetirizine was also reported to be effective against the chemotaxis of human eosinophils *in vitro* (De Vos *et al.*, 1989), which was not confirmed when guinea-pig eosinophils (R. Hidi, unpublished) were used *in vitro* as target cells. Since eosinophils are claimed to play a critical role in allergy and asthma (De Monchy *et al.*, 1985; Gleich & Adolphson, 1986), the anti-eosinophilotactic activity of cetirizine can be used as a tool, particularly since it is not shared by the histamine H_1-receptor antagonist, promethazine (Eidinger *et al.*, 1964).

To evaluate the mode of action of cetirizine on the inhibition of eosinophil tissue infiltration, the mechanism(s) of the eosinophil recruitment into inflammatory sites must be considered. We recently demonstrated that the intrathoracic injection of PAF (Martins *et al.*, 1989a; Silva *et al.*, 1989) or compound 48/80 (Martins *et al.*, 1990) induces a late pleural eosinophilia in rats. In both cases, dexamethasone or the protein synthesis inhibitors cycloheximide and actinomycin D suppressed eosinophilia, whereas anti-histamine drugs and the dual cyclo- and lipoxygenase inhibitor BW 755C were inactive (Silva *et al.*, 1989; 1991). Furthermore, the transfer of the 6 h PAF pleural washing from donor to recipient rats led to a selective eosinophilia (Silva *et al.*, 1989), by material newly generated in the pleural cavity of the donor rat, which is unrelated to PAF itself and is destroyed by heating (+100°C) or freezing (−20°C) (Silva *et al.*, 1990; 1991).

In the present study, we demonstrate that the topical administration of cetirizine prevents pleural eosinophilia in PAF- or compound 48/80-injected rats suggesting a local target, particularly since cetirizine failed to inhibit the *in vitro* eosinophil migration induced by the lipid. Additional evidence is given that cetirizine may not act directly upon eosinophils to abrogate the pleural eosinophilia.

Methods

Animals and pleurisy procedure

Male Wistar rats (150–200 g) bred in the Oswaldo Cruz Foundation Animal Department were used. They were anaesthetized with ether and injected intrathoracically with PAF (1 μg/cavity) or with compound 48/80 (25 μg/cavity) in a final volume of 100 μl. The agonists were injected through a 27.5-gauge needle about 3 mm in length. Sterile 0.9% NaCl solution (saline) was injected in the control groups. The animals were killed by terminal ether anaesthesia 24 h after the pleural stimulation, after which the thoracic cavity was rinsed with 3 ml of saline containing heparin (20 u ml^{-1}). In some experiments, the animals were killed at different times ranging from 1 to 120 h after stimulation.

Eosinophil counts

Cytospin preparations of pleural washing were stained with May-Grunwald-Giemsa for differential analysis, which was

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performed under an oil immersion objective. Total leucocyte counts were performed in Neubauer chambers by means of optical microscopy after dilution of the pleural fluid with Türk solution. Counts are shown as total of eosinophils ($\times 10^6$) per cavity.

Eosinophil chemotaxis in vitro

The *in vitro* migration experiments were carried out in a 48-well microchemotaxis chamber (Neuro Probe, Inc., U.S.A.) and with Toyo cellulose nitrate filters (3 μm pore), as previously described (Martins *et al.*, 1989b). Briefly, eosinophils from the rat peritoneal cavity were purified (80 to 90% purity) in a discontinuous metrizamide gradient and then resuspended in RPMI medium, pH 7.2 prepared with RPMI 1640, 30 mM HEPES buffer, 2 mg ml^{-1} sodium bicarbonate and 4 mg ml^{-1} ovalbumin. To test the potential interference of cetirizine with the eosinophil chemotaxis induced by 10^{-8} and 10^{-6} M PAF, cells were pre-incubated with 10^{-6} to 10^{-4} M cetirizine, or its vehicle, for 10 min at 37°C and transferred to the upper chemotaxis chamber. Chambers were incubated for 2 h at 37°C in a 5% CO_2:95% O_2 atmosphere. Then, the filters were fixed and stained as previously published (Richards & McCullough, 1984). The results are given as number of eosinophils present in 15 consecutive high-power fields on the immersion objective (100×) 40 μm from the upper filter surface.

Pleural washing transfer

Control and cetirizine-treated donors were killed 6 h after the intrathoracic (i.t.) injection of PAF (1 μg/cavity) and the pleural cavity washed with 0.5 ml of saline. The pleural washing (PPW) from PAF-injected donors was centrifuged (2500 r.p.m.) for 15 min at 4°C and the supernatant (200 μl) injected into the pleural cavity of recipient animals, as previously described (Silva *et al.*, 1989). The pleural eosinophil accumulation was evaluated in recipient animals 24 h after the transfer. Some recipient rats pretreated with cetirizine (30 mg kg^{-1}, i.p.), 1 h before, were injected with PPW from control donors. In other experiments, control rats were killed 6 h after i.t. injection of saline, and the pleural wash (SPW) injected into untreated recipient animals and eosinophil accumulation was evaluated 24 h after transfer.

Protocols for drug treatment

Cetirizine (5 to 30 mg kg^{-1}) or meclizine (40 mg kg^{-1}) were injected i.p. 1 h before the intrathoracic injection of the agonists. In another set of experiments, cetirizine (5 and 15 μg/cavity) was injected intrathoracically 5 min before PAF or compound 48/80, in a final volume of 100 μl. Control animals were treated with saline only.

Treatment with PAF receptor antagonists

BN 52021 and WEB 2086 (20 mg kg^{-1}) were administered i.p. 1 h before the intrathoracic injection of compound 48/80 (25 μg/cavity). Control animals were treated with saline only. The pleural eosinophilia was evaluated 24 h after administration of the agonist.

Materials

Cetirizine 2HCl was from UCB, Braine l'Alleud, Belgium. It was dissolved in saline and neutralized with 0.1 N sodium hydroxide. PAF(1-O-hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) was purchased from Bachem (Switzerland) and dissolved in saline-BSA 0.01%; compound 48/80, metrizamide and HEPES buffer were purchased from Sigma (U.S.A.); meclizine was from Pfizer (Brazil); BN 52021 (3-(1,1-dimethylethyl)-hexahydro-1,4,7b-trihydroxy-8- methyl-9H-1,7α (epoxymethano)-1H,6αH-cyclopenta (c)furo(2,3-b)furo(3',2':3, 4) cyclopenta(1,2-d)furan-5, 9,12(4H)-trione) was kindly provided by Dr P. Braquet, Institute Henri Beaufour (France); and WEB 2086 (3-(4-(2-chlorophenyl)-9- methyl-6H-thieno 2,2-f-(1,2,4)triazolo(4,3-a) (1,4)-diazepin-2-yl-1- (4-morpholinyl) -1-propanone) was kindly provided by Dr H. Heuer, Boehringer-Ingelheim (Federal Republic of Germany). All solutions were freshly prepared immediately before use.

Statistical analysis

The data were analysed statistically by microcomputer programme using analysis of variance (ANOVA) followed by Newman-Keuls Student's test. P values of 0.05 or less were considered significant. Results are presented as means with s.e.means.

Results

Time-course of the eosinophil accumulation caused by PAF and compound 48/80

As shown in Figure 1, the i.t. injection of PAF (1 μg/cavity) or compound 48/80 (25 μg/cavity) led to a significant long-lasting rat pleural eosinophil accumulation (PAF: $Fx,y = 16.9$, $DF_g = 13$, $DF_r = 68$, $P < 0.001$; compound 48/80: $Fx,y = 9.3$, $DF_g = 11$, $DF_r = 63$, $P < 0.001$). The effect was first noted 6 h after PAF (Figure 1a) and 24 h after compound 48/80 (Figure 1B) persisting, in both cases, for at least 96 h post-stimulation.

WEB 2086 (20 mg kg^{-1}) significantly reduced the PAF (1 μg/cavity)-induced eosinophil accumulation after 24 h

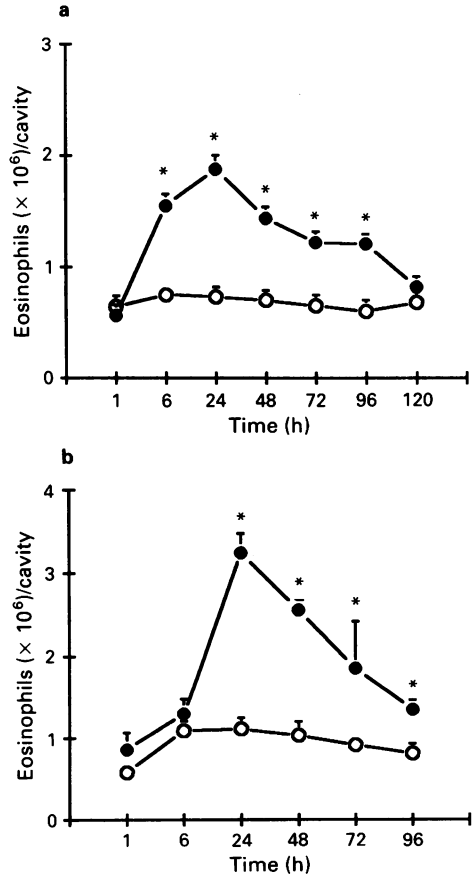


Figure 1 Time course of the pleural eosinophil accumulation induced by either PAF (1 μg/cavity) (a) or compound 48/80 (25 μg/cavity) (b). Each point indicates the mean from at least 6 animals; vertical bars show s.e.mean; values from agonist-(●) and vehicle-(○) injected animals, respectively. Statistically significant differences are indicated by an asterisk.

(absence WEB 2086: 1.95 ± 0.20 ($\times 10^6$ cells/cavity), $n = 7$; presence WEB 2086: 1.11 ± 0.05 ($\times 10^6$ cells/cavity), $n = 7$ ($P < 0.001$)). Under control conditions the eosinophil count from rats injected i.t. with saline was 0.75 ± 0.04 ($\times 10^6$ cells/cavity), $n = 6$. In contrast, pretreatment with the PAF receptor antagonists WEB 2086 and BN 52021 (20 mg kg^{-1}) failed to interfere with the increase in the pleural eosinophil counts elicited by compound 48/80 (Figure 2).

Interference of cetirizine with the eosinophilia by PAF and compound 48/80

Intraperitoneal pretreatment with cetirizine (5 to 30 mg kg^{-1}) 1 h before .stimulation with either PAF (Fx,y = 16.2, $P < 0.001$, Figure 3a) or compound 48/80 (Fx,y = 12.2, $P < 0.001$, Figure 3b) resulted in a significant inhibition of the pleural eosinophil accumulation. The analysis was performed 24 h after the agonists, showing ED_{50} values of 19 and 14 mg kg^{-1} for PAF and compound 48/80, respectively. In contrast, the histamine H_1-receptor antagonist, meclizine (also a piperazine derivative) (40 mg kg^{-1}) failed to modify the eosinophilia induced by PAF or compound 48/80 (Table 1).

As illustrated in Figure 4, topical (intrathoracic treatment with low concentrations of cetirizine (5 and 15 μg/cavity), 5 min before the agonists, also reduced dose-dependently the pleural eosinophilia induced by either PAF (Fx,y = 6.3, $P < 0.001$, Figure 4a) or compound 48/80 (Fx,y = 10.7, $P < 0.001$, Figure 4b) after 24 h.

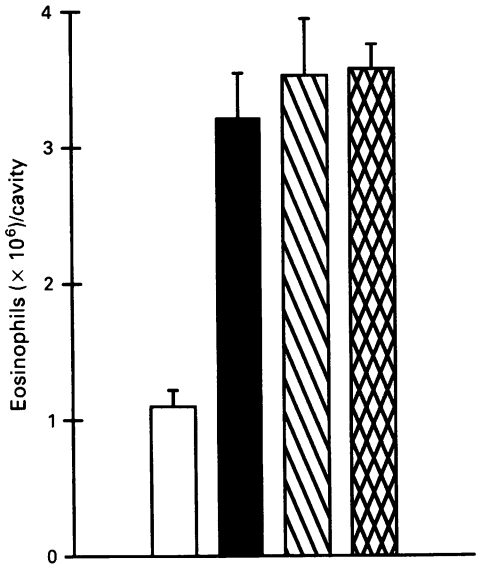


Figure 2 Lack of effect of PAF antagonists on the pleural eosinophil accumulation induced by compound 48/80 (25 μg/cavity); open and closed columns represent vehicle ($n = 8$) and compound 48/80-injected ($n = 7$) rats, respectively; hatched and cross-hatched columns represent compound 48/80-injected animals, which had received WEB 2086 ($n = 8$) and BN 52021, ($n = 4$) respectively. The values are expressed as the mean with s.e.mean shown by vertical bars.

Table 1 Interference of meclizine with the 24 h pleural eosinophilia caused by PAF (1 μg/cavity) or compound 48/80 (25 μg/cavity)

	Eosinophils × 10^6/*cavity*	
Agonist	*Untreated*	*Treated*
None	0.59 ± 0.10	
PAF	1.25 ± 0.09*	1.17 ± 0.13
Comp. 48/80	2.60 ± 0.11*	2.23 ± 0.26

Meclizine (40 mg kg^{-1}) was administered intraperitoneally 1 h before the agonists. The values are expressed as mean ± s.e.mean from at least 6 animals. $P < 0.05$ as compared to untreated non-stimulated group

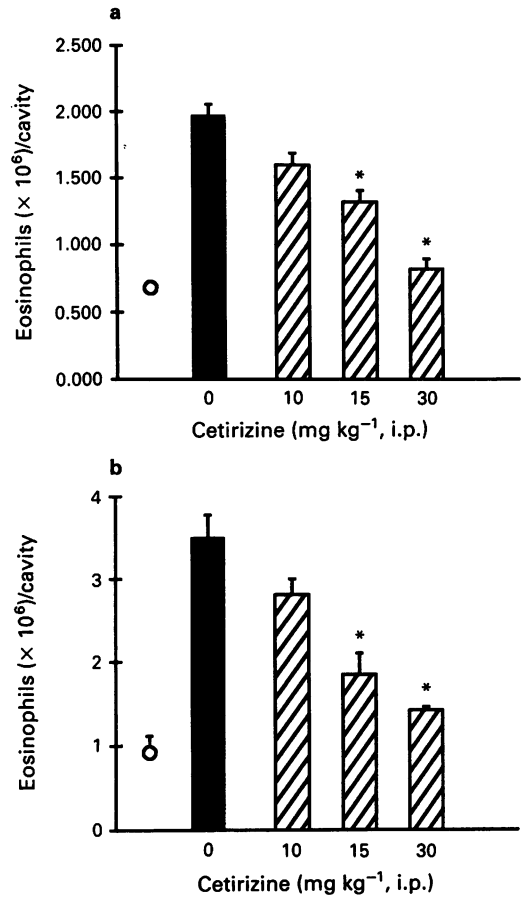


Figure 3 Effect of the intraperitoneal treatment with cetirizine (10 to 30 mg kg^{-1}) on the pleural eosinophil accumulation caused by PAF (a) or compound 48/80 (b). Columns represent the mean (with s.e.mean shown by vertical bars) from at least 6 animals. Circles and columns represent saline- and agonist-injected rats, respectively. Statistically significant differences are indicated by an asterisk.

Failure of cetirizine to block the in vitro *eosinophil chemotaxis by PAF*

The *in vitro* migration experiments were carried out in a 48-well microchemotaxis chamber, as previously described

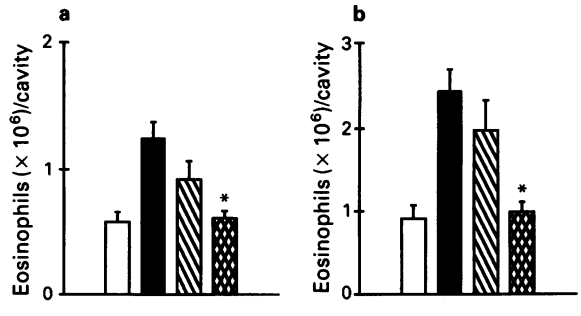


Figure 4 Effect of the intrathoracic treatment with low concentrations of cetirizine on the pleural eosinophil accumulation induced by PAF (a) or compound 48/80 (b). Open and closed columns represent saline- and agonist-injected animals, respectively; hatched and cross-hatched columns represent agonist-injected rats, which had received cetirizine 5 and 15 μg/cavity, respectively. The values are expressed as the mean (s.e.mean shown by vertical bars) from at least 7 animals. Statistically significant differences are indicated by an asterisk.

Table 2 Interference of cetirizine with the eosinophil chemotaxis *in vitro* induced by PAF

	Number of eosinophils per 15 fields		
Cetirizine (M)	*Vehicle)*	*PAF* 10^{-8} (M)	*PAF* 10^{-6} (M)
0	41 ± 4 (9)	111 ± 6 (13)*	180 ± 9 (12)*
10^{-6}	40 ± 5 (6)	122 ± 9 (7)	180 ± 22 (6)
10^{-5}	41 ± 6 (6)	123 ± 12 (7)	187 ± 11 (7)
10^{-4}	46 ± 6 (5)	117 ± 8 (10)	169 ± 11 (10)

Cells were pre-incubated for 10 min at 37°C with cetirizine in doses ranging from 10^{-6} to 10^{-4} M. Each value represents the mean ± s.e.mean from a number of independent experiments indicated by the figures in parentheses. * $P < 0.05$ as compared to vehicle-stimulated group.

(Martins *et al.*, 1989b). As shown in Table 2, previous coincubation of rat peritoneal eosinophils with cetirizine for 10 min at 37°C, in doses ranging from 10^{-6} to 10^{-4} M, failed to inhibit chemotaxis by PAF (10^{-8} and 10^{-6} M).

Interference of cetirizine with the transferable eosinophilotactic activity induced by PAF

As illustrated in Figure 5 and previously described (Silva *et al.*, 1989; 1991), the i.t. injection of PAF generates a marked and selective eosinophilotactic activity in the rat pleural fluid, which can be expressed in recipient animals. The pretreatment of recipients with cetirizine (30 mg kg^{-1}) significantly inhibited the pleural eosinophil accumulation detected 24 h after the transfer of the PAF pleural fluid. In contrast, when donors instead of recipients were treated, the transfer was maintained (Figure 5).

Discussion

The present findings confirm that the i.t. injection of PAF or compound 48/80 into the rat pleural cavity is followed by a late and long-lasting pleural eosinophilia, as previously

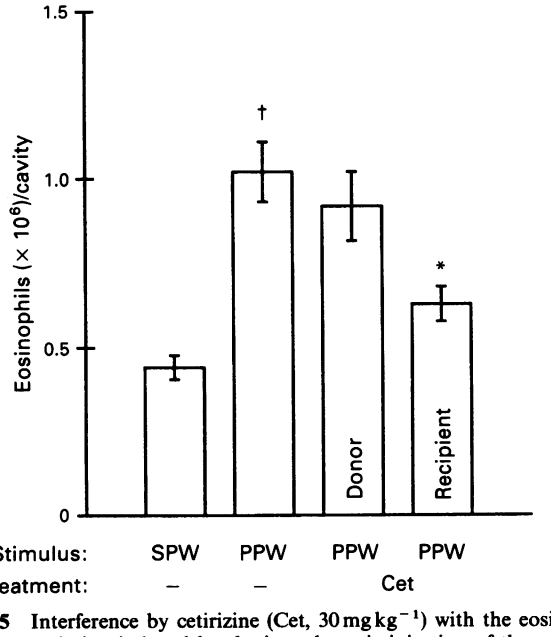


Figure 5 Interference by cetirizine (Cet, 30 mg kg^{-1}) with the eosinophil accumulation induced by the intrathoracic injection of the cell-free pleural fluid (PPW/200 µl) from PAF-injected rats. In controls, PPW was replaced by the cell-free pleural fluid from saline-injected rats (SPW/200 µl). As indicated, the drug was administered to either donor or recipient rats 1 h before the pleural stimulation. The values are expressed as the mean (with s.e.mean shown by vertical bars) from at least 7 animals. † $P < 0.05$ from the SSW group by Newman-Keuls-Student's test. * $P < 0.05$ from the cetirizine-untreated PPW group by Newman-Keuls-Student's test.

reported (Martins *et al.*, 1989a; 1990; Silva *et al.*, 1989). The increase in the eosinophil counts was noted within 6 h of PAF administration, peaked within 24 h and returned to the control basal levels only 120 h post-stimulation. In the case of compound 48/80, eosinophilia was first noted within 24 h and persisted significant for at least 96 h. We have shown that the i.p. administration of cetirizine dose-dependently inhibits the late pleural eosinophil accumulation induced by either PAF or compound 48/80, with ED_{50} values of 19 and 14 mg kg^{-1}, respectively. However, eosinophilia was not modified when the histamine H_1-receptor antagonist, meclizine, another piperazine derivative, was used for comparison. Taken together, these findings clearly indicate that the anti-eosinophilotactic activity of cetirizine is not accounted for by its ability to antagonize histamine H_1 receptors.

Previous studies have demonstrated that cetirizine inhibits the eosinophil infiltration during the cutaneous late-phase response induced in man by allergen (Fadel *et al.*, 1987; Michel *et al.*, 1988; Charlesworth *et al.*, 1989), compound 48/80 (Fadel *et al.*, 1987) or PAF (Fadel *et al.*, 1990). According to De Vos *et al.* (1989) cetirizine was able to abolish the human eosinophil chemotaxis elicited by either PAF or N-formyl-Met-Leu-Phe *in vitro*, suggesting that it might be acting directly upon eosinophils to impair the recruitment of these cells into the inflammatory focus. Nevertheless, this finding was not confirmed when guinea-pig eosinophils were used as target cells (R. Hidi, unpublished). Using eosinophils isolated from the rat peritoneal cavity, we also studied the potential interference of cetirizine with the *in vitro* eosinophil migration triggered by PAF. In contrast to what was observed by De Vos *et al.* (1989), our results show that the incubation of eosinophils and cetirizine fails to affect the *in vitro* eosinophil chemotaxis, under conditions where PAF receptor antagonists were clearly effective (Martins *et al.*, 1989b). This apparent discrepancy between the *in vitro* results with those obtained in man, on one hand, and rat and guinea-pig eosinophils, on the other, might involve species differences. Nevertheless, in our conditions, suppression of pleural eosinophilia by cetirizine should rather involve a mechanism other than its direct effect on eosinophils, since its i.t. injection, at doses from 5 to 15 µg/cavity, reduced the pleural eosinophilia caused by PAF or compound 48/80. These amounts are below the effective systemic doses; indeed, 15 mg kg^{-1} of cetirizine would be equivalent to approximately 3 mg/site, a more than 200 fold dose difference.

PAF is one of the most potent eosinophil chemoattractants in different experimental systems (Wardlaw *et al.*, 1986; Lellouch-Toubiana *et al.*, 1988; Martins *et al.*, 1989a). In spite of this, there is no certainty whether endogenous PAF is involved with tissue eosinophilia induced by other stimuli. Since cetirizine inhibited the pleural eosinophilia-induced by PAF, it might interfere with the effects of compound 48/80 by antagonizing its receptors. This hypothesis was not supported by our data, since specific PAF antagonists failed to modify the rise in eosinophil counts due to compound 48/80, under conditions where they were effective against PAF itself (Martins *et al.*, 1989c; Silva *et al.*, 1990; 1991).

A marked common feature in the rat pleural eosinophilia induced by either PAF or compound 48/80 is the great sensitivity to the local treatment with protein synthesis inhibitors (Silva *et al.*, 1989; Martins *et al.*, 1990). In fact, we have recently proposed that, in spite of being recognized as a potent eosinophil chemotaxin *in vitro* (Wardlaw *et al.*, 1986; Martins *et al.*, 1989b), PAF induces pleural eosinophilia in rats most probably by the secondary release of a soluble eosinophilotactic protein, which has been detected in the cell-free pleural fluid (Silva *et al.*, 1989; 1991). As described, the transfer of the pleural fluid supernatant from donor to recipient rats induces in the latter a dose-dependent and selective pleural eosinophilia, which was shown to be refractory to PAF antagonists, protein synthesis inhibitors and dexamethasone (Silva *et al.*, 1990; 1991). To understand the mode of action of cetirizine better, the transfer assay was performed

here under conditions where donor or recipient rats were treated. Treatment of recipients, but not of donors, inhibited the late eosinophilia due to the transfer of the PAF pleural washing, indicating that the expression of the eosinophilotactic activity, but not its generation, is prevented by cetirizine.

In conclusion, our findings demonstrate that the antihistaminic cetirizine inhibits both PAF and compound 48/80-induced pleural eosinophilia by a mechanism independent of histamine receptor blockade. Eosinophil chemotaxis *in vitro* was not impaired by cetirizine and it appears that cetirizine operates locally to suppress the effect, but not the generation of eosinophilotactic factors. We suggest that this drug prevents pleural eosinophil accumulation by affecting either the process of eosinophil anchorage and/or diapedesis or the potential local growth and differentiation of eosinophil progenitors. Further studies are now underway to clarify these points.

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Changes in plasma catecholamine and neuropeptide Y levels after sympathetic activation in dogs

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1 Plasma levels of noradrenaline (NA) and neuropeptide Y (NPY) were evaluated in two experimental models associated with an increase in sympathetic tone: conscious dogs which were subject to either sinoaortic denervation or acute administration of the α_2-adrenoceptor antagonist yohimbine.

2 Dogs that had undergone sinoaortic denervation exhibited a two fold increase in plasma NA without any change in NPY levels.

3 Yohimbine (0.05 mg kg^{-1} i.v. as a bolus) produced similar effects. A higher dose of yohimbine (0.5 mg kg^{-1} i.v.) increased both plasma NA (7 fold) and NPY (6.5 fold) levels.

4 The present results indicate that changes in plasma catecholamines and NPY are not always concomitant. They suggest that the simultaneous release of NA and NPY is only observed under *in vivo* conditions for a marked increase in sympathetic tone.

Keywords: Catecholamines; noradrenaline; neuropeptide Y; sympathetic nervous system; yohimbine; sinoaortic denervation

Introduction

Neuropeptide Y (NPY) is a 36-amino acid residue peptide which was originally isolated from the porcine brain. It possesses potent vasoconstrictor activity and is co-stored with noradrenaline (NA) in peripheral perivascular sympathetic nerves and the chromaffin cells of the adrenal medulla (see Lundberg *et al.*, 1989; 1990 for reviews).

Activation of the sympathetic nervous system has been shown to be associated *in vitro* with an increase in NPY release (Lundberg *et al.*, 1990). Cardiovascular diseases, such as acute myocardial infarction, angina pectoris and left heart failure are associated with sympathetic activation and elevated concentrations of circulating NPY (Lundberg *et al.*, 1990; Hulting *et al.*, 1990). However, the co-release of NPY and catecholamines has been poorly investigated in intact subjects.

Thus, the aim of the present study was to investigate whether an enhancement of sympathetic tone (reflected by plasma catecholamine levels) is necessarily associated with an increase in plasma NPY levels. Two different experimental models were selected: conscious dogs subject to sinoaortic denervation, a model associated with a marked increase in sympathetic tone (Damase-Michel *et al.*, 1987; Valet *et al.*, 1989a, b); and conscious dogs treated with yohimbine, a drug known to activate the sympathetic nervous system (Valet *et al.*, 1989c) through the blockade of α_2-adrenoceptors located on sympathetic nerve endings (Goldberg & Robertson, 1983).

Methods

Sinoaortic denervation

Six mongrel dogs of either sex weighing 16 to 24 kg were studied: sinoaortic denervation was performed as previously described (Damase-Michel *et al.*, 1987; Valet *et al.*, 1989a, b). Briefly, sinoaortic denervation involved two successive surgical procedures under α-chloralose (80 mg kg^{-1}, i.v.) anaesthesia. First, the carotid sinus nerve at the carotid bifurcation and the aortic depressor nerve in the cervical region were cut on the right side. The same procedure was repeated on the left side 4 weeks later. The effectiveness of baroreceptor denervation was assessed by the failure of noradrenaline (2 μg kg^{-1}, i.v.) and phenylephrine (0.1, 1.0 and 10.0 μg kg^{-1}, i.v.) to induce bradycardia. Moreover, nitroglycerin (1, 3, 10 and 30 μg kg, i.v.) induced a dose-dependent decrease in blood pressure in these animals without any change in heart rate, thus confirming the absence of any baroreceptor reflex. Each dog was studied before sinoaortic denervation and 1 month after sinoaortic denervation.

Effect of yohimbine

These experiments were performed on 6 or more mongrel dogs, each animal receiving two doses of yohimbine. The two experiments were separated by at least one week. No sham injection of saline was performed since previous studies have indicated that this had no effect on the cardiovascular parameters or on plasma catecholamine levels (Damase-Michel *et al.*, 1987).

Yohimbine hydrochloride (Sigma, Paris, France) was chosen for its ability to increase plasma catecholamine levels. Two doses (0.05 and 0.5 mg kg, i.v.) were selected. The drug was injected as a bolus after a 30 min rest period and blood pressure, heart rate, plasma NA, adrenaline (Ad) and NPY concentrations were measured before, and 15 min after injection.

Blood pressure and heart rate measurements in conscious dogs

Several days before any experiment, dogs were trained to stand still for 2 or 3 h on a Pavlov table and to become accustomed to i.v. infusion, recording and blood samplings. Systolic and diastolic blood pressures were recorded by means of a catheter, introduced into the abdominal aorta via the left femoral artery under local anaesthesia (xylocaine, 5% Lab. Roger Bellon, Neuilly sur Seine, France), connected to a Gould P23ID transducer and a Honeywell recorder. The heart rate was obtained with a heart period meter triggered by blood pressure.

Blood sampling and catecholamine assays

Arterial blood was collected from the catheter previously introduced into the abdominal aorta to prevent any stress. It was collected on lithium heparin with sodium metabisulphite (10 mM), centrifuged at 2000 *g* for 10 min at 0°C and plasma

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was stored at −80°C. Catecholamines were isolated selectively from the sample at 0°C, in darkness, by adsorption to activated alumina, then eluted with 0.1 M acetic acid. Dihydroxybenzylamine was used as internal standard. NA and Ad were assayed by a Waters high-pressure liquid chromatography apparatus using electrochemical detection: the working electrode potential was set at 0.65 V against a Ag/AgCl reference electrode. Catecholamines were separated on a C18 column (3.9 × 150 mm) at a constant flow rate of 1 ml min^{-1}. The electrochemical detector response was linear for concentrations ranging from 10 pg ml^{-1} to 100 ng ml^{-1}. In these conditions, the detection limit is 10 pg ml^{-1} (Valet *et al.*, 1989a).

Radioimmunoassay of neuropeptide

Plasma concentrations of NPY were determined by a sensitive and specific radioimmunoassay (Peninsula, England) using a specific antiserum, which was raised in rabbit against porcine NPY. Duplicate 100 µl aliquots of plasma were obtained from the dog as indicated above. We used arterial plasma since Howe *et al.* (1986) found no change in venous plasma NPY content from SHR stroke-prone rats but an increase in arterial plasma NPY concentrations. Plasma samples were incubated with [^{125}I]-NPY and a specific rabbit antiserum. Bound antigen was separated by a second antibody method, and its radioactivity was measured in a gamma counter. The antiserum shows no cross-reactivity to structurally related peptides, such as peptide YY or pancreatic polypeptide (respectively less than 0.003% and 0.002%). The response was linear for concentrations ranging from 5 to 1280 pg/100 µl. The detection limit was 5 pg/100 µl.

Statistical analysis

All data are presented as mean values ± s.e.mean. Statistical analysis was performed with the Wilcoxon test for paired comparisons. The level of significance was $P < 0.05$.

Results

Dogs with sinoaortic denervation

Sinoaortic denervation significantly increased systolic blood pressure (+45%), diastolic blood pressure (+79%) and heart rate (+69%) (Table 1). Under our experimental conditions, these cardiovascular values remained stable since they did not vary more than 30 mmHg and 40 beats min^{-1} respectively during a 3 h recording session (Valet *et al.*, 1989a). One month after sinoaortic denervation, NA plasma levels significantly rose (+83%) without any significant change in Ad values. Plasma NPY levels measured before and 1 month after sinoaortic denervation did not vary significantly (Table 1). In fact, they increased by 40% but because of the size of the standard error of the mean, this was not statistically significant.

Yohimbine administration

Acute administration of 0.05 mg kg^{-1} i.v. yohimbine significantly increased systolic (+30%) (but not diastolic) blood pressure, heart rate (+26%) and plasma NA (+120%) (but not Ad) levels. Plasma NPY levels did not change. The higher dose of yohimbine (0.5 mg kg^{-1} i.v.) increased systolic (+29%) and diastolic (+29%) blood pressures and also increased plasma NA (+500%), Ad (+377%) and NPY (+570%) levels (Table 1).

Discussion

The present study was undertaken in order to investigate whether NPY is always co-released with catecholamines when sympathetic tone increases *in vivo*. In fact, conflicting results occur in the literature since some authors demonstrate that there is differential release of NPY and catecholamines *in vivo* and that high frequencies of stimulation are required for NPY release *in vivo* in animals (Allen *et al.*, 1984; Zukowska-Grojec *et al.*, 1988; Lundberg *et al.*, 1989) or in man (Morris *et al.*, 1986; Pernow *et al.*, 1988). In contrast, Castagné *et al.* (1987) found that NPY release closely parallels changes in circulating NA during stress in the rat, whereas Lundberg *et al.* (1985) suggested that NPY is co-released together with NA upon sympathetic activation during physical exercise in man. More recently, increased plasma NPY and NA levels were described in hypertensive patients (Solt *et al.*, 1990) and a positive relationship was found between plasma NA and NPY levels during myocardial ischaemia in man (Ullman *et al.*, 1990).

Plasma variations of catecholamines (especially NA) are known to reflect changes in the activity of the sympathetic nervous system (Hjemdahl, 1988). Thus, in the present paper, two experimental conditions characterized by an increase in sympathetic tone (sinoaortic denervation or acute administration of yohimbine) were investigated. The present results clearly indicate that changes in plasma catecholamines and NPY are not always concomitant. In fact, in this study, there was no relationship between plasma NA and NPY levels whatever the experimental model used.

In dogs subject to sinoaortic denervation, the rise in sympathetic tone is not associated with a significant increase in NPY levels. To explain these results, three hypotheses can be proposed. Firstly, sinoaortic denervation could elicit an insufficient stimulation of sympathetic tone (2 fold) to induce an increase in NPY release. These results can be related to the data from Lundberg *et al.* (1990): NA and NPY are only co-released upon high frequency stimulation whereas low average frequency released only NA. Secondly, it is possible that the persistent rise in sympathetic tone observed in sinoaortically denervated dogs is unable to induce an increased NPY release

Table 1 Effect of sinoaortic denervation (SAD) or yohimbine administration on systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate (HR), noradrenaline (NA), adrenaline (Ad) and neuropeptide Y (NPY) plasma levels in conscious dogs

		SBP (mmHg)	*DBP* (mmHg)	*HR* (beats min^{-1})	*NA* (pg ml^{-1})	*Ad* (pg ml^{-1})	*NPY* (pg 100 μl^{-1})
SAD dogs	Before	185 ± 9	81 ± 3	77 ± 5	498 ± 56	181 ± 34	17 ± 9
	1 Month after SAD	269 ± 13*	145 ± 13*	130 ± 10*	910 ± 91*	239 ± 48	24 ± 16
Yohimbine 0.05 mg kg^{-1}, i.v.	0 min	174 ± 9	60 ± 3	72 ± 4	275 ± 48	234 ± 24	24 ± 4
	15 min	227 ± 11*	76 ± 4	91 ± 5*	605 ± 61*	369 ± 66	30 ± 5
Yohimbine 0.5 mg kg^{-1}, i.v.	0 min	180 ± 9	75 ± 5	80 ± 4	325 ± 75	242 ± 51	20 ± 8
	15 min	233 ± 14*	97 ± 6*	114 ± 17	2214 ± 645*	1155 ± 454*	134 ± 46*

Statistical analysis was performed by the Wilcoxon test for paired comparisons. Mean values ± s.e.mean are given. $n = 6$ aminals in each experiment.
*$P < 0.05$ when compared with control values.

which was shown to be preferentially facilitated by intermittent bursts of stimulation (Lundberg *et al.*, 1989). Finally, one can also postulate that a long term rise in sympathetic tone could lead to a depletion of the NPY pool in sympathetic nerve endings.

The second part of the study used yohimbine, an α_2-adrenoceptor antagonist. As already mentioned, the drug clearly increased NA and Ad levels (Valet *et al.*, 1989c) through the blockade of central and/or presynaptic α_2-adrenoceptors (Goldberg & Robertson, 1983). The higher dose ($0.5\,mg\,kg^{-1}$) induced a marked (7 fold) increase in plasma NA values associated with similar enhancement in plasma NPY values (6.5 fold). This agrees with the levels of yohimbine-induced NPY released in the pithed pig (Dahlöf *et al.*, 1986) and with the fact that α_2-adrenoceptors also regulate NPY release from the sympathetic nerve endings (Lundberg *et al.*, 1990). However, it should be noted that this dose of yohimbine elicited a very marked (7 fold) increase in plasma NA levels above the values measured in sinoaortically denervated dogs. Thus, we selected a lower dose ($0.05\,mg\,kg^{-1}$) in order to induce a 2 fold increase in plasma NA, i.e. a similar rise to that observed in the dogs with sinoaortic denervation. Under these conditions, plasma NPY values did not change.

In conclusion, these results demonstrate that, in the intact conscious dog, an increase in sympathetic tone is not necessarily associated with a rise in NPY release. They suggest that the simultaneous release of NA and NPY is only observed under *in vivo* conditions for a marked increase in sympathetic tone. These data suggest the existence of a threshold level of a sympathetic stimulation beyond which NA and NPY are co-released.

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Effects of inhibiting nitric oxide biosynthesis on the systemic and splanchnic circulation of rats with portal hypertension

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1 The effects of inhibiting endogenous nitric oxide (NO) synthesis with N^G-monomethyl-L-arginine (L-NMMA) on the systemic and splanchnic circulation have been investigated in rats with experimental chronic portal hypertension, anaesthetized with ketamine.

2 Portal hypertension was induced by partial portal vein ligation, 2 weeks prior to study. This procedure induced a reduction in systemic arterial blood pressure (MAP), an increase in cardiac output as measured by radiolabelled microspheres, a reduction in peripheral and splanchnic vascular resistance and an increased portal venous inflow (PVI) and portal pressure, as compared to control non-ligated rats.

3 L-NMAA (6.25 and 50 mg kg^{-1}, i.v.) dose-dependently increased MAP, reduced cardiac output and PVI, and increased peripheral and splanchnic vascular resistance. With L-NMMA (50 mg kg^{-1}), PVI and the vascular resistances returned to values comparable to those determined in control non-ligated anaesthetized rats under resting conditions.

4 Porto-collateral resistance was also increased by these doses of L-NMMA, whereas portal pressure was unchanged. The increase in renal blood flow and decrease in renal vascular resistance also seen in portal-hypertensive rats was reversed by L-NMMA (50 mg kg^{-1}).

5 These effects of L-NMMA (50 mg kg^{-1}) were inhibited by prior administration of L-arginine (300 mg kg^{-1}, i.v.).

6 These findings indicate that the chronic hyperdynamic circulatory characteristics following portal vein stenosis can be attenuated by L-NMMA. Thus, the excessive formation of endogenous NO may be implicated in the pathogenesis of the haemodynamic disturbances and splanchnic vasodilatation associated with chronic portal hypertension.

Keywords: Portal hypertension; splanchnic circulation; nitric oxide; N^G-monomethyl-L-arginine (L-NMMA)

Introduction

Chronic portal hypertension is associated with a hyperdynamic circulation, characterized by increased blood flow and reduced vascular resistance in the splanchnic and systemic circulation (Vorobioff *et al.*, 1983; Kravetz *et al.*, 1986). Such a hyperdynamic circulation observed in patients (Lebrec *et al.*, 1983; Bosch *et al.*, 1988) is reproduced in experimental models of portal hypertension such as that induced by chronic partial portal vein stenosis in the rat (Vorobioff *et al.*, 1983; Blanchet & Lebrec, 1982; Benoit *et al.*, 1984; Kravetz *et al.*, 1986). The mechanisms underlying the splanchnic vasodilatation under such conditions may reflect the reduced sensitivity of the vascular tissue to vasoconstrictor mediators (Kiel *et al.*, 1985; Pizcueta *et al.*, 1990) or the release of vasodilator factors, such as glucagon (Benoit *et al.*, 1984; 1986; Kravetz *et al.*, 1988).

The biological activity of endothelium-derived relaxing factor, (EDRF, Furchgott & Zawadzki, 1980; Furchgott, 1984) can be attributed to endogenous nitric oxide (NO) formed by vascular endothelial cells (Palmer *et al.*, 1987; Ignarro *et al.*, 1987; Khan & Furchgott, 1987; Kelm *et al.*, 1988). The biosynthesis of NO from its substrate amino acid, L-arginine (Palmer *et al.*, 1988a) is inhibited by N^G-monomethyl-L-arginine (L-NMMA) in endothelial cells and vascular tissue *in vitro* (Palmer *et al.*, 1988b; Rees *et al.*, 1989a). Furthermore, studies with L-NMMA *in vivo* have implicated NO in the physiological regulation of systemic arterial blood pressure in the rabbit, rat and guinea-pig (Rees *et al.*, 1989b; Compton *et al.*, 1989; Whittle *et al.*, 1989; Aisaka *et al.*, 1989), of peripheral vascular tone in man (Vallance *et al.*, 1989a,b) and of blood flow in the rat gastric, mesenteric and renal vascular beds (Piqué *et al.*, 1989; Gardiner *et al.*, 1990; Walder *et al.*, 1991).

While a reduction in endogenous NO biosynthesis may be involved in the pathogenesis of some forms of hypertension (Moncada *et al.*, 1991), excessive formation of NO may contribute to cardiovascular disease conditions involving reduced vascular resistance. In the present study therefore, the possible contribution of NO to the altered systemic and splanchnic circulation of the chronic portal hypertensive rat induced by partial portal vein ligation has been investigated by use of L-NMMA.

Methods

Animal preparation

The study was performed in male Sprague-Dawley rats, in which portal hypertension was induced by the ligation of the portal vein, as previously described in detail (Chojkier & Groszmann, 1981; Kravetz *et al.*, 1988). In brief, rats were anaesthetized with ketamine (100 mg kg^{-1}, i.m.), the portal vein isolated and a stenosis created by a single ligature of 3-0 silk placed around both the portal vein and 20 gauge blunt tipped needle. The needle was then removed from the ligature, thus leaving a calibrated constriction of the portal vein. The rats were allowed to recover and had free access to water and food until the day of the study, 2 weeks later, when the portal hypertension syndrome had fully developed.

The portal hypertensive animals were divided in 4 groups: a vehicle control group that received an intravenous bolus injection of saline (1 ml; $n = 9$; body weight 352 ± 18 g); a second group that received L-NMMA at a dose of 6.25 mg kg^{-1}, i.v. ($n = 9$; weight 372 ± 14 g); a third group that received L-NMMA at a dose of 50 mg kg^{-1}, i.v. ($n = 8$, weight 347 ± 12 g) and a fourth group in which L-arginine (300 mg kg^{-1}, i.v.; Sigma Chemical Company, Poole, Dorset, prepared as the acetate) dissolved in isotonic saline was

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administered 5 min before administration of L-NMMA (50 mg kg^{-1}, $n = 7$, weight 368 ± 15 g). These doses were chosen from preliminary dose-response studies on the effects of L-NMMA (1.25–100 mg kg^{-1}) on systemic arterial blood pressure (MAP) in portal hypertensive rats anaesthetized with ketamine, as those that gave submaximal and near maximal responses. L-NMMA (6.25 mg kg^{-1}) was the lowest dose that returned MAP to values comparable to those in control non-ligated animals.

In an additional study using control non-operated rats without portal ligation, one group received the vehicle, saline ($n = 8$, weight 368 ± 19 g) while in a further group, L-NMMA (50 mg kg^{-1}, i.v.; $n = 8$, weight 346 ± 14 g) was administered. L-NMMA as the acetate, prepared in the Dept. Medicinal Chemistry, Wellcome Research Labs, was dissolved freshly in isotonic saline before use.

Haemodynamic studies

The techniques used for the haemodynamic measurements have been described previously in detail (Groszmann *et al.*, 1982; Kravetz *et al.*, 1986; Pizcueta *et al.*, 1989). The rats were anaesthetized with ketamine HCl (100 mg kg^{-1}, i.m.). The left ventricle was catheterized via the right carotid artery with PE-50 tubing. Another PE-50 catheter was placed in the left femoral artery for arterial pressure measurements and for blood withdrawal. Through a 2-cm midline incision, the portal vein was catheterized via the ileocolic vein with tip chilled tubing. After verification that free backflow of blood was obtained, the catheter was fixed with cyanoacrylate glue and the abdomen wall was closed with silk sutures. This catheter was subsequently used for portal pressure measurements. Another PE-50 catheter was placed in the right atrium via the left external jugular vein and used for atrial pressure meaurement and drug infusion. All catheters were connected to pressure transducers, calibrated before each experiment, and blood pressures were registered on a multichannel recorder (Lectromed MT6-PX). The zero reference point was established 1 cm above the operating table. Rectal temperature was maintained at 37 ± 0.5°C throughout the study.

After obtaining baseline measurements of resting MAP, right atrial pressure and portal pressure, saline or L-NMMA (1 ml) was administered as a slow bolus injection through the catheter in the internal jugular vein. Ten minutes later, these pressures were measured again and cardiac output and regional blood flows were measured by a radioactive microsphere technique, as previously described (Kravetz *et al.*, 1986; Pizcueta *et al.*, 1989). A reference blood sample was obtained from the femoral artery catheter over a 75 s period at a rate of 1 ml min^{-1} using a continuous withdrawal pump. Approximately 5×10^4 microspheres labelled with ^{141}Ce (15 ± 3 μm diameter; specific activity: 10 mCi g^{-1}; New England Nuclear, Boston, MA, U.S.A.) were injected into the left ventricle, 15 s after beginning the blood withdrawal.

At the end of the experiments, the animals were injected intravenously with saturated KCl. The abdominal organs were dissected, blotted, weighed, cut into small pieces, and placed in counting tubes. The radioactivity (c.p.m.) of each organ was determined in a gamma-scintillation counter (Packard, 800c). The interference of Cr radioactivity (energy window: 240 to 400 keV) was corrected using Cr and Ce standards.

Cardiac output (ml min^{-1}) was calculated as follows:

$$\text{Injected radioactivity (c.p.m.)} \times \frac{\text{Reference blood flow (ml min}^{-1})}{\text{Reference blood radioactivity (c.p.m.)}}$$

Regional blood flows were calculated as:

$$\text{Organ blood flow (ml min}^{-1}) = \text{Organ radioactivity (c.p.m.)} \times \frac{\text{Reference blood flow (ml min}^{-1})}{\text{Reference blood radioactivity (c.p.m.)}}$$

Portal venous inflow (PVI), which represents the total blood flow entering the portal venous system, was calculated as the sum of blood flow to stomach, spleen, small and large intestines, pancreas and mesentery.

Resistance in each vascular bed was calculated from the ratio between perfusion pressure (P) and blood flow (Q) of each vascular territory. For the calculation of total peripheral vascular resistance, P was the value for mean arterial pressure minus the right atrial pressure and Q was the cardiac output, in the calculation of splanchnic vascular resistance, P was the value for mean arterial pressure minus the right atrial pressure and Q was the cardiac output; in the calculation of splanchnic vascular resistance, P was mean arterial pressure minus portal pressure and Q was the PVI. For the calculation of portocollateral resistance, P was portal pressure minus right atrial pressure and Q was the PVI.

Data analysis

All results are expressed as mean ± standard error of the mean. Student's *t* test for paired and non-paired data and the analysis of variance with contrasts were used in the statistical analysis of the results. Significance was taken at $P < 0.05$.

Results

Two weeks after ligation of the portal vein, the chronic portal hypertensive animals under resting conditions exhibited characteristic haemodynamic disturbances. Thus, there was a significantly ($P < 0.05$) decreased mean arterial pressure (MAP), increased cardiac output, and reduced total peripheral vascular resistance with splanchnic vasodilatation, as shown by lowered splanchnic vascular resistance as compared with anaesthetized control non-operated rats (Tables 1 and 2). Renal blood flow was also significantly increased with a

Table 1 Mean arterial pressure (MAP) and portal pressure (PP) under resting conditions and after the intravenous administration of N^G-monomethyl-L-arginine (L-NMMA, 50 mg kg^{-1}) or saline in control and chronic portal hypertensive rats; the effect of pretreatment with L-arginine (L-Arg; 300 mg kg^{-1}, i.v.) on the actions of L-NMMA in the portal ligated rats is also shown

	Control rats				*Portal hypertensive rats*				
	Resting	*Saline*	*Resting*	*L-NMMA*	*Resting*	*Saline*	*Resting*	*L-NMMA*	*L-NMMA + L-Arg*
MAP (mmHg)	129 ± 4	130 ± 41	121 ± 5	147 ± 4*	100 ± 5†	103 ± 5†	99 ± 6†	146 ± 7*	101 ± 5**
PP	6.3 ± 0.3	6.4 ± 0.3	7.3 ± 0.4	7.1 ± 0.4	11 ± 0.5†	11 ± 0.5†	11 ± 0.3†	12 ± 0.5†	11 ± 0.5†

Results, shown as MAP or PP (mmHg) under resting conditions or following saline or drug administration in control non-operated rats or following chronic portal vein ligation, are mean ± s.e.mean of 8–9 rats, where significant difference from corresponding resting values is given as * $P < 0.01$ and from the resting values in control rats as † $P < 0.05$. Inhibition of the effects of L-NMMA by L-arginine is shown as ** $P < 0.001$.

Table 2 Effects of saline or N^G monomethyl-L-arginine (L-NMMA, 50 mg kg^{-1}) on systemic and splanchnic haemodynamics in control and portal hypertensive rats: the effect of pretreatment with L-arginine (L-Arg; 300 mg kg^{-1}, i.v.) on the responses to L-NMMA in the portal ligated rats is also shown

	Control		*Portal hypertensive*		
	Vehicle ($n = 8$)	*L-NMMA* ($n = 8$)	*Vehicle* ($n = 9$)	*L-NMMA* ($n = 8$)	*L-NMMA + L-Arg* ($n = 7$)
Cardiac output (ml min^{-1} 100 g^{-1})	24.3 ± 2	23.6 ± 1.6	34.2 ± 2.9*	22.9 ± 2.4†	35.7 ± 3**
Peripheral vascular resistance (mmHg ml^{-1} min^{-1} 100 g^{-1})	5.5 ± 0.4	6.4 ± 0.6	3.2 ± 0.3*	6.2 ± 0.4†	3.4 ± 0.3*
Splanchnic vascular resistance (mmHg ml^{-1} min^{-1} 100 g^{-1})	36.2 ± 6	43.3 ± 4	14.6 ± 1.5*	29.7 ± 2.3†	18.7 ± 3.7**

Results, shown as the value of each parameter per 100 g body weight following intravenous administration of vehicle (saline) or L-NMMA or L-arginine and L-NMMA in control non-ligated or chronic portal hypertensive rats, are expressed as the mean ± s.e.mean of (n) studies. Significant difference from the corresponding control non-ligated group is shown as * $P < 0.05$ and from the vehicle (saline) group in the portal hypertensive rats as † $P < 0.05$. Significant inhibition by L-arginine of the actions of L-NMMA is shown as ** $P < 0.05$.

decrease in renal vascular resistance (Table 3). Portal venous inflow was likewise significantly elevated, as was portal pressure, while porto-collateral resistance was unchanged (Tables 1 and 3).

Effects of N^G-monomethyl-L-arginine in portal hypertensive rats

In portal hypertensive rats, intravenous administration of L-NMMA (6.25 and 50 mg kg^{-1}) induced a dose-dependent significant increase in MAP with an increase of 47 ± 7 mmHg ($n = 8$) at the highest dose (Figure 1). The change in MAP was observed within 1 min following administration of L-NMMA, reached its maximal values after 5–10 min, and was maintained for the duration of the study. The increase in MAP was accompanied by a decline in cardiac output, which was significant at the higher dose of L-NMMA (Figure 1).

L-NMMA (6.25 and 50 mg kg^{-1}) dose-dependently and significantly increased total peripheral vascular resistance and splanchnic vascular resistance (Figure 2). At the higher dose, L-NMMA caused a significant ($P < 0.05$) reduction in portal venous flow and increase in porto-collateral resistance, but there was no change in portal pressure induced by either dose of L-NMMA (Figure 3). In the renal circulation, L-NMMA (50 mg kg^{-1}) induced a significant decrease in renal blood flow and an increase in renal vascular resistance (Figure 4).

Comparison of the cardiovascular parameters following administration of the low dose of L-NMMA (6.25 mg kg^{-1}), indicated that MAP in these portal hypertensive rats was comparable to those in control animals under resting conditions (127 ± 3 and 129 ± 4 mmHg, respectively $P < 0.05$). Furthermore, following this dose of L-NMMA, cardiac output and total peripheral vascular resistance were likewise returned to values not significantly different from those in control animals under resting conditions.

Following administration of L-NMMA (50 mg kg^{-1}), the values for all the cardiovascular parameters measured in the portal hypertensive rats were not significantly different from those determined in control animals under resting conditions (Tables 2 and 3), except for MAP, porto-collateral resistance and portal pressure which were significantly higher (Tables 1 and 3).

Effects of L-arginine

Pretreatment of the portal hypertensive rats with L-arginine (300 mg kg^{-1}, i.v.) 5 min before L-NMMA (50 mg kg^{-1}), abolished ($P < 0.001$) its actions on MAP and all of the cardiovascular parameters measured (Tables 1, 2 and 3). L-Arginine

Table 3 Effects of saline or N^G monomethyl-L-arginine (L-NMMA, 50 mg kg^{-1}) on portal and renal haemodynamics in control and portal hypertensive rats: the effect of pretreatment with L-arginine (L-Arg; 300 mg kg^{-1}, i.v.) on the responses to L-NMMA in the portal ligated rats is also shown

	Control		*Portal hypertensive*		
	Vehicle ($n = 8$)	*L-NMMA* ($n = 8$)	*Vehicle* ($n = 9$)	*L-NMMA* ($n = 9$)	*L-NMMA + L-Arg* ($n = 7$)
Portal venous inflow (ml min^{-1} 100 g^{-1})	3.9 ± 0.5	3.3 ± 0.3	6.6 ± 0.5*	4.8 ± 0.5†	6.2 ± 0.8**
Porto-collateral resistance (mmHg ml^{-1} min^{-1} 100 g^{-1})	1.9 ± 0.3	2.3 ± 0.3	1.8 ± 0.2	2.7 ± 0.3†	1.9 ± 0.4**
Renal blood flow (ml min^{-1} 100 g^{-1})	3.6 ± 0.3	2.4 ± 0.3†	4.7 ± 0.2*	3.4 ± 0.3†	5.0 ± 0.5**
Renal vascular resistance (mmHg ml^{-1} min^{-1} 100 g^{-1})	37.3 ± 2.6	71.7 ± 9.1†	22.2 ± 1.4*	43.9 ± 2.8†	22.0 ± 0.3**

Results, shown as the value of each parameter per 100 g body weight following intravenous administration of vehicle (saline) or L-NMMA or L-arginine and L-NMMA in control non-ligated or chronic portal hypertensive rats, are expressed as the mean ± s.e.mean of (n) studies. Significant difference from the corresponding control non-ligated group is shown as * $P < 0.05$ and from the vehicle (saline) group in the portal hypertensive rats as † $P < 0.05$. Significant inhibition by L-arginine of the actions of L-NMMA is shown as ** $P < 0.05$.

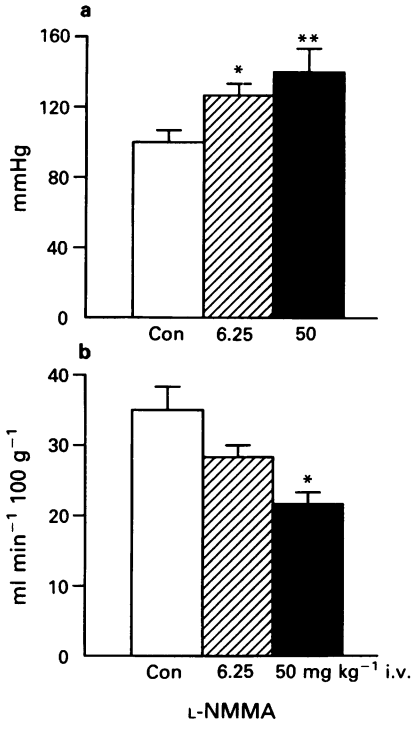


Figure 1 Effects of N^G-monomethyl-L-arginine (L-NMMA, 6.25 and 50 mg kg^{-1}, i.v.) on (a) systemic arterial blood pressure (mmHg) and (b) cardiac output (ml min^{-1} 100 g^{-1} body weight) in the portal hypertensive rat, 2 weeks after portal ligation. Results are shown as the mean of 8–9 experiments in each group, where vertical lines show s.e.mean. Significant difference from the resting values is shown as * $P < 0.05$; ** $P < 0.01$.

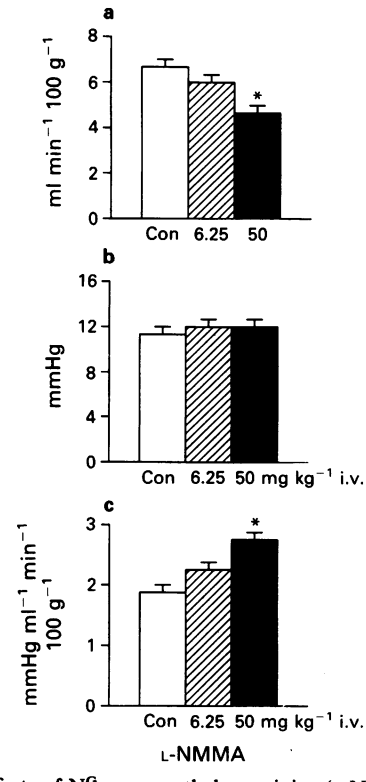


Figure 3 Effects of N^G-monomethyl-L-arginine (L-NMMA, 6.25 and 50 mg kg^{-1}, i.v.) on (a) portal venous inflow (ml min^{-1} 100 g^{-1} body weight) (b) portal pressure (mmHg) and (c) porto-collateral resistance (mmHg ml^{-1} min^{-1} 100 g^{-1} body weight) in the portal hypertensive rat, 2 weeks after portal ligation. Results are shown as the mean of 8–9 experiments in each group, where vertical lines show s.e.mean. Significant difference from the resting values is shown as * $P < 0.05$.

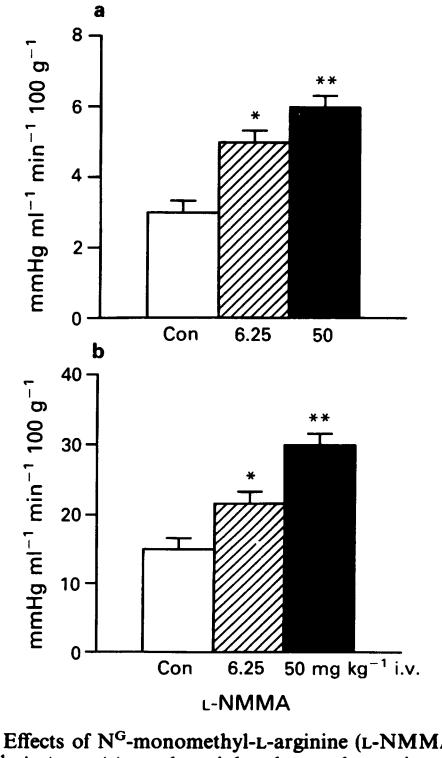


Figure 2 Effects of N^G-monomethyl-L-arginine (L-NMMA, 6.25 and 50 mg kg^{-1}, i.v.) on (a) total peripheral vascular resistance and (b) splanchnic vascular resistance (mmHg ml^{-1} min^{-1} 100 g^{-1} body weight) in the portal hypertensive rat, 2 weeks after portal ligation. Results are shown as the mean of 8–9 experiments in each group, where vertical lines show s.e.mean. Significant differences from the resting values is shown as * $P < 0.05$; ** $P < 0.01$.

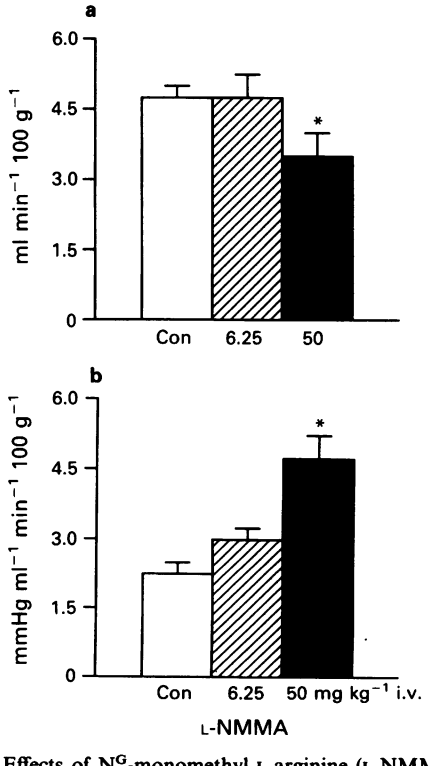


Figure 4 Effects of N^G-monomethyl-L-arginine (L-NMMA 6.25 and 50 mg kg^{-1}, i.v.) on (a) renal blood flow (ml min^{-1} 100 g^{-1} body weight) and (b) renal vascular resistance (mmHg ml^{-1} min^{-1} 100 g^{-1}) in the portal hypertensive rat, 2 weeks after portal ligation. Results are shown as the mean of 8–9 experiments in each group, where vertical lines show s.e.mean. Significant differences from the resting values is shown as * $P < 0.05$.

(300 mg kg^{-1}, i.v.) alone caused an initial inconsistent and transient fall in MAP but there was no significant effect on MAP when measured 5 min after administration, at the time of L-NMMA administration ($P > 0.05$, $n = 5$).

Effects of N^G-monomethyl-L-arginine in control rats

From preliminary dose-response studies with L-NMMA (6.25–100 mg kg^{-1}) in ketamine-anaesthetized control non-ligated rats, a dose of L-NMMA (50 mg kg^{-1}, i.v.) was selected for comparison of its actions on the haemodynamic parameters with those in the portal hypertensive rat.

L-NMMA (50 mg kg^{-1}) significantly increased resting MAP by 26 ± 4 mmHg ($n = 8$, $P < 0.05$) in these control rats, although this was significantly ($P < 0.05$) less than that observed in the portal hypertensive rats with this dose. As with the portal hypertensive rats, L-NMMA did not affect portal pressure in control rats (Table 1). However, unlike the findings in the portal hypertensive rats, the actions of this dose of L-NMMA on cardiac output, portal venous inflow or porto-collateral resistance did not reach statistical significance (Tables 2 and 3). Likewise, the overall changes in total peripheral and splanchnic vascular resistance did not reach significance although L-NMMA did significantly reduce renal blood flow and renal vascular resistance (Tables 2 and 3).

Discussion

Partial ligation of the portal vein in the rat induces an initial elevation of portal pressure and resistance and a decreased venous inflow (Sikuler *et al.*, 1985). However, 8 to 14 days later, extensive collaterals are formed, leading to the porto-systemic shunting of over 90% of the blood entering the portal venous system (Chojkier & Groszmann, 1981). This brings about a decrease of portal resistance to a level comparable to its resting value in non-ligated rats, while a substantial increase in venous inflow contributes to the sustained increase in portal pressure (Vorobioff *et al.*, 1983; Benoit *et al.*, 1984; Sikuler *et al.*, 1985; Kravetz *et al.*, 1986; 1988). The elevated inflow into the portal system is promoted by the accompanying splanchnic and renal vasodilatation, which is reflected by a fall in total peripheral, splanchnic and renal vascular resistance, with a reduction in systemic arterial blood pressure (BP) and increased cardiac output (Groszmann *et al.*, 1982; Sikuler *et al.*, 1985, Benoit *et al.*, 1986; Kravetz *et al.*, 1988). All of these cardiovascular changes were confirmed in the present study in the rat following 2-week partial portal ligation.

The processes underlying the splanchnic vasodilatation in chronic portal hypertension may involve a reduced vascular sensitivity to endogenous vasoconstrictors (Richardson & Withrington, 1976; Kiel *et al.*, 1985; Pizcueta *et al.*, 1990) or an increase in circulating levels of vasodilator factors, possibly due to their decreased hepatic metabolism as a result of porto-systemic shunting (Benoit *et al.*, 1984; 1986; Kravetz *et al.*, 1988). Although glucagon has been implicated (Benoit *et al.*, 1986; Kravetz *et al.*, 1988), studies with infusions of this peptide suggest that it could only account for 30–40% of the observed splanchnic vasodilatation observed in portal hypertensive rats (Benoit *et al.*, 1984; 1986). The present findings using L-NMMA suggest NO is a likely candidate for the mediator of vascular events following chronic portal ligation.

Intravenous administration of L-NMMA dose-dependently elevated BP in the portal hypertensive rat, as observed previously in the conscious or anaesthetized normotensive rat (Compton *et al.*, 1989; Whittle *et al.*, 1989; Gardiner *et al.*, 1990). This was accompanied by a decrease in the cardiac output and an increase in total peripheral, splanchnic and renal vascular resistance to values not significantly different from those observed under resting conditions in control non-ligated rats. In the control non-ligated rats, although BP was increased by L-NMMA, as was renal vascular resistance, the overall changes in total peripheral vascular resistance or total splanchnic resistance did not reach statistical significance. However, recent preliminary studies using similar radiolabelled microsphere techniques in non-operated rats have demonstrated differential changes in the various splanchnic vascular areas following this dose of L-NMMA (Pizcueta *et al.*, 1991). Thus, L-NMMA increased vascular resistance in the stomach, pancreas, spleen and mesenteric bed, but not in the small intestine and colon, indicating different sensitivities to the actions of this NO-synthase inhibitor in these splanchnic vascular beds (Pizcueta *et al.*, 1991). In contrast, in portal hypertensive rats, significant increases in vascular resistance were observed with L-NMMA in all of these vascular beds (Pizcueta *et al.*, 1991), as supported by the current findings of increased total splanchnic resistance. Previous studies with L-NMMA in normotensive chronically instrumented conscious rats have also demonstrated a reduction in regional blood flow, including vasoconstrictor actions on the mesenteric and renal vascular beds (Gardiner *et al.*, 1990), while reductions in blood flow to the gastric mucosa (Pique *et al.*, 1989) and renal cortex (Walder *et al.*, 1991) have been observed in the anaesthetized rat.

In previous studies in this model, a reduction in portal inflow by β-adrenoceptor blockade, or the administration of somatostatin was accompanied by a small but significant fall in the elevated portal pressure (Kravetz *et al.*, 1988; Pizcueta *et al.*, 1989; Piqué *et al.*, 1990). In the current study, the splanchnic vasoconstriction induced by L-NMMA was associated with a reduction in portal venous inflow in the ligated rats, but portal pressure was not significantly modified. This may be the consequence of the concurrent increase in porto-collateral resistance following administration of L-NMMA, which is likely to reflect venoconstriction in the porto-collateral vasculature.

The cardiovascular actions of L-NMMA in the portal hypertensive rats were abolished by pretreatment with L-arginine, as previously demonstrated in normotensive rats (Compton *et al.*, 1989; Whittle *et al.*, 1989; Gardiner *et al.*, 1990). The dose of L-arginine used had itself no significant effect on resting BP, measured 5 min after administration in these portal hypertensive rats. Thus, the mechanism by which L-NMMA could normalize BP, as well as vascular resistance and portal venous inflow, presumably reflects attenuation of the local synthesis of NO from L-arginine, suggesting an exaggerated local production of NO in these vascular beds under such conditions. Furthermore, by comparison with control non-ligated rats, portal hypertensive rats appeared to be more sensitive to the cardiovascular effects of L-NMMA. A detailed comparative evaluation of the sensitivity of these vascular beds to L-NMMA, and other NO synthesis inhibitors under such conditions is hence warranted in future studies. The present findings with L-NMMA also suggest that the porto-systemic collateral vasculature, which is developed from pre-existing venous channels (Vorobioff *et al.*, 1983), is capable of actively synthesizing NO. This contrasts with the findings of a relatively low production of NO by venous tissue under basal conditions (Vallance *et al.*, 1989b).

The factors that regulate substrate availability and enzyme activity involved in endogenous NO synthesis in these vascular beds are not yet fully known. Furthermore, although the calcium-dependent synthase responsible for NO production by endothelial cells is a constitutive enzyme, an inducible enzyme that forms NO from L-arginine and which is also inhibited by L-NMMA, has also been described in these cells (Radomski *et al.*, 1990). An inducible NO synthase, identified originally in the macrophage (Hibbs *et al.*, 1988; Marletta *et al.*, 1988; Stuehr *et al.*, 1989) has also been detected in vascular smooth muscle, lung and liver tissue (Busse & Mulsch, 1990; Knowles *et al.*, 1990; Rees *et al.*, 1990). Furthermore, it has been suggested recently that the vascular formation of NO by this inducible enzyme may contribute to the hyperdynamic

state seen in cirrhosis (Vallance & Moncada, 1991). It will therefore be important to investigate the enzymic characteristics of NO formation in endothelial cells and smooth muscle of the splanchnic vascular beds as well as in the newly developed porto-collateral venous system under the conditions seen in portal hypertension. Should an inducible NO synthase be involved in this systemic and splanchnic vasodilatation, then its selective attenuation would offer a novel and specific approach to the correction of vascular tone and blood flow under such pathological conditions with minimal effects on the physiological actions of NO formed by the constitutive enzyme.

The present findings that the hyperdynamic circulatory characteristics following portal vein stenosis can be attenuated by L-NMMA, thus provide initial evidence that an excessive formation of NO is implicated in the pathogenesis of the haemodynamic disturbances associated with chronic portal hypertension.

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Mechanisms contributing to the regional haemodynamic effects of neurotensin in conscious, unrestrained Long Evans rats

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1 The regional haemodynamic effects of i.v. bolus doses of neurotensin (10–1000 ng) were assessed in conscious, unrestrained Long Evans rats chronically instrumented with miniaturized, pulsed Doppler probes.

2 Neurotensin caused increases in blood pressure, together with dose-related tachycardias and constrictions in the renal, superior mesenteric and hindquarters vascular beds. The tachycardia elicited by the 1000 ng dose of neurotensin was preceded by a transient bradycardia.

3 In the presence of phentolamine, the pressor effect of neurotensin (1000 ng) was converted into a hypotensive effect, accompanied by reduced tachycardic and constrictor responses in the renal, superior mesenteric and hindquarters vascular beds. The tachycardia was not preceded by a bradycardia.

4 In the presence of phentolamine and propranolol, the pressor and bradycardic responses to neurotensin were unaffected, whereas the tachycardia was abolished. The renal vasconstrictor effect was smaller, while the constrictions in the superior mesenteric and hindquarters vasculars beds were not different from those in untreated rats.

5 In rats neonatally treated with capsaicin (50 mg kg^{-1}, s.c.), the pressor effects elicited by neurotensin (300 and 1000 ng) were reduced as were the constrictor responses in the renal (at the dose of 300 ng), superior mesenteric (at the dose of 300 ng) and hindquarters (at both doses) vascular beds. The bradycardia elicited by neurotensin (1000 ng) was absent, whereas the tachycardia was potentiated.

6 The results indicate that in conscious, intact rats neurotensin appears to exert cardiovascular influences through activation of sympathoadrenal mechanisms and also through non-adrenergic effects on the heart, renal, superior mesenteric and hindquarters vascular beds. The latter effects appear to involve capsaicin-sensitive nerves.

Keywords: Blood pressure; heart rate; neurotensin; regional haemodynamics; capsaicin

Introduction

Neurotensin (NT) is a tridecapeptide, first isolated from bovine hypothalami (Carraway & Leeman, 1973), and later found in various areas of the central nervous system, the gastrointestinal tract and in circulating blood in several animal species (Uhl & Snyder, 1976; 1981; Kobayashi *et al.*, 1977; Carraway & Leeman, 1978; Carraway & Bhatnagar, 1980; Hammer *et al.*, 1980; Manberg *et al.*, 1982; Shulkes *et al.*, 1982).

NT possesses a wide range of effects on both the central and peripheral nervous systems and on peripheral tissues (Carraway & Leeman, 1973; Bissette *et al.*, 1978; Brown & Miller, 1982). NT was found to produce contraction of some isolated blood vessels, such as the portal vein (Rioux *et al.*, 1980), coronary vasculature (Quirion *et al.*, 1979) and vessels of subcutaneous adipose tissue (Rosell & Rokaeus, 1981), and to have vasoconstrictor properties in the isolated, perfused hindquarters of the rat (Rioux *et al.*, 1982). The peptide also exerts potent positive inotropic and chronotropic effects on rodent isolated, spontaneously beating atria (Quirion *et al.*, 1978; Stene-Larsen & Helle, 1979). Intravenous administration of NT was found to induce hypotension in pentobarbitone anaesthetized rats, possibly through a histamine-mediated process (Quirion *et al.*, 1980), since pretreatment with compound 48/80, a well-known mast cell degranulating agent, was found not only to block the hypotensive effect of NT, but also to unmask hypertensive effects of high doses of NT (Quirion *et al.*, 1980). Using conscious rats, Sumners *et al.* (1982) reported that i.v. administration of NT elicited a fall in blood pressure which was sometimes preceded by a slight hypertensive effect. In pentobarbitone-anaesthetized guinea-pigs, NT was found to elicit dose-dependent pressor effects, which probably resulted from an influence of the peptide on the sympathetic nervous system, and a tachycardia, which was possibly due to a direct effect of the peptide on the sino-atrial node (Kerouac *et al.*, 1981). Recently, it has been reported that both effects were significantly reduced or abolished in guinea-pigs chronically treated with the neurotoxin, capsaicin (Bachelard *et al.*, 1987). Moreover, *in vitro* studies have shown that the positive chronotropic and inotropic effects of NT were abolished in atria taken from capsaicin-treated guinea-pigs (Bachelard *et al.*, 1987). Thus, it has been suggested that capsaicin-sensitive sensory neurones participate in the cardiovascular responses to NT in guinea-pigs (Bachelard *et al.*, 1987).

The present study was undertaken to investigate the regional haemodynamic effects produced by NT injected i.v. in conscious, unrestrained, rats, chronically instrumented with intravascular catheters and miniaturized pulsed Doppler probes to measure renal, superior mesenteric and hindquarters blood flows. The contribution of adrenoceptors to the cardiovascular responses to NT was examined by peripheral administration of α- and β-adrenoceptor antagonists. Moreover, to assess the involvement of capsaicin-sensitive sensory neurones in the cardiovascular responses to NT, some experiments were carried out in rats neonatally treated with capsaicin or with the vehicle for capsaicin.

Methods

The experiments were carried out on male Long Evans rats (350–400 g) bred in the Animal Unit in Nottingham. Details of the techniques used have been published elsewhere (Gardiner

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Table 1 Baseline values for heart rate, blood pressure and regional vascular conductance in conscious, untreated ($n = 8$), vehicle-treated (Veh-treated; $n = 9$) or capsaicin-treated (Cap-treated; $n = 9$) Long Evans rats

	Heart rate (beats min^{-1})	*Mean blood pressure* (mmHg)	*Doppler shift* (kHz)			*Vascular conductance* ([kHz mmHg^{-1}] 10^3)		
			Renal	Mesenteric	Hindquarters	Renal	Mesenteric	Hindquarters
Untreated	379 ± 12	101 ± 2	10.1 ± 0.9	8.3 ± 0.7	5.1 ± 0.6	99 ± 8	83 ± 7	51 ± 7
Veh-treated	361 ± 11	101 ± 3	9.3 ± 1.3	9.2 ± 1.0	6.2 ± 0.7	89 ± 11	91 ± 11	61 ± 7
Cap-treated	356 ± 11	104 ± 2	10.2 ± 0.9	8.0 ± 0.3	5.3 ± 0.8	98 ± 7	78 ± 2	51 ± 10

Values are mean ± s.e.mean.

et al., 1990a, b). Briefly, animals were anaesthetized (sodium methohexitone, 60 mg kg^{-1} i.p. supplemented as required) and had miniaturized pulsed Doppler probes (Haywood *et al.*, 1981) implanted around the left renal artery, the superior mesenteric artery and the distal abdominal aorta (below the level of the ileocaecal artery). The probe wires were tunnelled s.c. and exteriorized at the back of the neck, where they were sutured in place. Animals were given ampicillin (7 mg kg^{-1}, i.m., Penbritin, Beechams) and left to recover for at least 7 days. Thereafter, under brief anaesthesia (sodium methohexitone 40 mg kg^{-1}, i.p.), the probes wires were soldered into a microconnector (Microtech Inc., U.S.A.) and one intraarterial (distal abdominal aorta via the caudal artery) and two i.v. (right jugular vein) catheters were implanted. The catheters ran s.c. and emerged with the pulsed Doppler probe leads. The microconnector, soldered to the latter, was clamped in a harness worn by the rat and the catheters passed through a flexible spring (for protection) which was attached to the harness. Animals were returned to their home cages with free access to food and water. Experiments were not begun until the next day.

Throughout the experiments, continuous recordings were made of instantaneous heart rate, phasic and mean blood pressures and renal, mesenteric and hindquarters Doppler shift signals (Crystal Biotech VF-1 Doppler flowmeter, Holliston, MA, U.S.A.). Changes in Doppler shift signals were calculated as percentage of the baseline level. The latter have been shown to be a reliable index of volume flow (Haywood *et al.*, 1981; Wright *et al.*, 1987) and are referred to as flows in the text. The Doppler shift and corresponding mean arterial blood pressure signals were used to calculate percentage changes in regional vascular conductances (Gardiner *et al.*, 1990b).

At different times, varying between 0.5 and 30 min after peptide administration, measurements were made of heart rate, mean blood pressure and mean Doppler shift signals, to represent the full profile of the effects of the peptide, and related to the pre-drug baseline (absolute changes for the former two variables, percentages for the Doppler shifts). Before every experiment a 30 min baseline recording period was made. Animals had free access to water and food for the duration of the experiment. All i.v. injections were given in 100 μl isotonic saline (the dead space of the catheter). NT was dissolved in isotonic saline containing 1% bovine serum albumin (BSA).

The following experiments were performed:

Cardiovascular responses to neurotensin

Animals were used on 3 consecutive days during which they received 6 randomized i.v. injections of vehicle (0.1 ml isotonic saline containing 1% bovine serum albumin (BSA)) and NT at doses varying between 10 and 1000 ng; randomized between days, but the lower doses were always given before the higher doses on any particular day. Each animal received no more than 2 i.v. injections on the same day, separated by at least 120 min. Measurements were made before, during and for 30 min following i.v. injections of vehicle or NT. Injections were given over a period of 5 s.

Effect of pretreatment with phentolamine, or phentolamine and propranolol on responses to neurotensin

The α-adrenoceptor antagonist, phentolamine, was administered i.v. as a bolus (1 mg kg^{-1}, 0.1 ml) followed by continuous infusion of 1 mg kg^{-1} h^{-1} (0.3 ml h^{-1}) (Winn *et al.*, 1985) to a group of 11 rats. Fifteen min after the start of the phentolamine infusion the animals received an i.v. injection of NT (1000 ng).

The next day, in the same animals, a mixture of phentolamine (1 mg kg^{-1}, i.v. bolus, 1 mg kg^{-1} h^{-1}, infusion) and the β-adrenoceptor antagonist, propranolol, (1 mg kg^{-1}, i.v. bolus, 0.5 mg kg^{-1} h^{-1}, infusion) (Gardiner & Bennett, 1988) was administered and 15 min after onset of the coinfusion, NT (1000 ng) was injected i.v. Measurements were made before, during, and for 30 min following i.v. injection of NT.

Table 2 Peak cardiovascular changes following intravenous administration of saline-bovine serum albumin (saline-BSA, 1%) or increasing doses of neurotensin (NT) in conscious, untreated Long Evans rats

	Δ*Heart rate* (beats min^{-1})	Δ*Mean blood pressure* (mmHg)	Δ*Doppler shift* (%)			Δ*Vascular conductance* (%)		
			Renal	Mesenteric	Hindquarters	Renal	Mesenteric	Hindquarters
Saline-BSA 1%	+19 ± 6†	0 ± 1	−3 ± 2	+3 ± 4	+5 ± 5	−3 ± 1	−2 ± 3	−2 ± 4
NT 10 ng	+16 ± 6	+3 ± 2	0 ± 3	−3 ± 4	+5 ± 4	−2 ± 3	−2 ± 5	0 ± 6
NT 30 ng	+16 ± 8	+8 ± 2	−3 ± 2	+8 ± 7	−8 ± 2	−9 ± 2	−4 ± 4	−11 ± 3
NT 100 ng	+25 ± 10	+11 ± 1*	0 ± 2	−14 ± 3*	−11 ± 3	−8 ± 3	−17 ± 4*	−11 ± 4
NT 300 ng	+43 ± 20*	+14 ± 2*	−6 ± 2	−41 ± 5*	−28 ± 7*	−15 ± 2*	−45 ± 4*	−35 ± 6*
NT 1000 ng	−57 ± 24* +99 ± 12*	+15 ± 2*	−24 ± 4*	−52 ± 6*	−59 ± 5*	−25 ± 4*	−55 ± 4*	−62 ± 4*

Values are mean ± s.e.mean; $n = 8$, at least (some injections were given in the same animals).
*$P < 0.05$ versus control injection, analysis of variance followed by Fisher's test.
† $P < 0.05$ versus baseline, paired Student's t test for control injection of saline-BSA (1%).
With NT 1000 ng a biphasic effect was observed on heart rate; the first number indicates the initial change and below is the later change.

Cardiovascular responses to neurotensin in rats neonatally treated with capsaicin

At least 48 h after birth, Long Evans rats were anaesthetized with 1.5–2% halothane (in oxygen) and injected s.c. with either vehicle (10% ethanol, 10% Tween 80 in isotonic saline) or capsaicin (50 mg kg^{-1}) in a volume of 100 μl. After weaning (day 21–30 after birth) only male rats were selected for the experiments and housed in groups of 4 or 5 with free access to food and tap water.

About 20 weeks later, two groups of rats (vehicle-treated, $n = 9$ and capsaicin-treated, $n = 9$), previously instrumented with pulsed Doppler probes and intravascular catheters (as earlier described), were used on 3 consecutive days during which they received i.v. injections of vehicle (0.1 ml isotonic saline containing 1% BSA) and NT (300 and 1000 ng), in random order. Measurements were made before, during and for 30 min following i.v. injections of vehicle or NT.

Drugs

Neurotensin (Bachem), phentolamine mesylate (Ciba), propranolol hydrochloride (Imperial Chemical Industries) and 8 methyl-N-vanillyl-6-nonenamide (capsaicin; Sigma) were used.

Statistical analysis

Values are expressed as the mean ± s.e.mean; n is the number of animals. The paired Student's t test was used to analyse the effects of control injection of saline-BSA, while comparisons between groups were made by an analysis of variance followed by Fisher's test. A P value < 0.05 was taken to indicate a significant difference.

Results

The baseline values for cardiovascular variables are given in Table 1.

Haemodynamic responses to neurotensin

Control injections caused a slight tachycardia (significant at 1 min), but had no significant effect on the other measured or calculated variables (Table 2). Table 2 shows that the lowest doses of NT tested (10 and 30 ng) did not have effects significantly different from those of control injections. However, with the 100 ng dose of NT there was a slight increase in blood pressure (significant at 0.5 min) accompanied by falls in superior mesenteric flow (significant at 1–1.5 min) and vascular conductance (significant at 1–2 min); these effects were significantly different from those following control injections. There were no changes in heart rate or renal or hindquarters flows or vascular conductances.

NT at a dose of 300 ng produced a slight increase in blood pressure (significant at 0.5 and 1.5–5 min) and a tachycardia (significant at 4–10 min). There were long-lasting falls in superior mesenteric (significant at 1–30 min) and hindquarters flows (significant at 3–30 min), but no significant change in renal flow. These responses were associated with constrictions in renal (significant at 2–5 min), superior mesenteric (significant at 1–30 min) and hindquarters (significant at 2–30 min) vascular beds (Table 2).

The highest dose of NT (1000 ng) caused a slight increase in blood pressure (significant at 2–5 min) accompanied by a transient bradycardia (significant at 1 min) followed by a tachycardia (significant at 3–30 min), and reductions in renal (significant at 4–30 min), superior mesenteric (significant at 1–30 min) and hindquarters flows (significant at 0.5–30 min) (Figure 1 and Table 2). Hence, there were constrictions in renal (significant at 2–30 min), superior mesenteric (significant at 1–30 min) and hindquarters (significant at 0.5–30 min) vascular beds (Figure 2 and Table 2).

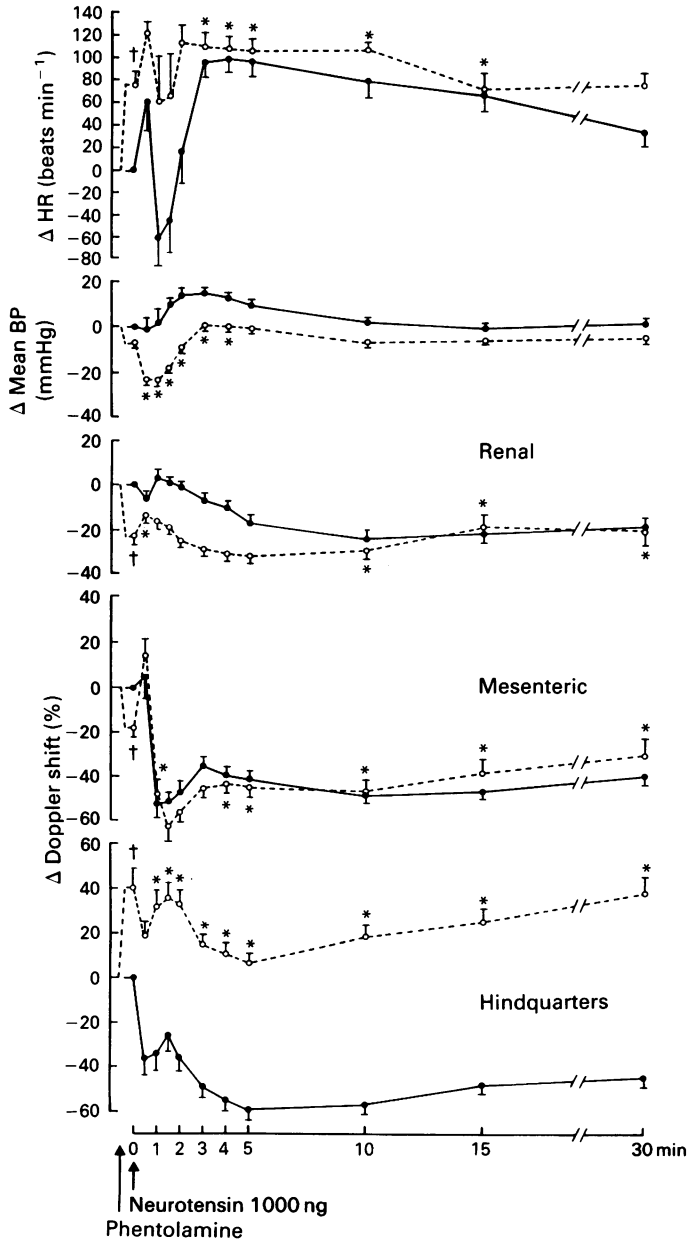


Figure 1 Cardiovascular responses to i.v. bolus injections of neurotensin (1000 ng) in conscious, unrestrained, Long Evans rats, in the absence, (●) or in the presence (○) of i.v. treatment with phentolamine (1 mg kg^{-1}, i.v. bolus, 1 mg kg^{-1} h^{-1}, infusion). Values are mean with s.e.mean shown by vertical lines. *$P < 0.05$ for changes in untreated group ($n = 15$) versus changes in treated group ($n = 11$) (analysis of variance followed by Fisher's test). †$P < 0.05$ versus pre-drug baseline (Student's t test for paired data). HR = heart rate; BP = blood pressure.

Haemodynamic responses to neurotensin (1000 ng) in the presence of phentolamine

Fifteen min after the onset of pretreatment with phentolamine there was no change in blood pressure, but there were persistent increases in heart rate, and hindquarters flow and reductions in renal and superior mesenteric flows. There were

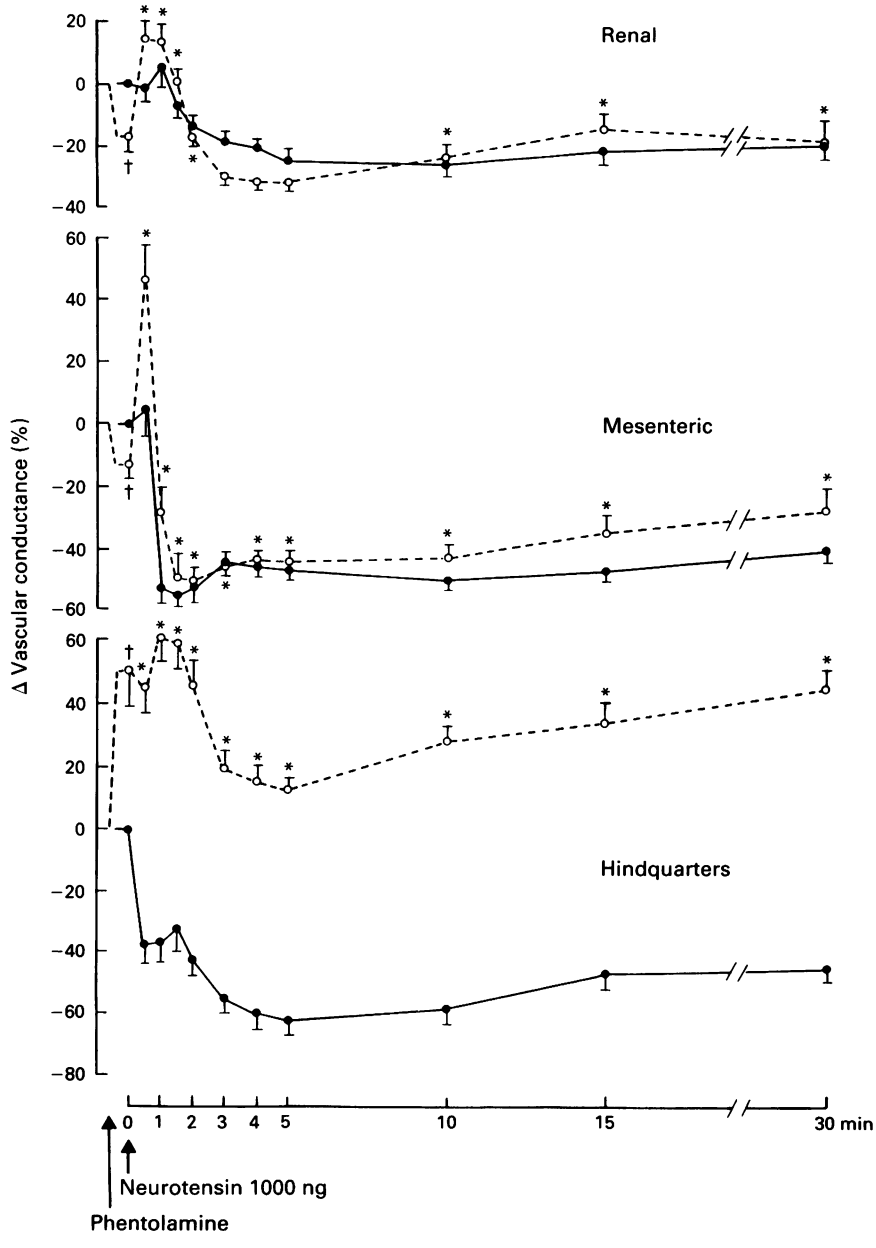


Figure 2 Changes in regional vascular conductances elicited by i.v. bolus injections of neurotensin (1000 ng) in conscious, unrestrained, Long Evans rats, in the absence, (●) or in the presence (○) of i.v. treatment with phentolamine (1 mg kg^{-1}, i.v. bolus, 1 mg kg^{-1} h^{-1}, infusion). These data were derived from those shown in Figure 1. Values are mean with s.e.mean shown by vertical lines. *$P < 0.05$ for changes in untreated group ($n = 15$) versus changes in treated group ($n = 11$) (analysis of variance followed by Fisher's test). † $P < 0.05$ versus pre-drug baseline (Student's t test for paired data).

reductions in renal and superior mesenteric vascular conductances and an increase in hindquarters vascular conductance (all significant, $P < 0.05$) (Figures 1 and 2).

In the presence of phentolamine, NT elicited a decrease in blood pressure (significant at 0.5–1.5 min), rather than an increase as observed in untreated rats, and a tachycardia (significant at 4–10 min), which was significantly less than in untreated animals. The reduction in renal flow observed in untreated rats was absent. There was an initial increase in superior mesenteric flow (significant at 0.5 min) followed by a reduction (significant at 1–15 min); the latter was significantly smaller than that in untreated animals. There was a decrease in hindquarters flow (significant at 0.5 and 3–15 min) that was also significantly less than in untreated rats. There were initial dilatations in the renal (significant at 0.5–1.5 min) and superior mesenteric vascular beds (significant at 0.5 min) followed by constrictions in both vascular beds (renal, significant at 4–5 min; superior mesenteric, significant at 1.5–15 min) (Figure 2). Both vasodilator responses were significantly different from those seen in untreated rats, while the constrictor responses in both vascular beds were significantly smaller than in untreated animals. The constriction in the hindquarters vascular bed (significant at 3–15 min) was significantly less than in untreated rats.

Haemodynamic responses to neurotensin (1000 ng) in the presence of phentolamine and propranolol

Fifteen min after the start of pretreatment with phentolamine and propranolol there were no significant changes in blood pressure, heart rate or renal flow or vascular conductance, but there was a persistent decrease in superior mesenteric flow and an increase in hindquarters flow, together with a mesenteric vasoconstriction and hindquarters vasodilatation (all significant, $P < 0.05$) (Figures 3 and 4).

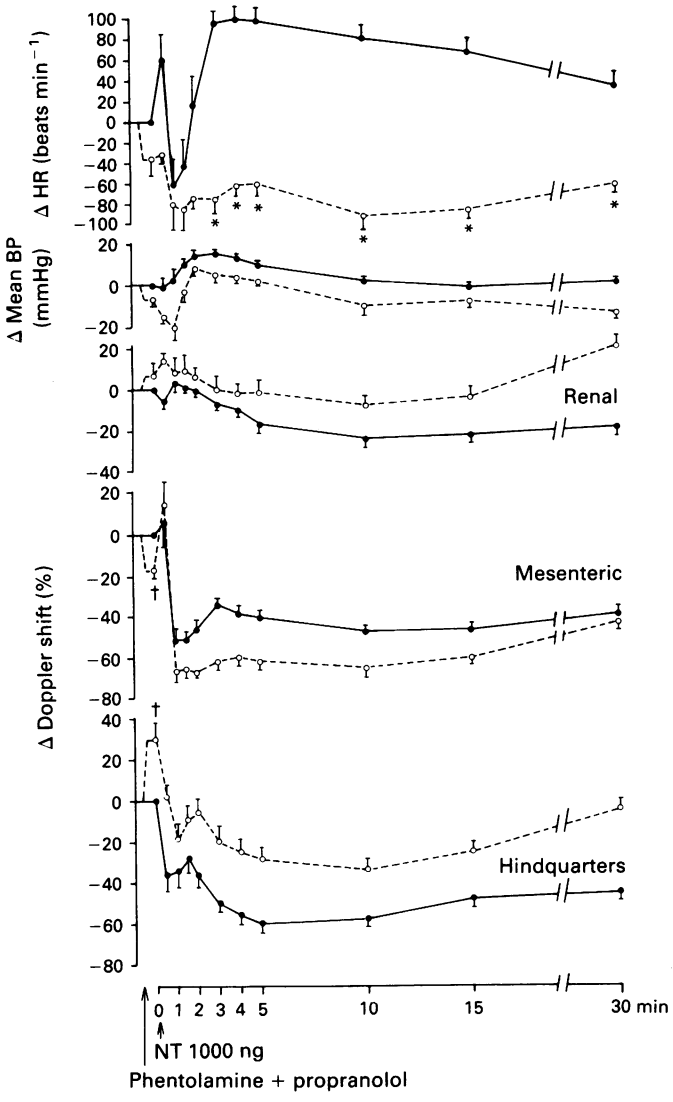


Figure 3 Cardiovascular responses to i.v. bolus injections of neurotensin (1000 ng) in conscious, unrestrained, Long Evans rats, in the absence (●) or in the presence (○) of i.v. treatment with phentolamine (1 mg kg^{-1}, i.v. bolus, 1 mg kg^{-1} h^{-1}, infusion) and propranolol (1 mg kg^{-1}, i.v. bolus, 0.5 mg kg^{-1} h^{-1}, infusion). Values are mean with s.e.mean shown by vertical lines. *$P < 0.05$ for changes in untreated group ($n = 15$) versus changes in treated group ($n = 11$) (analysis of variance followed by Fisher's test). †$P < 0.05$ versus pre-drug baseline (Student's t test for paired data). HR = heart rate; BP = blood pressure.

In the presence of phentolamine and propranolol, NT caused a slight decrease in blood pressure (significant at 0.5 min) followed by a modest increase (significant at 2–5 min); these changes were not significantly different from those in untreated rats (Figure 3). However, there was a persistent bradycardia (significant at 1–3 min) (Figure 3) and hence, the tachycardia observed in untreated animals was absent. There were decreases in renal (significant at 10 min) and hindquarters flows (significant at 0.5–30 min), while superior mesenteric flow initially showed an increase (significant at 0.5 min) followed by a decrease (significant at 1–30 min) (Figure 3); these effects were not different from those in untreated rats. However, the early dilatations in renal (significant at 0.5–1 min) and superior mesenteric vascular beds (significant at 0.5 min) were different from those in untreated rats (Figure 4). Furthermore, the subsequent renal vasoconstriction (significant at 3–5 min) was significantly less than in untreated animals, while the constriction in the superior mesenteric vascular bed (significant at 1–30 min) was not different (Figure 4). There was a constriction in the hindquarters vascular bed (significant at 0.5–30 min), which was not different from that in untreated rats (Figure 4).

Haemodynamic effects of neurotensin (300 and 1000 ng) in rats neonatally treated with capsaicin

The baseline values for cardiovascular variables in vehicle- and capsaicin-treated rats are given in Table 1. The resting values in vehicle- and capsaicin-treated rats were not different from those in untreated rats. Control injection (saline-BSA 1%) in vehicle-treated rats had no effect on blood pressure, but caused a slight increase in heart rate (significant at 0.5 min) and hindquarters flow (significant at 0.5 min), and vascular conductance (significant at 0.5 min) (Table 3). There was no change in renal or superior mesenteric flows or vascular conductances (Table 3). In capsaicin-treated rats, control injections had no effect on blood pressure, heart rate or renal or superior mesenteric flows, but caused an increase in hindquarters flow (significant at 0.5 min) (Table 3). However, there were no changes in renal, superior mesenteric or hindquarters vascular conductances (Table 3). The cardiovascular responses to control injections in capsaicin-treated rats were not different from those in vehicle-treated rats.

In vehicle-treated rats, NT (300 ng) caused a slight increase in blood pressure (significant at 0.5–5 min) and a tachycardia (significant at 0.5, 2 and 30 min) accompanied by decreases in superior mesenteric (significant at 1–10 min) and hindquarters flows (significant at 3–5 min), but no change in renal flow (Figure 5). Hence, there were constrictions in renal (significant at 2–4 min), superior mesenteric (significant at 1–5 min) and hindquarters vascular beds (significant at 3–5 min) (Figure 6).

Table 3 Peak cardiovascular changes following intravenous administration of saline-bovine serum albumin (saline-BSA 1%) (Control) or neurotensin (NT) in vehicle- (Veh) or capsaicin-treated (Cap) Long Evans rats

	Δ Heart rate (beats min^{-1})	*Δ Mean blood pressure* (mmHg)	*Δ Doppler shift* (%)			*Δ Vascular conductance* (%)		
			Renal	Mesenteric	Hindquarters	Renal	Mesenteric	Hindquarters
Control (Veh)	+22 ± 10†	0 ± 1	0 ± 1	+4 ± 3	+18 ± 6†	+1 ± 2	+2 ± 3	+23 ± 9†
NT 300 ng (Veh)	+111 ± 12*	+15 ± 2*	0 ± 2	−37 ± 8*	−19 ± 4*	−13 ± 2*	−42 ± 8*	−25 ± 4*
NT 1000 ng (Veh)	−72 ± 25*	+20 ± 2*	−3 ± 4	+33 ± 9*	−62 ± 4*	−18 ± 4*	+31 ± 11*	−67 ± 3*
	+78 ± 16*			−48 ± 3*			−56 ± 6*	
Control (Cap)	+2 ± 3	−3 ± 1	+2 ± 1	0 ± 2	+17 ± 7†	+3 ± 1	+1 ± 2	+4 ± 4
NT 300 ng (Cap)	+81 ± 16*	+8 ± 2*	−4 ± 2	−20 ± 3*	−7 ± 6	−4 ± 2	−25 ± 3*	−10 ± 7*
NT 1000 ng (Cap)	+149 ± 14*	+7 ± 2*	−28 ± 4*	+43 ± 8*	−49 ± 3*	−28 ± 4*	+42 ± 7*	−51 ± 3*
				−50 ± 4*			−50 ± 3*	

Values are mean ± s.e.mean; $n = 9$.
*$P < 0.05$ versus their respective controls, analysis of variance followed by Fisher's test.
†$P < 0.05$ versus baseline, paired Student's t test for control injections in vehicle- and capsaicin-treated rats.
With NT 1000 ng biphasic effects were observed on heart rate (vehicle-treated rats), superior mesenteric flow and vascular conductance; the first number indicates the initial change and below is the later change.

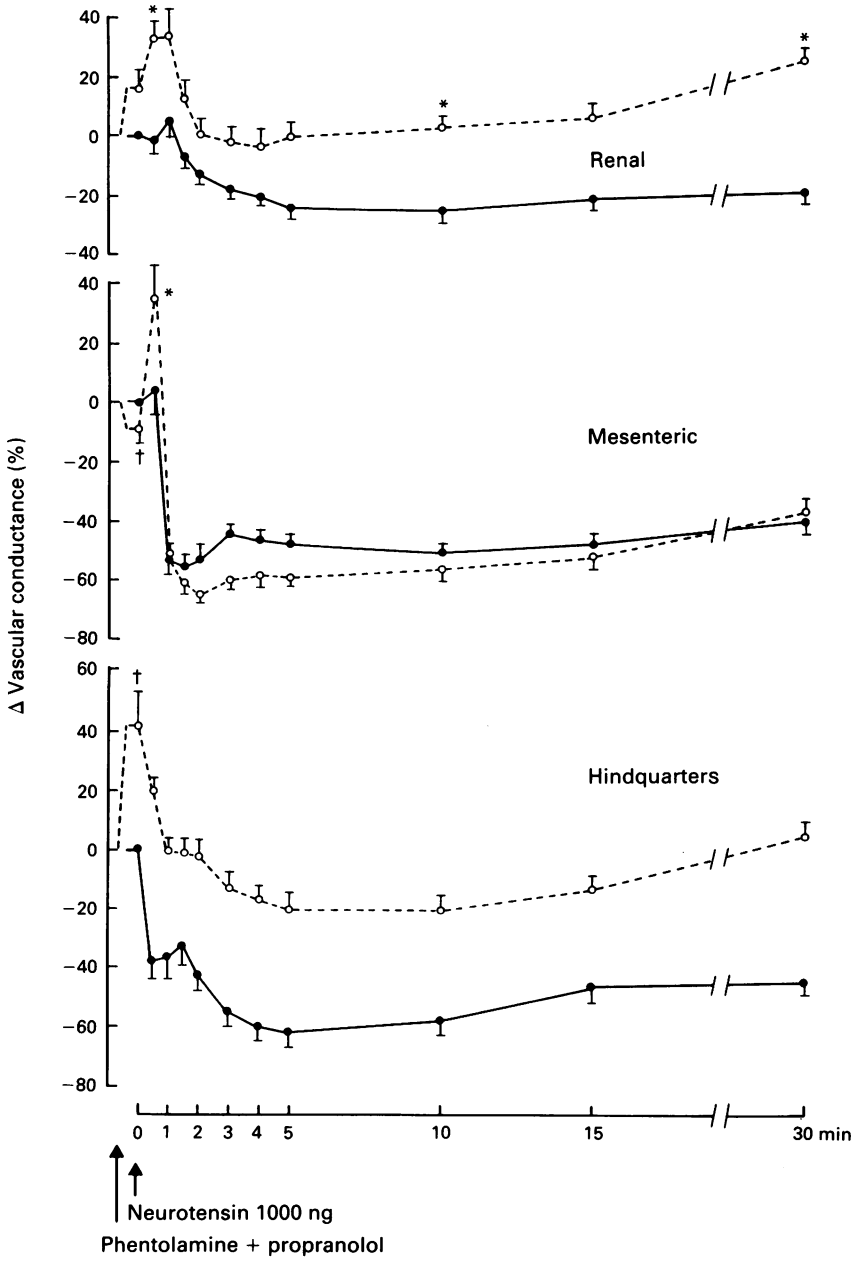


Figure 4 Changes in regional vascular conductances elicited by i.v. bolus injections of neurotensin (1000 ng) in conscious, unrestrained, Long Evans rats, in the absence (●) or in the presence (○) of i.v. treatment with phentolamine (1 mg kg^{-1}, i.v. bolus, 1 mg kg^{-1} h^{-1}, infusion) and propranolol (1 mg kg^{-1}, i.v. bolus, 0.5 mg kg^{-1} h^{-1}, infusion). These data were derived from those shown in Figure 3. Values are mean with s.e.mean shown by vertical lines. *$P < 0.05$ for changes in untreated group ($n = 15$) versus changes in treated group ($n = 11$) (analysis of variance followed by Fisher's test). †$P < 0.05$ versus pre-drug baseline (Student's t test for paired data).

In vehicle-treated rats, the higher dose of NT (1000 ng) produced an increase in blood pressure (significant at 1–10 min) and a bradycardia (significant at 1 min) followed by a tachycardia (significant at 3–30 min) (Figure 7). There was no significant change in renal flow, while there was an initial increase in superior mesenteric flow (significant at 0.5 min) followed by a decrease (significant at 1–30 min); there was a long-lasting decrease in hindquarters flow (significant at 0.5–30 min) (Figure 7). These changes were associated with constrictions in renal (significant at 2–10 and 30 min) and hindquarters vascular beds (significant at 0.5–30 min), while in the superior mesenteric vascular bed a dilatation (significant at 0.5 min) followed by a constriction (significant at 1–30 min) occurred (Figure 8).

In capsaicin-treated rats, the lower dose of NT (300 ng) caused a modest increase in blood pressure (significant at 0.5–1 and 3–4 min), which was significantly less, and a tachycardia (significant at 0.5 min) which was not different from that in vehicle-treated rats (Figure 5). As in the vehicle-treated rats, there was no change in renal flow, but a decrease in superior mesenteric flow (significant at 1–2 min), while the slight reduction in hindquarters flow, seen in response to NT in vehicle-treated animals was absent (Figure 5). Thus, there was no change in renal vascular conductance, and this was significantly different from the response in vehicle-treated rats; moreover, the vasoconstriction in the superior mesenteric vascular bed (significant at 1–3 min) was significantly smaller than in vehicle-treated rats and the hindquarters vasoconstriction observed in the latter was absent (Figure 6).

In capsaicin-treated animals, the higher dose of NT (1000 ng) had no effect on blood pressure; this was significantly different from the response seen in vehicle-treated rats (Figure 7). Furthermore, there was no bradycardia, although the tachycardia (significant at 0.5–30 min) was greater than in

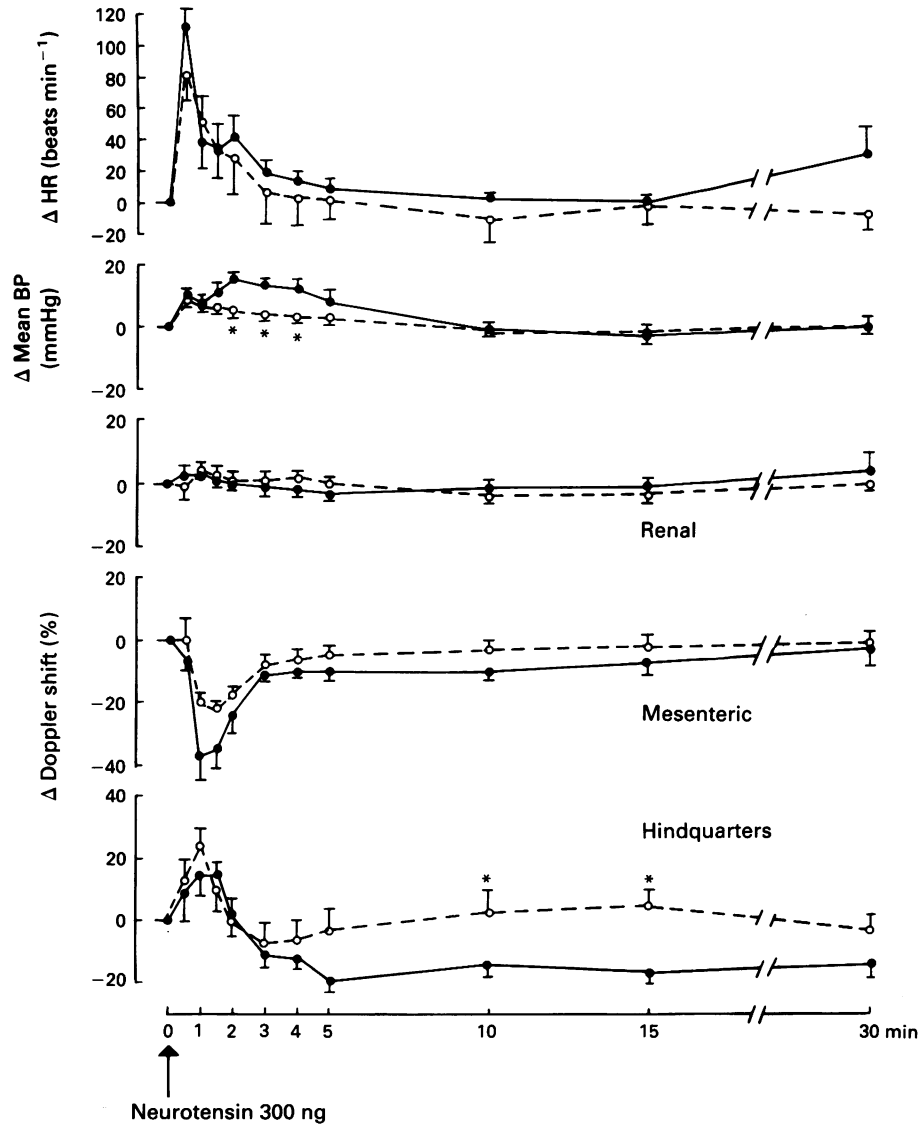


Figure 5 Cardiovascular responses to i.v. bolus injections of neurotensin in vehicle-treated (●) or capsaicin-treated (○) Long Evans rats. Values are mean with s.e.mean shown by vertical lines. *$P < 0.05$ for changes in vehicle-treated group ($n = 9$) versus changes in capsaicin-treated group ($n = 9$) (analysis of variance followed by a Fisher's test). HR = heart rate; BP = blood pressure.

vehicle-treated rats (Figure 7). There was a reduction in renal flow (significant at 3–30 min) which was greater than in vehicle-treated rats (Figure 7). There was an increase in superior mesenteric flow (significant at 0.5 min) followed by a decrease (significant at 1–30 min); these effects were not different from those in vehicle-treated rats. The reduction in hindquarters flow (significant at 2–30 min) was smaller than in vehicle-treated rats (Figure 7). Hence, there were constrictions in the renal (significant at 3–30 min) and superior mesenteric vascular beds (significant at 1–30 min), but the superior mesenteric vasoconstriction was preceded by a vasodilatation (significant at 0.5 min) (Figure 8). These responses were not significantly different from those in vehicle-treated rats. However, there was a constriction in the hindqarters vascular bed (significant at 2–30 min) which was significantly smaller than that in vehicle-treated rats (Figure 8).

Discussion

The present work has shown that i.v. administration of the neuropeptide, neurotensin, can have substantial haemodynamic effects in conscious Long Evans rats. As noted in Table 2, NT caused a slight but significant pressor effect and a dose-related tachycardia which was preceded, at the higher dose of NT (1000 ng), by a transient bradycardia. These changes were accompanied by dose-related reductions in renal, superior mesenteric and hindquarters flows and dose-related constrictions in these 3 vascular beds (Table 2).

Pressor effects of neurotensin

The finding of a pressor response to NT contrasts with previous studies carried out in pentobarbitone-anaesthetized rats, showing that NT produced hypotensive effects, probably through a histamine-mediated mechanism, and possibly involving other endogenous vasoactive substances (Quirion *et al.*, 1980). Since pentobarbitone anaesthesia reduces sympathetic nerve activity (Holmes & Scheider, 1973), it is feasible that this would inhibit any pressor effects of NT mediated through the sympathetic nervous system. However, in a study carried out in conscious Sprague-Dawley rats, hypotensive effects were reported following injection of NT (0.1–2.0 $\mu g\,kg^{-1}$) (Sumners *et al.*, 1982).

Although a strain-dependent difference in sensitivity to NT could explain the discrepancy between these results and ours, the results in Table 2 show that, in spite of substantial dose-related vasoconstrictor responses in the renal, superior mesenteric and hindquarters vascular beds, the increase in blood pressure was small and not dose-related. One explanation of

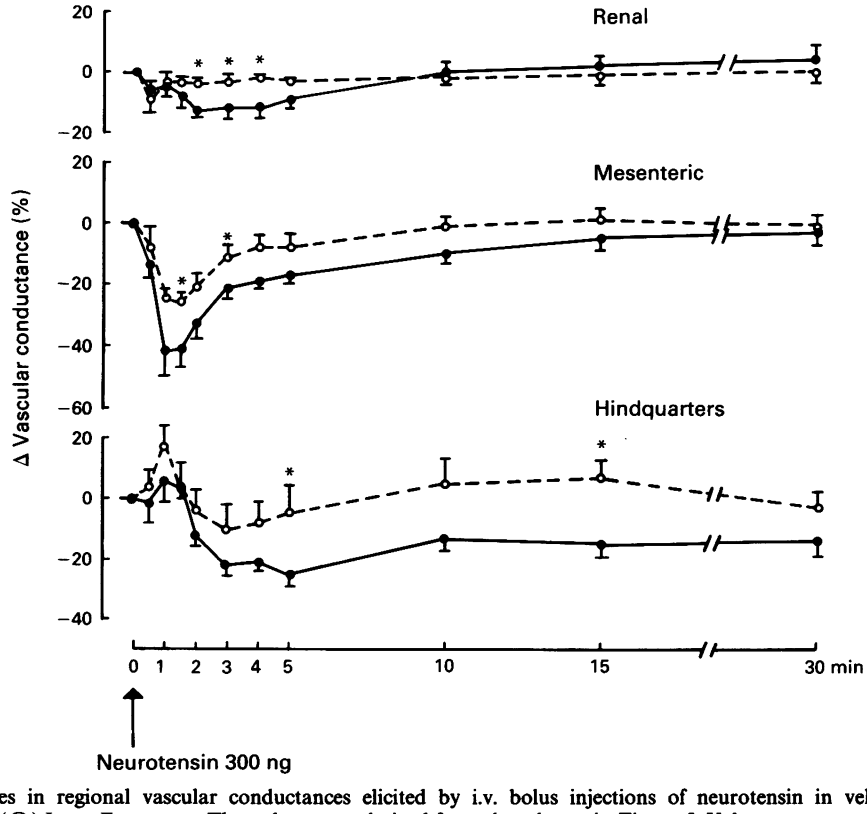


Figure 6 Changes in regional vascular conductances elicited by i.v. bolus injections of neurotensin in vehicle-treated (●) or capsaicin-treated (○) Long Evans rats. These data were derived from that shown in Figure 5. Values are mean with s.e.mean shown by vertical lines. $*P < 0.05$ for changes in vehicle-treated group ($n = 9$) versus changes in capsaicin-treated group ($n = 9$) (analysis of variance followed by Fisher's test).

these findings could be that NT caused increases in conductance in other vascular beds through release of endogenous vasodilator substances (Chahl, 1979; Chahl & Walker, 1981; Carraway *et al.*, 1982; Kerouac *et al.*, 1982). Such putative vasodilator effects could counterbalance the vasoconstrictor effects of NT and obscure any dose-relatedness of the pressor influence of the peptide. However, it is also feasible that NT reduced cardiac output and hence prevented any larger pressor effect resulting from the vasoconstriction.

In the presence of both propranolol and phentolamine, the pressor response to NT was similar to that seen in the unblocked state (although the haemodynamic profile was slightly different) (Figures 3 and 4). Hence, the hypotensive effect of NT in the presence of phentolamine alone (Figure 1) was probably due to an enhancement of a β-adrenoceptor-mediated vasodilator response (phentolamine causing both antagonism of prejunctional α-adrenoceptor autoinhibitory effects and suppression of postjunctional α-adrenoceptor vasoconstrictor influences). A similar observation has been made following bombesin administration in conscious Long Evans rats (Janssen *et al.*, 1991). As in that study we elected to use phentolamine and propranolol, rather than selective α_1-, α_2-, β_1- and β_2-adrenoceptor antagonists, since it was our intention simply to obtain evidence for the involvement of α- or β-adrenoceptors in the responses to neurotensin. Moreover, in our experience selective α_1-, α_2-, or β_2-adrenoceptor antagonists can, themselves, produce complex haemodynamic changes in conscious rats (Gardiner & Bennett, 1988).

Heart rate effects of neurotensin

Phentolamine reduced the tachycardia and converted the pressor effect of NT into a depressor effect (Figure 1). The latter effect would have been expected to cause a greater baroreflex-mediated tachycardia, but it is possible that the increased heart rate in the presence of phentolamine was responsible for the reduced tachycardic response to NT. On the other hand, it is also possible that cardiac α-adrenoceptors (Bennett & Kemp, 1978; Flavahan & McGrath, 1982; Tung *et al.*, 1985) were involved in the tachycardic response to NT.

Propranolol together with phentolamine had no effect on the bradycardia produced in response to NT, but abolished the tachycardia (Figure 3). Therefore, it is likely that neurally released and/or circulating catecholamines were involved in the tachycardic response to NT through activation of β- and, possibly, α-adrenoceptor-mediated mechanisms. The bradycardic response to NT might have been mediated by activation of cardiac vagal cholinergic neurones (or nerve terminals). This interpretation is supported by previous studies showing that, in guinea-pig isolated hearts, the tachycardia elicited by NT was sometimes preceded by transient bardycardia. The incidence and amplitude of the bradycardia were increased by neostigmine, but reduced by atropine, suggesting that receptors for NT exist on terminal branches of the vagus nerves, at least in guinea-pig hearts (Bachelard *et al.*, 1985). In the present work it is also feasible that NT induced a bradycardia through a vagovagal reflex (see later).

Haemodynamic effects of neurotensin in the hindquarters

In response to NT alone, the hindquarters vascular bed showed a vasoconstriction associated with a reduction in flow (Figures 1 and 2 and Table 2). In the presence of phentolamine, both responses were reduced (Figures 1 and 2). However, in the additional presence of propranolol the hindquarters vasoconstrictor response to NT, as well as the reduction in blood flow, were not different from those seen in untreated rats. Collectively, these results suggest that part of the hindquarters vasoconstrictor response to NT was due to activation of postjunctional α-adrenoceptor-mediated mechanisms and,

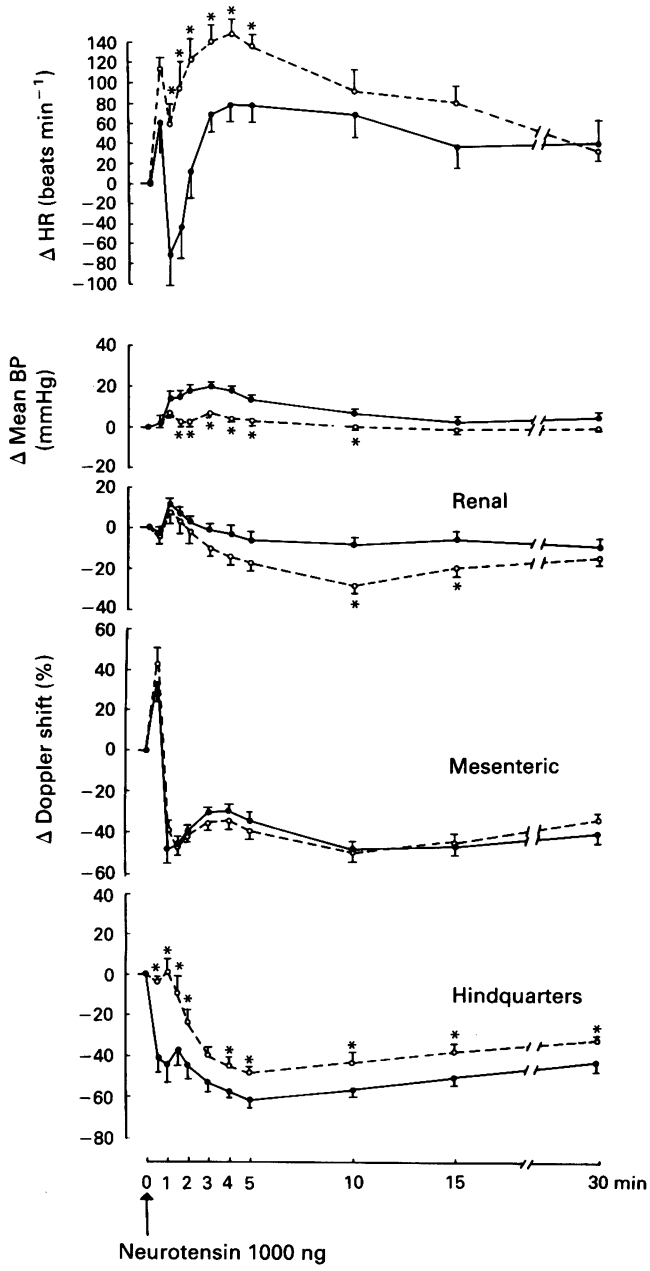


Figure 7 Cardiovascular responses to i.v. bolus injections of neurotensin in vehicle-treated (●) or capsaicin-treated (○) Long Evans rats. Values are mean with s.e.mean shown by vertical lines. *$P < 0.05$ for changes in vehicle-treated group ($n = 9$) versus changes in capsaicin-treated group ($n = 9$) (analysis of variance followed by a Fisher's test). HR = heart rate; BP = blood pressure.

following blockade of these, and prejunctional autoinhibitory α-adrenoceptors, β-adrenoceptor-mediated hindquarters vasodilator mechanisms were augmented and contributed to the attenuation of the constrictor and pressor responses to NT seen in presence of phentolamine. Since in the additional presence of propranolol the hindquarters vasoconstrictor effect of NT was not different from that seen in untreated rats it is feasible that, in the presence of phentolamine alone, any non-adrenergic vasoconstrictor effects of NT were masked by β-adrenoceptor-mediated vasodilatation (Gardiner *et al.*, 1991).

A possible candidate for mediating non-adrenergic vasoconstrictor effects of NT is neuropeptide Y. This peptide has been shown to cause a pressor effect associated with vasoconstrictions in the renal, superior mesenteric and hindquarters vascular beds of conscious rats (Gardiner *et al.*, 1988). Moreover, neuropeptide Y has been shown to be co-localized with noradrenaline in postganglionic sympathetic neurones (Pernow, 1988). Therefore, it is possible that stimulation of afferent nerves by NT (see later) causes reflex activation of the sympathetic nervous system, and induces the release of noradrenaline together with neuropeptide Y. However, further studies are required to substantiate this hypothesis.

Renal haemodynamic effects of neurotensin

In the renal vascular bed, phentolamine alone caused a reduction of the constrictor effect of NT, which suggests an involvement of α-adrenoceptor mechanisms in the renal vasoconstriction following NT alone. Furthermore, the finding that in the presence of phentolamine and propranolol the renal vasoconstrictor response to NT was less than in untreated rats could indicate an involvement of the renin angiotensin system in the response seen in the latter. Thus, if NT caused sympathoadrenal activation and consequent β-mediated stimulation of renin release, this would increase circulating levels of angiotensin II. Angiotensin II has been shown to cause a pressor response associated with vasoconstrictions in the renal and superior mesenteric vascular beds in conscious rats (Gardiner *et al.*, 1988). Hence, propranolol, by inhibiting renin release, would attenuate these effects.

In the presence of phentolamine alone, the renal vasoconstrictor effect of NT was preceded by a slight vasodilatation which was propranolol-resistant and absent in untreated rats. It is feasible that this was due to an indirect effect of NT mediated through nonadrenergic-noncholinergic mechanisms, possibly involving the release of an endogenous vasodilator substance, such as histamine. However, further studies are required to substantiate this possibility.

Superior mesenteric haemodynamic effects of neurotensin

In the presence of phentolamine alone, the superior mesenteric vasoconstrictor effect of NT was reduced. Therefore, the constrictor effect of NT in untreated rats was probably partially mediated through α-adrenoceptors. However, in the presence of phentolamine and propranolol the constrictor response to NT was not different from that seen in the untreated state. This might indicate that, in the presence of phentolamine alone, β-adrenoceptor-mediated vasodilatation (as discussed earlier) could have masked any nonadrenergic vasoconstrictor effects of NT.

In the presence of phentolamine alone, the superior mesenteric vasoconstrictor response to NT was preceded by a vasodilatation which was propranolol-resistant and absent in untreated animals. As discussed above, it is feasible that this was due to NT causing the release of some endogenous vasodilator substance (Chahl, 1979; Chahl & Walker, 1981; Carraway *et al.*, 1982; Kerouac *et al.*, 1982).

Haemodynamic effects of neurotensin in capsaicin-treated rats

Previous studies have demonstrated that some effects of NT are profoundly altered following treatment of animals (guinea-pig) or isolated preparations (heart or atria of guinea-pigs) with the neurotoxin, capsaicin (Rioux *et al.*, 1986; 1988; Bachelard *et al.*, 1987). In the present work we found that, in rats neonatally treated with capsaicin, the pressor response to NT (300 and 1000 ng) was reduced, as were the constrictor responses in the renal (at the dose of 300 ng), superior mesenteric (at a dose of 300 ng) and hindquarters (at both doses) vascular beds. It is likely that the reduced vasoconstrictor effects were responsible for the decrease in the pressor response to NT. Moreover, in capsaicin-treated animals the bradycardic response elicited by the higher dose of NT was absent whereas the tachycardia was potentiated. Together

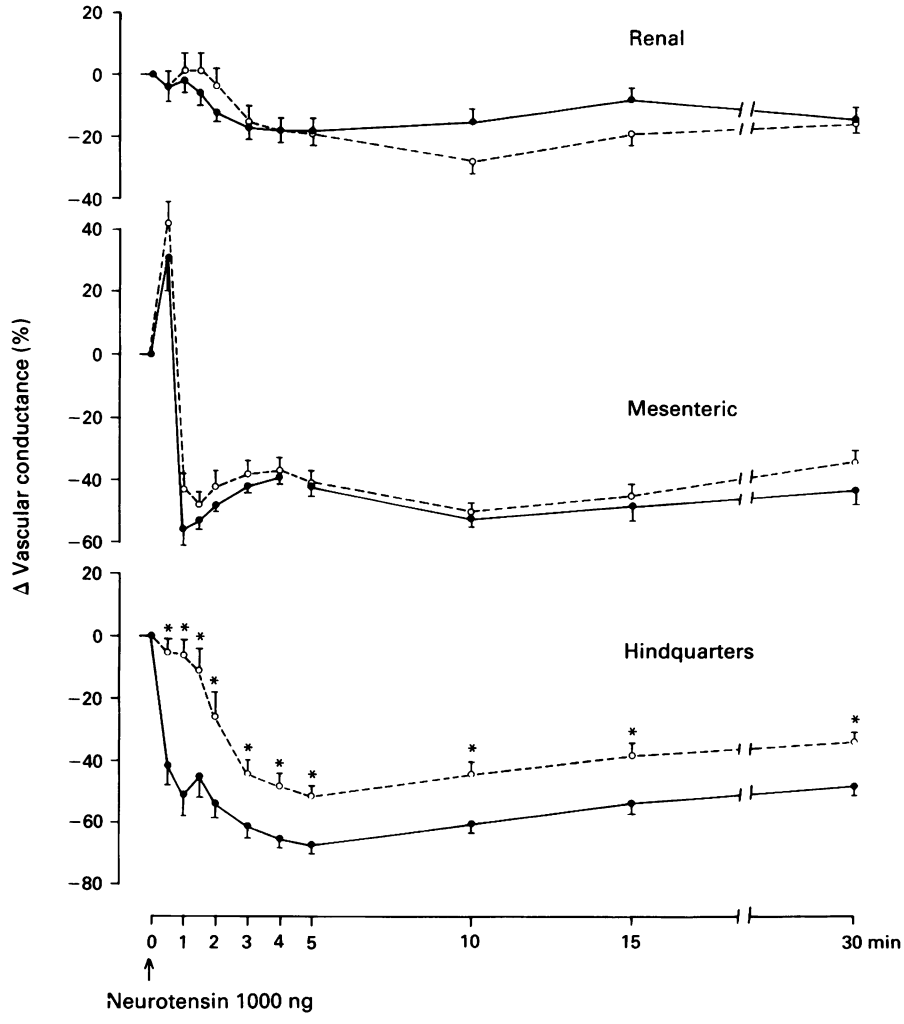


Figure 8 Changes in regional vascular conductances elicited by i.v. bolus injections of neurotensin in vehicle-treated (●) or capsaicin-treated (○) Long Evans rats. These data were derived from those shown in Figure 7. Values are mean with s.e.mean shown by vertical lines. *$P < 0.05$ for changes in vehicle-treated group ($n = 9$) versus changes in capsaicin-treated group ($n = 9$) (analysis of variance followed by Fisher's test).

these results suggest an involvement of capsaicin-sensitive neurones in the cardiovascular responses to NT.

Other studies have demonstrated that NT has the ability to stimulate capsaicin-sensitive primary afferents and to trigger cardiovascular reflexes following its i.p. administration or topical application to the surface of the heart in anaesthetized guinea-pigs (Rioux *et al.*, 1986; 1990). Thus, it is feasible that activation of capsaicin-sensitive neurones contributed to the pressor and vasoconstrictor effects of NT in normal animals. For example, activation of capsaicin-sensitive primary afferents by NT might lead to reflex excitation of the efferent vasomotor outflows to renal, superior mesenteric and hindquarters vascular beds.

As far as the bradycardic response to NT is concerned, a likely explanation is that this effect was due to a reflex activation of the parasympathetic nervous system in control animals. Neonatal treatment with capsaicin has been shown to induce degeneration of at least a portion of the vagal afferent cell bodies of the nodose ganglion and of the terminal projections in the nucleus of the solitary tract (Helke & Eskay, 1985) which is the central nervous system site of termination of most vagal afferents. Thus, one would expect that if NT produced its bradycardic effect through an activation of capsaicin-sensitive afferents, neonatal treatment with capsaicin might so alter the heart rate response.

Conclusion

The present results indicate that NT exerts influences on the cardiovascular system in conscious rats through sympathoadrenal mechanisms and possibly also through direct and/or indirect non-adrenergic effects on the heart, and on the renal, superior mesenteric and hindquarters vascular beds. The findings are consistent with an involvement of neuropeptide-containing, capsaicin-sensitive sensory neurones in part of the cardiovascular response to NT, in conscious, unrestrained rats.

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Involvement of capsaicin-sensitive neurones in the haemodynamic effects of exogenous vasoactive peptides: studies in conscious, adult Long Evans rats treated neonatally with capsaicin

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1 The regional haemodynamic effects of i.v. bolus injections of bradykinin (0.05 or 0.5 nmol), cholecystokinin (0.175 or 1.75 nmol), substance P (0.01 or 0.1 nmol) and calcitonin gene-related peptide (0.05 or 0.5 nmol) were assessed in conscious, adult Long Evans rats that had been treated neonatally with either capsaicin (50 mg kg^{-1}, s.c.) or vehicle.

2 In vehicle-treated rats, both doses of bradykinin were without effect on blood pressure, but caused tachycardia and hindquarters vasodilatation. Moreover, after the higher dose there were dilatations in the renal and superior mesenteric vascular beds. In capsaicin-treated rats the hindquarters vasodilator effects elicited by both doses of bradykinin were significantly reduced, while the tachycardia and responses in the renal and superior mesenteric vascular beds were unchanged.

3 In vehicle-treated rats, cholecystokinin caused dose-dependent increases in blood pressure accompanied by renal, superior mesenteric and hindquarters vasoconstriction followed, after the higher dose, by a hindquarters vasodilatation. The lower dose produced a tachycardia, while there was a bradycardia followed by a tachycardia after the higher dose. In capsaicin-treated rats, the pressor response, as well as the renal vasoconstrictor effects of cholecystokinin, were greater than in vehicle-treated rats, while the heart rate, superior mesenteric or hindquarters responses were not different.

4 In vehicle-treated rats, substance P produced a dose-dependent depressor response and tachycardia accompanied by dilatations in the renal and hindquarters vascular beds and constriction in the superior mesenteric vascular bed. In capsaicin-treated rats, the responses to the lower dose of substance P were not different from those in vehicle-treated rats, while the depressor response to the higher dose of substance P was slightly less than in vehicle-treated rats and the renal vasodilatation was absent.

5 In vehicle-treated rats, calcitonin gene-related peptide caused dose-dependent hypotensive and tachycardic effects associated with dilatations in renal and hindquarters vascular beds and a constriction in the superior mesenteric vascular bed. After the higher dose, the renal vasodilatation was followed by a modest vasoconstriction. In capsaicin-treated rats, the depressor responses to both doses of calcitonin gene-related peptide were slightly more prolonged than in vehicle-treated animals, whereas the heart rate and renal and mesenteric vascular conductance changes were not significantly different. However, there was a more sustained hindquarters vasodilator response to the higher dose of calcitonin gene-related peptide in the capsaicin-treated rats.

6 The results suggest that peripheral, capsaicin-sensitive neurones are involved in the cardiovascular responses to exogenous bradykinin and cholecystokinin in conscious rats. It does not appear that the extent of involvement of these neurones is underestimated on account of development of marked supersensitivity to the peptides they normally release, since responses to such peptides (e.g. substance P and calcitonin gene-related peptide) are relatively normal in capsaicin-treated rats.

Keywords: Regional haemodynamics; capsaicin; blood pressure; heart rate; peptides

Introduction

Capsaicin (8 methyl-N-vanillyl-6-nonenamide), the pungent algesic substance contained in many red peppers, is a valuable pharmacological tool for studying a specific subset of primary afferent neurones (Buck & Burks, 1986; Maggi & Meli, 1988). It is now well documented that some unmyelinated primary afferent neurones sensitive to capsaicin contain a variety of peptides, including substance P (SP) and calcitonin gene-related peptide (CGRP) (Skofitsch & Jacobowitz, 1985; Buck & Burks, 1986; Franco-Cereceda *et al.*, 1987), which have potent cardiovascular effects (Pernow, 1983; Sigrist *et al.*, 1986; Dipette *et al.*, 1987; Lappe *et al.*, 1987; Gardiner *et al.*, 1988; 1989a,b,c). In several animal species, it has been demonstrated that the peripheral endings of capsaicin-sensitive, primary afferents are widely distributed throughout peripheral tissues, usually in association with blood vessels (Furness *et al.*, 1982; Barja *et al.*, 1983; Rozsa *et al.*, 1984; Lundberg *et al.*, 1985; Uddmann *et al.*, 1986; Wharton *et al.*, 1986; Wharton & Gulbenkian, 1987; Martling *et al.*, 1988; Hottenstein *et al.*, 1991). This observation raises the possibility that these neurones may be involved with the regulation of vascular tone.

There is evidence indicating that the neuropeptides contained in the capsaicin-sensitive primary afferent neurones can be released in response to stimuli, such as the nonapeptide, bradykinin (BK), and that other peptides, such as cholecystokinin (CCK), cause activation of capsaicin-sensitive afferents (Geppetti *et al.*, 1988; 1990; McCann *et al.*, 1988; Manzini *et al.*, 1989). Hence, release of the neuropeptides contained in these afferent fibres could contribute, at least partly, to some of the *in vivo* effects of BK and CCK.

Capsaicin given to newborn rats is known to cause permanent and selective degeneration of up to 90% of peripheral unmyelinated primary sensory neurones (Nagy *et al.*, 1981). Parallel to the destruction of these unmyelinated fibres it has

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been shown by radioimmunoassay that the levels of SP and CGRP are substantially, and irreversibly, reduced in regions of the peripheral and central nervous system normally containing projections of primary sensory neurones (Gamse *et al.*, 1980; Cuello *et al.*, 1981; Nagy *et al.*, 1981; Skofitsch & Jacobowitz, 1985; Hammon & Ruda, 1989). Therefore, mechanisms dependent on the integrity of these sensory fibres, and possibly mediated by local release of the associated neuropeptides, might be significantly affected following this treatment.

The purpose of the present study was to assess the influence of capsaicin-sensitive primary afferent neurones on the regional haemodynamic effects of BK and CCK by comparing responses to these peptides in control rats and in rats treated neonatally with capsaicin. However, since degeneration of the neuropeptide-containing, capsaicin-sensitive primary afferents could alter sensitivity to neuropeptides contained in these fibres (for instance, SP and CGRP), the cardiovascular responses to exogenous SP and CGRP were also evaluated in the same control rats and rats treated neonatally with capsaicin that were challenged with BK and CCK.

Methods

Long Evans rats were anaesthetized with 1.5–2% halothane (in oxygen) on day 2 after birth and injected subcutaneously with either vehicle (10% ethanol, 10% Tween 80 in 0.9% w/v NaCl solution) or capsaicin (50 mg kg^{-1}) in a volume of 100 μl. After weaning (day 21–30 after birth) male rats were selected and housed in groups of 4 or 5 with free access to food and tap water.

At least 20 weeks after birth, rats (350–400 g) were anaesthetized with sodium methohexitone (Lilly, 60 mg kg^{-1}, i.p., supplemented as required) and had pulsed Doppler flow probes implanted as previously described (Haywood *et al.*, 1981; Gardiner *et al.*, 1988; 1990b,c). After operation, animals were given ampicillin trihydrate (Penbritin, Beecham, 7 mg kg^{-1}, i.m.) and housed singly in cages with free access to food and water.

At least 7 days after this operation, the animals that were healthy (i.e. gaining weight and feeding, drinking and grooming normally) were re-anaesthetized (sodium methohexitone; 40 mg kg^{-1}, i.p., with supplemental doses). The probe wires were soldered into a microconnector and the quality of the signals from the implanted probes was checked via a pulsed Doppler monitoring system (VF-1 mainframe, Crystal Biotech), modified to operate with a pulse repetition frequency of 125 kHz (Gardiner *et al.*, 1990a). Any animal in which good signals (signal: noise ratio > 20:1) were not obtained from all 3 probes was rejected from the study. Animals that met this criterion had 3 i.v. catheters (right jugular) and 1 intra-arterial catheter (distal abdominal aorta, via caudal artery) implanted. We used 3 i.v. catheters so the peptides could be injected through a fresh catheter on each experimental day (see below). The catheters ran subcutaneously and emerged at the same point as the pulsed Doppler probe leads. A microconnector (Microtech Inc. USA), soldered to the probe leads, was clamped in a harness worn by the rat and the catheters passed through a flexible spring (for protection) attached to the harness. Animals were left until the next day before experiments were begun.

Throughout the experiment, recordings were made of heart rate, phasic and mean blood pressures and renal, superior mesenteric and hindquarters Doppler shift signals. The phasic Doppler shift signals were monitored to ensure their acceptability (Gardiner *et al.*, 1990b,c). Heart rate, mean blood pressure and mean Dopple shifts were averaged (by eye) over 10 s at time points selected to represent the full profile of the effects of the peptide. Changes (absolute changes for the former two variables, percentages for the Doppler shifts) and vascular conductances were calculated relative to the pretreatment baseline value. Before every experiment baseline measurements were made over a period of 30 min. Animals (fully conscious and unrestrained) were allowed free access to water and food for the duration of the experiment.

Two groups of animals (vehicle-treated, $n = 9$ and capsaicin-treated, $n = 9$) were used on 3 consecutive days, during which they received i.v. injections of peptide vehicle (0.1 ml isotonic saline containing 1% bovine serum albumin (BSA)), bradykinin (BK) (0.05 or 0.5 nmol), substance P (SP) (0.01 or 0.1 nmol), calcitonin gene-related peptide (CGRP) (0.05 or 0.5 nmol) and cholecystokinin (CCK) (0.175 or 1.75 nmol), in random order, although low doses were always given before high doses. Injections of low doses of peptides were separated by at least 60 min; the intervals separating the high doses were sufficient to allow variables to return to baseline (up to 2 h). Measurements were made before, during and for 15 min following i.v. injections of vehicle or peptides. Since the results obtained with the two doses of each peptide were consistent, only the data relating to the effects of the higher doses are given in the Results.

Drugs and peptides

Substance P (Bachem), rat α-calcitonin gene-related peptide (Bachem), bradykinin (Bachem), cholecystokinin (26–33), sulphated (Bachem), and capsaicin (Sigma) were used. Peptides were dissolved in isotonic saline containing 1% BSA. All i.v. injections were given as 100 μl boluses which were flushed in with 100 μl isotonic saline (the dead spaces of the catheters).

Statistical analysis

Values are expressed as the mean ± s.e.mean; n is the number of animals. Results were analysed for statistical significance by a paired Student's t test or an analysis of variance (ANOVA) followed by Fisher's test. The paired Student's t test was used to evaluate the responses to injection of saline-BSA, while the ANOVA was used to compare the responses to peptide injections with those to saline-BSA injections, or to compare the responses to peptide injections in vehicle-treated and capsaicin-treated rats. In the text the word significant means $P < 0.05$ (at least).

Results

The baseline values for cardiovascular variables in vehicle- and capsaicin-treated rats are given in Table 1. The resting values in capsaicin-treated rats were not different from those in vehicle-treated animals. The i.v. injection of saline-BSA in vehicle-treated rats had no effect on mean arterial blood pressure, but caused a slight, but significant, increase in heart rate and hindquarters flow, while there were no significant changes in renal or superior mesenteric flows (Table 2). Hence, there was a dilatation of the hindquarters vascular bed but no significant effect on renal or superior mesenteric vascular conductances (Table 2).

Table 1 Baseline values for heart rate, blood pressure and regional vascular conductance in vehicle- or capsaicin-treated Long Evans rats

Treatment	*Heart rate* (beats min^{-1})	*Mean blood pressure* (mmHg)	*Doppler shift* (kHz) *Renal*	*Mesenteric*	*Hindquarters*	*Vascular conductance* ([kHz mmHg^{-1}] 10^3) *Renal*	*Mesenteric*	*Hindquarters*
Vehicle	381 ± 10	103 ± 3	10.0 ± 1.5	8.8 ± 1.1	5.0 ± 0.4	96 ± 13	87 ± 11	50 ± 5
Capsaicin	354 ± 17	109 ± 5	10.3 ± 1.4	7.9 ± 0.4	5.2 ± 0.6	94 ± 11	73 ± 5	50 ± 7

Values are mean ± s.e.mean; $n = 9$ for both groups.

Table 2 Peak cardiovascular changes following intravenous administration of saline-bovine serum albumin (1%) or peptides in vehicle-treated (Veh) or capsaicin-treated (Cap) Long Evans rats

		Δ *Heart rate* (beats min^{-1})	Δ *Mean blood pressure* (mmHg)	Δ *Doppler shift* (%)			Δ *Vascular conductance* (%)		
				Renal	*Mesenteric*	*Hindquarters*	*Renal*	*Mesenteric*	*Hindquarters*
Veh	Saline-BSA (1%)	+29 ± 10†	0 ± 3	+1 ± 1	−4 ± 5	+23 ± 6†	+1 ± 2	−5 ± 4	+24 ± 8†
Cap	Saline-BSA (1%)	+10 ± 7	+2 ± 1	−1 ± 2	−1 ± 3	+18 ± 6†	−3 ± 1	−3 ± 3	+16 ± 7
Veh	BK (0.5 nmol)	+98 ± 11*	−10 ± 8	+8 ± 4	+28 ± 11*	+49 ± 18*	+25 ± 11* −8 ± 1*	+44 ± 12* −7 ± 3*	+72 ± 15*
Cap	BK (0.5 nmol)	+98 ± 9*	+4 ± 7	+12 ± 3*	+14 ± 8*	+35 ± 10	+10 ± 7 −4 ± 2	+46 ± 14*	+30 ± 8#
Veh	CCK (1.75 nmol)	−60 ± 17* +51 ± 15*	+32 ± 2*	+9 ± 3*	−57 ± 3*	+42 ± 11*	−27 ± 3*	−67 ± 2*	−10 ± 14* +34 ± 7*
Cap	CCK (1.75 nmol)	−55 ± 19* +53 ± 8*	+45 ± 3*#	−10 ± 6	−62 ± 2*	+43 ± 6*	−38 ± 3*#	−74 ± 1*	−14 ± 13* +31 ± 6*
Veh	SP (0.1 nmol)	+128 ± 12*	−28 ± 2*	−13 ± 2*	−50 ± 4*	+73 ± 9*	+21 ± 5*	−30 ± 5* +14 ± 5*	+140 ± 12*
Cap	SP (0.1 nmol)	+133 ± 9*	−21 ± 2*#	−16 ± 4*	−51 ± 2*	+74 ± 9*	+6 ± 5#	−38 ± 4* +13 ± 7	+119 ± 10*
Veh	CGRP (0.5 nmol)	+157 ± 15*	−34 ± 2*	−19 ± 5*	−40 ± 7*	+38 ± 15	+65 ± 11* −13 ± 2*	+16 ± 18 −27 ± 9*	+100 ± 22*
Cap	CGRP (0.5 nmol)	+134 ± 13*	−37 ± 2*	−33 ± 3*#	−37 ± 4*	+34 ± 5	+43 ± 9* −18 ± 3*	+18 ± 6* −16 ± 3*	+99 ± 9*

Values are mean ± s.e.mean, $n = 9$ in both groups. BK = bradykinin, CCK = cholecystokinin, SP = substance P, CGRP = calcitonin gene-related peptide.
† $P < 0.05$ versus baseline, paired Student's t test for injection of saline-bovine serum albumin (saline-BSA). * $P < 0.05$ versus response to injection of saline-BSA, analysis of variance followed by Fisher's test. # $P < 0.05$ versus vehicle-treated responses, analysis of variance followed by Fisher's test. Some responses were biphasic; in those cases the first value represents the initial change and below is the later change.

In the capsaicin-treated rats the injection of saline-BSA 1% had no effect on blood pressure, heart rate or renal or superior mesenteric flows but caused a slight increase in hindquarters flow (Table 2). There were no significant changes in renal, superior mesenteric or hindquarters vascular conductances (Table 2). The cardiovascular responses following injection of saline-BSA in capsaicin-treated rats were not significantly different from those in vehicle-treated animals (Table 2).

Haemodynamic effects of bradykinin

Vehicle-treated rats BK (0.5 nmol) had no significant effect on blood pressure, but produced tachycardia (significant at 0.5–1 min). There were increases in superior mesenteric (significant at 0.5 min) and hindquarters flow (significant at 0.5–1 min), but no significant change in renal flow (Figure 1, Table 2). These responses were associated with initial increases in renal and superior mesenteric vascular conductances followed by modest decreases (Table 2). There was only vasodilatation in the hindquarters vascular bed (Table 2).

Capsaicin-treated rats BK (0.5 nmol) had no significant effect on blood pressure, but produced tachycardia (significant at 0.5–1 min) that was not different from that in vehicle-treated rats (Figure 1, Table 2). There was a modest increase in renal flow (significant at 0.5–1 min) and a marked increase in superior mesenteric flow (significant at 0.5 min). These responses were not significantly different from those seen in vehicle-treated rats. The increase in hindquarters flow was not different from that in vehicle-treated rats, or from the response following control injection of saline-BSA (Figure 1, Table 2). There was no significant change in renal vascular conductance, whereas the superior mesenteric vascular bed showed a dilatation (Table 2). The latter was not followed by a constriction, in contrast to that seen in vehicle-treated rats, but these changes were not significantly different from those in vehicle-treated rats (Table 2). There was an increase in hindquarters vascular conductance which was significantly less than the response evoked in vehicle-treated rats and not different from the response to control injection of saline-BSA (Table 2).

Haemodynamic effects of cholecystokinin

Vehicle-treated rats CCK (1.75 nmol) produced an increase in blood pressure (significant at 0.5–2 min) accompanied by an initial bradycardia (significant at 0.5–1 min) followed by a tachycardia (significant at 3–5 min) (Figure 2, Table 2). Renal and hindquarters flows increased (renal, significant at 2–3 min; hindquarters, significant at 1–4 min), while a decrease in superior mesenteric flow occurred (significant at 0.5–3 min) (Figure 2, Table 2). There were constrictions in renal (significant at 0.5 min) and superior mesenteric (significant at 0.5–3 min) vascular beds (Figure 3, Table 2). There was a modest constriction in the hindquarters vascular bed (significant at 0.5 min), followed by a dilatation (significant at 1–5 min) (Figure 3, Table 2).

Capsaicin-treated rats CCK (1.75 nmol) caused a pressor response (significant at 0.5–4 min) that was slightly greater, and an initial bradycardia (significant at 0.5–1 min) followed by a tachycardia (significant at 3–15 min), which were not different, from those seen in vehicle-treated rats (Figure 2, Table 2). There was no significant change in renal flow, while a reduction in superior mesenteric flow (significant at 0.5–3 min) and an increase in hindquarters flow (significant at 1–4 min) occurred (Figure 2, Table 2). These responses were not significantly different from those in vehicle-treated rats (Figure 2, Table 2). There were constrictions in renal (significant at 0.5–1 min), superior mesenteric (significant at 0.5–3 min) and hindquarters (significant at 0.5 min) vascular beds, followed by a dilatation in the hindquarters vascular bed (significant at 2–3 min) (Figure 3, Table 2). The response in the renal vascular bed was greater, while the responses in the two other vascular beds were not different from those in vehicle-treated rats (Figure 3, Table 2).

Haemodynamic effects of substance P

Vehicle-treated rats SP (0.1 nmol) produced a decrease in blood pressure (significant at 0.5–1 min) and a marked tachycardia (significant at 0.5–2 min) accompanied by reductions in renal (significant at 0.5 min) and superior mesenteric (significant at 0.5 min) flows and a substantial increase in hindquarters flow (significant at 0.5 min) (Figure 4, Table 2).

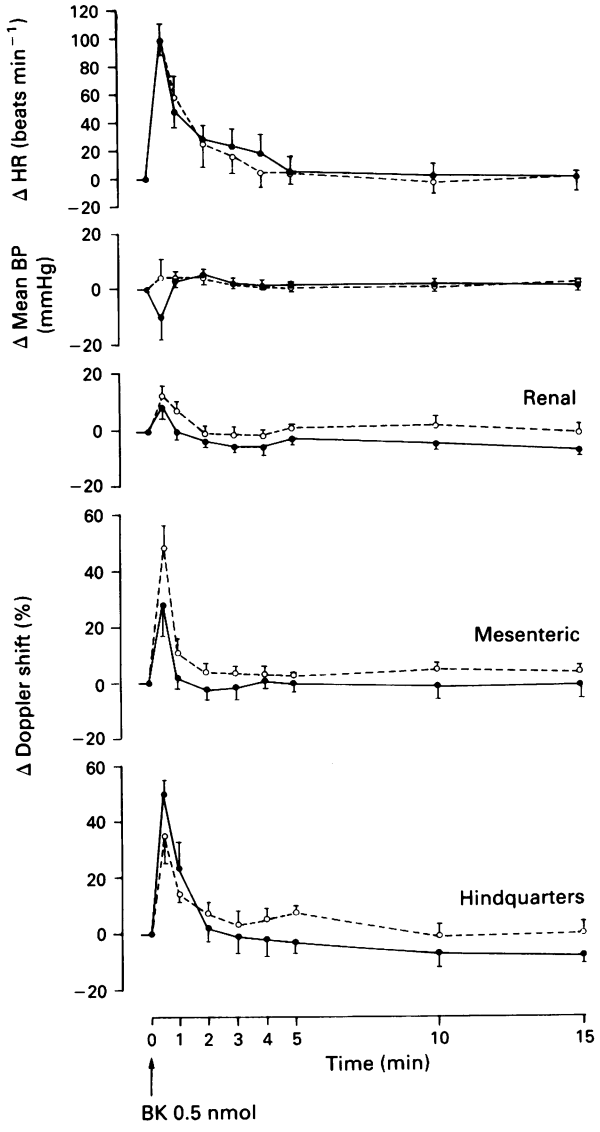


Figure 1 Cardiovascular responses to i.v. bolus injections of bradykinin (BK, 0.5 nmol) in vehicle- (●) or capsaicin-treated (○) Long Evans rats. Values are mean with s.e.mean shown by vertical lines. * $P < 0.05$ vehicle-treated group ($n = 9$) versus capsaicin-treated group ($n = 9$), (analysis of variance followed by Fisher's test). HR = heart rate; BP = blood pressure.

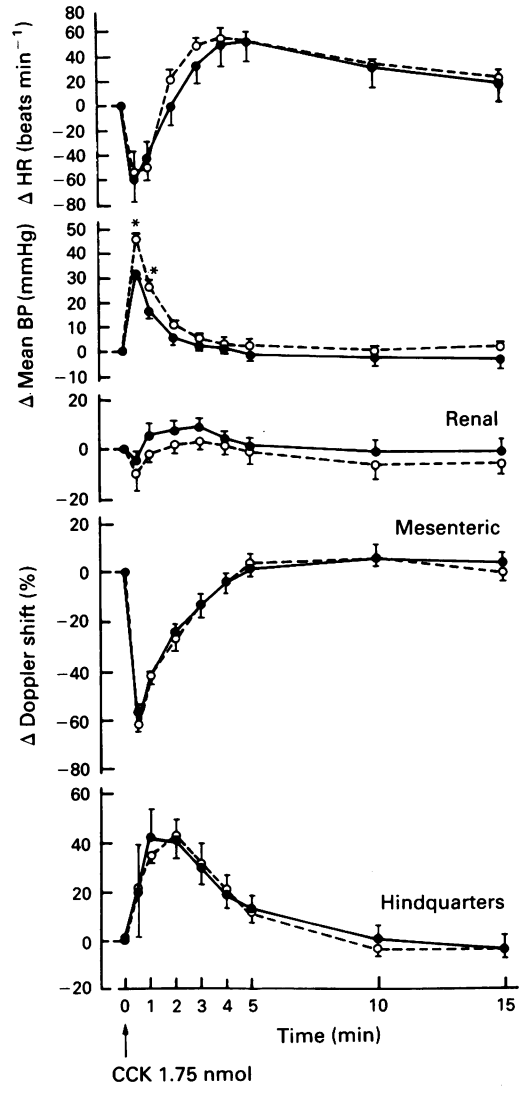


Figure 2 Cardiovascular responses to i.v. bolus injections of cholecystokinin (CCK, 1.75 nmol) in vehicle- (●) or capsaicin-treated (○) Long Evans rats. Values are mean with s.e.mean shown by vertical lines. * $P < 0.5$ vehicle-treated group ($n = 9$) versus capsaicin-treated group ($n = 9$), (analysis of variance followed by Fisher's test). HR = heart rate; BP = blood pressure.

These responses were associated with a moderate renal dilatation (significant at 0.5 min) but a marked dilatation in the hindquarters vascular bed (significant at 0.5 min) (Figure 5, Table 2). The superior mesenteric vascular bed showed an initial constriction (significant at 0.5 min) followed immediately by a dilatation (significant at 1 min) (Figure 5, Table 2).

Capsaicin-treated rats SP (0.1 nmol) caused a decrease in blood pressure (significant at 0.5 min) which was slightly, but significantly, less and a tachycardia (significant at 0.5–2 min), which was not different, from that in vehicle-treated rats (Figure 4, Table 2). There were reductions in renal (significant at 0.5 min) and superior mesenteric (significant at 0.5 min) flows and a substantial increase in hindquarters flow (significant at 0.5 min); these were not different from the responses seen in vehicle-treated animals (Figure 4, Table 2). The renal vasodilatation observed in vehicle-treated animals was absent, although there was a substantial dilatation in the hindquarters vascular bed (significant at 0.5 min) which was not different from that in vehicle-treated animals. As in vehicle-treated animals, the superior mesenteric vascular bed also showed an initial decrease (significant at 0.5 min) followed immediately by an increase in conductance (significant at 1 min) (Figure 5, Table 2).

Haemodynamic effects of calcitonin gene-related peptide

Vehicle-treated rats CGRP (0.5 nmol) caused a decrease in blood pressure (significant at 0.5–15 min) and a marked tachycardia (significant at 0.5–15 min) accompanied by a delayed reduction in renal flow (3–15 min) and an early-onset reduction in superior mesenteric flow (significant at 1–15 min); the increase in hindquarters flow was not different from the response following control injection of saline-BSA (Figure 6, Table 2). The renal vascular bed showed an early dilatation (significant at 0.5–2 min) followed by a late constriction (significant at 10–15 min) (Figure 7, Table 2). There was a delayed constriction in the superior mesenteric vascular bed (significant at 2–4 min) and a substantial dilatation in the hindquarters vascular bed (significant at 0.5–3 and 5–15 min) (Figure 7, Table 2).

Capsaicin-treated rats CGRP (0.5 nmol) caused a decrease in blood pressure (significant at 0.5–15 min) which was more prolonged, and a tachycardia (significant at 0.5–15 min) which was not different from that in vehicle-treated animals (Figure 6, Table 2). The reduction in renal flow (significant at 3–15 min) was slightly greater, while the reduction in the

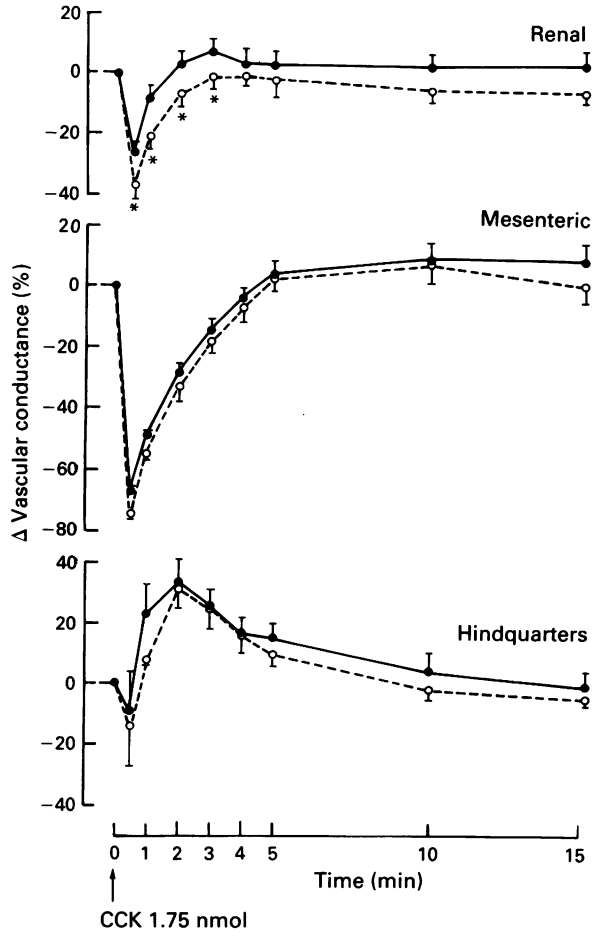


Figure 3 Changes in regional vascular conductances elicited by i.v. bolus injections of cholecystokinin (CCK, 1.75 nmol) in vehicle-treated (●) or capsaicin-treated (○) Long Evans rats. These data were derived from the data shown in Figure 2. Values are mean with s.e.mean shown by vertical lines. * $P < 0.05$ vehicle-treated group ($n = 9$) versus capsaicin-treated group ($n = 9$), (analysis of variance followed by Fisher's test).

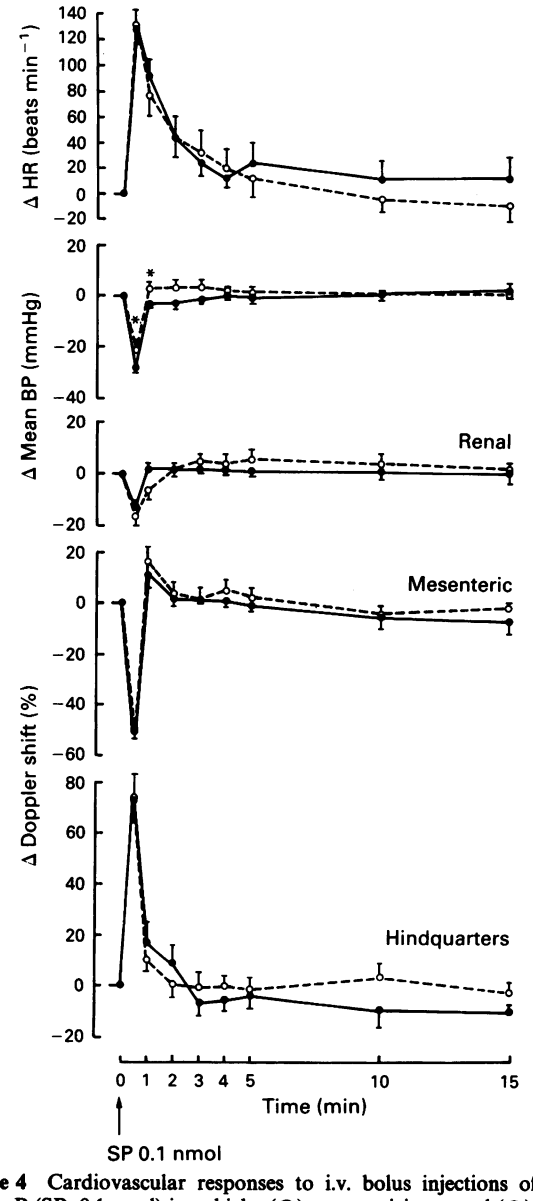


Figure 4 Cardiovascular responses to i.v. bolus injections of substance P (SP, 0.1 nmol) in vehicle- (●) or capsaicin-treated (○) Long Evans rats. Values are mean with s.e.mean shown by vertical lines. * $P < 0.05$ vehicle-treated group ($n = 9$) versus capsaicin-treated group ($n = 9$), (analysis of variance followed by Fisher's test). HR = heart rate; BP = blood pressure.

superior mesenteric flow (significant at 1–15 min) was not different from that in vehicle-treated rats (Figure 6, Table 2). The increase in hindquarters flow was not different from that in vehicle-treated rats or from the response to saline-BSA (Figure 6, Table 2). There was a dilatation followed by constriction in the superior mesenteric vascular bed (significant at 3–10 min) and a marked hindquarters dilatation (significant at 0.5–15 min) (Figure 7, Table 2). The renal vascular bed showed an early dilatation (significant at 0.5–2 min) followed by a late constriction (significant at 5–15 min). The hindquarters dilatation was more prolonged than that seen in vehicle-treated rats (Figure 7), but the renal and mesenteric responses were not different from those in vehicle-treated rats (Figure 7, Table 2).

Discussion

The first objective of the present work was to delineate the influence of neonatal treatment with capsaicin on the cardiovascular responses to exogenous BK and CCK. Since there is evidence that some of the *in vivo* effects of these peptides are attributable to stimulation, and/or sensitization of neuropeptide-containing, capsaicin-sensitive sensory neurones, we reasoned that destruction of the majority of these primary afferents, by neonatal treatment with capsaicin, ought to affect responses to BK and CCK. Our second objective was to determine if neonatal treatment with capsaicin produced any supersensitivity to CGRP and/or SP, i.e. two peptides contained in capsaicin-sensitive neurones. Although we did not make any assessments of the extent of capsaicin-induced lesions in the present study, the protocol we followed has been found to cause a selective and permanent destruction of a large percentage of the peripheral, unmyelinated fibres (Nagy *et al.*, 1981).

In capsaicin-treated rats the resting values for mean arterial pressure, heart rate, and renal, superior mesenteric and hindquarters flows and vascular conductances were not significantly different from those of control rats. These results corroborate previous findings also in conscious rats (Bennett & Gardiner, 1985a,b).

Haemodynamic effects of bradykinin

In rats treated neonatally with capsaicin the heart rate and blood pressure responses to BK were similar to those seen in vehicle-treated animals. This finding could be interpreted as indicating a lack of involvement of capsaicin-sensitive neurones in the cardiovascular responses to BK. However, examination of the regional haemodynamic changes revealed that

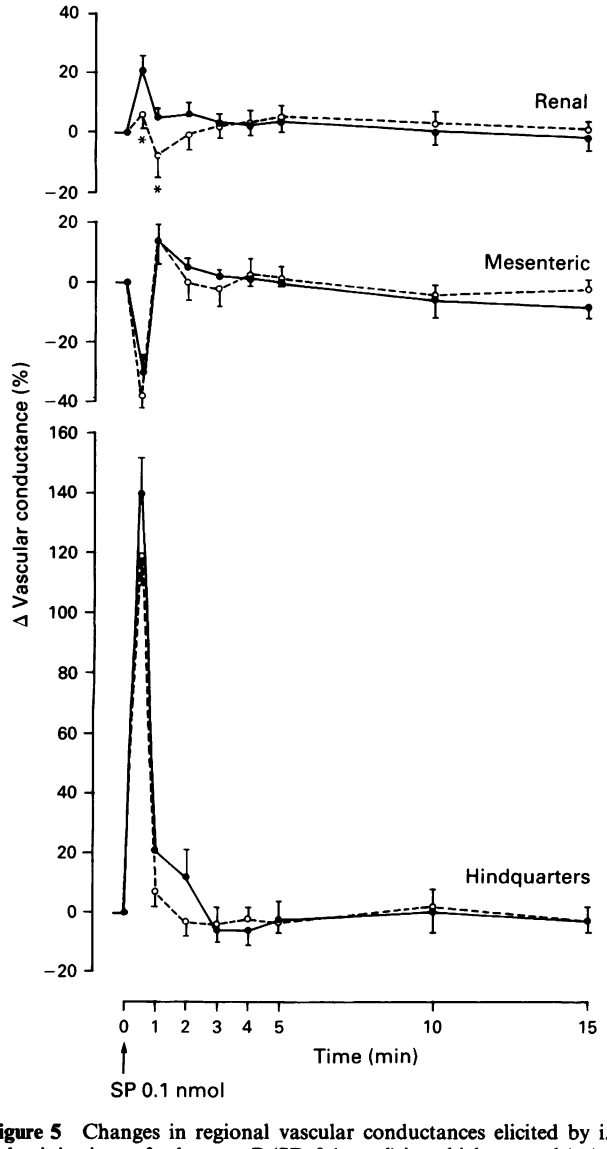


Figure 5 Changes in regional vascular conductances elicited by i.v. bolus injections of substance P (SP, 0.1 nmol) in vehicle-treated (●) or capsaicin-treated (○) Long Evans rats. These data were derived from the data shown in Figure 4. Values are mean with s.e.mean shown by vertical lines. * $P < 0.05$ vehicle-treated group ($n = 9$) versus capsaicin-treated group ($n = 9$), (analysis of variance followed by Fisher's test).

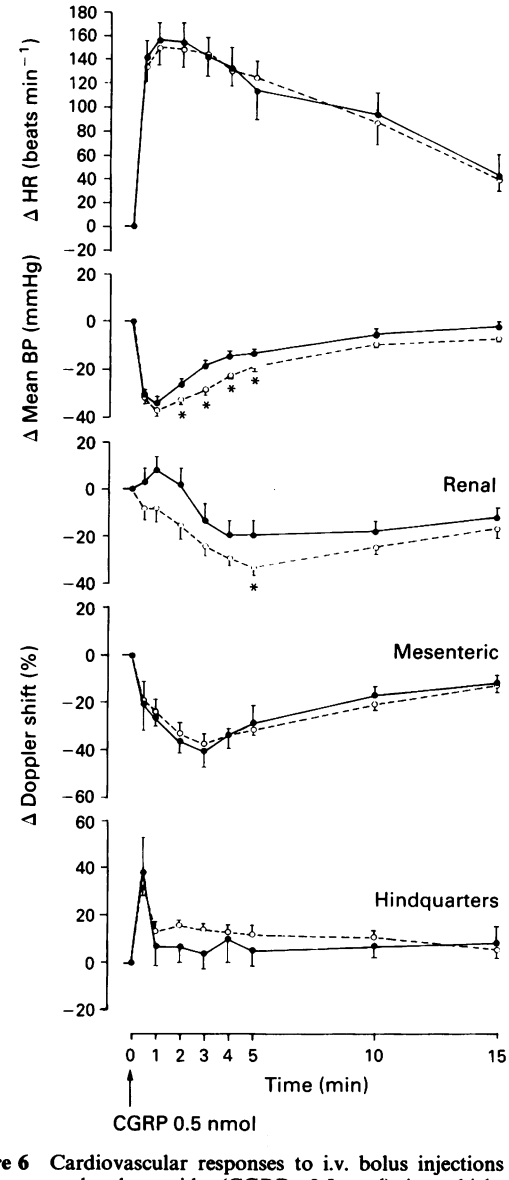


Figure 6 Cardiovascular responses to i.v. bolus injections of calcitonin gene-related peptide (CGRP, 0.5 nmol) in vehicle- (●) or capsaicin-treated (○) Long Evans rats. Values are mean with s.e.mean shown by vertical lines. * $P < 0.05$ vehicle-treated group ($n = 9$) versus capsaicin-treated group ($n = 9$), (analysis of variance followed by Fisher's test). HR = heart rate; BP = blood pressure.

the increase in hindquarters vascular conductance elicited by BK was significantly smaller in capsaicin-treated, than in vehicle-treated, rats. This finding is compatible with the hindquarters vasodilator response in vehicle-treated animals being the result of stimulation of capsaicin-sensitive nerve fibres, evoking local release of vasodilator substances (e.g. SP, CGRP) from the nerve fibres. Previous studies have demonstrated that BK can cause the release of SP and CGRP from capsaicin-sensitive sensory fibres in the heart (Geppetti *et al.*, 1988; 1990; Manzini *et al.*, 1989). However, in conscious Long Evans rats, we have found that the hindquarters vasodilator effect of BK is converted into a vasoconstriction in the presence of a β_2-adrenoceptor antagonist (Gardiner *et al.*, unpublished). Therefore, one explanation of the present results is that the hindquarters vasodilator response to BK results from stimulation of capsaicin-sensitive neurones causing the release of adrenaline from the adrenal medulla (Feldberg & Lewis, 1964; Warashina *et al.*, 1990). This interpretation is supported by the findings of previous studies indicating that capsaicin-sensitive neurones innervating the adrenal medulla are involved in modulating the neurogenic secretion of catecholamines induced by selective stressors, such as histamine, insulin, cold and capsaicin itself (Khalil *et al.*, 1984; 1987; Watanabe *et al.*, 1988; Zhou *et al.*, 1990; Zhou & Livett, 1991). While it is feasible that bradykinin could act directly on adrenal medullary chromaffin cells to release adrenaline, it is not obvious why neonatal treatment with capsaicin should influence such a process.

It is interesting to note that besides the hindquarters vasodilator effects of BK, none of the other cardiovascular responses were significantly affected by the neonatal treatment with capsaicin. Thus, it is likely that in vehicle-treated rats, the overall action of BK would be the resultant not only of the stimulation of capsaicin-sensitive sensory nerve endings, but also of direct effects, as well as indirect effects via the release of other mediators (such as histamine, prostaglandins and nitric oxide) (Johnson & Erdos, 1973; Goldberg *et al.*, 1976; Warren *et al.*, 1987; Gardiner *et al.*, 1990c).

Haemodynamic effects of cholesytokinin

In rats neonatally-treated with capsaicin the heart rate responses to CCK were not different from, while the pressor

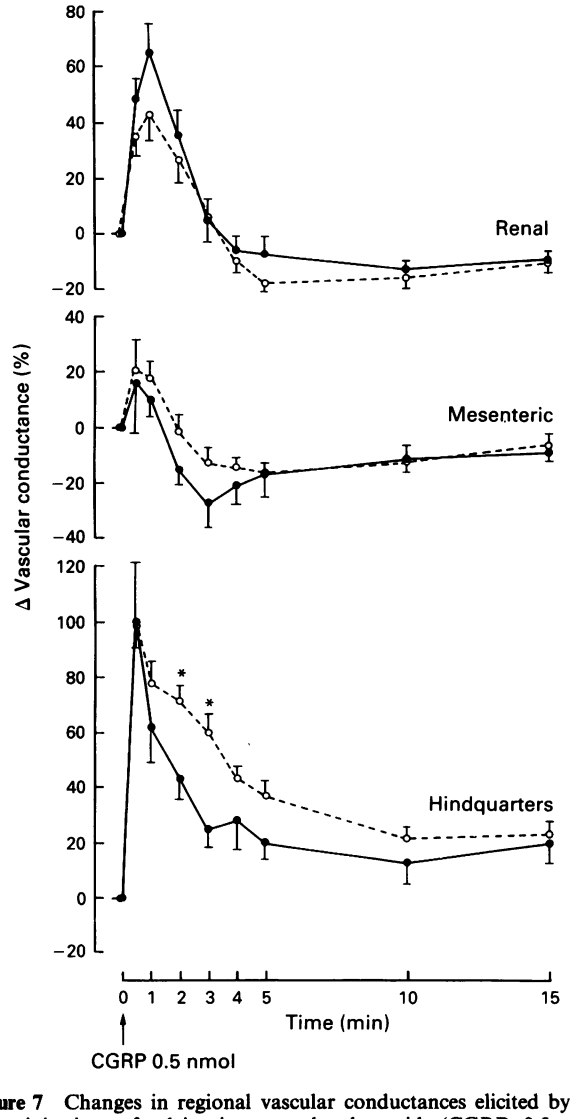


Figure 7 Changes in regional vascular conductances elicited by i.v. bolus injections of calcitonin gene-related peptide (CGRP, 0.5 nmol) in vehicle-treated (●) or capsaicin-treated (○) Long Evans rats. These data were derived from the data shown in Figure 6. Values are mean with s.e.mean shown by vertical lines. * $P < 0.05$ vehicle-treated group ($n = 9$) versus capsaicin-treated group ($n = 9$), (analysis of variance followed by Fisher's test).

responses elicited by both doses of CCK were slightly greater than, those in vehicle-treated rats. Hence, these results suggest that capsaicin-sensitive neurones modulate the pressor response to exogenous CCK in control animals. The blood pressure and heart responses to CCK in capsaicin-treated rats were associated with a greater renal vasoconstriction, while the superior mesenteric and hindquarters vasoconstrictor effects were not different from those seen in vehicle-treated rats. However, the possibility that the greater renal vasoconstrictor response to CCK was responsible for the potentiation of the pressor response in capsaicin-treated rats seems unlikely since, under both conditions (i.e., in vehicle- and in capsaicin-treated rats) the pressor response to CCK was not accompanied by any significant changes in renal flow (Table 2). Therefore, these results suggest the decrease in renal vascular conductance was autoregulatory, rather than indicating direct or indirect effects of CCK on the renal vasculature. The potentiation of the pressor response to CCK in capsaicin-treated rats raises the possibility that there was a greater increase in cardiac output in these animals than in vehicle-treated rats. However, we cannot exclude the possibility that in the capsaicin-treated rats CCK evoked greater vasoconstrictor responses in vascular beds not examined.

Haemodynamic effects of substance P

Carter & Lightman (1983) observed an increased sensitivity of the SP receptors in the nucleus tractus solitarius in capsaicin-treated rats. It has also been reported that neonatal treatment with capsaicin causes a supersensitivity to SP in laminae I and II of the lumbar spinal cord (Mantyh & Hunt, 1985). These observations constitute a rational basis for investigating the effects of neonatal treatment with capsaicin on the cardiovascular responses to SP.

Administration of SP produced a dose-related depressor response and tachycardia in both vehicle- and capsaicin-treated rats, accompanied by dilatations in the renal and hindquarters vascular beds and constriction in the superior mesenteric vascular bed (Miller & Scott, 1988; Gardiner *et al.*, 1989c). In capsaicin-treated rats, the depressor response to SP was slightly less, and the renal vasodilatation was absent compared to vehicle-treated rats. However, the magnitudes of the tachycardia and the dilatations in the superior mesenteric and hindquarters vascular beds observed in capsaicin-treated rats were not different from those in vehicle-treated rats. It is likely that the inhibition of the renal vasodilatation in capsaicin-treated rats was responsible for the slight attenuation of the hypotensive response to SP. Although the explanation of these effects is unclear, the present results do not indicate any increase in sensitivity of the vasculature to SP in capsaicin-treated rats.

Haemodynamic effects of calcitonin gene-related peptide

The characteristics of the cardiovascular responses to CGRP we observed in vehicle-treated rats are consistent with those of previous studies carried out in conscious, untreated rats (Gardiner *et al.*, 1988; 1989a,b), and as in those studies, the lack of a mesenteric vasodilator response to CGRP was probably due to activation of reflex and neurohumoral mechanisms.

In rats treated neonatally with capsaicin the depressor response to CGRP was more prolonged than in vehicle-treated rats, but the heart rate and renal and mesenteric conductance changes were not significantly different from those in vehicle-treated rats. However, the prolongation of the depressor response to the high dose of CGRP in the capsaicin-treated rats was associated with a significant prolongation of the rise in hindquarters vascular conductance. Thus, although the potentiation of the depressor and hindquarters vasodilator responses to CGRP in capsaicin-treated rats might have been due to neonatal treatment with capsaicin causing a small increase in CGRP sensitivity, this effect was not sufficient to show up as an increase in all regional vasodilator responses to the peptide.

Conclusions

From the present results it appears that neuropeptide-containing, capsaicin-sensitive neurones make a contribution to the cardiovascular responses to exogenous BK and CCK in conscious Long Evans rats. Considering the cardiovascular responses to exogenous SP and CGRP, it is feasible that release of these peptides, from capsaicin-sensitive neurones, following administration of BK or CCK contributes to the haemodynamic effects of the latter peptides. However, we have been unable to inhibit the haemodynamic responses to exogenous BK by pretreatment with the CGRP antagonist, human α-CGRP [8–37] (Gardiner, S.M. *et al.*, unpublished observations). Nonetheless, further studies are required to assess the possibility that local release of neuropeptides contributes to the effects of BK or CCK, since capsaicin-sensitive sensory neurones are known to contain vasoactive peptides in addition to SP and CGRP.

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Mechanisms of coronary vasodilatation produced by ATP in guinea-pig isolated perfused heart

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1 Isolated hearts of guinea-pigs were perfused *in vitro* with a physiological salt solution via a retrograde aortic cannulation (Langendorff preparation) at constant perfusion pressure. Bolus intra-arterial injections of various vasodilator drugs were made and the coronary flow responses were measured with an electromagnetic flow probe placed in the arterial inflow circuit. Inhibitory drugs were infused intra-arterially.

2 Nitro-L-arginine (NLA; 500 μM), an NO synthesis inhibitor, decreased coronary baseline flow by 16 $\pm$ 0.8%, converted acetylcholine-induced coronary vasodilatation to vasoconstriction and had no effect on coronary flow responses to adenosine or papaverine. Sodium nitroprusside-induced responses were enhanced during NLA infusion by 46 $\pm$ 11%.

3 Adenosine 5'-triphosphate (ATP) increased coronary flow but coronary flow responses to ATP were not altered by infusion of NLA.

4 ATP-induced coronary dilatation was not significantly attenuated by infusion of the adenosine receptor antagonist XAC, (xanthine amine congener; 2 μM), whereas XAC decreased coronary flow responses to adenosine by 75% $\pm$ 5%.

5 ATP-induced coronary flow responses were reduced by only 31 $\pm$ 4% during indomethacin infusion (2.8 μM) whereas indomethacin completely eliminated the initial vasoconstriction phase and greatly attenuated the peak flow and duration of the later vasodilatation phase seen in response to arachidonic acid (0.75 nmol). Indomethacin had no effect on vasodilatations produced by adenosine or prostaglandin I_2.

6 These results indicate that ATP-induced coronary dilatation in the isolated, perfused heart of the guinea-pig is not dependent upon NO production or upon degradation of ATP to adenosine. The coronary dilator action of ATP may be partially dependent (~30%) upon the production of vasodilator prostaglandins.

Keywords: Nitric oxide; nitro-L-arginine; indomethacin; adenosine; xanthine amine congener; prostaglandins; coronary vasodilatation

Introduction

Vasodilatation induced by adenosine 5'-triphosphate (ATP) has been shown to be be wholly or partially endothelium-dependent in several types of isolated blood vessel (De Mey & Vanhoutte, 1981; Cassis *et al.*, 1987) and *in vivo* microcirculation preparations (Koller *et al.*, 1991), in which the endothelium is easily removed or damaged. The endothelium-dependence of the vascular actions of ATP has proved more difficult to evaluate in whole-organ preparations, such as the isolated heart, because of the problem of selectively damaging the endothelium in such preparations. A role for endothelium-derived relaxing factor (EDRF), thought to be nitric oxide (NO) or a nitrosyl compound, in ATP-induced coronary dilatation has been proposed. This suggestion is based upon the measurement of NO release from isolated hearts stimulated by ATP (Kelm & Schrader, 1990) and upon the inhibitory effects of hydroquinone (Hopwood *et al.*, 1989) and electrolytically generated oxygen radicals (Lee *et al.*, 1990) on the coronary response to ATP. Although hydroquinone and oxygen radicals are known to inactivate NO, the specificity of these interventions is uncertain. Thus, this evidence is not conclusive. ATP also causes the release of prostaglandin I_2 (PGI_2) from the heart (Needleman *et al.*, 1974), presumably in large part from the endothelium (Needham *et al.*, 1987). Therefore, two possible endothelium-derived mediators of ATP-induced coronary vasodilatation are NO and PGI_2. It has also been proposed that some of the vasodilatation produced by ATP is, in fact, due to its degradation to adenosine (Moody *et al.*, 1984).

This study was designed to test the hypothesis that ATP-induced coronary vasodilatation is mediated by NO, prostaglandins and adenosine. This was accomplished by use of specific inhibitory agents: nitro-L-arginine (an inhibitor of NO synthase), indomethacin (a cyclo-oxygenase inhibitor) and xanthine amine congener (XAC, an adenosine receptor antagonist).

Our results show that ATP-induced coronary vasodilatation in guinea-pig isolated heart is not mediated by NO or adenosine. A portion of the response (about one-third) may be due to the production of vasodilator prostaglandins.

Methods

Isolated heart preparation

Male guinea-pigs (275–375 g) were anaesthetized with sodium pentobarbitone (65 mg ml^{-1} or to effect, i.p.). Heparin (400 u) was injected intravenously. After the thorax was opened, the heart was quickly excised, placed in ice-cold saline and immediately mounted, via the ascending aorta, onto a perfusion apparatus. The hearts were perfused with a non-recirculating perfusate at a constant hydrostatic pressure of 75–80 cmH_2O (Langendorff preparation). The perfusate was a modified Krebs-Henseleit solution containing (in mM): NaCl 127, KCl 4.7, $MgSO_4$ 1.1, KH_2PO_4 1.2, glucose 5.5, pyruvate 2.0, $CaCl_2$ 2.5. The perfusate was equilibrated with 5% CO_2:95% O_2 at 37°C and the pH was adjusted with $NaHCO_3$ (20–25 mM) to 7.40. The perfusate was filtered (0.45 μm) before use. The perfusate in the reservoir was continuously pumped through a 3 μm filter to prevent particulate matter from entering the coronary circulation. The pericardium and adherent lung tissue were removed. The pulmonary artery was cannulated to allow coronary venous drainage. A saline-filled catheter was attached at one end to a pressure transducer (Gould P23ID)

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and at the other to a balloon which was inserted into the left ventricle for the measurement of left ventricular (LV) pressure (P). The balloon was inflated to achieve an end-diastolic LVP of 5–10 mmHg. Heart rate and LV *dP/dt* were obtained via electronic differentiation from the pressure signal using a biotachometer (Grass 7P411) and analog differentiation amplifier (Grass 7P20C), respectively. Hearts were paced at 275–285 beats min^{-1} (Grass Model S44 Stimulator, Stimulus Isolation Unit SIU5A, and Constant Current Unit CCU1A) via two Teflon-coated stainless steel wires inserted into the right ventricular myocardium. Coronary flow was measured with a square wave electromagnetic flowmeter (Carolina) and a cannulating transducer placed in the aortic inflow line. All meters and transducers were calibrated daily. Coronary flow, heart rate, LVP and LV *dP/dt* were recorded continuously on a Grass Model 7E Polygraph. A 30 min equilibration period was allowed before the experiment was begun.

Vasodilator drugs were given by intra-arterial bolus injection (0.25 ml followed by a 0.5 ml flush with arterial perfusate) through a stopcock-controlled injection port above the flow transducer. Inhibitors were infused (Harvard Infusion Pump, Model 22) intra-arterially at 1/50 the total inflow rate through an infusion port in the flow transducer.

Preparations were not used for experiments if the spontaneous (unpaced) heart rate was less than 190 beats min^{-1}, if baseline coronary flow was less than 3 ml min^{-1} g^{-1} or if coronary flow failed to increase by at least 100% following a 30-s period of inflow occlusion. All experiments were completed in less than 3 h. Thirty-second occlusions were performed periodically throughout each experiment to verify the capacity of the preparation to vasodilate.

Responses to vasodilator drug injections were measured first under control conditions, and again during infusion of one of the inhibiting agents used in this study. Thus each heart served as its own control. During the control run, several doses of each vasodilator were tested. A dose of each vasodilator agent was chosen that produced a response equivalent to 50–75% of the maximum flow response to that drug in each heart. This dose was used as the challenge dose for the remainder of the experiment. There were small variations among the hearts within an experimental series in the dose of the vasodilator agent chosen as the challenge dose by this criterion. The range of doses used as the challenge in each series is given in the text. Maximal coronary flow was measured as the peak flow following release of a 30 s inflow occlusion.

Drugs

Acetylcholine, sodium nitroprusside and papaverine (Sigma) and ATP and adenosine (Boehringer-Mannheim) were dissolved in 0.9% NaCl. Nitro-L-arginine (Sigma) was dissolved in 0.9% NaCl by sonication for 40–60 min at 30°C. Arachidonic acid (Nu-Chek, 10 mg ml^{-1}) was dissolved in ethanol, and aliquots of this stock were diluted 1:10 with 100 mM Na_2HPO_4 and further diluted with 0.9% NaCl to achieve the injectate concentration. Indomethacin (Merck Sharpe & Dohme, 1 mg ml^{-1}) was dissolved in 100 mM Na_2CO_3 (pH 7.4–7.5) and diluted with 0.9% NaCl (final pH 7.1–7.2). PGI_2 (Upjohn, 50 μg) was dissolved in 1 ml of Tris buffer (100 mM, pH 8) and diluted with 0.9% NaCl. XAC (Research Biochemicals) was dissolved in 0.1 M NaOH, then diluted 1:100 with 0.9% NaCl.

Statistics

Baseline and peak flow responses to each vasodilator, before and during infusion of the test agent, were evaluated by analysis of variance (ANOVA), using a model that accounted for repeated measurements in an individual heart. *Post-hoc* intergroup analysis was performed using the F-test of Sokal & Rohlf (1969). A $P < 0.05$ was taken as indicating statistical significance. Means ± 1 s.e.mean are given in the text and figures.

Use of animals

All procedures used in this study that were related to the use and care of laboratory animals were reviewed and approved in advance by the Institutional Animal Care and Use Committee (IACUC) of New York Medical College, according to the guidelines set forth in the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. Moreover, all experiments were conducted in accord with the Guiding Principles in the Care and Use of Animals as approved by the Council of the American Physiological Society.

Results

Effects of nitro-L-arginine

Under control conditions, acetylcholine induced a 2.6 ± 0.5 ml min^{-1} g^{-1} increase in coronary flow from a baseline of 6.2 ± 0.03 ml min^{-1} g^{-1}. During nitro-L-arginine (NLA) infusion (500 μM) the acetylcholine-induced coronary dilatation was completely abolished. Most of the hearts (10 of 14), in fact, displayed a vasoconstriction response to acetylcholine (−0.7 ± 0.4 ml min^{-1} g^{-1}) during NLA (Figure 1). This indicates the successful blockade by NLA of the NO synthesis that mediates the dilator action of acetylcholine. Smaller concentrations of NLA (50–200 μM) were found in pilot experiments to attenuate, but not abolish, the coronary dilator effect of acetylcholine. This inhibition was specific in that coronary flow responses to adenosine and papaverine were not inhibited by NLA (Figure 1). ATP-induced coronary dilatation was not affected by NLA (Figure 1). Coronary dilatation in response to sodium nitroprusside was actually increased by 46 ± 11% over the control response ($P < 0.05$).

Effects of indomethacin

Arachidonic acid produced a biphasic coronary response, an initial transient vasoconstriction followed by vasodilatation, which lasted several minutes. As shown in Figures 2 and 3, indomethacin (2.8 μM) completely eliminated the vasoconstriction phase of the arachidonic acid response. The peak flow in the vasodilatation phase of the response was reduced by 71 ± 13% (flow increase of 0.5 ± 0.2 ml min^{-1} g^{-1} vs. control response of 2.0 ± 0.4 ml min^{-1} g^{-1}, $P < 0.05$, Figure 3) and the duration of this phase was greatly shortened (Figure 2). Indomethacin did not affect adenosine or PGI_2-induced vasodilatations, indicating the specificity of its action as a cyclooxygenase inhibitor. ATP-induced coronary dilatation

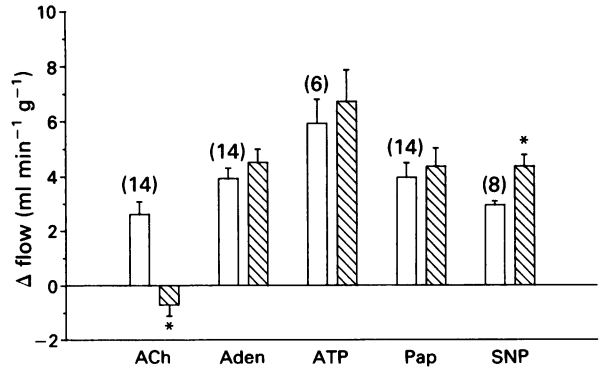


Figure 1 Peak change in coronary flow (ml min^{-1} g^{-1}) following bolus injection of acetylcholine (ACh, 2.5–7.5 nmol), adenosine (Aden, 25–75 nmol), ATP (25 nmol), papaverine (Pap, 25–75 nmol) or sodium nitroprusside (SNP, 25 nmol) before (open columns) and during nitro-L-arginine (NLA, 500 μM) infusion (hatched columns). The number of hearts is indicated in parentheses. * $P < 0.05$ vs. control (pre-NLA infusion). Each error bar = 1 s.e.mean.

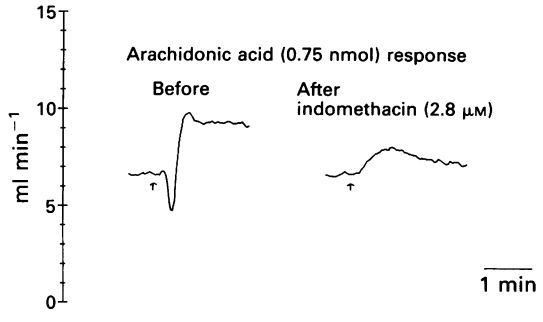


Figure 2 A representative biphasic response of coronary flow in the guinea-pig isolated heart to bolus injection of arachidonic acid (0.75 nmol, AA) before and during indomethacin (2.8 μM) infusion. ↑ = AA injection; $n = 7$ hearts. Note that in the control response (before), flow has returned only slightly towards control by the end of the tracing depicted whereas the post-indomethacin response is virtually complete within the same time frame.

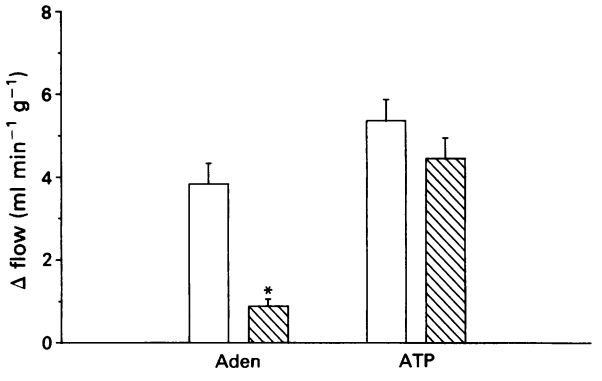


Figure 4 Peak change in coronary flow in response to adenosine (Aden, 25–75 nmol) and ATP (25 nmol) before (control, open columns) and during xanthine amine congener infusion (2 μM, hatched columns). $n = 7$ hearts; * $P < 0.05$ vs. control.

responses, however, were reduced 31 ± 4% during indomethacin infusion (control response of 3.9 ± 0.3 ml min^{-1} g^{-1} vs. 2.7 ± 0.2 ml min^{-1} g^{-1} during indomethacin, $P < 0.05$, Figure 3). Infusion of the indomethacin vehicle (5 mM Na_2CO_3 in isotonic saline) had no effect on baseline flow or any of the flow responses to the vasodilators used in this study.

Effects of xanthine amine congener

XAC, a potent adenosine receptor blocker, was infused at a concentration of 2 μM. At this concentration, XAC inhibited adenosine-induced coronary vasodilatation by 75 ± 5% (flow response of 0.9 ± 0.2 ml min^{-1} g^{-1} during XAC infusion vs. control response of 3.8 ± 0.5 ml min^{-1} g^{-1}, $P < 0.05$, Figure 4). ATP responses, however, were not significantly changed (4.5 ± 0.5 ml min^{-1} g^{-1} vs. control response of 5.4 ± 0.5 ml min^{-1} g^{-1}). The XAC vehicle (1 mM NaOH in isotonic saline), had no effect on baseline flow or any of the flow responses.

Effects of inhibitors on baseline coronary flow

The inhibitor drugs used in this study each had a slight, but statistically significant, effect on baseline coronary flow. As shown in Figure 5, NLA (500 μM) decreased baseline flow by 16 ± 0.8% from 6.2 ± 0.03 to 5.2 ± 0.05 ml min^{-1} g^{-1} ($n = 14$ hearts, $P < 0.05$). XAC (2 μM) decreased baseline flow by 25 ± 0.7% from 5.2 ± 0.05 to 3.9 ± 0.07 ml min^{-1} g^{-1} ($n = 7$ hearts, $P < 0.05$). Indomethacin (2.8 μM) caused a slight increase in baseline flow, by 8 ± 1% from 6.2 ± 0.2 to 6.7 ± 0.2 ml min^{-1} g^{-1} ($n = 7$ hearts, $P < 0.05$).

Discussion

The major conclusions reached in this study are that (1) ATP is a coronary vasodilator in the guinea-pig isolated, perfused heart; (2) nitric oxide (NO) and adenosine appear to exert a basal influence on the resting coronary flow in this preparation; (3) ATP-induced coronary vasodilatation in the guinea-pig isolated heart is not dependent on NO; (4) ATP-induced coronary vasodilatation is partially (about one-third) due to prostaglandin production; and (5) ATP-induced coronary vasodilatation is not due to the degradation of ATP to adenosine, but rather to the direct action of ATP, presumably, on specific ATP receptors.

It has been demonstrated that the vasodilator action of ATP in a variety of experimental preparations is at least partly dependent upon the endothelium (De Mey & Vanhoutte, 1981; Cassis *et al.*, 1987; Hopwood *et al.*, 1989; Lee *et al.*, 1990; Radermacher *et al.*, 1990; Koller *et al.*, 1991). Many

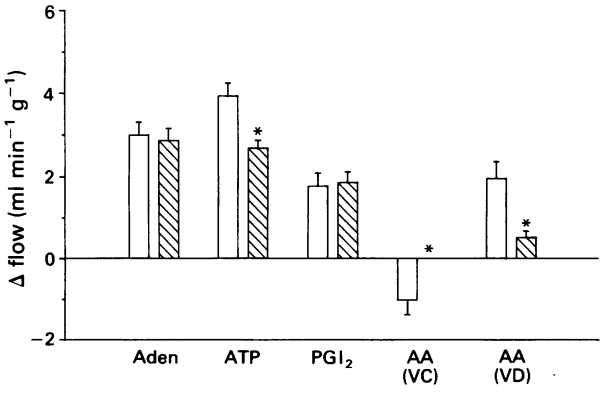


Figure 3 Peak change in coronary flow in response to bolus injections of adenosine (Aden, 7.5–25 nmol), ATP (7.5–25 nmol), prostacyclin (PGI_2, 3.35 nmol) and arachidonic acid (AA, 0.75 nmol) before (control, open columns) and during indomethacin infusion (2.8 μM, hatched columns). $n = 7$ hearts; * $P < 0.05$ vs. control; vasoconstrictor (VC) and vasodilator (VD) phases of arachidonic acid response are depicted separately.

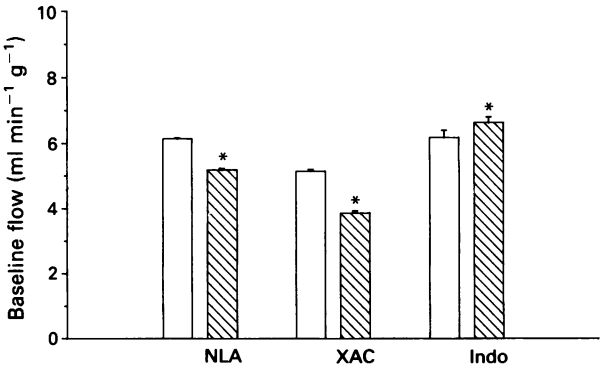


Figure 5 Baseline coronary flow before infusion (control, open columns) and during infusion of nitro-L-arginine (NLA, 500 μM, $n = 14$ hearts), xanthine amine congener (XAC, 2 μM, $n = 7$ hearts) or indomethacin (Indo, 2.8 μM, $n = 7$ hearts) (hatched columns). * $P < 0.05$ vs. control.

isolated conduit blood vessels show a diminished relaxation response to ATP after the endothelium has been physically or chemically removed (De Mey & Vanhoutte, 1981; De Mey et al., 1982; Cassis et al., 1987). Because it is difficult to damage the endothelium selectively in intact vascular beds, the endothelium-dependence of the microvascular vasodilator action of ATP is less well established.

In the perfused rabbit hindlimb, the vasodilator effects of acetylcholine, ATP and substance P, but not those of adenosine, nitroglycerin or PGE_2 were reduced after gossypol (Pohl et al., 1987; Dezsi et al., 1990). Although gossypol was regarded in these studies as an inhibitor of endothelium-mediated vasodilatation, this drug may have other actions (Ye et al., 1987). Therefore, the implication of these studies that ATP is an endothelium-dependent or NO-dependent dilator in the rabbit hindlimb remains unproven. Koller et al. (1991), using a light-dye method to create focal endothelial damage, have demonstrated the endothelium-dependence of ATP-induced arteriolar dilatation in the rat cremaster muscle microcirculation without defining the specific mediator involved.

No comparably definitive study has been reported for the coronary circulation, although several studies have suggested the involvement of an endothelium-derived relaxing factor (EDRF) in the coronary dilator action of ATP. For example, coronary dilator responses to ATP were attenuated by electrical stimulation of the perfusate (Lee et al., 1990) and by hydroquinone (Hopwood et al., 1989). Both electrical current and hydroquinone generate superoxide anions, which may then inactivate NO. It is possible, however, that these oxygen radicals might attenuate vasodilatation in some way other than by attacking NO. Thus, although there is suggestive evidence that ATP acts via the endothelium to cause vasodilatation in the coronary and skeletal muscle microcirculations, the identity of the endothelium-derived mediator is not certain. Despite these suggestive results, our finding that NLA fails to attenuate the coronary dilator action of ATP allows us to reject the hypothesis that NO is the endothelium-derived mediator of this response. NLA is superior to hydroquinone or electrolytic generation of oxygen radicals for use as a test of this hypothesis because, unlike oxygen radicals, arginine analogues such as NLA specifically inhibit NO synthesis (Palmer et al., 1988; Moore et al., 1990). Our finding that the dilator effect of acetylcholine is abolished, whereas the coronary responses to adenosine and papaverine are preserved, further supports the specificity of the action of NLA.

A number of recent studies support our present conclusion that NO may have little or nothing to do with ATP-induced vasodilatations in many vasculator beds. In studies using guinea-pig pulmonary artery (Sata et al., 1990), and isolated, perfused rabbit (Pohl et al., 1991) and guinea-pig (Kelm & Schrader, 1990) hearts, dilator actions of acetylcholine and ATP were differentially affected by a variety of agents, including a spin-trapping agent, NLA and oxyhaemoglobin. Since the vasorelaxant or vasodilator effects of acetylcholine were blocked by these agents whereas those of ATP were not, these studies suggest that mediators other than NO are involved in ATP-induced vasorelaxation in these preparations. In contrast, NO synthase inhibitors antagonized the rabbit hepatic arterial vascular responses to both ATP and acetylcholine in a recent study (Mathie et al., 1991), indicating that the mechanisms of ATP-induced vasodilatation are tissue-specific.

It also remains possible that NO may interact synergistically with prostaglandins or other vasodilator metabolites and thus contribute to the coronary dilator response to ATP. Such synergism must be either small or compensated for when absent however, because blockade of NO synthase had no apparent antagonistic effect on ATP-induced coronary dilatation in our experiments.

In our study, the responses to SNP were potentiated by NLA, in keeping with other reports (Busse et al., 1989; Mathie et al., 1991; Moncada et al., 1991; Mugge et al., 1991). It is possible, that basal release of NO may slightly diminish the vasodilator reserve of the coronary bed, thus decreasing the responses elicited by superimposed vasodilator stimuli. However, this effect may be specific for interactions with guanylate cyclase (Moncada et al., 1991), rather than just a general baseline effect, because flow responses to other dilator agents (adenosine, papaverine) were not significantly potentiated.

ATP stimulates the production of a vasodilator prostaglandin (PGI_2) from the heart (Minkes et al., 1973; Needleman et al., 1974) and from cultured vascular endothelial cells (Needham et al., 1987). In our experiments with guinea-pig hearts, arachidonic acid produced a biphasic response in agreement with previous observations (Talesnik, 1986). Indomethacin, which is a specific inhibitor of cyclo-oxygenase, inhibits the biosynthesis of both vasodilator and vasoconstrictor prostaglandins and thromboxanes. Our indomethacin dose greatly attenuated both vasoconstrictor and vasodilator phases of the arachidonic acid response. ATP-induced vasodilatation, however, was reduced by less than one-third. Therefore, while vasodilator prostaglandins, most likely PGI_2, are involved in the mediation of the coronary response to ATP in the guinea-pig isolated heart, they do not provide a full explanation of the vascular response. In previous work, indomethacin greatly attenuated ATP-induced coronary dilatation in some studies (Minkes et al., 1973; Needleman et al., 1974), but not in others (Lee et al., 1990; Pohl et al., 1991). This discrepancy could be due in part to species differences and to differences in the physiological status of the various preparations. A prior study of guinea-pig heart found no effect of indomethacin on the coronary dilator action of ATP, but that study used a K^+-arrested heart (Lee et al., 1990). In the present study, in contrast, we used a beating, pressure developing heart preparation.

It was also possible that ATP-induced coronary dilatation might be mediated by adenosine formed from the rapid degradation of ATP by ectoenzymes on both endothelium and vascular smooth muscle (Pearson et al., 1980; Moody et al., 1984). Indeed, Pohl et al. (1991) found that less than 5% of the 1 μM ATP infused into rabbit isolated hearts appeared in the coronary effluent after one pass through the heart. We found that the potent adenosine receptor antagonist, XAC, had no significant effect on ATP-induced dilator responses despite the fact that XAC attenuated adenosine-induced vasodilator responses by more than 75%. Therefore, degradation of ATP to adenosine was not responsible for the observed increases in coronary flow produced by ATP.

Baseline coronary flow was reduced by NLA, as has been found previously not only for the heart, but also for other vascular beds (Amezcua et al., 1989; Kelm & Schrader, 1990; Moore et al., 1990; Radermacher et al., 1990; Mugge et al., 1991). This suggests that in the guinea-pig isolated heart, there is significant basal production of NO and further suggests that NO contributes a dilator influence in the regulation of basal vasomotor tone and vascular resistance in this preparation.

In a similar fashion, XAC reduced baseline coronary flow, thus implying a basal release of adenosine, which would contribute to the regulation of basal vascular resistance in this preparation. Wei et al. (1988) also found consistent and reproducible reductions in basal coronary flow in this same preparation after infusion of adenosine deaminase, the enzyme that converts adenosine to vasoinactive inosine. In contrast, Kroll & Feigl (1985) found no decrease in basal coronary following adenosine deaminase infusion. Thus the importance of adenosine in the regulation of basal coronary blood flow might be species-dependent.

Baseline flow changes with indomethacin administration seem to be variable in the literature. Small increases (Sunahara & Talesnik, 1979; present study), no change (Harlan et al., 1978; Lee et al., 1990) or small decreases (Hintze & Kaley, 1977) in basal coronary flow or vascular conductance with indomethacin have been described.

The changes in baseline flow with these inhibitors did not affect the conclusions of the present study as the changes in

baseline flow were small. Moreover, the inhibitions of the vasodilator responses to acetylcholine by NLA, to adenosine by XAC, and to arachidonic acid by indomethacin were specific in that the vasodilator responses to other dilator agents were not affected. Therefore, non-specific changes in the responses due to baseline flow changes may be ruled out.

The question that is raised by this study is, if NO and adenosine do not contribute to ATP-induced vasodilatation and prostaglandins (PGI_2) mediate less than one-third of ATP-induced vasodilatation, what factor(s) do provide for most of the coronary vasodilator response produced by ATP?

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Oxidative modification of low-density lipoproteins and the inhibition of relaxations mediated by endothelium-derived nitric oxide in rabbit aorta

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1 The mechanism by which Cu^{2+}-oxidized low-density lipoproteins (oxLDL) inhibit acetylcholine (ACh)-evoked relaxations mediated by endothelium-derived nitric oxide (EDRF) in rabbit aortic rings was investigated. The proposed role of lysophosphatidylcholine (LPC) in the inhibition was also studied.

2 The kinetics of lipid peroxidation of native low-density lipoproteins (LDL) from individual donors, as measured by changes in conjugated diene concentration, were related to the inhibitory effects of the resultant oxLDL. It was found that the more susceptible LDL was to oxidation, the greater the inhibition.

3 No correlation was found between the inhibitory effects of oxLDL and LPC content.

4 Synthetic 1-palmitoyl LPC produced an inhibition of ACh-induced relaxations and when added to precontracted rings evoked nitric oxide-mediated endothelium-dependent relaxation. This latter effect was not elicited by oxLDL.

5 Synthetic 1-palmitoyl (10 μM) had no effect on relaxations evoked by glyceryl trinitrate in endothelium-denuded aortic rings in contrast to the inhibition found previously for oxLDL.

6 Concentrations of oxLDL and phospholipase A_2-treated LDL which inhibited relaxation contained very different LPC concentrations. Unlike oxLDL, the inhibitory effects of phospholipase A_2-treated LDL preparations were independent of the donors and showed no lag period.

7 We sugggest that there are differences in the mechanisms by which oxLDL and 1-palmitoyl LPC exert their inhibitory effects on relaxation.

8 The inhibition of relaxation by oxLDL (1–2 mg protein ml^{-1}) was prevented by the presence of high-density lipoproteins (HDL; 1–2 mg protein ml^{-1}).

9 It is proposed that prevention of the inhibition of relaxation by HDL is consistent with the inhibitory factor(s) being lipophilic constituents of oxLDL. However, variations in the inhibitory effects of oxLDL preparations are not due to differences in their LPC content and factors other than LPC must contribute to the inhibition.

Keywords: Endothelium-derived nitric oxide; low-density and high-density lipoproteins; lysophosphatidylcholine; atherosclerosis; oxidation; rabbit aorta

Introduction

Elevated plasma concentrations of low-density lipoproteins (LDL) are recognised as leading to the development of atherosclerosis whereas high-density lipoproteins are believed to play a protective role in the aetiology of the disease. The initiation of artherosclerotic lesions is thought to occur through oxidative modification of LDL by exposure to cells of the intima (Steinberg *et al.*, 1989). Studies *in vitro* have shown that the modified lipoproteins can be taken up by macrophages to form foam cells, the precursor of atherosclerotic lesions. Evidence for this pathway has come from the finding that oxidatively modified LDL are present in atherosclerotic lesions and have undergone similar changes to those of LDL modified *in vitro* which include lipid peroxidation of polyunsaturated fatty acids and the formation of conjugated dienes, deacylation of phosphatidylcholine (PC) to lysophosphatidylcholine (LPC) and the generation of new epitopes on the apolipoprotein B that are recognised by macrophages. These modifications can be simulated in the absence of cells by the oxidation of LDL by Cu^{2+} ions. The kinetics of the peroxidation of the fatty acids in LDL can be monitored by the absorbance change at 234 nm due to the formation of conjugated dienes (Esterbauer *et al.*, 1989) or by the increase in relative fluorescence intensity at 420 nm, a change which is related to the extent of apolipoprotein B modification (Esterbauer *et al.*, 1990). By use of the former method, a variation in the susceptibility to oxidation of LDL from individual human donors, has been found (Esterbauer *et al.*, 1990).

There is now convincing evidence that relaxations mediated by endothelium-derived nitric oxide (EDRF) are impaired in arteries from hypercholesterolaemic and atherosclerotic animals (Verbeuren *et al.*, 1986; Shimokawa & Vanhoutte, 1989). Impairment has been reported to be reversed in the aorta of hypercholesterolaemic rabbits by exposure *in vivo* to exogenous L-arginine, the precursor of nitric oxide, implying that its synthesis may be reduced (Cooke *et al.*, 1991). Similar dysfunctions in the relaxation of human coronary arteries have also been correlated with the incidence of hypercholesterolaemia (Vita *et al.*, 1990).

We have suggested that the impairment of EDRF responses is caused by the accumulation of LDL or their modified forms in the arterial wall (Plane *et al.*, 1989; Jacobs *et al.*, 1990). This is supported by the finding that LDL which have been oxidatively modified by Cu^{2+} ions (Plane *et al.*, 1989; Jacobs *et al.*, 1990; Yokoyama *et al.*, 1990) or by endothelial cells (Kugiyama *et al.*, 1990) inhibit relaxations mediated by EDRF. This inhibition has been attributed to LPC generated in the LDL during modification (Kugiyama *et al.*, 1990; Yokoyama *et al.*, 1990). In our previous study, we found that oxidized low-density lipoproteins (oxLDL) from human donors varied in the inhibitory effects they had on the EDRF responses evoked by acetylcholine (ACh) (Jacobs *et al.*, 1990). These variations ranged from a shift in the sensitivity to ACh

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to a complete irreversible inhibition. In view of the differences in the susceptibility of LDL from different donors to oxidation, we have examined here the possibility that the variation in the inhibitory effects of oxLDL preparations is related to the oxidisability of the original native LDL and the amount of lysophosphatidylcholine that is generated during oxidative modification. The effects of high-density lipoproteins (HDL) on EDRF responses and their inhibition by oxLDL are also investigated, as HDL is known to oppose the cytotoxic effects of LDL (Hessler *et al.*, 1979).

Methods

Preparation of lipoproteins

Fresh plasma was obtained from apparently healthy volunteers (donors A-K) with informed consent. LDL (density 1.019–1.063 g ml^{-1}) and HDL (density 1.063–1.21 g ml^{-1}) were isolated from individual plasma samples by discontinuous density gradient ultracentrifugation (Jacobs *et al.*, 1990). LDL from different donors was kept separate whereas the HDL was usually pooled. The isolated lipoproteins were dialysed for 18 h against modified Tyrode buffer (composition (mM): NaCl 0.13, $NaHCO_3$ 0.012, $NaHPO_4$ 0.0042, KCl 0.0027). All buffers contained 0.3 mM EDTA to prevent auto-oxidation of the lipoproteins. Native LDL prepared by this method showed no significant oxidation as measured by thiobarbituric acid reactive substance assays (TBARS; Hessler *et al.*, 1983). Pooled HDL was further purified from contaminating apolipoprotein B containing proteins by heparin-sepharose chromatography (Weisgraber & Mahley, 1980). All lipoproteins were extensively dialysed against Tyrode buffer containing EDTA before use.

Oxidative modification of low-density lipoptoteins

Freshly prepared LDL (5 mg protein ml^{-1}) were dialysed for 24 h to remove EDTA and then oxidatively modified by incubation with 5 μM $CuSO_4$ in Tyrode buffer for 24 h at 20°C. Oxidation was terminated by the addition of 1.5 mM EDTA followed by dialysis against the latter buffer containing 0.30 mM EDTA.

Measurement of the susceptibility of low-density lipoproteins to oxidation

The monitoring of the oxidation of LDL was performed by measurement of the u.v. absorption at 234 nm during an incubation of LDL with Cu^{2+} (Esterbauer *et al.*, 1989). LDL without Cu^{2+} plus 1.5 mM EDTA were used in the reference cuvette. The increase in absorption at 234 nm is due to the formation of conjugated dienes during the peroxidation of polyunsaturated fatty acids. Absorbance at 234 nm shows an initial slower increase (lag phase) as antioxidants are destroyed and then increases more rapidly (propagation phase) reaching a plateau phase at which the absorbance is maximal as the rate of formation of dienes approaches their rate of decomposition (Esterbauer *et al.*, 1990). After this time, the dienes decompose and absorbance is no longer solely due to them. Because of the rapid rate of oxidation of some of our donors (Figure 1), we have adopted the time (in min) required to reach 50% of the maximum absorbance, described here as the half-time (Figure 1) as a measure of the susceptibility of LDL to oxidation, to which it is inversely related, instead of the lag time and maximum rate of conjugated diene formation used by Esterbauer *et al.* (1989). The maximum change in absorbance at 234 nm was defined as the increase in absorbance from zero-time to the maximum absorbance at the plateau (Figure 1).

The modification of the apolipoprotein B moiety of LDL, another measure of oxidative modification, was determined after 24 h incubation with Cu^{2+} by the increase in relative fluorescence intensity at 420 nm (excitation wavelength 350 nm; Esterbauer *et al.*, 1987) as described by Dunn *et al.* (1988).

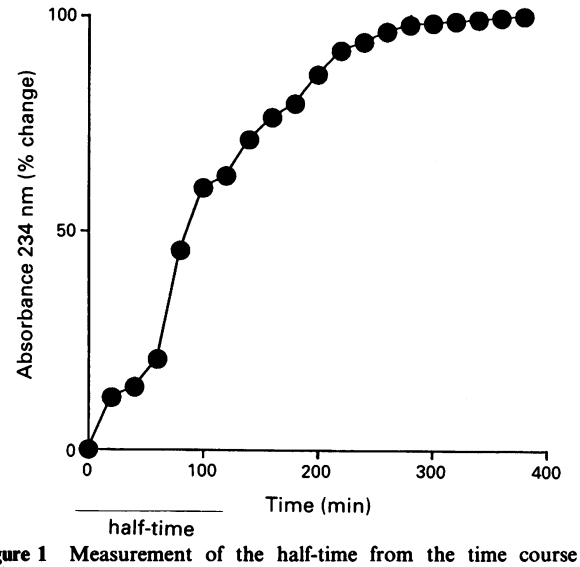


Figure 1 Measurement of the half-time from the time course of Cu^{2+}-induced oxidation of low-density lipoproteins (LDL). Oxidation was determined by monitoring the change in absorption at 234 nm due to increase in conjugated diene formation for 400 min. LDL was incubated with 5 μM $CuSO_4$ in Tyrode buffer. The reference blank contained LDL without $CuSO_4$ in buffer supplemented with 1.5 mM EDTA. The time required to reach 50% of the maximum absorbance at 234 nm, described as the half-time was measured as shown.

Preparation of phospholipase A_2-treated low-density lipoprotein

Phospholipase A_2-treated LDL were prepared by the incubation of native LDL (1 mg protein ml^{-1}) with 10 u ml^{-1} agarose-bound phospholipase-A_2 (Sigma, from *Naja naja* venom) in 100 mM Tris buffer (pH 7.4) containing 2 mM $CaCl_2$ for 2 h at 37°C. The phospholipase A_2-bound to agarose was removed from the incubation mixture by a 5 min centrifugation at 5000*g*. Control incubations were performed using incubation mixtures without the addition of enzyme or without LDL.

Determination of lysophosphatidylcholine content of low-density lipoptotein

Lipids were extracted from LDL samples (100–500 μg protein) with chloroform/methanol (2:1 v/v) by the method of Bligh & Dyer (1959). The phospholipids were separated by thin layer chromatography (t.l.c.) on silica gel G (Schleicher & Schull, Gmbh) with chroroform/methanol/acetic acid/water (75:45:12:8 v/v) as solvent and LPC spots were visualised with iodine vapour, scraped from the plates and assayed for phosphorus (Skipski *et al.*, 1964).

Determination of endothelium derived relaxing factor (NO)-dependent relaxations

EDRF-dependent and independent relaxations of rabbit aortic rings were determined as described by Jacobs *et al.* (1990). Tissues were mounted under 2 g tension in jacketed water baths at 37°C containing oxygenated Krebs buffer, for isometric force measurements. After 60 min equilibration, the tension was reset to 2 g and the tissues left for a further 30 min. Rings were precontracted with phenylephrine (PE, 0.05–0.1 μM) and then relaxed to cumulative doses of ACh or glyceryl trinitrate (GTN). After washout and equilibration for 30 min, the tissues were preincubated with oxLDL, HDL, serum albumin, phospholipase A_2-treated LDL or synthetic 1-palmitoyl-LPC for 0–30 min and the contraction/relaxation procedure was repeated. Controls were performed with the

appropriate buffer or phospholipase A_2 (1–2 u ml^{-1}). After washout, relaxations were reassessed to determine the reversibility of any effects. % inhibition of maximal relaxation was calculated from the maximal relaxation before and after treatment with lipoproteins or lipids. Control tissues were used to correct for any time-dependent changes in responses. In some experiments, precontracted intact and endothelium-denuded rings were relaxed by cumulative concentrations of 1-palmitoyl LPC with and without pre-incubation with 100 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) or N^{G}-nitro-L-arginine for 10 minutes. The integrity of the endothelium was confirmed after each experiment by *en face* silver staining (Poole *et al.*, 1958).

Statistical analysis

Results are expressed as mean ± s.e. mean for n separate experiments. Values of half-times, % inhibition of maximal relaxation, maximum relaxation and maximum absorbance obtained in experiments with preparations from the same donor and those between donors were tested for significance by two-way analysis of variance (ANOVA). The independent variable was the donor. In addition differences in mean values of these properties between donors were tested for significance by the use of Student's t test for unpaired samples where $P < 0.05$ was considered significant.

Drugs and chemicals

Phenylephrine, 1-palmitoyl-LPC, phosphatidylcholine, serum albumin (fatty acid free) and agarose-bound phospholipase A_2 (*Naja naja*), N^{ω}-nitro-L-arginine methyl ester and N^{G}-nitro-L-arginine were obtained from Sigma, Poole, Dorset and other chemicals were obtained from BDH (U.K.)

Results

Kinetics of oxidation and the inhibitory properties of oxidised low density lipoprotein

LDL from 9 donors (A–I) were oxidised with Cu^{2+} ions for 24 h. The susceptibility of each LDL to oxidation was determined and then the oxLDL of the same donors tested for their inhibitory effects on the endothelium-dependent relaxations evoked by cumulative concentrations of ACh in rabbit aortic rings. The susceptibility of the LDL from each donor to oxidation was characterized by the half-time (see Methods and Figure 1). The more susceptible the LDL was to oxidation, the shorter the half-time. Significant differences in half-time (ANOVA $P < 0.001$) were found between donors as shown in Table 1 (column 1). After 6 h, the absorbance at 234 nm for all the LDL preparations had reached a similar plateau which was not significantly different between donors (2.4 ± 0.13 absorption units, $n = 36$, $P > 0.05$). The end point of oxidation as measured after 24 h by relative fluorescence intensity at 420 nm, which is due to apolipoprotein B modification, was not significantly different between donors (4.9 ± 1.1, $n = 30$, $P > 0.05$).

The inhibitory properties of oxLDL preparations were also found to vary between donors as previously reported (Jacobs *et al.*, 1990). The % inhibition of maximal relaxation caused by the various oxLDL preparations are summarised in Table 1 (column 2) and showed significant differences (ANOVA, $P < 0.001$). The inhibitory properties of oxLDL were unaffected by increasing the time of oxidation to 48 h instead of the standard 24 h (unpublished result). Table 1 (column 1 and 2) shows a comparison of the susceptibility of LDL to oxidation (half-time) and % inhibition of maximum relaxation. A negative trend in the susceptibility to oxidation (increasing half-time) from donor A–I was parallelled by a similar trend in the inhibition of relaxation. The inhibition showed a negative correlation with half-time (correlation coefficient, 0.993) which fitted a straight line (regression coefficient, 0.63).

Table 1 The relationship between half-time, inhibition of relaxation and lysophosphatidylcholine (LPC) content

Donor	*Half-time* (min)	*% inhibition maximal relaxation*	*LPC in oxLDL* (μmol mg^{-1})
A	40 ± 4.5	97.0 ± 2.5 (i)	0.20 ± 0.045
B	45 ± 1.7	93.0 ± 4.1 (i)	0.17 ± 0.056
C	49 ± 3.4	89.0 ± 3.4 (i)	ND
D	54 ± 3.2	84.0 ± 3.7 (p.r)	0.21 ± 0.057
E	69 ± 3.9	79.0 ± 1.9 (i)	0.26 ± 0.083
F	120 ± 5.1	51.3 ± 3.0 (r)	0.23 ± 0.040
G	135 ± 4.3	41.7 ± 2.1 (r)	0.25 ± 0.080
H	155 ± 7.3	21.0 ± 1.3 (r)	0.18 ± 0.057
I	160 ± 4.6	15.0 ± 1.4 (r)	0.25 ± 0.051

Half-time, % inhibition of maximal relaxation and LPC content determined as in Methods. After washout: i: irreversible inhibition; p.r: partially reversible; r: reversible inhibition. Values of half-time and % inhibition of maximal relaxation (mean ± s.e.mean, $n = 4$) showed significant variations between many donors (ANOVA, $P < 0.001$). Exceptions, donors A and B, A and C, B and C, C and D, for both parameters; for half-time only: G and H, H and I; for % inhibition of maximal relaxation only: B and D, D and E, F and G, G and H. Values of LPC content of the oxidised low-density lipoprotein (oxLDL) of different donors (mean ± s.e.mean, $n = 3$–4) showed no significant variation (ANOVA, $P > 0.1$). ND not done.

Lysophosphatidylcholine content of oxidised low-density lipoprotein does not correlate with inhibitory potency

It has been proposed that lysophosphatidylcholine in oxLDL accounts for the inhibition of relaxation (Kugiyama *et al.*, 1990; Yokoyama *et al.*, 1990). It follows that the amount of LPC in oxLDL preparations from different donors (A, B, D–H) should be related to their ability to inhibit relaxation. Table 1 (columns 2 and 3) shows the LPC content and inhibition of maximal relaxation for the oxLDL of these donors. The inhibition of relaxation varies from 15–97% and was significantly different between donors (ANOVA, $P < 0.001$). In contrast, a range of values from 0.17–0.26 μmol mg^{-1} protein was found for the LPC content of oxLDL. These were not significantly different between donors (mean value 0.22 ± 0.025 μmol mg^{-1} protein, $P > 0.1$). The LPC content was therefore unrelated to the inhibition of relaxation.

1-Palmitoyl lysophosphatidylcholine and relaxations mediated by endothelium-derived NO

The effect of 1-palmitoyl LPC on vascular tone was investigated and compared to the effects that were previously found for oxLDL (Jacobs *et al.*, 1990). Figure 2 shows the cumulative dose-response curve for the relaxations evoked by 1-palmitoyl LPC (0.1–50 μM) in pre-contracted aortic rings containing endothelium. Maximal relaxation (68.9 ± 6.7%, $n = 4$) was achieved at 50 μM LPC; above this concentration the rings began to re-contract as a result of the loss of the endothelium. Relaxations evoked by 1-palmitoyl-LPC were almost abolished in the presence of 100 μM L-NAME or N^{G}-nitro-L-arginine (not shown), suggesting that the relaxations were mediated by the release of EDRF (Figure 2). No relaxations were evoked by 1-palmitoyl LPC in the absence of endothelium or when 1,2 dipalmitoyl phosphatidylcholine was used instead of 1-palmitoyl LPC (not shown). The relaxation by 1-palmitoyl LPC is in contrast to the failure of oxLDL (2 mg protein ml^{-1}) to evoke relaxations in precontracted rings either immediately or during a 30 min incubation.

1-Palmitoyl LPC (10 μM), added immediately before the tissues were contracted with PE, caused inhibition of the relaxations evoked by cumulative concentrations of ACh (Figure 3) whereas dipalmitoyl phosphatidylcholine was ineffective (not shown). The inhibition of maximal relaxation by

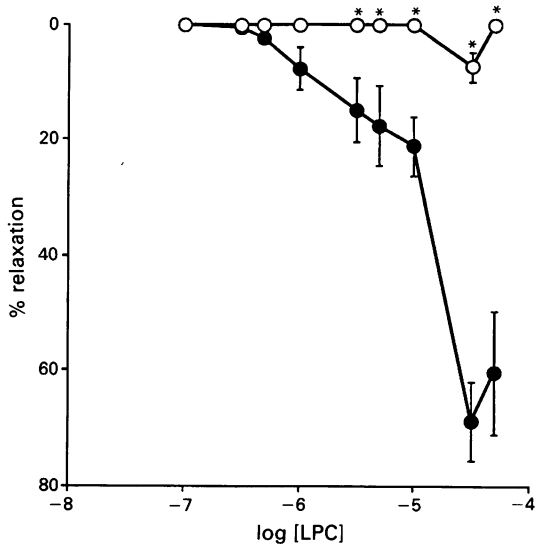


Figure 2 Dose-response curves showing the relaxations evoked by cumulative concentrations of 1-palmitoyl lysophosphatidylcholine (LPC). Relaxations expressed as % of precontraction. Relaxations were evoked in precontracted intact aortic rings by cumulative concentrations of 1-palmitoyl LPC. Without 100 μM N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) (●); with L-NAME (○). Each point is the mean of 4 experiments with s.e.mean shown by vertical bars. Differences in mean values were tested by Student's t test for unpaired samples. *$P < 0.05$ was considered significant.

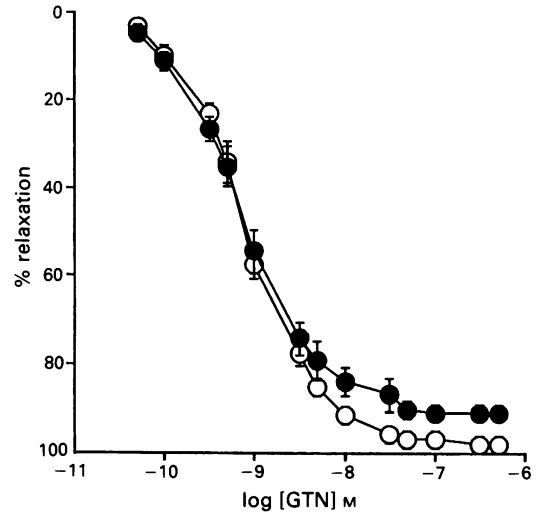


Figure 4 Dose-response curve showing the effect of lysophosphatidylcholine (LPC) on endothelium-independent relaxations evoked by glyceryl trinitrate (GTN). Endothelium-denuded aortic rings were contracted with phenylephrine (0.05 μM) and relaxed with cumulative concentrations of glyceryl trinitrate. The contraction and relaxation cycle was repeated in the presence of LPC (10 μM). Relaxations are expressed as % of precontraction. Without LPC (10 μM) (○); with LPC (●). Each point is the mean of 4 experiments with s.e.mean shown by vertical bars. Differences in mean values were tested by Student's t test for unpaired samples. *$P < 0.05$ was considered significant.

1-palmitoyl-LPC was 94 ± 5.9% and this inhibition was reduced to 10 ± 2.0% after its removal by washing the tissues. At lower concentrations of LPC (1 μM), preincubation for 30 min was required to cause an inhibition of maximal relaxation (23 ± 3%, $n = 4$). 1-Palmitoyl-LPC (10 μM) had no effect on relaxations evoked by cumulative concentrations of glyceryl trinitrate in precontracted aortic rings denuded of endothelium (Figure 4).

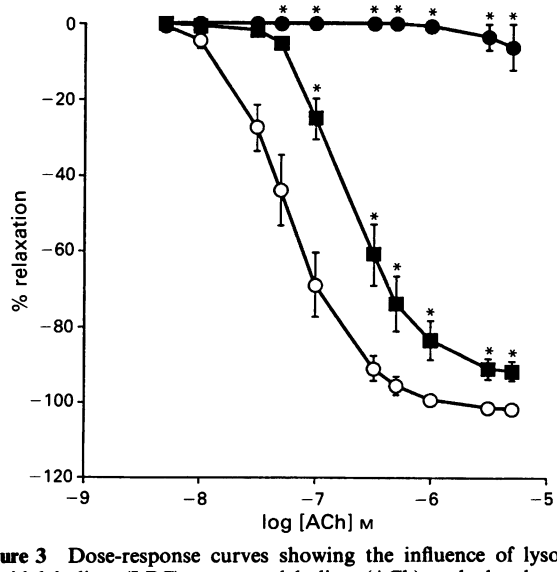


Figure 3 Dose-response curves showing the influence of lysophosphatidylcholine (LPC) on acetylcholine (ACh)-evoked relaxations. Intact aortic rings were contracted with phenylephrine (0.05 μM) in the presence or absence of 10 μM LPC and relaxed by cumulative concentrations of ACh. After removal of the LPC by washing, the contraction relaxation cycle was repeated. Relaxations are expressed as % of precontraction. Control, no LPC (○); +LPC (10 μM) (●); after washout (■). Each point is the mean of 4 experiments with s.e.mean shown by vertical bars. Differences in mean values were tested by Student's t test for unpaired samples. *$P < 0.05$ was considered significant.

The inhibition of relaxation by phospholipase A_2-treated low-density lipoproteins

The inhibitory effects of phospholipase A_2-treated LDL preparations (0.01–2 mg protein ml^{-1}) on relaxations evoked by cumulative concentrations of ACh, were determined. The threshold concentration for the inhibition was 0.01 and was maximum at 0.1 mg protein ml^{-1}. Figure 5 shows the influence of phospholipase A_2-treated LDL (0.1 mg protein ml^{-1}) on the dose-response relationship for ACh-evoked relaxation. The maximal inhibition achieved was not significantly different between donors (65.4 ± 2.1%; $P > 0.05$), in contrast to the variations found with oxLDL (Table 1). The inhibition was reduced to 33.7 ± 2.5% after washout. Phospholipase A_2-treated LDL 0.05 mg protein ml^{-1} caused 21 ± 4.5% inhibition of maximal relaxation ($n = 4$) and this effect was completely reversed. Addition of phospholipase A_2 alone or untreated LDL (Jacobs *et al.*, 1990) had no effect on relaxations.

The time course of inhibition by oxLDL (donors J and K) and phospholipase A_2-treated LDL from the same donors is shown in Figure 6. The inhibition by phospholipase A_2-treated LDL occurred without a lag period whereas that by oxLDL required 15–20 min before the maximum effect was attained.

Phospholipase A_2-treated LDL showed no significant differences in LPC content between donors (0.35 ± 0.05 μmol LPC mg^{-1} protein, $P > 0.1$). Table 2 shows a comparison of the lysophospholipid concentration of 1-palmitoyl LPC, phospholipase A_2-treated LDL and oxLDL (donor E) in the organ bath and their corresponding inhibition of the maximal relaxation induced by ACh. 1-Palmitoyl LPC and phospholipase A_2-treated LDL inhibited maximal relaxation by 94 and 64% respectively, compared with 79% by oxLDL, the concentrations of their lysophospholipids were approximately 50 and 15 fold less than that of oxLDL.

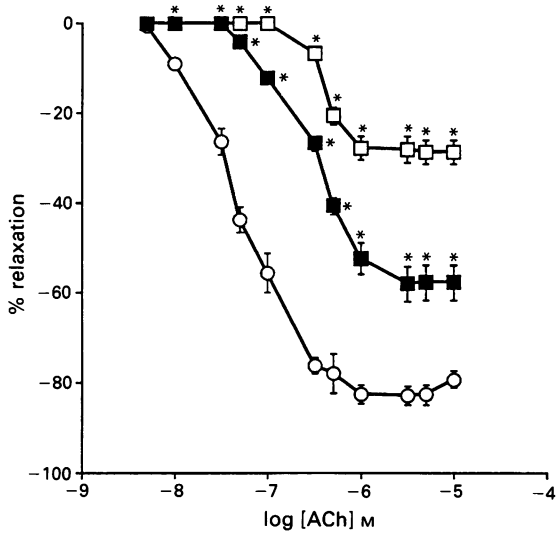


Figure 5 Dose-response curve showing the influence of phospholipase-treated low-density lipoprotein (LDL) on acetylcholine (ACh)-evoked relaxation. Intact aortic rings were contracted with phenylephrine in the presence or absence of phospholipase A_2-treated LDL (0.1 mg protein ml^{-1}) and relaxed by cumulative concentrations of ACh. Relaxations are expressed as % of precontraction. Control, no phospholipase A_2-treated-LDL (○); with phospholipase A_2-treated-LDL (□); after washout (■). Each point is the mean of 19 determinations in 4 separate experiments using 6 different donors; s.e.mean shown by vertical bars. Differences in mean values were tested by Student's t test for unpaired samples. $*P < 0.05$ was considered significant.

High-density lipoprotein and serum albumin reduce the inhibitory effect of oxidised low-density lipoprotein

The effects of HDL on endothelium-dependent relaxation and its inhibition by oxLDL, were investigated. Relaxations evoked by cumulative concentrations of ACh in aortic rings were unaffected by concentrations of HDL from 0.5–5 mg protein ml^{-1}. For example, in the presence of HDL alone (2 mg protein ml^{-1}), maximal relaxations at 5 μM ACh were 93 ± 1.4% and were not significantly different from the control tissues (96 ± 1.9%, $n = 4$). oxLDL (2 mg protein ml^{-1}) inhibited maximal relaxation by 70 ± 2.9% (Figure 7). The inhibition of maximal relaxation was reduced to 74 ± 3.8% in the presence of 1 mg protein ml^{-1} of HDL and abolished by 2 mg protein ml^{-1}. Similarly, serum albumin (fatty acid-free) at a concentration of 1 and 2 mg protein ml^{-1} also reduced significantly the inhibition by oxLDL to 50 ± 1.8% and 29.7 ± 2.1% respectively ($P < 0.05$, $n = 4$) whereas serum albumin alone had no effect on relaxations (not shown).

Table 2 The influence of lysophosphatidylcholine (LPC) concentration on inhibition of maximal acetylcholine induced relaxations

Modified lipoproteins	*% inhibition of maximal relaxation*	*LPC* (μmol ml^{-1})
oxLDL	79.0 ± 1.9	0.520 ± 08
1-Palmitoyl-LPC	94.0 ± 5.9	0.010
Phospholipase A_2-treated LDL	65.4 ± 2.1	0.035 ± 05

Data on oxidised low-density lipoprotein (oxLDL) (2 mg protein ml^{-1}, donor E), 1-palmitoyl-LPC (10 μM) and phospholipase A_2-treated LDL (0.1 mg protein ml^{-1}) were compiled from Table 1, Figures 3 and 5 respectively.

Discussion

The importance of oxLDL in the pathogenesis of atherosclerosis has been underlined by its immunological identification

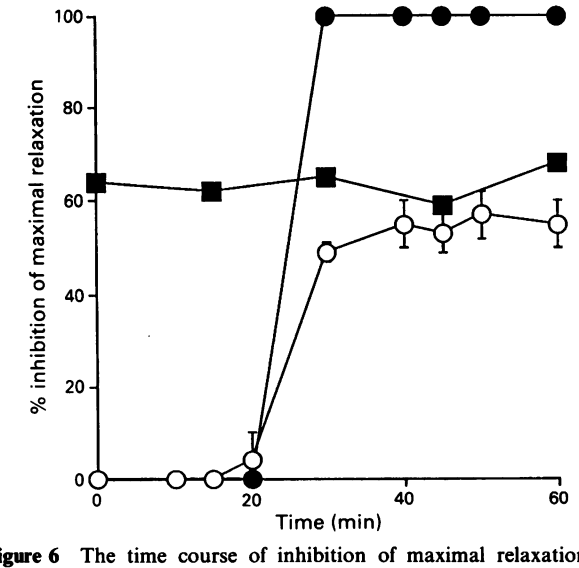


Figure 6 The time course of inhibition of maximal relaxation by phospholipase A_2-treated low-density lipoprotein (LDL) and oxidised LDL (oxLDL). Relaxations were evoked by cumulative concentrations of acetylcholine (ACh) in intact aortic rings which had been precontracted with 0.1 μM phenylephrine before and after incubation with oxLDL (2 mg protein ml^{-1}) or phospholipase A_2-treated LDL for 0–30 min. % inhibition of maximal relaxation was determined from maximal relaxation before and after incubation with the modified lipoproteins. oxLDL (donor, J) (○); oxLDL (donor K) (●); phospholipase A_2-treated LDL (donor J and K) (■). Each point is the mean of 4 experiments (oxLDL) and pooled data of 6 experiments with two donors (phospholipase A_2-treated LDL) with s.e.mean shown by vertical bars.

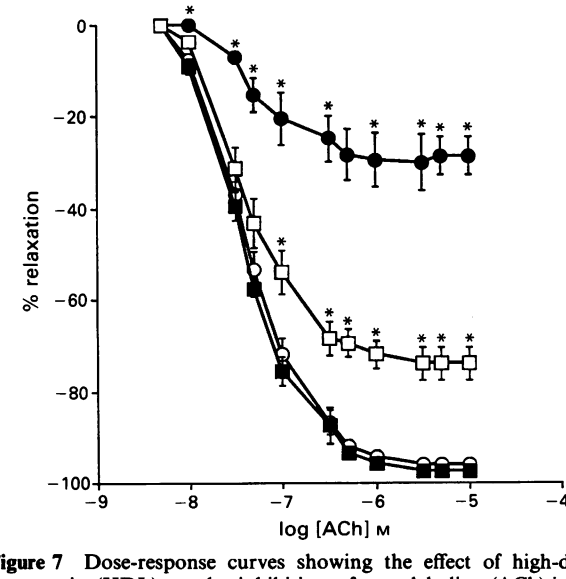


Figure 7 Dose-response curves showing the effect of high-density lipoprotein (HDL) on the inhibition of acetylcholine (ACh)-induced relaxations by oxidised low-density lipoprotein (oxLDL). Intact aortic rings were contracted with phenylephrine and relaxed by cumulative concentrations of ACh. The contraction-relaxation cycle was repeated after a preincubation with oxLDL (2 mg protein ml^{-1}) in the presence or absence of HDL. Relaxations are expressed as % of precontraction. Control, HDL alone (no lipoproteins was identical) (○); oxLDL alone (2 mg protein ml^{-1}) (●); oxLDL, (2 mg protein ml^{-1}) + HDL, (2 mg protein ml^{-1}) (■); oxLDL, (2 mg protein ml^{-1}) + HDL, (1 mg protein ml^{-1}) (□). Each point is the mean of 4 experiments with s.e.mean shown by vertical bars. Differences in mean values were tested by Student's t test for unpaired samples. $*P < 0.05$ was considered significant.

in focal lesions in the arterial wall (Steinberg *et al.*, 1989). The juxtaposition of the oxLDL in the atherosclerotic plaque makes the modified lipoproteins prime candidates for interfering with the generation and actions of EDRF. The concentrations of oxLDL which inhibit relaxation (0.5–2 mg protein ml^{-1}; Jacobs *et al.*, 1990) are consistent with concentrations that might be predicted to occur in human atherosclerotic lesions. LDL concentrations in the human intima are estimated as four fold higher than found in plasma of normal and hypercholesterolaemic individuals (0.4–3 mg protein ml^{-1}) and may be even higher in the extracellular fluid of atherosclerotic arteries (Smith & Ashall, 1983), where it can become oxidised and accumulate in foam cells in an undegraded form (Sparrow *et al.*, 1989; Rosnfeld *et al.*, 1991).

Our previous study has shown that Cu^{2+}-oxidised LDL inhibit EDRF responses (Jacobs *et al.*, 1990). The inhibitory effects were found to vary reproducibly with the donor of the original LDL. This observation can be explained by the fact that the LDL of each individual varies in composition and this influences the susceptibility to oxidation and possibly the amount and nature of the products formed (Esterbauer *et al.*, 1990). We show here that the susceptibility to oxidation of the original LDL as quantified by the half-time is positively related to the inhibition of relaxation by oxLDL i.e. the shorter the half-time, the greater the potency of the inhibition (Table 1). Since the same maximum absorbance is reached irrespective of half-time, this implies that a shorter half-time reflects a greater turnover of conjugated dienes and presumably lipid hydroperoxides. The relationship between half-time and inhibition can then be explained if the inhibitors of relaxation are produced as a result of reactions which are initiated by the turnover of lipid hydroperoxides. Thus a greater susceptibility to oxidation will result in higher concentration of inhibitor(s). In addition, the properties of LDL which promote a greater susceptibility to oxidation might foreseeably yield oxidative products that have greater inibitory effects on relaxation.

The inhibition of EDRF-mediated relaxations by oxLDL has now been demonstrated in many laboratories (Plane *et al.*, 1989; Jacobs *et al.*, 1990; Kugiyama *et al.*, 1990; Yokoyama *et al.*, 1990; Simon *et al.*, 1990; Galle *et al.*, 1991). The effects observed, range from an abolition of relaxation (Kugiyama *et al.*, 1990; Yokoyama *et al.*, 1990) to lesser effects (Simon *et al.*, 1990; Galle *et al.*, 1991). The results from different laboratories are difficult to compare because the conditions used for the preparation and modification of the LDL vary. For example, different additives: enzyme inhibitors, antioxidants and antibacterial agents, are used during the preparation of LDL, which may alter or even contribute to the vasoactivity. The fact that the properties of 'oxidised LDL' are not uniquely defined but will always depend on the point at which the oxidation is terminated and the conditions of oxidation (Esterbauer *et al.*, 1990) are another source of variation. Furthermore, most of the above studies have been performed with LDL prepared from pooled plasma, which introduces the possibility of variations in the susceptibility to oxidation and hence differences in the inhibitory properties due to the selection of donors. It is notable that the variations in inhibitory effects between different laboratories are similar to those found in our studies using oxLDL from individual donors. Nevertheless, it should be recognised that by using pooled LDL, exchange of antioxidants and lipids between LDL particles during modification may mask the inhibitory effects of the LDL of individual donors and yield preparations with totally different properties.

The inhibition of relaxation by oxLDL has been attributed to LPC generated during oxidative modification. This conclusion was reached because 1-palmitoyl-LPC (Yokoyama *et al.*, 1990; Kugiyama *et al.*, 1990) and phospholipase A_2-treated LDL inhibited endothelium-dependent relaxation. Although these observations have been confirmed in this study, we have reason to doubt that LPC is the only factor which contributes to the inhibition. This is because the inhibitory effects of oxLDL were unrelated to their LPC content (Table 1). This conclusion demonstrates that LPC cannot account for the variation in inhibitory properties of oxLDL preparations from different donors. Other constituents of oxLDL may well be of greater significance in causing inhibition by the more potent preparations whereas LPC may be more important for the effects of the less potent and more easily reversible preparations (Table 1).

Previous studies failed to recognise the dual effects of 1-palmitoyl-LPC in evoking both endothelium-dependent relaxation mediated by EDRF (Saito *et al.*, 1988) as well as the inhibition of the relaxation elicited by ACh (Kugiyama *et al.*, 1990; Yokoyama *et al.*, 1990) as is reported here (Figures 2 and 3). One possible explanation for these dual actions is that LPC activates the endothelial cells and elevates intracellular Ca^{2+} to such an extent that they become refractory to stimulation by ACh. Since oxLDL and in preliminary studies, phospholipase A_2-treated LDL, failed to induce relaxation in precontracted aortic rings, alternative or additional mechanisms of inhibition are therefore likely to operate with these proteins.

Other observations indicate differences in the properties of oxLDL and LPC. It was previously found that oxLDL reduced the sensitivity of rabbit aortic rings to the relaxing effects of the endothelium-independent vasodilators, GTN and nitric oxide (Jacobs *et al.*, 1990). The lack of a similar attenuation of the relaxations evoked by GTN (Figure 4) or nitric oxide (unpublished result) by 1-palmitoyl LPC suggests that it does not inhibit guanylate cyclase, sequestrate or inactivate nitric oxide, mechanisms which might contribute to the effects of oxLDL (Jacobs *et al.*, 1990; Galle *et al.*, 1991).

The inhibitory properties of phospholipase A_2-treated LDL also differ in several respects from those of oxLDL. Preincubation for 15–20 min is required to cause the inhibition by oxLDL whereas phospholipase A_2-treated LDL from the same donor inhibited immediately (Figure 6). Furthermore, there was a discrepancy in the concentration of LPC in preparations of phospholipase A_2-treated LDL and oxLDL that produce inhibition (Table 2). These results can again be explained by the presence of other inhibitory substances in oxLDL or by tighter binding of the LPC in the oxidised lipoproteins.

Our study with HDL has given some indication as to the mechanism of inhibition by oxLDL. The relaxations evoked by ACh were not affected by the presence of HDL alone as they were by LDL or oxLDL (Jacobs *et al.*, 1990). Inhibition by the latter lipoproteins is therefore unlikely to result solely from the sequestration of nitric oxide which would be expected to be just as soluble in the lipid core of HDL. The prevention of the inhibition of relaxation by HDL (Figure 7) and serum albumin implicates the lipophilic molecules in oxLDL. Both HDL and serum albumin prevented the inhibition by 1-palmitoyl-LPC and phospholipase A_2-treated LDL (unpublished result) as predicted from the well-known high avidity which LPC has for these former proteins. However, there are many other cytotoxic substances in oxLDL besides LPC (Thomas & Jackson, 1991) that bind to these particular proteins which warrant further investigation as potential inhibitors.

Hessler *et al.* (1979) found that HDL protect cultured endothelial cells from the cytotoxic effects of oxLDL. It is possible that this cytotoxicity resulted from the same mechanism as prevents the inhibition of EDRF responses, namely by binding lipid components generated during the oxidation of LDL. The concentrations of HDL (Figure 7) which prevented the inhibition of EDRF responses by oxLDL overlaps with their normal plasma concentrations which can be up to 1.5 mg protein ml^{-1}. It is therefore feasible that this protective effect of HDL might operate *in vivo* unless overcome by excessive accumulation of oxLDL. The concentrations of serum albumin in plasma. although somewhat lower in the human intima, are far higher than the concentrations of serum albumin (which was fatty acid-free) used in these experiments.

However, serum albumin *in vivo* has normally bound fatty acids which may limit its capacity to further bind the inhibitory lipid(s) of oxLDL.

The variations in the susceptibility of LDL from different donors to oxidation that are shown here, may also occur *in vivo* and determine the cytotoxic effects of the lipoproteins including their ability to inhibit EDRF-mediated relaxation. Conversely, antioxidant drugs and the elevation of plasma HDL might be expected to prevent the cytotoxicity.

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Platelet-activating factor relaxes ferret tracheal smooth muscle and reduces transepithelial potential difference *in vitro*

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1 The effects of platelet activating factor (PAF) were examined on the smooth muscle tone, mucus volume, lysozyme and albumin outputs and potential difference (PD) across the ferret tracheal wall.

2 PAF (0.1–10 μM) had no direct effect on mucus volume, lysozyme or albumin output from the ferret trachea. PAF produced concentration-dependent relaxations of the tracheal smooth muscle and reductions in PD across the tracheal wall. There was no change in the histological appearance of the trachea after exposure to PAF.

3 The PAF-induced smooth muscle relaxation was not affected by FPL55712, a combination of mepyramine and cimetidine, or by a combination of the oxygen free-radical scavengers catalase and superoxide dismutase (SOD); but was abolished by indomethacin or the PAF-receptor antagonist WEB2086.

4 The PAF-induced reduction in PD was not affected by indomethacin, FPL55712 or mepyramine and cimetidine, but was prevented by catalase and SOD, and by WEB2086.

5 We conclude that PAF relaxes ferret tracheal smooth muscle *in vitro* by receptor-mediated release of a bronchodilator prostaglandin, possibly PGE_2. PAF also reduces PD across the trachea suggesting changes in epithelial function; however, there is no histological epithelial damage after PAF. The reduction in PD with PAF is probably produced by receptor-mediated release of oxygen free-radicals. The cellular source of these free-radicals and of the dilator prostaglandin is unclear.

Keywords: Platelet activating factor; trachea; mucus; smooth muscle; albumin; potential difference

Introduction

Platelet-activating factor (PAF) 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (Benveniste *et al.*, 1979) is a derivative of phosphatidylcholine which is released from a variety of inflammatory cells including basophils, mast cells, platelets, macrophages and neutrophils (Camussi *et al.*, 1981; Benveniste *et al.*, 1982).

When released, PAF has potent effects on the airways including bronchoconstriction *in vivo* in both experimental animals and man (Morley *et al.*, 1984; Cuss *et al.*, 1986) increased microvascular permeability (O'Donnell & Barnett, 1987; Evans *et al.*, 1987) and chemotaxis and activation of inflammatory cells (Morley & Page, 1984). In contrast to its bronchoconstrictor action *in vivo*, PAF does not contract airway smooth muscle *in vitro*; it either has no effect (Schellenberg, 1987) or produces a relaxation (Brunelleschi *et al.*, 1987), suggesting the *in vivo* action is indirect and possibly occurs predominantly through the release of bronchoconstrictor mediators from platelets (Schellenberg, 1987). The effects of PAF on airway mucus secretion have not been examined in detail, although high doses of PAF increase the secretion of radiolabelled macromolecules from ferret trachea *in vivo* (Hahn *et al.*, 1985) and explants of human airways (Goswami *et al.*, 1987; Rogers *et al.*, 1990; Lundgren *et al.*, 1990).

The ferret whole trachea *in vitro* is a preparation which allows the simultaneous assessment of smooth muscle tone and submucosal gland secretion (Webber & Widdicombe, 1987; Webber, 1989). Lysozyme is a bactericidal enzyme localized specifically to serous cells (Tom-Moy *et al.*, 1983) and is a useful marker for secretion from these cells. Albumin is actively transported across the ferret tracheal epithelium *in vitro* (Webber & Widdicombe, 1989) and the measurement of this transport together with the measurement of potential difference (PD) across the tracheal wall provides a good indication of epithelial function and integrity.

In the present study we have investigated the effects of PAF on tracheal smooth muscle tone, submucosal gland secretion, epithelial albumin transport, and transepithelial PD in the ferret trachea *in vitro*.

Methods

The ferret trachea in vitro

Ferrets of either sex, weighing 0.5–1.5 kg, were anaesthetized by an intraperitoneal injection of sodium pentobarbitone (Sagatal, May & Baker, 50 mg kg^{-1}). The trachea was exposed and cannulated about 5 mm below the larynx with a perspex cannula containing a conical collecting well (Webber & Widdecombe, 1987). The ferret was then killed with an overdose of sodium pentobarbitone injected into the heart. The chest was opened along the midline and the trachea exposed to the carina, cleared of adjacent tissue, removed and cannulated just above the carina. The trachea was mounted, laryngeal end down, in a jacketed organ bath with Krebs-Henseleit buffer restricted to the submucosal side. The composition of the Krebs-Henseleit solution was (mM): NaCl 120.8, KCl 4.7, KH_2PO_4 1.2, $MgSO_47H_2O$ 1.2, $NaHCO_3$ 24.9, $CaCl_2$ 2.4, glucose 5.6. The buffer was maintained at 37°C and gassed with 95% O_2:5% CO_2. The lumen of the trachea remained air-filled. Secretions were carried by gravity and mucociliary transport to the lower cannula, where they pooled and could be withdrawn periodically into a polyethylene catheter which was inserted into the lower cannula to form an airtight seal. The catheters containing the secretions were sealed at both ends with bone wax, numbered and stored frozen until required.

After defrosting, the secretions were washed out of the catheters into labelled plastic vials with 0.5 ml distilled H_2O. The vials were frozen and stored for use in the albumin and lysozyme assays. Preliminary experiments had shown that frozen storage for up to 6 months does not affect the enzymatic activity of lysozyme or the albumin content. Secretion volumes were estimated by the differences in weights of the catheters with secretions and dried without secretions, and the secretion rates were expressed as μl min^{-1} (assuming 1 g of secretion is equivalent to 1 ml).

During an experiment the carinal cannula was attached to a pressure transducer which was connected to a pen-recorder. Changes in smooth muscle tone produced changes in tracheal pressure which were registered by the pressure transducer and

recorded on the pen-recorder, thus enabling assessment of changes in smooth muscle tone of the trachea during mucus collection.

The electrical potential difference (PD) across the tracheal wall was measured with two calomel reference electrodes. These were filled with 3.8 M KCl and placed in separate beakers of the same solution. Electrical contact was made with the preparation by use of two agar bridges. These were constructed from polyethylene tubing (0.5 mm internal diameter) filled with 3.8 M KCl in 2.5% w/v agar solution. One bridge was placed in the buffer on the submucosal side of the trachea and the second inserted into a second hole in the perspex cannula used to collect the mucus. Electrical contact between this bridge and the tracheal luminal wall was maintained by the mucus collecting in the perspex cannula. Output from the two electrodes was into a high input impedance buffer amplifier and then displayed on a digital voltmeter. The two agar bridges were initially placed together in 0.15 M NaCl to confirm that this produced a stable potential difference close to 0 V. Any residual voltage measured here was subtracted from subsequent measurements of potential difference made in the preparation.

Assay for lysozyme

The lysozyme concentrations of the mucus samples were measured by a turbidimetric assay which relies on the ability of lysozyme to break down the cell wall of the bacterium *Micrococcus lysodeikticus*. Addition of lysozyme to a solution of the bacteria reduces the turbidity of the solution, thereby leading to a fall in optical density (OD) measured at 450 nm.

A stock suspension of *M. lysodeikticus* of 3 mg ml^{-1} was prepared. When diluted 10 fold (the dilution in the assay) this suspension gives an OD of approximately 0.6 at 450 nm. To produce a standard curve, various concentrations of hen egg white lysozyme (0.5 to 100 ng ml^{-1}) were incubated in duplicate in 1.5 ml potassium phosphate buffer (50 mM, pH 7.4) containing *M. lysodeikticus* (0.3 mg ml^{-1}), sodium azide (1 mg ml^{-1}) and bovine serum albumin (BSA, 1 mg ml^{-1}). The BSA was included in the assay for its protein stabilizing effects and the sodium azide was added to prevent the growth of bacteria in the incubating solutions. The reaction mixtures were incubated for 18 h at 37°C. After incubation the OD of each solution was measured at a wavelength of 450 nm with potassium phosphate buffer pH 7.4 containing BSA (1 mg ml^{-1}) as a blank. The standard curve was constructed by plotting the fall in OD (reduction in turbidity) against the concentration of lysozyme in the solution.

To estimate the concentration of lysozyme in a mucus sample, 20 μl of sample were incubated in 1.5 ml potassium phosphate buffer (50 mM, pH 7.4), exactly as described above for the known concentrations of lysozyme used in the preparation of the standard curve. The lysozyme concentrations (equivalent to hen egg white lysozyme) of the 20 μl samples and hence of the original mucus samples were estimated from the standard curve. The rate of output of lysozyme was then calculated by dividing the total amount of lysozyme in a mucus sample by the time over which the sample accumulated.

Albumin transport

To examine the effect of PAF on methacholine-induced transport of albumin across the ferret trachea, BSA was added to the buffer bathing the submucosal surface of the trachea in a concentration of 4 mg ml^{-1}. Fluorescent BSA (0.02–0.03 mg ml^{-1}) was also added to the buffer as a marker and enabled an estimate to be made of the total amount of albumin which appeared in the mucus samples.

The fluorescence of the mucus samples was measured with a fluorimeter, at an excitation wavelength of 490 nm and an emission wavelength of 550 nm. The fluorescent albumin concentration of the mucus samples was estimated from a standard curve relating fluoresence (arbitrary units) to the concentration of fluorescent albumin (range 25 ng ml^{-1} to 3 μg ml^{-1}). The total concentration of albumin in the mucus samples was obtained by multiplying the fluorescent albumin concentration (estimated from the standard curve) by the ratio of non-fluorescent to fluorescent albumin used in the experiment. The rate of output of albumin was determined by dividing the total amount of albumin in a mucus sample by the time over which that sample accumulated. Previous studies have shown that the measurement of fluorescence provides an accurate indication of the total albumin concentration in the mucus samples (Webber & Widdicombe, 1989).

Experimental protocol

Tracheae were set-up in pairs. After a 30 min equilibration period the control PD across both tracheae was recorded and any mucus produced was removed and processed as described above. All tracheae were then exposed to three concentrations of PAF (0.1, 1 and 10 μM) singly and in a random sequence. Each concentration of PAF was left in contact with the trachea for 30 min and during this time any change in intraluminal pressure was recorded. After 30 min the PD was recorded and any secretion produced was withdrawn and processed. The tracheae were then washed twice and fresh buffer containing no PAF was placed in the organ bath. Three control periods of 30 min were allowed between each addition of PAF in order for the PD to recover towards a value similar to that before addition of PAF. After three concentrations of PAF had been added the tracheae were allowed to rest for four periods of 30 min. The buffer surrounding one of the tracheae (test trachea) was then replaced with buffer containing one of the pharmacological antagonists. This buffer was left in contact with the trachea for 30 min and any change in PD or intraluminal pressure was recorded. Any mucus produced after 30 min was withdrawn. The other trachea (control trachea) was bathed by buffer containing no antagonist. PAF (1 μM) was then added to both tracheae for 30 min. The control trachea was exposed to PAF in normal buffer and the test trachea to PAF in buffer containing the antagonist. Any changes in PD and intraluminal pressure produced by PAF were recorded and any mucus produced was collected at the end of the 30 min. This was followed by three control periods with no PAF present in order for the PD to recover to a value not significantly different from that before PAF. The antagonists tested were the cyclo-oxygenase inhibitor indomethacin (1 μM), the leukotriene receptor antagonist FPL55712 (1 μM), a combination of the H_1-antagonist mepyramine (1 μM) and the H_2-antagonist cimetidine (10 μM), a combination of the oxygen free radical scavengers catalase (500 u ml^{-1}) and superoxide dismutase (40 u ml^{-1}), and the PAF-receptor antagonist WEB2086 (10 μM). Only one antagonist or combination of antagonists was tested on each test trachea.

Analysis of results

The concentration-response curves and bar charts presented in Results were obtained by pooling the results from a group of experiments. All values shown are means ± s.e.means. Differences between PAF-induced responses and baseline values were analysed for statistical significance using a one-way analysis of variance. Differences between PAF-induced responses in control tracheae and those obtained in the presence of a pharmacological antagonist were analysed by Student's unpaired t test. In all cases $P < 0.05$ was taken as significant.

Histology

In each experiment samples of trachea were taken for histological examination before and at the end of experiments. These samples were fixed in formal saline and sections (5 μm thick) were cut and prepared for light microscopy with hae-

matoxylin and eosin staining. Changes in the thickness of the epithelium were assessed by measuring the depth of the epithelium (including basement membrane) at ten points selected at random in each of twelve pre- and post-PAF samples. Changes in gland and acini number were assessed by manual counting in twelve pre- and post-PAF samples.

Results

Baseline values

The basal mucus volume, lysozyme and albumin outputs before the first addition of PAF in all tracheae were ($n = 24$) $0.04 \pm 0.03\,\mu l\ min^{-1}$, $25 \pm 13\,ng\,min^{-1}$ and $0.24 \pm 0.07\,\mu g\,min^{-1}$ respectively. The basal PD before addition of PAF was -8.6 ± 0.5 mV.

Effects of PAF

PAF (0.1–10 μM) had no significant effect on basal mucus volume output, lysozyme output or albumin output (Figure 1).

PAF produced a concentration-dependent reduction in intraluminal tracheal pressure (Figure 2) indicating tracheal smooth muscle relaxation. PAF also produced a concentration-dependent reduction in the negativity of the PD across the trachea (Figure 3). These PD changes produced by PAF were long-lasting and the PD was still depressed in the control period after PAF-removal (Figure 3). However, the PD recovered before the next addition of PAF (Figure 3) and, because the concentrations of PAF were applied in a random sequence, there are no significant differences between the control values for PD before addition of each concentration of PAF (Figure 3).

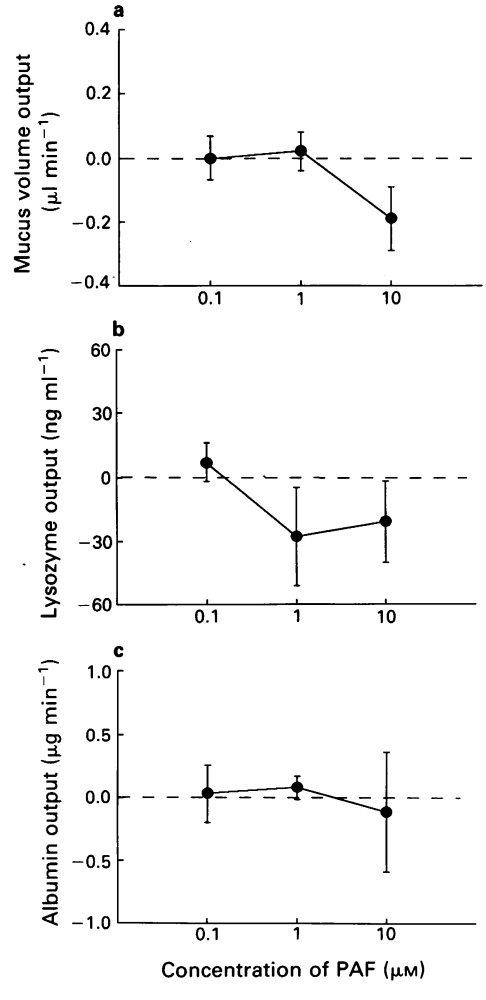


Figure 1 Concentration-response effects of PAF on (a) baseline mucus volume, (b) lysozyme and (c) albumin outputs from the ferret trachea. Points are means of 12–16 determinations with s.e.means shown as vertical lines.

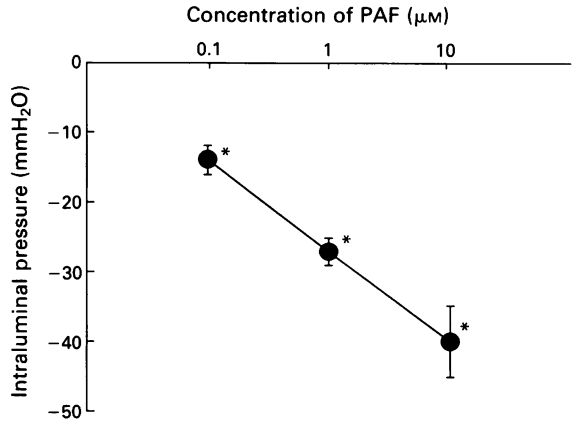


Figure 2 Concentration-response curve for the effect of PAF on intraluminal pressure. PAF reduces intraluminal pressure indicating tracheal smooth muscle relaxation. Points are means of 12–16 determinations with s.e.means shown as vertical lines. * Significant reduction in intraluminal pressure.

Effects of antagonists on responses to PAF

None of the antagonists had any significant effect on tracheal intraluminal pressure. Furthermore no antagonist affected basal mucus volume, lysozyme or albumin outputs, or the PD across the trachea (Table 1). PAF (1 μM) reduced intraluminal tracheal pressure in all control tracheae (not exposed to an antagonist, Table 2). This reduction in intraluminal pressure was not significantly different in test tracheae exposed to FPL55712 (7-[3-(4-acetyl-3-hydroxy-2-propylphenoxyl-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid), mepyramine and cimetidine, and catalase and SOD (Table 2). However, the PAF-induced smooth muscle relaxation was virtually abolished in test tracheae exposed to indomethacin or the PAF-antagonist WEB2086 (3-(4-(2-chlorophenyl) - 9 - methyl - 6H - thieno - (3,2-f)(1,2,4) - triazolo - (4,3-a)(1,4)-diazepine-2-yl)-1-(4-morpholinyl)-1-propanone) (Table 2). It is possible that the tracheal smooth muscle is not able to relax after indomethacin or WEB2086. However, in separate experiments the β_2-adrenoceptor agonist salbutamol produced reductions in intraluminal pressure of 52 ± 4 and

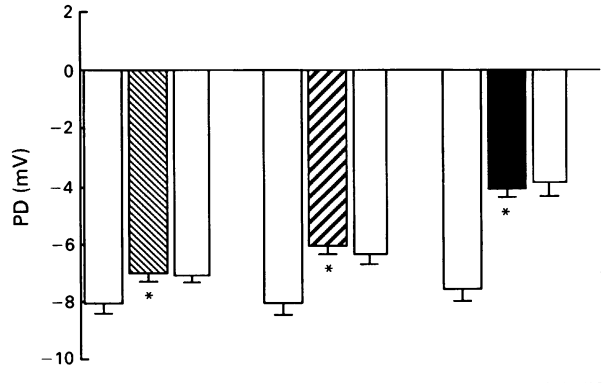


Figure 3 Concentration-response effect of PAF on potential difference (PD) across the ferret trachea. (□) Control PD, the PD values after PAF are those in the third control period after PAF exposure and demonstrate that the PD did recover; PD after PAF 0.1 μM (▨), 1 μM (▨) and 10 μM (■). The responses are the means of 12–16 determinations with s.e.means shown as vertical lines. * Significantly different ($P < 0.05$) from control PD before PAF.

Table 1 The effect of pharmacological antagonists on baseline mucus volume, lysozyme and albumin outputs, and potential difference (PD) across the ferret trachea

	Mucus volume ($\mu l\,min^{-1}$)		*Lysozyme* ($ng\,min^{-1}$)		*Albumin* ($\mu g\,min^{-1}$)		*PD* (mV)	
Antagonist	*Control*	*+Antagonist*	*Control*	*+Antagonist*	*Control*	*+Antagonist*	*Control*	*+Antagonist*
Indomethacin	0.04 ± 0.03	0.05 ± 0.03	27 ± 11	23 ± 7	0.27 ± 0.14	0.31 ± 0.16	−7.6 ± 0.3	−7.9 ± 0.4
FPL55712	0.06 ± 0.04	0.04 ± 0.03	19 ± 4	26 ± 12	0.22 ± 0.13	0.20 ± 0.09	−8.2 ± 0.5	−7.8 ± 0.3
Mepyramine + cimetidine	0.07 ± 0.04	0.05 ± 0.03	31 ± 17	19 ± 6	0.32 ± 0.12	0.27 ± 0.16	−8.0 ± 0.6	−7.7 ± 0.3
Catalase + SOD	0.03 ± 0.06	0.07 ± 0.05	22 ± 13	12 ± 6	0.18 ± 0.09	0.23 ± 0.07	−8.0 ± 0.6	−8.0 ± 0.5
WEB2086	0.04 ± 0.03	0.04 ± 0.03	16 ± 6	22 ± 7	0.29 ± 0.06	0.37 ± 0.12	−7.2 ± 0.2	−7.5 ± 0.6

Values given are the means (±s.e.means) of 6 determinations.
SOD: superoxide dismutase.

54 ± 8 mmH_2O before and after indomethacin (1 μM), and of 64 ± 6 and 61 ± 3 mmH_2O before and after WEB2086 (10 μM) ($n = 6$ in all cases). Therefore neither indomethacin or WEB 2086 affect the ability of the tracheal muscle to relax to agents other than PAF.

PAF (1 μM) reduced PD across all control tracheae (Table 3). This reduction in PD to PAF was not changed in test tracheae exposed to indomethacin, FPL55712 or mepyramine and cimetidine, but was abolished in test tracheae exposed to catalase and SOD, or to WEB2086 (Table 3).

Histology

From histological observation of the trachea at the beginning and end of experiments it was clear that PAF produced no changes in the appearance of the epithelium or submucosal tissues. There was no evidence of PAF-induced stripping of epithelial cells or mucus granule discharge. Quantitatively, the thickness of the epithelium (including basement membrane) in tracheal samples taken before and after exposure to PAF was 27 ± 3 μm ($n = 12$) and 28 ± 4 μm ($n = 12$) respectively. The number of glands in pre- and post-PAF samples was 27 ± 4 ($n = 12$) and 29 ± 6 ($n = 12$) per circumference, and these consisted of 13 ± 2 and 12 ± 2 acini per gland.

Table 2 The effect of pharmacological antagonists on the reduction in intraluminal pressure produced by PAF (1 μM)

	Intraluminal pressure Change (mmH_2O)	
Antagonist	*PAF alone*	*PAF + antagonist*
Indomethacin	−27 ± 1*	−6 ± 3†
FPL55712	−25 ± 2*	−26 ± 2*
Mepyramine + cimetidine	−32 ± 5*	−32 ± 2*
Catalase + SOD	−27 ± 1*	−29 ± 1*
WEB2086	−24 ± 5*	−3 ± 2†

Values shown are the means (±s.e.means) of 6 determinations.
* Significant ($P < 0.05$) reduction in intraluminal pressure produced by PAF.
† Significant ($P < 0.05$) inhibition of the PAF-induced reduction in intraluminal pressure. Only one antagonist or combination of antagonists was tested on each trachea.
SOD: superoxide dismutase.

Discussion

Mucus secretion from the ferret trachea is almost entirely from mucous and serous cells in the submucosal glands, as there are very few goblet or other secretory cells in the surface epithelium (Robinson *et al.*, 1986). The bactericidal enzyme lysozyme is a specific marker for serous cell secretion (Tom-Moy *et al.*, 1983). In the present study PAF had no significant effect on either mucus volume output or lysozyme output from the ferret trachea suggesting that PAF has no direct action on mucous or serous cells in the submucosal glands. Similarly, PAF had no effect on fucose output (a marker for mucus glycoprotein) into the guinea-pig lumen *in vivo* (Rogers *et al.*, 1990). In contrast PAF did increase mucus glycoprotein release in the absence of platelets from cat and human airways *in vitro* (Goswami *et al.*, 1987; Lundgren *et al.*, 1990), ferret trachea *in vitro* (Hahn *et al.*, 1985) and tracheal explants in organ culture from guinea-pig, rat, rabbit and ferret (Adler *et al.*, 1986). In all these cases mucous glycoprotein release was determined by measuring $^{35}SO_4$-labelled macromolecule output and in each case very high doses of PAF were used. It is possible that, at high doses, PAF may weakly stimulate the output of radiolabelled macromolecules with no appreciable change in mucus volume, or that the macromolecules do not come from submucosal gland secretions (Hahn *et al.*, 1990). These effects would not be seen in the present study which was based on the collection of submucosal gland secretion. PAF also stimulates mucus glycoprotein release from cat tracheal submucosal glands in the presence, but not in the absence of platelets (Sasaki *et al.*, 1987). This effect is likely to be indirect via the release of a secretagogue from platelets. Additionally, PAF had a potent stimulatory action on mucus

Table 3 The effect of pharmacological antagonists on the reduction in potential difference produced by PAF (1 μM)

	Potential difference (mV)					
	PAF alone			*PAF + antagonist*		
Antagonist	*Control*	*+PAF*	*Control*	*Control*	*+PAF*	*Control*
Indomethacin	−7.6 ± 0.5	−4.8 ± 0.4*	−7.8 ± 0.2	−7.3 ± 0.4	−4.2 ± 0.3*	−7.3 ± 0.4
FPL55712	−7.4 ± 0.5	−5.2 ± 0.4*	−7.7 ± 0.4	−9.4 ± 2.3	−5.9 ± 0.3*	−8.9 ± 1.2
Mepyramine + cimetidine	−7.6 ± 0.8	−4.8 ± 0.4*	−7.1 ± 1.1	−9.3 ± 1.3	−5.6 ± 0.2*	−9.1 ± 0.8
Catalase + SOD	−12.9 ± 0.9	−7.3 ± 0.4*	−11.8 ± 1.2	−12.5 ± 0.8	−11.6 ± 1.2†	−13.1 ± 2.1
WEB2086	−8.3 ± 1.1	−5.2 ± 0.4*	−7.9 ± 0.4	−9.4 ± 1.6	−9.1 ± 1.2†	−8.8 ± 0.7

Values shown are the means (±s.e.means) of 6 determinations. The control PD values after PAF are those in the third control period after PAF exposure and demonstrate that the PD did return to a pre-PAF value.
* Significant ($P < 0.05$) reduction in PD from control before PAF.
† Significant ($P < 0.05$) inhibition of the PAF-induced reduction in PD. Only one antagonist or combination of antagonists were tested on each trachea.
SOD: superoxide dismutase.

volume from the pig trachea *in vitro* (Steiger *et al.*, 1987). It is not clear why PAF stimulates fluid output from the pig trachea, but not from the ferret trachea, but there may be a species difference in the types of secretory cells present and PAF may stimulate secretion from cells in the pig trachea (perhaps in the epithelium) that are not present in the ferret trachea.

PAF is a potent bronchoconstrictor agent *in vivo* in both experimental animals and man when administered by aerosol or intravenously (Morley *et al.*, 1984; Cuss *et al.*, 1986; Rubin *et al.*, 1987). This *in vivo* bronchoconstriction is probably secondary to platelet activation (Vargaftig *et al.*, 1980). PAF has a variable effect on airway smooth muscle *in vitro*. It contracts canine tracheal smooth muscle, but only if platelets are added to the organ bath (Popovich *et al.*, 1988). If PAF is injected into guinea-pig isolated perfused lungs and the perfusate collected and added to isolated strips of airway tissues from guinea-pigs a contraction is observed in the absence of platelets (Jancar *et al.*, 1989). This contraction is due to the release of spasmogens (probably leukotrienes as the response is blocked by lipoxygenase inhibitors) from the isolated lungs. In contrast PAF had no effect on human isolated bronchi (Schellenberg, 1987) or guinea-pig lung parenchymal strips (Vargaftig *et al.*, 1981). In the present study, PAF relaxed the ferret trachea in the absence of platelets. Similar *in vitro* relaxations to PAF have been reported with the guinea-pig trachea (Prancan *et al.*, 1982; Brunelleschi *et al.*, 1987; Conroy *et al.*, 1990). The relaxation to PAF in the present study was abolished by indomethacin suggesting it is mediated by release of dilator cyclo-oxygenase products. It was also abolished by the PAF-receptor antagonist WEB2086 indicating that release of the dilator cyclo-oxygenase product is receptor-mediated and not due to a non-specific detergent effect of PAF. PAF-induced relaxations of the guinea-pig trachea *in vitro* are also prevented by indomethacin (Brunelleschi *et al.*, 1987; 1989) and WEB2086 (Brunelleschi *et al.*, 1989; Conroy *et al.*, 1990).

The nature of the dilator cyclo-oxygenase product released by the ferret trachea in the present study and the cellular source of this mediator are unclear. However, a significant increase in the levels of prostaglandin E_2 (PGE_2), which is known to relax airway smooth muscle preparations (Gardiner, 1975), was found in the perfusate surrounding the guinea-pig trachea on addition of PAF, and removal of the epithelium prevented both the release of PGE_2 and the relaxant effect of PAF (Brunelleschi *et al.*, 1987; Conroy *et al.*, 1990). Thus it seems possible that the PAF-induced relaxations of the ferret trachea observed in the present study are produced by receptor-mediated release of PGE_2 from the epithelium.

Transepithelial potential difference (PD) provides an indication of the function and condition of the tracheal epithelium. A normal value for the ferret *in vitro* trachea is about 8.0 mV, with the lumen negative with respect to the submucosa (Robinson *et al.*, 1989). This PD is governed by the electrical conductance across the epithelium and the extent of ion transport across epithelial cells. The conductance is determined mainly by the size of the paracellular pathways between epithelial cells (an increase in the size will produce an increase in conductance and a reduction in negativity of the PD). The ferret trachea has an epithelium which absorbs sodium from the lumen under baseline conditions; however active chloride secretion into the lumen can be promoted pharmacologically (Corrales *et al.*, 1986). Both of these ion transport processes will increase the luminally negative PD. In the present study exposure of the trachea to PAF reduced PD by between 30 and 80% and this response depended on the concentration of PAF. This reduction in PD is in contrast to Widdicombe *et al.* (1989) who found that PAF had no effect on short circuit current (I_{sc}) across the canine trachea, and to Sestini *et al.* (1990) who suggested that PAF markedly increased I_{sc} in rat trachea. The results of the present study suggest that PAF is either enhancing the conductance across the trachea by increasing the size of the paracellular pathways, or is reducing ion transport. In contrast the increased I_{sc} found with PAF in rat (Sestini *et al.*, 1990) and guinea-pig (Rogers *et al.*, 1990) trachea suggests stimulation of ion transport (either Na^+ absorption or Cl^- secretion) leading to an increased PD. The reduction in PD in the present study could be explained by stimulation of Na^+ secretion into the lumen which is known to occur with other mediators (Corrales *et al.*, 1986). PAF increases lung epithelial permeability in guinea-pigs (Macquin-Mavier *et al.*, 1990) and increases conductance across guinea-pig trachea *in vitro* (Rogers *et al.*, 1990). Both of these effects, and the reduction in PD in the present study, could be explained by a PAF-induced increase in the size of the paracellular pathways between epithelial cells. Indeed PAF increases the paracellular permeability of the ferret trachea to luminal DTPA (Hanafi *et al.*, 1990) and of the guinea-pig airways to mannitol (Rogers *et al.*, 1990). Thus the reduction in PD with PAF in the present study is best explained by PAF increasing the size of the paracellular pathways between epithelial cells and that this effect outweighs any increase in PD produced by stimulation of ion transport.

The reduction in PD with PAF was prevented by WEB2086 suggesting it is mediated by PAF receptors and is not due to PAF having a non-specific detergent effect on the epithelium and damaging or stripping the epithelial cells. This is supported by the histological studies which showed that the epithelium of tracheae exposed to PAF was intact and undamaged. Furthermore the changes in PD produced by PAF were not permanent and the PD tended to recover, albeit slowly, after removal of PAF. Similarly, intravenous PAF did not damage the epithelium of guinea-pig tracheae even though there was a large increase in permeability (Rogers *et al.*, 1990). The reduction in PD is not due to a direct effect of PAF since it was also blocked by a combination of the free radical scavengers catalase and SOD. This suggests that PAF is acting on specific receptors to release free-radicals which are, in some way, opening up the paracellular pathways. These free-radicals have no effect on basal submucosal gland secretion or epithelial albumin transport, since there was no change in these variables on application of PAF. Similarly, the smooth muscle relaxation to PAF was not affected by catalase and SOD, and so was not due to release of free-radicals. In contrast, direct application of the free-radical H_2O_2 produces weak contractions of guinea-pig isolated trachea (Rhoden & Barnes, 1989) and rat bronchi (Szarek & Schmidt, 1990), and production of O_2^- by a mixture of xanthine and xanthine oxidase elicits a contraction of guinea-pig isolated trachea (Nishida *et al.*, 1985). The most likely explanations for this apparent anomaly is that the free-radicals released by PAF in the present study have no effect on ferret tracheal smooth muscle; alternatively any contractile effect they have may be masked by the relaxant effect of the dilator prostaglandin released by PAF; however, if this were the case, catalase and SOD should potentiate the smooth muscle relaxation to PAF.

Albumin is actively transported across the ferret and rabbit tracheal epithelium from the submucosal side into the lumen (Webber & Widdicombe, 1989; Price *et al.*, 1989). Unexpectedly PAF had no effect on the baseline epithelial transport of albumin into the tracheal lumen. If PAF is increasing the size of the epithelial paracellular pathways one might expect that it would alter the permeability of the tracheal epithelium to albumin. The most likely explanations for the lack of effect of PAF are that baseline albumin transport is very low and any effect of PAF is too small to be detected, that the increase in paracellular pathways is not large enough to permit more albumin through, or that albumin is transported through the epithelial cells rather than between them.

The responses to PAF in the present study were obtained in the absence of a circulation and thus, largely in the absence of platelets. It is possible that some platelets may still be present in the preparation although it was thoroughly washed. However the number of platelets present is likely to be low and secondary release of mediators (such as free-radicals) from them is unlikely to account for the responses observed to PAF. It is not clear what effect the re-addition of platelets

would have on the responses to PAF *in vitro* and such experiments are not easy to assess because the platelets will stick to everything including the glass organ baths. Thus, the effect of platelets on the secretory and epithelial responses to PAF can only be adequately assessed with *in vivo* models.

In summary, PAF has no direct effect on mucus volume, lysozyme or albumin output into the ferret trachea, but produces a concentration-dependent relaxation of tracheal smooth muscle which is mediated by receptor-induced release of a dilator cyclo-oxygenase product, possibly PGE_2. PAF also reduces the transepithelial PD by receptor-mediated release of oxygen free-radicals which may increase the size of the epithelial paracellular pathways. The cellular source of the dilator cyclo-oxygenase product and the free-radicals is at present unknown.

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PAF-induced muscarinic cholinoceptor hyperresponsiveness of ferret tracheal smooth muscle and gland secretion *in vitro*

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1 The effects of exposure of the ferret trachea *in vitro* to platelet activating factor (PAF) were examined on methacholine-induced smooth muscle contraction, mucus volume and lysozyme outputs, and albumin transport across the tracheal epithelium.

2 Methacholine (0.1–30 μM) produced concentration-dependent increases in tracheal smooth muscle tone and mucus volume, lysozyme and albumin outputs from the trachea.

3 The concentration-response curves for methacholine-induced smooth muscle contraction, mucus volume and lysozyme outputs were all shifted upwards after exposure of the trachea to PAF (1 μM) with a significant increase in maximum response for each variable. The EC_{50} values for methacholine-induced smooth muscle contraction and mucus volume output were significantly reduced after PAF exposure suggesting an increase in the potency of methacholine. The concentration-response curve for methacholine-induced albumin output was shifted downwards after PAF exposure with a greatly reduced maximum but no change in the EC_{50} for methacholine.

4 PAF-induced hyperresponsiveness of methacholine-induced smooth muscle contraction, mucus volume and lysozyme outputs was not affected by indomethacin, FPL55712, or mepyramine and cimetidine, but was prevented by catalase and superoxide dismutase (SOD), and by WEB2086. Similarly, PAF-induced inhibition of methacholine-stimulated albumin output was prevented by catalase and SOD, and by WEB2086.

5 We conclude that PAF induces hyperresponsiveness of ferret tracheal smooth muscle and submucosal gland secretion (including lysozyme secretion from serous cells) to methacholine. This hyperresponsiveness is probably produced by receptor-mediated release of oxygen free-radicals. The inhibition of methacholine-induced albumin flux suggests a loss of epithelial function which is also probably mediated by release of free-radicals. The mechanism by which the free-radicals produce the changes in responsiveness to methacholine, and the cellular source of the free-radicals, remain to be established.

Keywords: Platelet activating factor; methacholine; mucus; smooth muscle; albumin; catalase; superoxide dismutase; WEB2086

Introduction

In the previous paper we described the direct effects of platelet activating factor (PAF) on ferret tracheal smooth muscle, submucosal gland secretion, epithelial albumin transport and potential difference (PD) across the trachea (Webber *et al.*, 1992). Perhaps the most important property of PAF is its ability to induce a potent and long-lasting hyperresponsiveness of airway smooth muscle *in vivo* (Mazzoni *et al.*, 1985; Cuss *et al.*, 1986; Robertson & Page, 1987). This hyperresponsiveness, like PAF-induced bronchoconstriction, may be dependent on the presence of circulating platelets (Mazzoni *et al.*, 1985) and may involve the release of mediators from eosinophils (Sanjar *et al.*, 1990). However, whether PAF can induce a hyperresponsiveness of airway smooth muscle *in vitro*, predominantly in the absence of circulating inflammatory cells, has not been studied systematically. Furthermore, it is not known whether PAF changes the responsiveness of other airway effector tissues such as submucosal glands and epithelium in a way similar to that with airway smooth muscle.

The muscarinic agonist, methacholine, potently contracts tracheal smooth muscle and stimulates submucosal gland secretion from the ferret trachea *in vitro* (Webber & Widdicombe, 1987). Albumin is actively transported across the ferret tracheal epithelium and this transport is stimulated by methacholine (Webber & Widdicombe, 1989). In the present study we have investigated the effect of exposing the ferret trachea *in vitro* to PAF on the responses of the smooth muscle, submucosal gland secretion (using lysozyme as a specific marker for serous cells) and epithelial albumin transport produced by methacholine.

Methods

The description of the ferret trachea *in vitro* and of the assays for lysozyme and albumin were described in detail in the previous paper (Webber *et al.*, 1992).

Experimental protocol

In the first set of experiments the effects of a single concentration of PAF (1 μM) on concentration-response curves for methacholine-induced tracheal smooth muscle contraction and mucus volume, lysozyme and albumin outputs was examined. In the second set of experiments we investigated the effect of increasing PAF concentrations (0.1–10 μM) on the responses to a single concentration of methacholine (1 μM).

In the first set of experiments, after an initial 30 min control period, three or four different concentrations of methacholine, covering at least a tenfold concentration range, were added to the submucosal buffer bathing the trachea singly and in random sequence. Each concentration was left in contact with the trachea for 30 min and during this time any increase in smooth muscle tone was recorded. The trachea was then washed twice and fresh buffer containing no methacholine was placed in the organ bath. One or two control periods of 30 min were allowed between addition of methacholine, depending on how quickly the secretion rate returned to a basal level.

After three or four concentrations of methacholine had been added to the trachea, the buffer surrounding the trachea was replaced with buffer containing PAF (1 μM). This buffer was left in contact with the trachea for 1 h and was replaced every 30 min. Any change in smooth muscle tone during this period

was recorded, and any mucus produced was withdrawn at the end of each 30 min. After a 30 min control period without PAF the same three or four concentrations of methacholine were added to the trachea in the same sequence as before PAF exposure. Methacholine was added to the trachea in a buffer which did not contain PAF. All mucus samples were assayed for lysozyme and albumin (Webber *et al.*, 1992).

In the second set of experiments, tracheae were set up in pairs, in separate organ baths. One trachea was exposed twice to methacholine (1 μM) for 30 min with one or two control periods of 30 min between each addition of methacholine. Any changes in smooth muscle tone or mucus volume output produced by methacholine were recorded. After methacholine (1 μM) had been added to the trachea twice, the buffer surrounding the trachea was replaced with buffer containing PAF (0.1 μM). This buffer was left in contact with the trachea for 1 h. The trachea was then washed and left for a 30 min control period in buffer containing no PAF. Methacholine (1 μM) was then added to the trachea and the changes in smooth muscle tone and mucus volume output were recorded. The procedure described above was repeated twice with the trachea being exposed to PAF (1 μM) then PAF (10 μM) and responses to methacholine (1 μM) after each PAF exposure were recorded.

The second trachea acted as a control. Exactly the same procedure as described for the test trachea was carried out except that the second trachea was exposed not to PAF but to buffer containing no PAF.

Effects of antagonists

To examine the effects of antagonists on PAF-induced changes in responsiveness to methacholine, tracheae were again set up in pairs. Exactly the same protocol was used for both tracheae except the buffer used for the test trachea contained one of the antagonists. Both tracheae were exposed to methacholine (1 μM) for 30 min and any changes in smooth muscle tone or mucus volume output were recorded. After two control periods the tracheae were then exposed to PAF (1 μM) for 1 h. The tracheae were then washed thoroughly and left for a further control period of 30 min. The responsiveness of the tracheae to methacholine (1 μM) was then re-assessed. The antagonists tested were indomethacin (1 μM), FPL55712 (1 μM), a combination of mepyramine (1 μM) and cimetidine (10 μM), a combination of catalase (500 u ml^{-1}) and superoxide dismutase (40 u ml^{-1}), and WEB 2086 (10 μM).

An exposure time for PAF of 1 h was used in all experiments because this was shown to be optimal in preliminary studies. Thus, the maximum degree of hyperresponsiveness occurred with a 1 h exposure and there was no further increase in the effect of PAF with exposure times greater than 1 h. For example, in the case of smooth muscle tone, the increase in response to methacholine (1 μM) was 42 $\pm$ 5% ($n = 6$) with a 30 min exposure to PAF, 71 $\pm$ 6% ($n = 6$) with a 1 h exposure and 68 $\pm$ 4% ($n = 6$) with a 2 h exposure.

Analysis of results

The concentration-response curves and bar charts presented in Results were obtained by pooling the results from groups of experiments. In the first set of experiments, the EC_{50} values and maximum responses ($\pm$95% confidence limits) for methacholine were estimated by fitting concentration-response curves to the data points by a computerised, non-linear, least squares estimate. EC_{50} is the concentration of methacholine which produced 50% of the maximum response to methacholine. Differences between EC_{50} values or maximum responses for methacholine before and after exposure of the trachea to PAF were analysed for statistical significance by Student's unpaired t test. In the second set of experiments differences between control responses (means $\pm$ s.e.mean) to methacholine and responses to methacholine after PAF were analysed for significance by Student's paired t test. The effects of antagonists on the PAF-induced changes in responsiveness to methacholine were also analysed for signifiance by Student's paired t test. In all cases $P < 0.05$ was taken as significant.

Results

Effect of PAF (1 μM) on concentration-response curves for methacholine

Intraluminal pressure Methacholine (0.1–30 μM) produced a concentration-dependent increase in intraluminal pressure (Figure 1). This concentration-response curve was shifted upwards and leftwards after exposure of the trachea to PAF (1 μM, Figure 1) with a significantly reduced EC_{50} value (3 fold, Table 1) and a significant increase in maximum response (32%, Table 1).

Mucus volume output The mucus volume output in control periods before addition of methacholine was 0.07 $\pm$ 0.05 μl min^{-1} ($n = 24$). Methacholine produced a concentration-dependent increase in mucus volume output from this control level (Figure 1). This concentration-response curve for methacholine was shifted upwards and leftwards after exposure of the trachea to PAF (1 μM) with a significantly reduced EC_{50} value (3.3 fold, Table 1) and a significantly increased maximum response (38%, Table 1).

Lysozyme output The lysozyme output in control periods before addition of methacholine was 16 $\pm$ 11 ng min^{-1} ($n = 24$). Methacholine produced a concentration-dependent increase in lysozyme output from these control levels (Figure 2). This concentration-response curve for methacholine was shifted upwards after exposure of the trachea to PAF with a significantly increased maximum response (73%, Table 1), but no significant change in the EC_{50} (Table 1).

Albumin output The albumin output in controls before methacholine was 0.32 $\pm$ 0.14 μg min^{-1} ($n = 24$). Methacholine produced a concentration-dependent increase in albumin output from this control level (Figure 2). After exposure of the trachea to PAF, the maximum response for methacholine was greatly reduced (50%, Table 1) but there was no significant change in the EC_{50} value (Table 1).

Concentration-related effects of PAF (0.1–10 μM) on responses to methacholine (1 μM)

Baseline values in control and test tracheae The baseline mucus volume output in control tracheae (tracheae which did

Table 1 EC_{50} values and maximum responses ($\pm$95% confidence limits) for methacholine before and after exposure of the trachea to PAF (1 μM)

	EC_{50} (μM)		Maximum response	
	Before PAF	*After PAF*	*Before PAF*	*After PAF*
Intraluminal pressure (mmH_2O)	1.8 (1.6–2.0)	0.6 (0.4–0.8)*	127 (121–133)	168 (162–174)*
Mucus volume output (μl min^{-1})	1.3 (1.1–1.5)	0.4 (0.3–0.5)*	1.62 (1.56–1.68)	2.24 (2.21–2.27)*
Lysozyme output (ng min^{-1})	1.2 (0.9–1.5)	1.0 (0.8–1.2)	395 (380–410)	570 (520–620)*
Albumin output (μg min^{-1})	0.9 (0.6–1.2)	1.0 (0.7–1.3)	2.43 (2.33–2.53)	1.22 (1.18–1.26)*

* Significantly different ($P < 0.05$) from value before exposure to PAF using Student's unpaired t test.

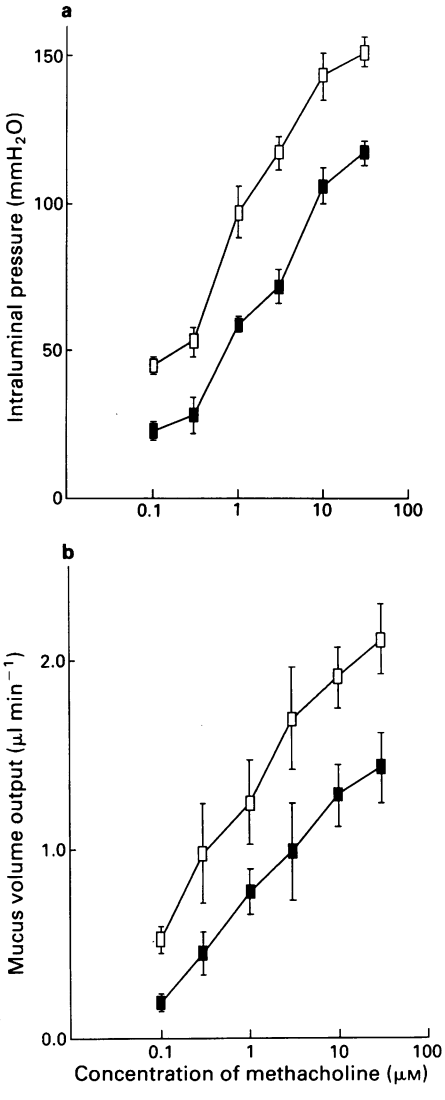


Figure 1 Concentration-response curves for methacholine-induced increases in (a) intraluminal pressure and (b) mucus volume output from the ferret trachea *in vitro*. (■) Responses to methacholine before exposure of the trachea to PAF (1 μM); (□) responses to methacholine after PAF exposure. Points are means of 4–6 determinations with s.e.means shown as vertical lines.

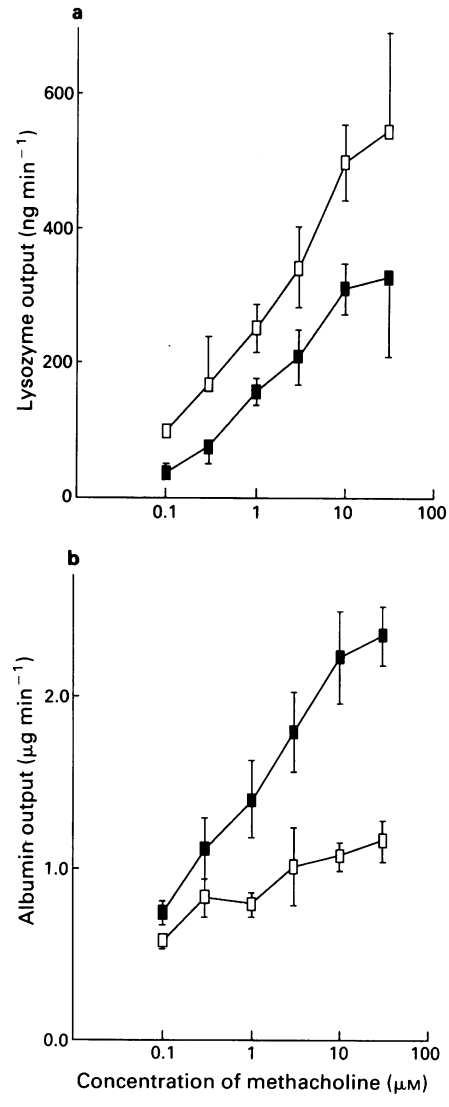


Figure 2 Concentration-response curves for methacholine-induced increases in (a) lysozyme and (b) albumin outputs from the ferret trachea *in vitro*. (■) Responses to methacholine before exposure of the trachea to PAF (1 μM); (□) responses to methacholine after PAF exposure. Points are means of 4–6 determinations with s.e.means shown as vertical lines.

not receive PAF) before addition of any drugs was $0.04 \pm 0.04\,\mu l\,min^{-1}$ ($n = 6$), compared to $0.06 \pm 0.03\,\mu l\,min^{-1}$ ($n = 6$) in test tracheae (tracheae before exposure to PAF). Similarly the baseline lysozyme outputs in control and test tracheae were 27 ± 12 and $21 \pm 6\,ng\,min^{-1}$ respectively, and the baseline albumin outputs were 0.18 ± 0.09 and $0.27 \pm 0.16\,\mu g\,min^{-1}$ respectively. The PD's across control and test tracheae before addition of drugs were -8.2 ± 0.4 and -7.9 ± 0.6 mV respectively. There are no significant differences between baseline values in control and test tracheae for any of these variables.

Intraluminal pressure Methacholine (1 μM) increased intraluminal pressure by $75 \pm 5\,mmH_2O$ ($n = 6$) in control tracheae and by $63 \pm 9\,mmH_2O$ in test tracheae. These responses are not significantly different. Exposure of the trachea to PAF (0.1–10 μM) produced a concentration-dependent enhancement of the increase in intraluminal pressure produced by methacholine (Figure 3). By contrast there was no significant change in the tracheal smooth muscle response to methacholine in control tracheae not exposed to PAF (Figure 3).

Mucus volume output Methacholine (1 μM) increased mucus volume output from 0.06 ± 0.04 and $0.05 \pm 0.04\,\mu l\,min^{-1}$ to 1.05 ± 0.15 and $1.13 \pm 0.19\,\mu l^{-1}$ in control and test tracheae respectively. The responses in control and test tracheae were not significantly different. Exposure of the trachea to PAF (0.1–10 μM) produced a concentration-dependent enhancement of the methacholine-induced increase in mucus volume output (Figure 3). By contrast, there was no change in the methacholine-induced increase in mucus volume output in control tracheae not exposed to PAF (Figure 3).

Lysozyme output Methacholine (1 μM) increased lysozyme output from 23 ± 6 and $21 \pm 9\,ng\,min^{-1}$ to 214 ± 80 and $158 \pm 39\,ng\,min^{-1}$ in control and test tracheae respectively. These responses to methacholine are not significantly different. PAF (0.1–10 μM) concentration-dependently enhanced the methacholine-induced increase in lysozyme output (Figure 4). There was no change in methacholine-induced lysozyme output in the control tracheae not exposed to PAF (Figure 4).

Albumin output Methacholine (1 μM) increased albumin output from 0.23 ± 0.07 and $0.33 \pm 0.06\,\mu g\,min^{-1}$ to 5.5 ± 1.9 and $6.1 \pm 1.6\,\mu g\,min^{-1}$ in control and test tracheae respectively. These responses to methacholine are not significantly different. PAF (0.1–10 μM) produced a concentration-

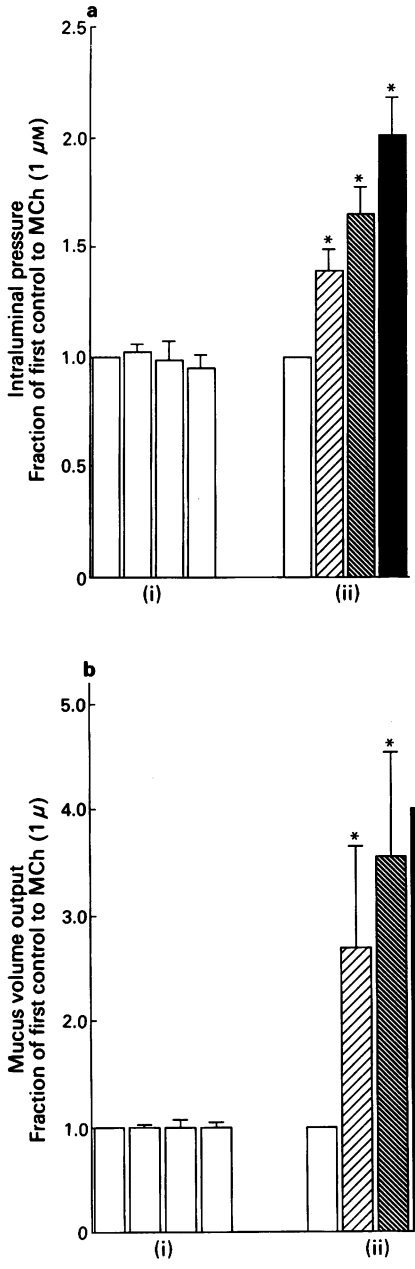


Figure 3 Concentration-response effects of tracheal exposure to PAF (0.1–10 μM) on (a) the increase in intraluminal tracheal pressure and (b) mucus volume output produced by methacholine (1 μM). Responses to methacholine have been expressed as a fraction of the first control response to methacholine. (i) Control tracheae which were not exposed to PAF; (ii) tracheae exposed to PAF. (□) Control responses to methacholine; responses to methacholine after exposure to PAF, 0.1 μM (▨); 1 μM (▧) and 10 μM (■). Responses are the means of six determinations with s.e.means shown by vertical lines. * Significantly different ($P < 0.05$) form first control response to methacholine.

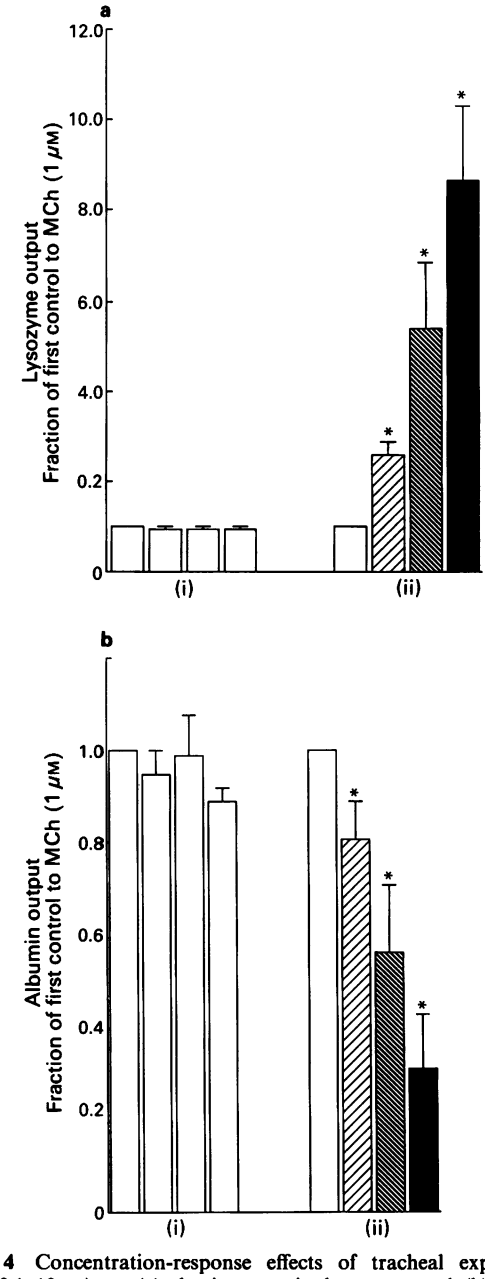


Figure 4 Concentration-response effects of tracheal exposure to PAF (0.1–10 μM) on (a) the increase in lysozyme and (b) albumin output produced by methacholine (1 μM). Responses to methacholine have been expressed as a fraction of the first control response to methacholine. (i) Control tracheae not exposed to PAF; (ii) tracheae exposed to PAF. (□) Control responses to methacholine; responses to methacholine after exposure to PAF, 0.1 μM (▨), 1 μM (▧) and 10 μM (■). Responses are the means of six determinations with s.e.means shown by vertical lines. * Significantly different ($P < 0.05$) from first control to methacholine.

dependent inhibition of the methacholine-induced increase in albumin output (Figure 4b). There was no change in methacholine-induced albumin output in control tracheae (Figure 4).

The concentration-related effects of PAF are not simply due to an additive effect of the drug since in preliminary experiments, repeated exposure to the same concentration of PAF resulted in the same increase in responsiveness to methacholine. For example, in the case of smooth muscle, the control response to methacholine (1 μM) was $+68 \pm 6$ mmH_2O ($n = 6$); this was increased by 72 ± 4, 69 ± 6, 75 ± 7 and 69 ± 5% with four successive exposures to PAF (1 μM).

Effects of antagonists

None of the antagonists themselves had any significant effect on the responses produced by methacholine; the smooth muscle contraction, and mucus volume, lysozyme and albumin outputs produced by methacholine in control and test tracheae were not significantly different (Table 2).

Indomethacin, FPL55712 (7-[(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid) and mepyramine with cimetidine had no effect on the PAF-induced increase in smooth

Table 2 The effect of pharmacological antagonists on the responses to methacholine (1 μM)

Antagonist	*Luminal pressure* (mmH_2O)		*Mucus volume output* ($\mu l\,min^{-1}$)		*Lysozyme output* ($ng\,min^{-1}$)		*Albumin output* ($\mu g\,min^{-1}$)	
	Control	*+Antagonist*	*Control*	*+Antagonist*	*Control*	*+Antagonist*	*Control*	*+Antagonist*
Indomethacin	73 ± 8	71 ± 4	0.56 ± 0.05	0.56 ± 0.20	123 ± 35	132 ± 33	3.5 ± 0.3	3.5 ± 0.2
FPL55712	68 ± 2	60 ± 7	0.42 ± 0.14	0.51 ± 0.06	206 ± 46	252 ± 54	4.4 ± 0.9	3.7 ± 0.7
Mepyramine + cimetidine	64 ± 3	70 ± 3	0.40 ± 0.11	0.42 ± 0.08	215 ± 22	192 ± 23	3.3 ± 0.5	3.3 ± 0.1
Catalase + SOD	70 ± 9	68 ± 5	0.65 ± 0.20	0.50 ± 0.15	316 ± 28	357 ± 17	4.5 ± 0.4	4.1 ± 0.6
WEB2086	75 ± 6	73 ± 5	0.44 ± 0.09	0.47 ± 0.03	84 ± 9	86 ± 5	6.3 ± 2.5	6.1 ± 2.0

Values given are means ± s.e.means with $n = 6$. None of the antagonists has had any effect on the increased luminal pressure or mucus, lysozyme and albumin outputs produced by methacholine. SOD: superoxide dismutase.

muscle contraction, mucus volume and lysozyme output to methacholine, or on the PAF-induced inhibition of methacholine-induced albumin output (Table 3). A combination of catalase and SOD significantly inhibited the PAF-induced increase in responsiveness of smooth muscle, mucus volume and lysozyme, and the decreased albumin output to methacholine (Figure 5). Similarly the PAF-antagonist WEB 2086 (3-(4-(2-chlorophenyl)-9-methyl-6H-thieno-(3,2-f) (1,2,4)-triazolo-(4,3-a) (1,4)-diazepine-2-yl)-1-(4-morpholinyl)-1-propanone) abolished the changes in methacholine-induced responsiveness produced by PAF (Figure 6).

Discussion

PAF induces a non-selective and long lasting increase in bronchial smooth muscle responsiveness to several mediators including histamine, methacholine and 5-hydroxytryptamine in intact guinea-pigs (Mazzoni *et al.*, 1985; Robertson & Page, 1987; Fitzgerald *et al.*, 1987), dogs (Chung *et al.*, 1986), sheep (Christman *et al.*, 1987) and healthy human subjects (Cuss *et al.*, 1986). The hyperresponsiveness to intravenous PAF in guinea-pigs seems to depend on platelets, since platelet depletion with specific cytotoxic agents abrogates the hyperresponsiveness (Mazzoni *et al.*, 1985). However the hyperresponsiveness to inhaled PAF in guinea-pigs is platelet-independent (Lefort *et al.*, 1984; Sanjar *et al.*, 1990), whereas that in rabbits is again dependent on platelets (Coyle *et al.*, 1990). Clearly there are species and route of administration differences in the role of platelets in PAF-induced smooth muscle hyperresponsiveness. The exact mechanism by which PAF produces hyperresponsiveness of airway smooth muscle *in vivo* is unclear but may involve the release of thromboxane from inflammatory cells (Chung *et al.*, 1986), or other mediators from eosinophils (Coyle *et al.*, 1990). In platelet-dependent responses it may be the platelets themselves that are regulating the influx and activation of other inflammatory cells such as the eosinophil (Coyle *et al.*, 1990).

In the present *in vitro* study, the concentration-response curve for methacholine-induced tracheal smooth muscle contraction was shifted to the left and upwards after exposure to PAF with an increase in both the potency of methacholine and the maximum contraction produced by methacholine. Thus, PAF induces a hyperresponsiveness of ferret tracheal smooth muscle to methacholine *in vitro*. This enhancement in response to methacholine is unlikely to be due to an additive effect of PAF as PAF has a direct relaxant effect on the ferret tracheal smooth muscle *in vitro* (Webber *et al.*, 1992). This hyperresponsiveness depends on the concentration of PAF that the trachea is exposed to and no change in responsiveness to methacholine was seen in tracheae which were not exposed to PAF. PAF also induces increased tracheal responsiveness to parasympathetic stimulation in the dog, and this may be due to a direct hyperresponsiveness to acetylcholine (Bethel *et al.*, 1989). In contrast to the *in vivo* studies described above, the hyperresponsiveness to PAF *in vitro* cannot depend on platelets; it is also unlikely to be due to release of mediators from inflammatory cells since histological analysis showed there are very few such cells in the *in vitro* trachea. The hyperresponsiveness is also not due to release of cyclo-oxygenase products, leukotrienes C_4 and D_4, or histamine since indomethacin, FPL55712 and, mepyramine and cimetidine were without effect. In contrast, the hyperresponsiveness was significantly attenuated by catalase and SOD suggesting that it is probably mediated, at least in part, by the release of oxygen free-radicals. This free-radical release is likely to be mediated by specific PAF-receptors since the hyperresponsiveness was also abolished by WEB2086. Leukotriene D_4-induced hyperresponsiveness of guinea-pig tracheal smooth muscle to histamine is also prevented by pretreatment with SOD (Weiss & Bellino, 1986). Direct application of oxygen free-radicals also induces hyperresponsiveness of cat airway smooth muscle to acetylcholine *in vivo* (Katsumata *et al.*, 1990), and H_2O_2 produces hyperresponsiveness of rat bronchi to electrical field stimulation *in vitro* (Szarek & Schmidt, 1990). In contrast, there was no hyperresponsiveness to histamine of guinea-pig trachea *in vitro* after exposure to H_2O_2 (Rhoden & Barnes, 1989). The reason for these conflicting reports is not clear but may represent a species difference.

In the present study there was also an increase in the responses of the submucosal glands (mucus volume and lysozyme output) to methacholine after PAF exposure. The concentration-response curves for methacholine-induced mucus volume and lysozyme output were both shifted upwards after PAF exposure and the maximum responses were increased. There was also an increase in the potency of methacholine in stimulating mucus volume output. There was no increase in potency for lysozyme output, but at each concentration of methacholine the response was significantly enhanced after PAF exposure. Thus PAF induces a hyperresponsiveness of ferret submucosal gland secretion to methacholine and since lysozyme output was also increased at least part of this hyperresponsiveness is secretion from serous cells. As with tracheal smooth muscle the degree of hyperresponsiveness was dependent on the concentration of PAF that the trachea was exposed to and no hyperresponsiveness of submucosal gland secretion was seen in tracheae not exposed to PAF. As with smooth muscle, the enhanced response to methacholine produced by PAF cannot be due to an additive effect of this mediator as PAF itself has no effect on baseline mucus volume or lysozyme output. The hyperresponsiveness of submucosal gland secretion after PAF is a novel response which has not previously been reported even *in vivo* in the presence of platelets. The submucosal gland secretion hyperresponsiveness, like that of the smooth muscle, is not due to release of cyclo-oxygenase products, leukotrienes or histamine as the respective antagonists were without effect, but is mediated by PAF receptor-induced release of oxygen radicals since it was prevented by catalase and SOD, and by WEB2086. There have been no previous studies on the effects of free-radicals on mucus secretion.

Methacholine produced a concentration-dependent increase in the output of albumin into the ferret tracheal lumen suggesting stimulation of epithelial albumin transport. The concentration-response curve for methacholine-induced albumin output was shifted downwards after PAF exposure with a greatly reduced maximum response. Although there was no change in the potency of methacholine, at each concentration of methacholine albumin output was significantly

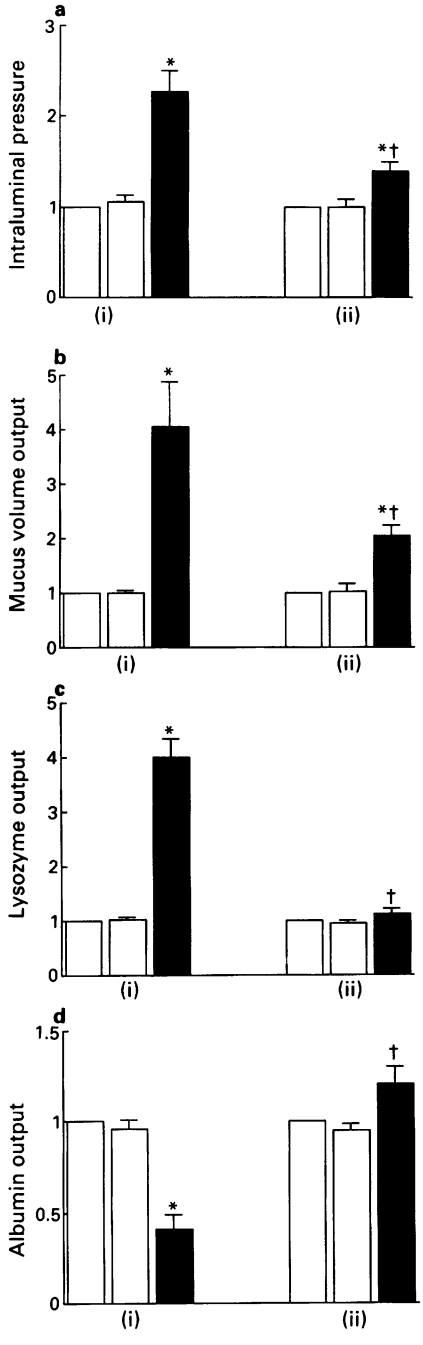


Figure 5 The effect of catalase and superoxide dismutase (SOD) on (a) the changes in responsiveness of tracheal smooth muscle and (b) mucus, (c) lysozyme and (d) albumin outputs to methacholine (1 μM) produced by PAF (1 μM). (i) Control tracheae where catalase and SOD were absent; (ii) test tracheae with catalase and SOD present. (□) Control response to methacholine; (■) response to methacholine after exposure to PAF. All responses to methacholine have been expressed as a fraction of the first control response to methacholine. $n = 6$. * Significant change in response to methacholine after PAF. † Significant inhibition by catalase and SOD of the change in responsiveness produced by PAF.

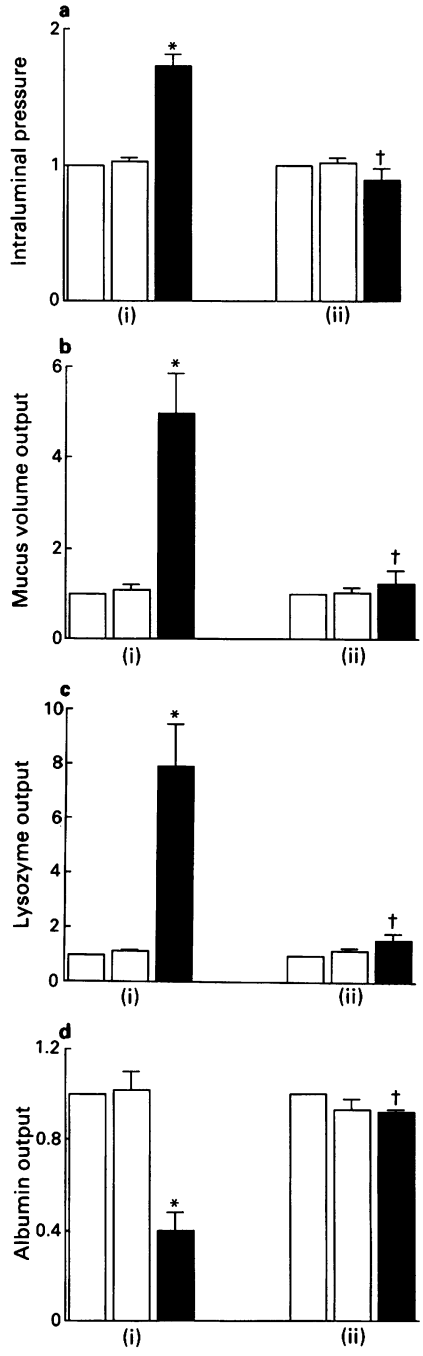


Figure 6 The effects of WEB2086 on (a) the changes in responsiveness of tracheal smooth muscle and (b) mucus, (c) lysozyme and (d) albumin outputs to methacholine produced by PAF (1 μM). (i) Control tracheae where WEB2086 was absent; (ii) test tracheae where WEB2086 was present. (□) Control response to methacholine; (■) response to methacholine after exposure to PAF. All responses to methacholine have been expressed as a fraction of the first control response to methacholine. $n = 6$. * Significant change in response to methacholine after PAF. † Significant inhibition by WEB2086 of the change in responsiveness produced by PAF.

reduced after PAF exposure. Thus PAF exposure inhibits the stimulation of epithelial albumin transport produced by methacholine. This loss of epithelial function is consistent with the reduction in PD across the trachea seen with PAF (Webber *et al.*, 1992). As with the reduction in PD, and the smooth muscle and submucosal gland hyperresponsiveness, the PAF-induced inhibition of albumin output to methacholine was attenuated by catalase and SOD, and abolished by WEB2086 again suggesting the involvement of receptor-mediated release of free-radicals.

In summary, the addition of PAF to the ferret trachea *in vitro* leads to the production of a cyclo-oxygenase product which immediately relaxes the smooth muscle and oxygen free-radicals which reduce the PD indicating a change in epithelial function (Webber *et al.*, 1992). Neither the cyclo-oxygenase product or the free-radicals have any effect on basal submucosal gland secretion or epithelial albumin transport (Webber *et al.*, 1992). However, the results from the study described here suggest the free-radicals induce a hyperresponsiveness of submucosal gland secretion as well as

Table 3 The effect of indomethacin, FPL55712 and, mepyramine and cimetidine on the changes in responsiveness of tracheal smooth muscle and mucus, lysozyme and albumin outputs to methacholine (1 μM) produced after exposure to PAF (1 μM)

	Indomethacin				*FPL55712*				*Mepyramine + cimetidine*			
	Control tracheae		*Test tracheae*		*Control tracheae*		*Test tracheae*		*Control tracheae*		*Test tracheae*	
	Cont	*After PAF*	*Cont*	*After PAF*	*Cont*	*After PAF*	*Cont*	*After PAF*	*Cont*	*After PAF*	*Cont*	*After PAF*
Intraluminal pressure (mmH_2O)	99 ± 5	185 ± 4*	105 ± 3	193 ± 18*	104 ± 6	207 ± 8*	101 ± 10	216 ± 17*	106 ± 6	188 ± 11*	95 ± 5	197 ± 14*
Mucus volume output ($\mu l\,min^{-1}$)	103 ± 7	348 ± 28*	94 ± 15	382 ± 89*	113 ± 11	432 ± 66*	98 ± 8	498 ± 72*	109 ± 11	394 ± 20*	110 ± 5	368 ± 12*
Lysozyme output ($ng\,min^{-1}$)	93 ± 9	420 ± 73*	99 ± 8	486 ± 116*	93 ± 6	493 ± 129*	92 ± 5	590 ± 134*	121 ± 24	572 ± 144*	119 ± 21	461 ± 63*
Albumin output ($\mu g\,min^{-1}$)	102 ± 4	36 ± 3*	103 ± 6	32 ± 6*	102 ± 5	35 ± 7*	102 ± 5	32 ± 5*	100 ± 5	30 ± 2*	100 ± 10	24 ± 4*

Control tracheae are those where no antagonist was present and test tracheae are those where an antagonist was present. The values given are the mean ± s.e.mean responses to methacholine. $n = 6$. * Significant change in response to methacholine after exposure to PAF.

smooth muscle contraction to the muscarinic agonist methacholine; but they inhibit methacholine-stimulated epithelial albumin transport. The hyperresponsiveness of smooth muscle and submucosal gland secretion occurs *in vitro* in the absence of circulating platelets or other inflammatory cells demonstrating that these cells are not essential for PAF-induced changes in responsiveness. The cellular source of the dilator cyclooxygenase product and the free-radicals is not known but the epithelium is a likely candidate. Similarly the exact mechanism behind the free radical-induced changes in responsiveness to methacholine are not known.

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Adenosine receptors in post-mortem human brain

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1 Adenosine A_2-like binding sites were characterized in post-mortem human brain membranes by examining several compounds for their ability to displace [^{3}H]-CGS 21680 (2[*p*-(2 carboxyethyl)-phenethylamino]-5'-N-ethylcarboxamido adenosine) binding.

2 Two A_2-like binding sites were identified in the striatum.

3 The more abundant striatal site was similar to the A_{2a} receptor previously described in rat striatum, both in its pharmacological profile and striatal localization.

4 The less abundant striatal site had a pharmacological profile similar to that of the binding site characterized in the other brain regions examined. This was intermediate in character between A_1 and A_2 and may represent another adenosine receptor subtype.

5 The co-purification of [^{3}H]-CGS 21680 binding during immunoisolation of human striatal cholinergic membranes was used to assess the possible cholinergic localization of A_2-like binding sites in the human striatum. Only the more abundant striatal site co-purified with cholinergic membranes. This suggests that this A_{2a}-like site is present on cholinergic neurones in the human striatum.

Keywords: Adenosine receptors; human brain; CGS 21680

Introduction

Adenosine is known to modulate neuronal function through receptor-mediated mechanisms including effects on second messenger systems (Londos & Wolff, 1977; Daly *et al.*, 1983), transmembrane ion fluxes (Dunwiddie, 1985) and neurotransmitter release (Richardson *et al.*, 1987; Fredholm & Dunwiddie, 1988). Adenosine receptors are classified as A_1 and A_2, a designation which is based on the relative potencies of a number of selective ligands, e.g. at A_1 receptors N^6-**R**-phenylisopropyl adenosine (**R**-PIA) is more potent than 5'-N-ethylcarboxamido adenosine (NECA) whereas the reverse is true at A_2 receptors (Bruns *et al.*, 1986). Sensitivity to adenosine agonists also distinguishes between the receptors, with adenosine itself having nanomolar affinity for A_1 receptors and micromolar affinity for A_2 receptors (van Calker *et al.*, 1979; Daly *et al.*, 1981). A previous classification, based on inhibition and stimulation of adenylate cyclase mediated by A_1 and A_2 receptors respectively (van Calker *et al.*, 1979), is less satisfactory since adenosine receptors may be coupled to other effector systems including plasma membrane ion channels (West *et al.*, 1987; Isenberg *et al.*, 1987) and phosphatidylinositol turnover (Petcoff & Cooper, 1987).

A_2 receptors have been further subdivided into high affinity (A_{2a}) and low affinity (A_{2b}) subtypes (Premont *et al.*, 1979; Londos *et al.*, 1982). The high affinity A_{2a} receptor is localized to the striatum, olfactory tubercle and nucleus accumbens in the rat (Bruns *et al.*, 1986; Jarvis *et al.*, 1989). Indeed this receptor appears to be localized to the cholinergic neurones of the striatum (Brown *et al.*, 1990). The recent development of the A_{2a} selective agonist CGS 21680 (2[*p*-(2-carboxyethyl)-phenethylamino]-5'-N-ethyl carboxamido adenosine) has greatly facilitated the study of the A_{2a} receptor since it shows a 185 fold selectivity for the A_2 over the A_1 receptor (Jarvis *et al.*, 1989) and appears not to bind to the A_{2b} receptor (Lupica *et al.*, 1990).

In contrast to the rat, relatively little information is available on adenosine receptors in human brain. Quantitative autoradiography has demonstrated a heterogeneous distribution of A_1 binding sites, similar to that of A_1 receptors in rat brain (Goodman & Snyder, 1982; Fastbom *et al.*, 1987). The study of Dodd *et al.* (1986) suggested the presence of A_2-like receptors in post-mortem human cerebral cortex, while Reynaud *et al.* (1990) presented a preliminary characterization of A_2 receptors in post-mortem pineal gland. These studies on the human A_2-receptor, like those of Stone *et al.* (1988) used [^{3}H]-NECA, an agonist which is equally potent at A_1 and A_2 receptors (Bruns *et al.*, 1987). Blockade of A_1 receptor binding was achieved by the inclusion of 50 nM cyclopentyladenosine. Most recently, autoradiography using the selective ligand CGS 21680 has shown that in human brain, A_2 receptors are exclusively localized to the caudate, putamen, nucleus accumbens, olfactory tubercle and lateral segment of the globus pallidus (Martinez-Mir *et al.*, 1991). This study also showed that A_2 receptor density is decreased in the basal ganglia of patients of Huntington's chorea, suggesting that A_2 receptors may be localized on the neurones which undergo degeneration in this condition, namely the small and medium spiny neurones in the striatum. In the present study we have attempted to characterize the human A_{2a} receptor using the selective ligand CGS 21680, and to determine whether either of the two previously reported A_2-like binding sites (Stone *et al.*, 1988) in the human brain are present on cholinergic membranes.

Methods

Preparation of membranes

Brain tissue was obtained at a post-mortem examination from cases with no history of neurological or psychiatric disorders. Case details are shown in Table 1. Pieces of grey matter (approximately 1–5 g) were dissected free of surrounding structures and white matter, immersed in ice-cold 50 mM Tris-HCl (pH 7.7 at 25°C) and then frozen slowly as described by Hardy *et al.* (1983). Tissue was taken from the following brain regions: corpus striatum (caudate and putamen), hippocampus, thalamus, cerebellar cortex and frontal, temporal and parietal lobes of cerebral cortex. Freezing and subsequent storage at −70°C for up to 8 weeks resulted in no detectable loss of CGS 21680 binding activity. When needed, the tissue was thawed rapidly by immersion in a waterbath at 37°C and transferred immediately to ice-cold Tris buffer. Subsequent membrane preparation procedures were carried out at 4°C.

The method of membrane preparation was essentially that described by Bruns *et al.* (1986). Briefly, the tissue was homog-

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Table 1 Details of patients from which brain tissue was obtained for use in this study

Patient	*Age* (years)	*Sex*	*Time from death to post mortem examination* (h)	*Cause of death*	*Specific binding of* [^{3}H]*-CGS 21680 in striatum* (pmol g^{-1} original tissue)
1	32	M	72	Myocardial infarction	2.2 ± 0.1 (17)
2	75	M	48	Ruptured abdominal aortic aneurysm	2.2 ± 0.2 (10)
3	69	F	48	Congestive cardiac failure	2.1 ± 0.1 (9)
4	58	M	24	Myocardial infarction	2.1 ± 0.1 (4)

[^{3}H]-CGS 21680 (7 nM) binding to membranes prepared from striatum was carried out as described in Methods.

enized in a tight-fitting Teflon-glass motor-driven homogenizer, diluted to 10% (w/v) with Tris buffer and then disrupted for 30 s with a Polytron PT 10-35 homogenizer (Kinematica GmbH Lissau) at setting 8. The suspension was then centrifuged at 47,800 *g* for 20 min (all centrifugation steps were carried out in a Sorval RC5B centrifuge), and the supernatant discarded. The pellet was resuspended in Tris buffer with a Polytron (as above) at 1 g 10 ml^{-1}, and incubated with 1.0 unit ml^{-1} (1 unit: 1.0 μmol min^{-1} at pH 7.5 at 25°C) of adenosine deaminase (Sigma) for 1 h at 4°C. It was then recentrifuged, resuspended at 1 g 2.5 ml^{-1} in Tris buffer with 50 mM $MgCl_2$ and 10 units ml^{-1} of adenosine deaminase, and immediately used in the binding assay. The protein concentration was determined by the method of Lowry *et al.* (1951) in the presence of 0.5% sodium dodecyl sulphate, against bovine serum albumin standards.

Binding assays

All incubations were in duplicate. The incubation mixture consisted of 50 mM Tris-HCl (pH 7.7 at 25°C), 10 mM $MgCl_2$, 2 units ml^{-1} adenosine deaminase, 7.0 nM [^{3}H]-CGS 21680 or 7.0 nM [^{3}H]-Me-2-phenylisopropyladenosine (PIA), and 40 mg of original tissue weight of human brain membranes (11 ± 1 mg protein ml^{-1}) in a total volume of 0.5 ml. The concentration of [^{3}H]-CGS 21680 was selected as being consistent with that used in the characterization of this compound as a selective A_2 ligand in rat brain (Jarvis *et al.*, 1989). 5'Guanylyl-imidodiphosphate (Gpp(NH)p) 10 μM, was included in some experiments. Test compounds for competition studies were dissolved in water or dimethylsulphoxide (DMSO). Control incubations, to determine total binding, received an equal volume of water (50 μl) or DMSO (2.5 μl); the resulting concentration of DMSO had no effect on specific binding. Non-specific binding was determined in assays with 0.1 mM NECA. Specific binding was calculated as the difference between total and non-specific binding. Reactions were terminated after 90 min at 25°C by vacuum filtration through Whatman GF/B glass fibre filters, which were washed three times with 4 ml of ice-cold Tris buffer. The damp filters were put into scintillation vials, 8 ml of Emulsifier-Safe scintillation fluid (Packard) was added and the vials were left for 36 h, shaken, and counted in a liquid scintillation counter.

Co-purification of striatal binding sites

Human striatal membranes were prepared as described above, using 0.32 M sucrose, 4 mM HEPES, 0.1 mM EDTA pH 7.4, 1 mg ml^{-1} bovine serum albumin, in place of Tris buffer. The cholinergic nerve terminal membranes were sensitized with anti-(Chol-1) antibodies. These antibodies were prepared from a sheep antiserum raised against synaptic membranes from the electric organ of *Torpedo marmorata*, by precipitation with 40% (v/v) saturation ammonium sulphate and subsequent dialysis against 0.15 M NaCl, 10 mM sodium phosphate pH 7.4. These antibodies recognize two gangliosides present on cholinergic membranes in mammalian brain (Richardson *et al.*, 1982; Derrington *et al.*, 1989). A 1:100 dilution of the antibodies was incubated with membranes derived from 0.4 g of original tissue in 10 ml of sucrose buffer for 30 min at 4°C. The sensitized membranes were subsequently isolated on a cellulose immunoadsorbent bearing a mouse monoclonal anti-(sheep IgG) antibody, as previously described (Richardson *et al.*, 1984; Richardson, 1986). Adenosine deaminase, 1.0 unit ml^{-1}, was included in the incubation of the membrane preparation with the immunoadsorbent. Binding assays were carried out as described above, using the cholinergic nerve terminal membranes still bound to the immunoadsorbent and with 14 nM [^{3}H]-CGS 21680 and 10 u ml^{-1} adenosine deaminase in the incubation mixture. The percentage recovery of acetylcholinesterase (AChE) and 5'-nucleotidase were determined, by the methods of Ellman (1961) and Newby *et al.*, (1975) respectively. In each experiment, non-immune [^{3}H]-CGS 21680 binding and percentage recovery of AChE and 5'-nucleotidase were determined in parallel using immunoadsorbent pre-incubated with striatal membranes which had been treated with non-immune sheep antibodies. The [^{3}H]-CGS 21680 binding, AChE activity and 5'-nucleotidase activity due to immunoadsorbent alone were also determined.

Drugs used

The following drugs were used: 5'-N-ethylcarboxamidoadenosine (NECA, Sigma), N^6-(R-phenylisopropyl)-adenosine (R-PIA, Sigma), N^6-(S-phenylisopropyl)-adenosine (S-PIA, Boehringer Mannheim) and (2[*p*-(2 carboxyethyl)-phenethylamino]-5'-N-ethylcarboxamido adenosine (CGS 21680, Ciba-Geigy) dissolved in water; CGS 21680 (carboxyethyl-^{3}H[N], New England Nuclear Research Products), specific activity 48.6 Ci mmol^{-1}, dissolved in 70% ethanol; 4-amino-1-phenyl-[1,2,4] triazolo [4,3-a] quinoxaline (CP 66, 713, Pfizer), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, Research Biochemicals Incorporated) and 8-cyclopentyl theophylline (CPT, Research Biochemicals Incorporated) dissolved in DMSO.

Data analysis

All results are expressed as mean values ± s.e.mean.

Binding data were analysed and concentration-inhibition curves constructed by use of the non-linear regression curve-fitting programme Graph PAD InPlot, Version 3.0 (Motulsky, 1989). The equations used describe the competition of a ligand for binding to one type of receptor, and to two types of receptor for which the competing ligand has different affinities and the radioligand has identical affinities. Goodness of fit was assessed by the sum of the square of the distance of each point from the curve, i.e. the data points were not weighted. A partial *F*-test ($P < 0.05$) was used to determine whether the binding data were best described by the one-site or two-site binding model.

Data obtained after copurification of [^{3}H]-CGS 21680 binding with striatal cholinergic membranes was analysed by use of Student's *t* test for unpaired data. Two groups of data were considered to be significantly different when $P < 0.05$.

Results

Regional distribution of [^{3}H]*-CGS 21680 binding*

The regional distribution of A_2 receptors in human brain was assessed by measuring the binding to membranes derived

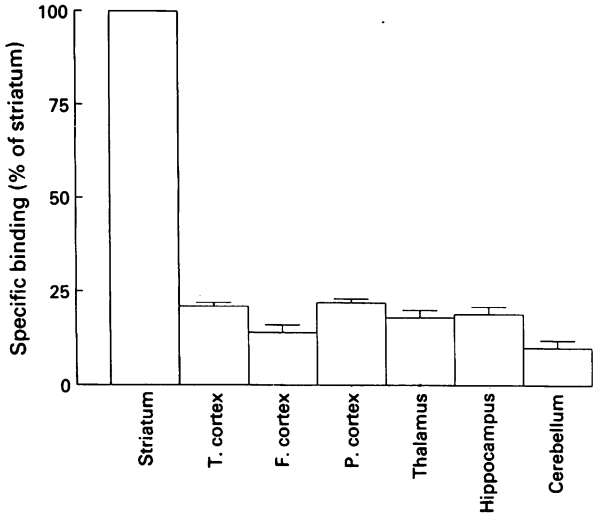


Figure 1 Regional distribution of [^{3}H]-CGS 21680 binding: [^{3}H]-CGS 21680 (7 nM) binding to membranes prepared from seven areas of post mortem human brain was carried out as described in the Methods. The results are means of between 5 and 12 determinations and are expressed as % of that observed in the striatum, which amounted to 2.1 ± 0.1 pmol g^{-1} wet weight of tissue; s.e.mean shown by vertical bars.

from seven different areas (Figure 1). It is apparent that this binding was concentrated in the striatum, with lower levels of specific binding in the other brain regions investigated. The binding observed with 7.0 nM [^{3}H]-CGS 21680 was approximately the same in the striatum of each brain, irrespective of age and post mortem delay prior to freezing (Table 1).

[^{3}H]-CGS 21680 binding in the striatum

In order to characterize further the striatal binding site, the ability of a number of ligands to displace the binding was investigated. Three of the ligands tested showed complex displacement curves which could be resolved into two binding sites. Figure 2 shows the displacement of [^{3}H]-CGS 21680 by the A_1/A_2 agonist NECA, the A_1 agonist **R**-PIA and the A_2 antagonist CP66,713. For both agonists the binding site with lower affinity represented the greater proportion of total binding (58 ± 6% for NECA and 76 ± 3% for **R**-PIA). The derived K_I values are shown in Table 2 and the Hill coefficients in Table 3. In contrast the A_2 selective antagonist CP66,713 displaced 63 ± 1% of the binding with high affinity (K_I = 21 ± 7 nM), but was unable to displace completely the binding to the remaining site at concentrations up to 10 μM.

Table 2 K_I values (nM) for the inhibition of [^{3}H]-CGS 21680 binding to human brain membranes

	Striatum		
Ligand	*Site 1*	*Site 2*	*Temporal cortex*
Agonists			
CGS 21680	125 ± 17 (8)		350 ± 58 (7)
NECA	3 ± 1 (12)	202 ± 39 (12)	1.0 ± 0.6 (8)
R-PIA	4 ± 1 (10)	615 ± 144 (10)	4 ± 1 (10)
S-PIA	ND	ND	19 ± 3 (4)
Antagonists			
CP66,713	>20,000 (8)	21 ± 7 (8)	>20,000 (6)
CPT	260 ± 48 (4)		63 ± 28 (4)
DPCPX	2190 ± 740 (4)		260 ± 87 (10)

K_I values are means ± s.e.mean with the number of determinations in parentheses. Site 1 in the striatum corresponds to the less abundant site. ND not determined. For other abbreviations, see text.

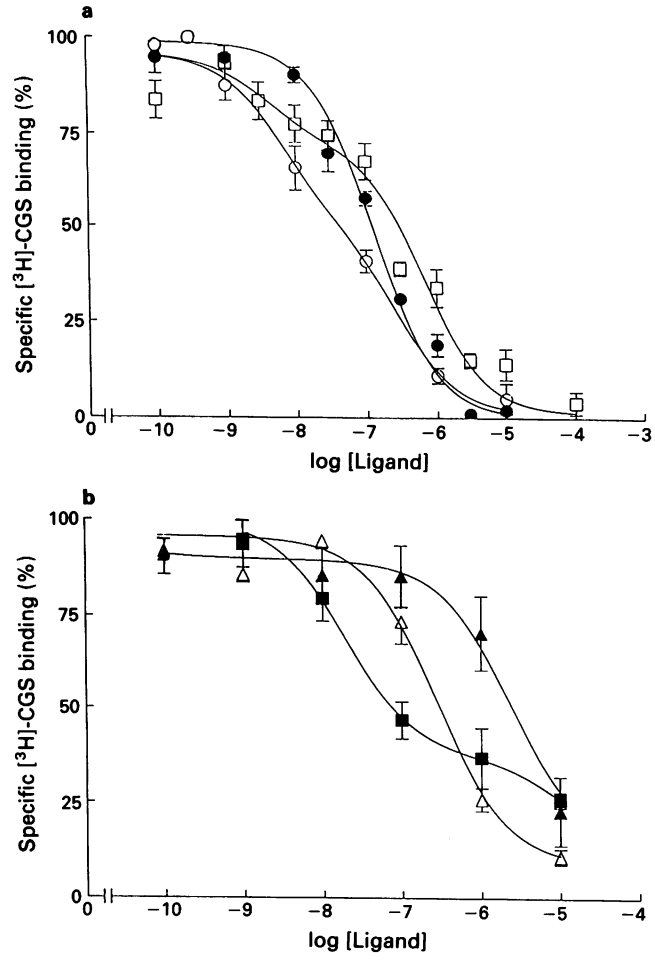


Figure 2 Displacement of [^{3}H]-CGS 21680 binding from striatal membranes: the displacement of 7.0 nM [^{3}H]-CGS 21680 binding from striatal membranes by adenosine receptor agonists (a) and antagonists (b) was carried out as described in the Methods. The results are means of the number of determinations shown in parentheses; (○) NECA (12); (●) CGS 21680 (6); (□) **R**-PIA (6); (■) CP66,713 (7); (△) CPT (4); (▲) DPCPX (4); s.e.means shown by vertical bars except when less than the size of the symbol used. For abbreviations, see text.

This may be due to the relative insolubility of this compound in aqueous buffer at concentrations above 1 μM (Sarges *et al.*, 1990). The A_2-selective agonist, CGS 21680, and the A_1 antagonist, CPT, which shows a 133 fold selectivity for the A_1 over the A_2 receptor (Bruns *et al.*, 1986), fitted a single site model, being unable to distinguish between the two sites (Figure 2, Tables 2 and 3). The highly selective A_1 antagonist, DPCPX (740 fold selective for A_1 over A_2; Bruns *et al.*, 1987) inhibited the binding with very low affinity.

Table 3 Hill coefficients for the inhibition of [^{3}H]-CGS 21680 binding to human striatal membranes

Ligand	
Agonists	
CGS 21680	0.9 ± 0.1 (8)
NECA	0.6 ± 0.1 (12)
R-PIA	0.6 ± 0.1 (10)
Antagonists	
CP66,713	0.4 ± 0.1 (8)
CPT	1.0 ± 0.1 (4)
DPCPX	1.2 ± 0.2 (4)

Values are means ± s.e.mean with the number of determinations in parentheses. For abbreviations, see text.

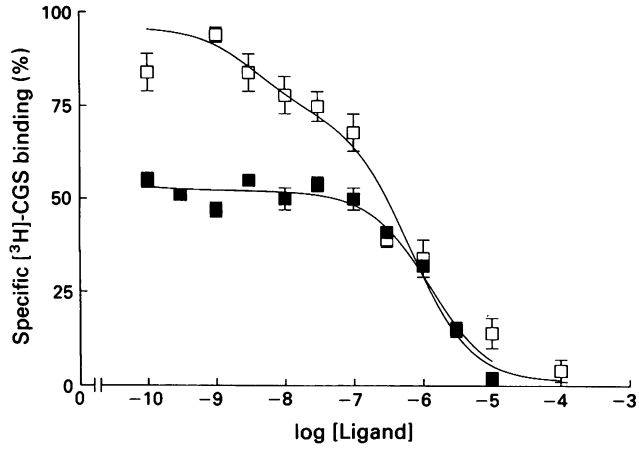


Figure 3 Displacement of [^{3}H]-CGS 21680 binding from striatal membranes: the displacement of 7.0 nM [^{3}H]-CGS 21680 from striatal membranes by **R**-PIA was carried out in the presence (■) and absence (□) of 10 μM Gpp(NH)p. The results are means of 4–6 determinations; s.e.means shown by vertical bars except when less than the size of the symbol used. For abbreviations, see text.

The order of potency of the adenosine ligands tested at these two sites was therefore as follows: for the less abundant site (Site 1) NECA = **R**-PIA > CGS 21680, and CPT > DPCPX ⩾ CP66,713, while at the more abundant site (Site 2) CGS 21680 > NECA > PIA and CP66,713 > CPT > DPCPX.

The inclusion of 10 μM 5'-guanylylimidodiphosphate in the binding assay inhibited the binding of 7 nM [^{3}H]-CGS 21680 by 43 ± 2% (n = 6). Subsequent displacement of this binding with **R**-PIA revealed that site 1 was no longer detectable while the K_I for **R**-PIA at site 2 was increased to 1.20 ± 0.02 μM (Figure 3). Binding of [^{3}H]-CGS 21680 to site 2, in the presence of 30 nM **R**-PIA to block site 1, was reduced by 17 ± 3%.

[^{3}*H*]-*CGS 21680 binding in other brain regions*

In order to determine whether the non-striatal binding sites corresponded to either of the striatal ones, adenosine ligands were tested for their ability to displace [^{3}H]-CGS 21680 binding to temporal cortex membranes. Analysis of binding data indicated that in the concentration-range examined, only one site was recognized by these ligands, the relative order of potency being NECA > **R**-PIA > **S**-PIA > CGS 21680 and CPT > DPCPX (Figure 4). As in site 1 of the striatum, CP66,713 was unable to displace more than 50% of the binding at concentrations up to 10 μM.

Displacement of [^{3}H]-CGS 21680 binding by NECA and **R**-PIA was also investigated in the hippocampus, thalamus, and frontal and parietal lobes of cerebral cortex. NECA recognized one binding site in all regions examined which had an affinity for NECA similar to that of the binding site characterized in the temporal cortex, and site 1 in the striatum (Figure 5). Due to limited availability of tissue, it was not possible to determine full concentration-inhibition curves with **R**-PIA in all brain regions. However, displacement of [^{3}H]-CGS 21680 binding was investigated in hippocampus, thalamus, and parietal and frontal lobes of cerebral cortex with 0.1 nM and 100 nM concentrations of **R**-PIA. In these brain regions, [^{3}H]-CGS 21680 binding was inhibited by 63–88% in the presence of 100 nM **R**-PIA, compared to 80 ± 7% in the temporal cortex and 32 ± 5% in the striatum (Figure 6). This indicates the presence of a high affinity site for **R**-PIA, as well as for NECA, in these brain regions, which is comparable to the site characterized more fully in the temporal cortex. Specific binding of [^{3}H]-CGS 21680 in the cerebellar cortex was too small to be characterized in this way.

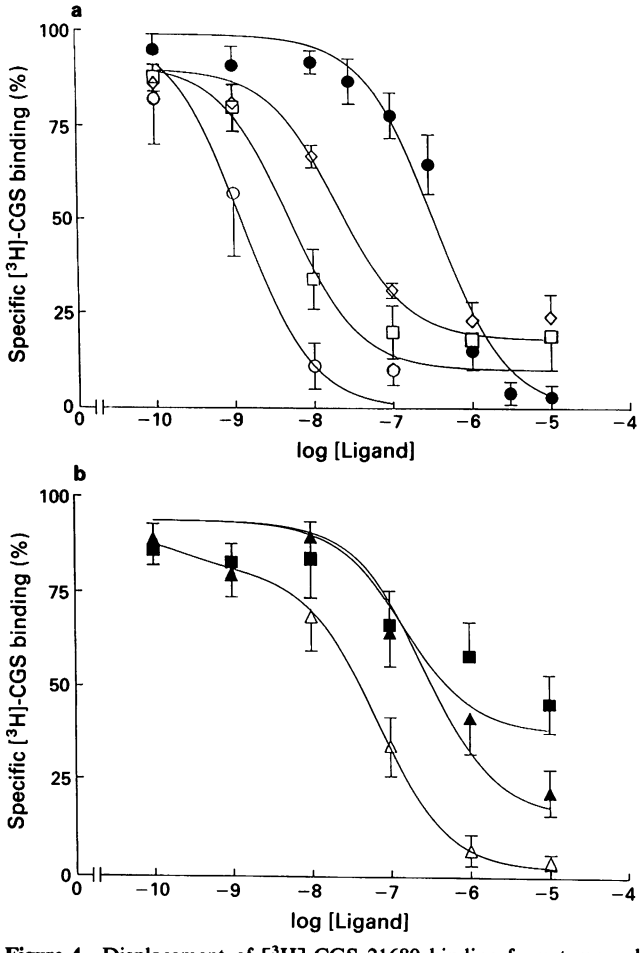


Figure 4 Displacement of [^{3}H]-CGS 21680 binding from temporal cortex membranes: the displacement of 7.0 nM [^{3}H]-CGS 21680 from temporal cortex membranes by adenosine receptor agonists (a) and antagonists (b) was carried out as described in the Methods. The results are means of the number of determinations shown in parentheses: (○) NECA (4); (●) CGS 21680 (6); (□) **R**-PIA (8); (◇) **S**-PIA (4); (■) CP66,713 (7); (△) CPT (4) and (▲) DPCPX (10); s.e.means shown by vertical bars except when less than the size of the symbol used. Total binding amounted to 0.5 ± 0.05 pmol g^{-1} wet weight of tissue. For abbreviations, see text.

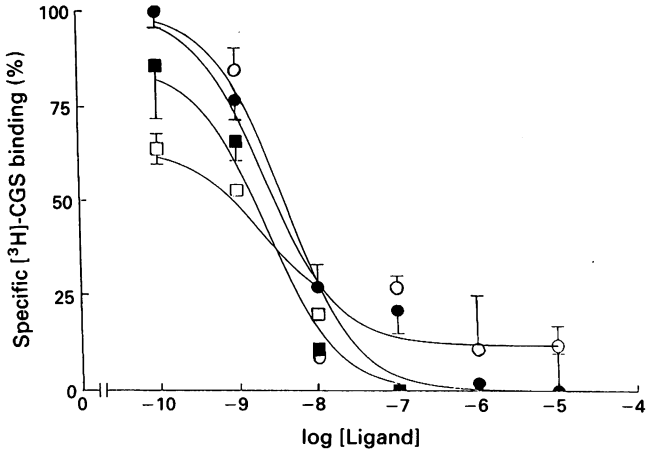


Figure 5 Displacement of [^{3}H]-CGS 21680 binding by NECA from membranes of different brain regions: the displacement of 7.0 nM [^{3}H]-CGS 21680 from membranes was carried out as described. The results are means of the number of determinations shown in parentheses: (○) hippocampus (4); (■) thalamus (4); (●) parietal cortex (4); (□) frontal cortex (2); s.e.mean shown by vertical bars except when less than the size of the symbol used. The total binding was (pmol g^{-1} of tissue): hippocampus 0.4 ± 0.1, thalamus 0.4 ± 0.1, parietal cortex 0.5 ± 0.1, frontal cortex 0.3 ± 0.1. K_I values were: hippocampus 2.0 ± 0.4 nM, thalamus 2.0 ± 0.1 nM, parietal cortex 4.0 ± 1.0 nM, frontal cortex 2.0 ± 0.4 nM.

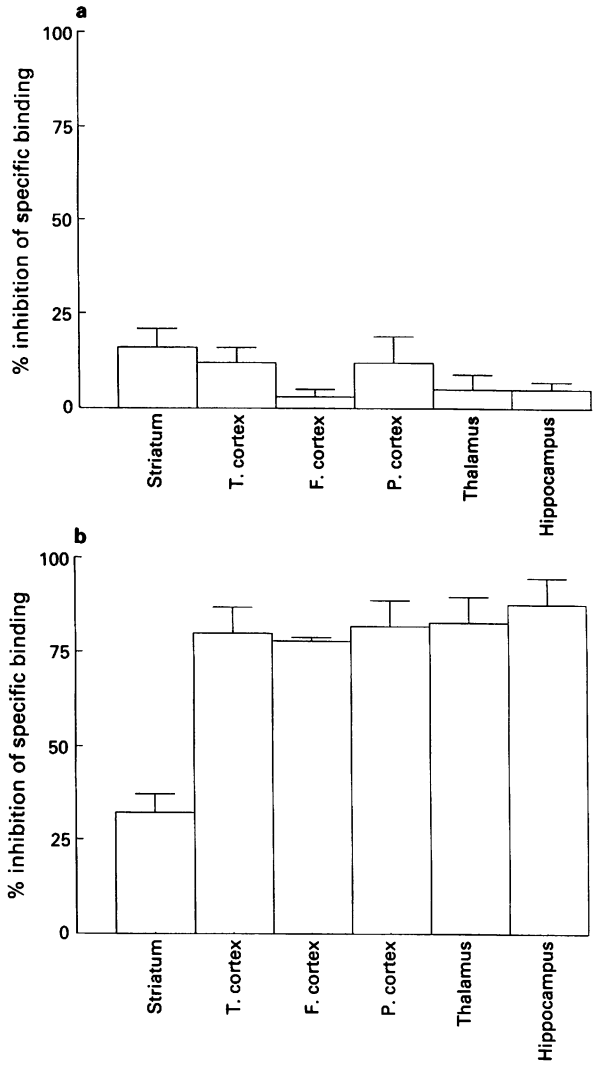


Figure 6 Inhibition of [^{3}H]-CGS 21680 binding by (a) 0.1 nM R-PIA, and (b) 100 nM R-PIA: The displacement of [^{3}H]-CGS 21680 binding was carried out as described. The results are expressed as % inhibition, and are the mean of between 6 and 13 determinations; s.e.mean shown by vertical bars. The total binding of 7.0 nM [^{3}H]-CGS 21680 in the absence of other ligands was the same as that shown in the legends to Figures 4 and 5. For abbreviations see text.

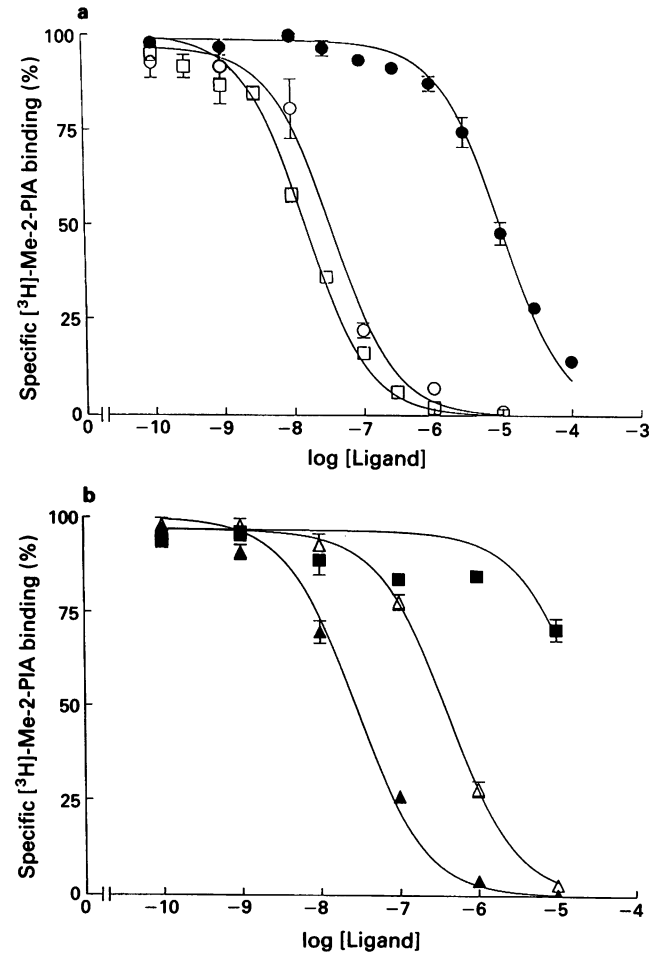


Figure 7 Inhibition of [^{3}H]-PIA binding to temporal cortex membranes: the displacement of 7.0 nM [^{3}H]-PIA binding by adenosine receptor agonists (a) and antagonists (b) was carried out as described. The results are means with the number of determinations shown in parentheses: (□) R-PIA (6); (○) NECA (4); (●) CGS 21680 (5); (▲) DPCPX (4); (△) CPT (4) and (■) CP66,713 (6). S.e.mean shown by vertical bars except when less than the size of the symbol used. The K_I values were 14 ± 1 nM for R-PIA, 34 ± 6 nM for NECA, 9460 ± 1280 nM for CGS 21680, 29 ± 2 nM for DPCPX, 370 ± 14 nM for CPT and >20,000 nM for CP66,713. Total binding of [^{3}H]-PIA was 5.6 ± 0.1 pmol g^{-1} tissue.

The effect of Gpp(NH)p on [^{3}H]-CGS 21680 binding in a brain region other than striatum was investigated in the hippocampus. Specific binding was found to be reduced by $52 \pm 15\%$ ($n = 6$) in the presence of 10 μM Gpp(NH)p.

In order to ascertain whether this ubiquitous CGS 21680 binding site corresponded to the A_1 receptor, the ability of a number of ligands to displace [^{3}H]-R-PIA binding from temporal cortex membranes was investigated. Figure 7 illustrates the displacement curves obtained, the relative order of potencies being R-PIA > NECA > CGS 21680 and DPCPX > CPT > CP66,713.

Immunoaffinity purification of striatal cholinergic membranes

Cholinergic membranes bearing the Chol-1 antigens (Ferretti & Borroni, 1986) were affinity purified; the yield of acetylcholinesterase being used as a marker for cholinergic membranes, and the yield of 5'-nucleotidase as a more general membrane marker. R-PIA, 30 nM was used to distinguish between the two CGS 21680 binding sites in these membranes, and it can be seen from Table 4 that the yield of the PIA-resistant site, i.e. site 2, was comparable to that of acetylcholinesterase and greater than that of the PIA-sensitive site, i.e. site 1 ($P < 0.01$). Non-specific binding to the immunoadsorbent was not detectable for CGS 21680 binding and 5'-nucleotidase, and amounted to 0.8% of the total acetylcholinesterase activity present.

Table 4 Copurification of [^{3}H]-CGS 21680 binding with striatal cholinergic membranes

[^{3}H]-CGS 21680 binding	% yield
Total	4.2 ± 0.9 (5)
R-PIA-sensitive (site 1)	0.9 ± 0.4 (5)
R-PIA-resistant (site 2)	6.7 ± 1.6 (5)
5'-Nucleotidase	2.2 ± 0.8 (3)
Acetylcholinesterase	6.1 ± 0.9 (2)

The amount bound to the immunoadsorbent is expressed as % of the initial activity, after subtraction of the non-specific binding seen with membranes sensitized with non-immune sheep immunoglobulin, and are means ± s.e.mean with the number of experiments in parentheses. The initial activity present in each purification tube was: [^{3}H]-CGS 21680 binding 14.4 ± 4.0 fmol, of which the R-PIA-resistant amounted to 9.0 ± 3.0 fmol, while the balance was R-PIA-sensitive. 5'-Nucleotidase 5.8 ± 1.5 nmol h^{-1}; acetylcholinesterase 1.1 ± 0.3 μmol min^{-1}.

Discussion

The results presented in this study suggest that there are at least two discrete sub-types of [^{3}H]-CGS 21680 binding sites in the human brain. Both sites were detectable in the striatum where they were distinguished by the agonists NECA and PIA as well as the antagonist CP66,713, which, of the ligands tested, showed the greatest difference in affinities for the two sites. This compound has been shown to antagonize competitively NECA- and CGS 21680-stimulated acetylcholine release from rat striatal nerve terminals (Kirkpatrick, K.A. & Richardson, P.J., unpublished results). The most abundant site (site 2), which consisted of between 60 and 76% of the total binding, showed an agonist order of potency similar to that described for the A_{2a} receptor in the rat striatum, i.e. CGS 21680 > NECA > PIA (Jarvis *et al.*, 1989). The non-hydrolysable analogue of GTP, GppNHp, inhibited CGS 21680 binding to this site (in the presence of 30 nM **R**-PIA to block site 1) by 17 ± 3% and reduced the affinity of **R**-PIA twofold. This is consistent with the effect of GTP on CGS 21680 binding in the rat striatum (Jarvis *et al.*, 1989), and suggests that this site is linked to a G-protein. The antagonist order of potency was CP66,713 > CPT > DPCPX, which is consistent with the 13 fold selectivity of CP66,713 for A_{2a} (over A_1) receptors (Sarges *et al.*, 1990) and the 740 fold selectivity of DPCPX for A_1 over A_{2a} receptors (Bruns *et al.*, 1986) in all of the regions tested. This A_{2a}-like site was detectable only in the striatum, which is consistent with the distribution of the A_{2a} receptor in the rat brain (Bruns *et al.*, 1987; Jarvis *et al.*, 1989; Parkinson & Fredholm, 1990).

In contrast, site 1 had different relative orders of potency for both agonists (NECA = **R**-PIA > CGS 21680) and antagonists (CPT > DPCPX > CP66,713). GppNHp (10 μM) rendered this site undetectable in the striatum. This could be consistent with this site being a high affinity form of site 2. However, given the different antagonist affinities for the two sites, it could also be due to the GppNHp-mediated inhibition of binding rendering the site too small to detect in the presence of site 2. If, as seems likely, site 1 is the same as that in other areas of the brain, then a 50% inhibition of binding by GppNHp was to be expected. This would have resulted in this site contributing approximately 13% to the total binding in striatal membranes.

The study of Nanoff *et al.* (1990) showed that guanine nucleotides induce a 50% reduction in maximal specific agonist binding to rabbit striatal membranes that possess partially digested A_2 adenosine receptor protein, but a small to insignificant decrease in agonist binding to membranes containing intact, undigested receptor protein. It is suggested that unless proteolysis occurs, the A_2 adenosine receptor is tightly associated with a G-protein and displays minimal guanine nucleotide modulation of agonist binding. If this is the case, then the enhanced GppNHp effect seen in our study at site 1 in the striatum may be consistent with this site representing a proteolytic product of site 2. This seems unlikely, however, since only site 2 co-purifies with cholinergic membranes (Table 4). In the study of Martinez-Mir *et al.* (1991), in which human brain A_2 receptors were visualized autoradiographically and shown to be localized to some parts of the basal ganglia, it is likely that both sub-types of receptor were labelled. The failure to detect site 1 in other brain areas may have been due to the relatively short exposure time (21 days), given that the density of site 1 in these other brain areas is significantly less than in the striatum.

The similarity of site 1 to that characterized in the temporal cortex and elsewhere is shown in the similar nature of the relative agonist affinities (NECA > **R**-PIA > CGS 21680) and antagonist affinities (CPT > DPCPX > CP66,713). The inhibition of binding by 10 μM GppNHp (53%) was so great that analysis of the resultant binding site was not possible. This ubiquitous CGS 21680 binding site is unlikely to be the A_1 receptor since it is present in low abundance (B_{max} = 0.7 ± 0.3 pmol mg^{-1} protein), the **R**-PIA:**S**-PIA affinity ratio was only 5 (Bruns *et al.*, 1986) and the relative orders of potency for agonists and antagonists were different from that observed for [^{3}H]-PIA displacement. The displacement of [^{3}H]-PIA from temporal cortex membranes showed the characteristic relative orders of potency expected for the A_1 receptor (Bruns *et al.*, 1986). It is also unlikely that the ubiquitous site corresponds to the A_{2b} receptor since CGS 21680 has been shown to have little effect on these receptors in the rat (Lupica *et al.*, 1990), and the **R**-PIA:**S**-PIA affinity ratio implies binding to A_{2a} rather than A_{2b} (Bruns *et al.*, 1986).

We have previously shown that the rat brain A_{2a} receptor may be localized to cholinergic neurones in the striatum (Brown *et al.*, 1990). In an attempt to ascertain whether the human A_{2a} receptor was also present on cholinergic neurones, human striatal cholinergic membranes were affinity purified (Richardson, 1988). In the absence of a specific marker other than the Chol-1 gangliosides, acetylcholinesterase was used as an index of the yield of cholinergic plasma membrane. As expected, the yield of acetylcholinesterase was greater than that of the more general plasma membrane marker 5'-nucleotidase, an enzyme known to be present at the rat striatal cholinergic synapse (Richardson *et al.*, 1987) as well as on other plasma membranes (Kreutzberg *et al.*, 1986). The CGS 21680 binding site resistant to 30 nM PIA (i.e. site 2) copurified with the cholinergic membranes, whereas site 1 did not. This suggests that only the more abundant, A_{2a}-like, CGS 21680 binding site is present on cholinergic neurones. It is however not possible to determine from these data whether this site is restricted to cholinergic neurones. Martinez-Mir *et al.* (1991) found that A_2 receptors are localized on striatal neurones which use γ-aminobutyric acid, enkephalin and substance P as neurotransmitters. It would be of further interest to determine which of the two sub-types of A_2 receptor identified in this study is present on these neurones.

In summary, two A_2-like binding sites have been described in human brain, one of which is similar to the rat A_{2a} receptor and is striatal specific, while the other is more widely distributed and has intermediate A_1 and A_2 characteristics. This may represent another subtype of the adenosine A_2 receptor. The A_{2a}-like receptor has been shown to be present on human cholinergic neurones.

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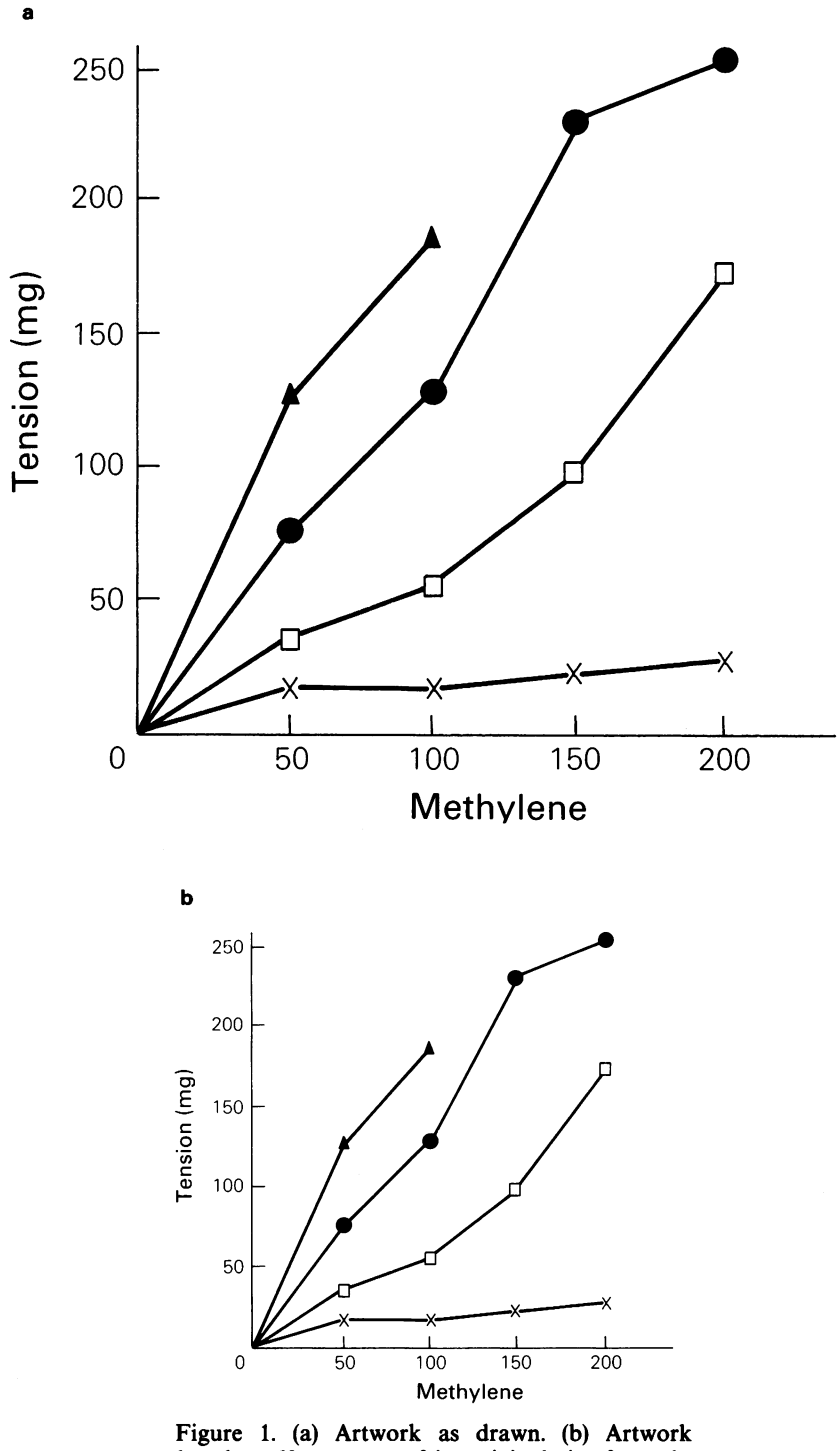


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Physico-chemical quantities

The *British Journal of Pharmacology* uses the SI symbols for units. The following prefixes for multiples of units should be used:

Multiplier	*Prefix*	*Symbol*
10^{-1}	deci	d
10^{-2}	centi	c
10^{-3}	milli	m
10^{-6}	micro	μ
10^{-9}	nano	n
10^{-12}	pico	p
10^{-15}	femto	f
10^{-18}	atto	a
Multiplier	*Prefix*	*Symbol*
10^{3}	kilo	k
10^{6}	mega	M
10^{9}	giga	G
10^{12}	tera	T

Thus, micron = μm; ångstrom = 0.1 nm. Mixed prefixes are not permissible, thus mμg should be ng. The symbols d (10^{-1}) and c (10^{-2}) should be restricted to those occasions on which there is a strongly felt need for them (e.g. cm).

Use of the solidus

The solidus should be avoided as far as possible and the negative index substituted, e.g. $mg\,kg^{-1}$ rather than mg/kg; $pmol\,mm^{-2}\,min^{-1}$ rather than pmol/mm^2/min.

SYMBOLS

Symbols denoting physical quantities are usually printed as italic capitals (indicated by single underline in typescript). A dash over the symbol indicates a mean value; a dot over the symbol indicates a time derivative. Suffixes may be used to indicate 'where' and 'what'. They are printed as inferiors on the line. Multiple suffixes should be avoided if a simpler symbol adequately defined is unambiguous, but if necessary should be separated by commas e.g. P_{A,CO_2} denotes partial pressure of CO_2 alveolar air.

CHEMICAL AND BIOLOGICAL ABBREVIATIONS

Authors should also consult *Nomenclature Guidelines for Authors* contained in this issue of the Journal. The abbreviations listed may be used without definition *except* those for chemicals, drugs and enzymes which must be written in full at first mention in the title, summary and again in the text. At first mention they should be followed by the abbreviation in brackets. Subsequently, the abbreviation alone may be used.

The list of abbreviations for chemical, drug and enzyme names is clearly not comprehensive and includes only a few commonly used examples.

Use abbreviations sparingly as extensive use can make the text hard to follow.

Physico-chemical quantities

Quantity	*Preferred unit*	*Symbol*
Amount (of substance)	mole	mol
Capacitance	farad	F
Concentration	moles per litre	M or $mol\,l^{-1}$
Current	ampere	A
Electrical conductance	siemens	S
Electromotive force	volt	V
Flow (blood or other liquid)	litres per second (or min)	$1\,s^{-1}$ or $1\,min^{-1}$
Flow (air or other gas)	litres per second (or min)	$1\,s^{-1}$ or $1\,min^{-1}$
Force	newton	N
Frequency of regular event	hertz	Hz
Length	metre	m
Mass	gram	g
Power	watt	W
Pressure (or partial pressure)	pascal*	Pa
Radioactivity	becquerel or curie	Bq (60 d.p.m.) or Ci (3.7×10^{10} Bq)
Resistance (electrical)	ohm	Ω
Temperature	degree celsius	°C
Time	second (preferred)	s
	minute	min
	hour	h
Volume (blood or other liquid)	litre	l
Volume (air or other gas)	litre	l
Work	joule	J

* mm of mercury (mmHg) are allowed if conventional, and if mercury manometer is used for calibration.

Chemical and biological abbreviations

acetylcholine	ACh
acetylcholinesterase	AChE
adenosine 3′ : 5′-cyclic monophosphate	cyclic AMP
adenosine 5′-phosphate	AMP
adenosine triphosphatase	ATPase
γ-aminobutyric acid	GABA
analysis of variants	F
adrenaline	Ad
analytical standard of reagent purity	A.R.
anhydrous	anhyd.
approximate(ly)	approx.
approximately equals	≈
aqueous	aq.
arg-vasopressin	AVP
boiling point	b.p.
bovine serum albumin	BSA
cardiovascular system	CVS
catechol-*O*-methyl transferase	COMT
central nervous system	CNS
cerebrospinal fluid	CSF
chi-squared (statistics)	χ^2
clearance	*c*
coenzyme A	CoA
concentrated	conc.
correlation coefficient	*r*
cubic	cu.
degree of freedom (statistics)	d.f.
deoxyribonucleic acid	DNA
deoxyribonuclease	DNase
dextro-(absolute configuration)	D-
dextro-(optical rotation)	(+)-
diameter	diam.
diameter, inside	i.d.
diameter, outside	o.d.
diffusion coefficient	*D*
3,4-dihydroxyphenylalanine	DOPA
3,4-dihydroxyphenylethylamine	dopamine
direct current	d.c.
disintegration per minute	d.p.m.
dissociation constant	K_D
dissociation constant, negative logarithm of	pK
distilled	dist.
dry ice	solid CO_2
edition	edn
editor(s)	ed.
effective concentration	EC_{50}
effective dose, median	ED_{50}
electrocardiogram	ECG
electrocorticogram	ECoG
electroconvulsive therapy	ECT
electroencephalogram	EEG
electromyogram	EMG
electron spin resonance	e.s.r
endothelial-derived relaxing factor	EDRF
epithelial-derived relaxing factor	EpDRF
equilibrium constant	*K*
equivalent (general use)	equiv.
erythrocyte	r.b.c.
erythrocyte sedimentation rate	ESR
ethylenediaminetetracetic acid	EDTA
excitatory postsynaptic potential	e.p.s.p.
experiment	expt
experimental	exptl

fatty acids, nonesterified	NEFA
figure(s) (with reference number)	Figure(s)
figure (diagram)	figure
gas–liquid chromatography	g.l.c.
glomerular filtration rate	GFR
haemoglobin	Hb
half-life	$t_{1/2}$
high-frequency	h.f.
high performance liquid chromatography	h.p.l.c.
human serum albumin	HSA
hydrogen-ion concentration	$[H^+]$
hydrogen-ion activity, negative logarithm of (hydrogen-ion exponent)	pH
6-hydroxydopamine	6-OHDA
5-hydroxyindoleacetic acid	5-HIAA
5-hydroxytryptamine	5-HT
immunoglobulins	IgA, IgD, IgE, IgG, IgM
inhibitor constant	K_i
inhibitory concentration	IC_{50}
inhibitory postsynaptic potential	i.p.s.p.
insoluble	insol.
international unit	iu
intra-arterial	i.a.
intracellular fluid	ICF
intradermal	i.d.
intramuscular	i.m.
intraperitoneal	i.p.
intracerebroventricular	i.c.v.
intravenous	i.v.
isotope (atomic mass) e.g. iodine-131	^{131}I
isotopically substituted compounds e.g.	[^{14}C]-ethanol
laevo-(absolute configuration)	L-
laevo-(optical rotation)	(−)-
lethal dose, median	LD_{50}
leukotriene	LT
logarithm to base e	$\log_e$ or ln
logarithm to base 10	$\log_{10}$
maximum	max.
mean arterial pressure	MAP
mean value of (statistics)	$\bar{x}$
melting point	m.p.
meta	*m*-
Michaelis constant	K_M
minimum	min.
mobility (electrophoresis)	*m*
monoamine oxidase	MAO
noradrenaline	NA
nuclear magnetic resonance	n.m.r.
number	no. or No.
number of observations (statistics)	*n*
ortho	*o*-
packed cell volume	PCV
page/pages	p./pp.
para-	*p*-
paragraph	para. or ¶
parts per million	p.p.m.
per cent	%
platelet activating factor	PAF
posterior	post.
probability (significance level in a statistical test)	*P*
radioimmunoassay	RIA
rectus (configuration by the sequence rule)	**R**
red blood corpuscle	RBC
relative band speed to front (chromatography)	R_F
relative molecular mass	M_r
relative retention time (gas chromatography)	t_r
renal plasma flow	RPF
resistance (respiratory)	*R*
respiratory conductance	Sgaw
revolutions per minute	r.p.m.
ribonucleic acid	RNA
section	§
sedimentation coefficient (ultracentrifugation)	*s*
sinister (configuration by the sequence rule)	**S**
soluble	sol.
solution	soln.
Spearman rank coefficient	r_s
standard deviation: (of observed sample)	s.d.
standard error (of estimate mean value)	s.e.mean
standard error (of sampling)	s.e.
standard temperature and pressure	STP
subcutaneous	s.c.
sum (statistical): of hypothetical population	Σ
of observed sample	S or Σ
temperature	temp.
thin layer chromatography	t.l.c.
time clock—24 h clock used e.g. 18 h 30 min	*t*
time constant	τ
2-amino-2-hydroxymethyl-propan-1,3-diol	Tris
ultraviolet	u.v.
unit	u
vacuum	vac.
valency	e.g. Fe^{2+}; Fe(II) protoporphyrin
volume by volume	v/v
wavelength	λ
weight	wt.
weight by volume	w/v

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NOMENCLATURE GUIDELINES FOR AUTHORS

With effect from 1 January 1992

The Nomenclature Working Party (NWP) of the Editorial Board of the *British Journal of Pharmacology* has consulted many acknowledged experts in an effort to clarify and standardize receptor and other nomenclature systems for use by Editors until the recommendations of the IUPHAR Commission on Receptor Nomenclature and Classification are made known.

NWP is unanimous in its view that, with rare exceptions, the Journal should use spellings, names and abbreviations that have been chosen by international bodies or specialist groups specially convened for the purpose.

For receptor nomenclature, with few exceptions, the Journal generally follows the guidelines laid down in the TIPS Receptor Nomenclature Supplement (1992).

1 *Definition of receptors and subtypes*

Receptors and their subtypes are defined in relation to structural information where this is available and on the basis of functional studies. With the latter, they are defined in terms of the relative potencies of agonists and selectivities of antagonists and by the binding of such ligands, without reference to second (or other) messenger systems.

2 *Format of receptor names*

It was agreed that, until the IUPHAR Commission on Receptor Nomenclature and Classification make their recommendations:

(a) Editors will permit with reluctance new nomenclature systems in papers accepted for publication if and only if there are compelling reasons to introduce a new terminology (or modify an accepted one). The criteria upon which the new receptor type or subtype is defined must be given, together with adequate explanations of the relationship between the previous nomenclature (fully referenced) and the proposed one.

N.B. The new nomenclature should not appear in the Title, Short Title or Keywords, unless qualified by the adjective putative (e.g. ... mediated by the putative β_3-adrenoceptor).

(b) Only well-established and universally accepted subtype names (e.g. muscarinic and nicotinic cholinoceptors; α- and β-adrenoceptors) will be acceptable without any reference to the originator of these terms. In cases of controversy concerning further subdivision of the subtype, full referencing must be given.

(c) Receptor subtypes should be designated by means of a subscript numeral or capital letter. Some double subscripts (i.e. numerical plus letter) have been introduced but, where possible, further introductions should be avoided and must be fully referenced.

3 *Types of receptor*

(a) *Acetylcholine receptors* (see Cholinoceptors).

(b) *Adrenoceptors* The principal subtypes are α_1-, α_2-, β_1- and β_2-adrenoceptors. Reference to either 'atypical' β-adrenoceptor or a putative β_3-adrenoceptor would be permitted, provided fully referenced.

(c) *Angiotensin receptors* At present only the AT_1 receptor is recognised. The AT_2 binding site should be fully referenced.

(d) *Bombesin receptors* No subtypes are recognised. Proposed subtypes may be used as discussion points, but a full explanation is required.

(e) *Bradykinin receptors* B_1 and B_2 receptors are recognised. Possible additional types and subtypes of bradykinin receptors should not be designated, except as discussion points and should be fully referenced.

(f) *Calcitonin gene related peptide (CGRP) receptors* No subtypes are recognised. Proposed subtypes may be used as discussion points, but a full explanation is required.

(g) *Cholecystokinin (CCK) receptors* The principal subtypes are CCK_A and CCK_B receptors, CCK_B receptors being known also as gastrin receptors.

(h) *Cholinoceptors* The two principal subtypes are muscarinic and nicotinic cholinoceptors (the term acetylcholine receptors is acceptable).

Muscarinic cholinoceptors Nomenclature should be confined to only three subtypes, namely M_1, M_2 and M_3 cholinoceptors, where M_2 refers to the cardiac subtype and M_3 includes both smooth muscle and glandular subtypes.

Note that the style m1, m2, etc. refers to nomenclature for a receptor with pharmacology similar to native receptors but which is encoded by a cloned gene/cDNA.

The abbreviation mAChR and variants are not acceptable.

Nicotinic cholinoceptors The principal subtypes currently accepted are muscle-type and neuronal-type receptors. The abbreviation nAChR and variants are not acceptable.

(i) *Dopamine receptors* D_1 and D_2 dopamine receptors are currently recognised. Other subtypes must be very clearly defined and fully referenced. DA_1 and DA_2 should not be used for peripheral dopamine receptors.

(j) *Endothelin receptors* The style to be used is ET_1 and ET_2 and should be fully referenced.

(k) *Excitatory amino acid receptors* At present, no subdivisions of the receptor for N-methyl-D-aspartate (NMDA; see 6(c) below) are permitted except as discussion points.

Four non-NMDA receptors have been established and are named:

(i) AMPA receptors.
(ii) Kainate receptors.
(iii) 2-Amino-4-phosphobutyrate receptors (also sometimes known as ABP or AP4 receptors) to be abbreviated L-AP4 receptors.
(iv) Metabotropic receptors.

(l) *γ-Aminobutyric acid (GABA) receptors* The principal subtypes are $GABA_A$ and $GABA_B$ receptors. Any other is to be used only as a discussion point and to be fully referenced.

(m) *Histamine receptors* The only histamine receptor subtypes that are acceptable without a need for very clear definition and full referencing are H_1-, H_2 and H_3-, although in the last case a definition and references are desirable.

(n) *Receptors for 5-hydroxytryptamine* The name 5-hydroxytryptamine (5-HT) is preferred to serotonin (see 4(b) below). The principal subtypes recognised are $5\text{-HT}_{1A, 1B, 1C, 1D}$, 5-HT_1-like, 5-HT_2, 5-HT_3 and 5-HT_4.

Other types may be debated in the Discussion section but, until there is international agreement on the nomenclature, these subtypes must be referenced.

(o) *Leukotriene receptors* When first mentioned, the style leukotriene (LT) receptor should be used, thereafter LT receptor. Receptors should be designated according to the leukotriene that selectively or preferentially binds to them. All leukotriene receptor types should be fully referenced.

(p) *Neuropeptide Y receptors* No subtypes are recognised. Proposed subtypes may be used as discussion points, but a full explanation is required.

(q) *Opioid receptors* The principal subtypes are μ-, δ- and κ-opioid receptors. Other possible subtypes (e.g. ε) are acceptable only as discussion points and must be referenced.

(r) *Oxytocin receptors* (see Vasopressin and oxytocin receptors).

(s) *Prostanoid receptors* The principal types are DP, EP, FP, IP and TP receptors. These should be introduced as prostanoid XP receptors, thereafter simply as XP receptors (where X denotes the type). If subtypes exist, they would be referred to as XP_n, (e.g. EP_1, EP_2, EP_3) receptors.

(t) *Purinoceptors* The principal subtypes permissible are P_1 and P_2. Subdivisions of P_1 into A_1 and A_2 types and of P_2 into P_{2X} and P_{2Y} types are acceptable. Proposed subtypes may be used as discussion points, but a full explanation is required.

(u) *Tachykinin receptors* Except as discussion points, only the following tachykinin receptor subtypes are acceptable at present: NK_1, NK_2 and NK_3.

(v) *Vasoactive intestinal peptide (VIP) receptors* No subtypes are recognised. Proposed subtypes may be used as discussion points, but a full explanation is required.

(w) *Vasopressin and oxytocin receptors* The principal subtypes are designated V_1, V_2, V_3 and OT receptors; V_3 has sometimes been known as V_{1B} but the original term V_3 is preferred (see 2(c)).

4 *Naming of nerve fibres*

Many nerve fibres are now known to release more than one transmitter, and future work may show that this is in fact the general rule. In that case, the concept of the same transmitter being released either at different developmental stages or under various experimental conditions would no longer hold, and single adjectives that imply this (e.g. cholinergic, noradrenergic) would become inappropriate when applied to nerve fibres, as distinct from transmitter functions. For the present, those nerve fibres that are known to function by releasing more than one identified transmitter may be described accordingly; for example, noradrenergic-purinergic, cholinergic-peptidergic (in alphabetical order, the order implying no priority of function).

N.B. The suffix 'ergic' should continue to be applied only to nerve fibres and to the transmission event, in accordance with Dale's intentions. For example, 'cholinergic' indicates that the nerve fibre, or the transmission, functions under particular conditions through the release of a choline-like substance. The suffix should not be used loosely to mean 'pertaining to'. Hence, for example, the expression 'cholinergic receptor' (rather than cholinoceptor) is an inappropriate use of the term. The term *nitrergic* may be used to describe the transmission event but may not be used to describe nerves.

(a) *Catecholamine releasing nerve fibres* The adjective to be applied to nerve fibres that release dopamine as a transmitter is dopaminergic (not DAergic).

Nerve fibres that are known to function by releasing noradrenaline are to be described as noradrenergic. The term adrenergic should be reserved for either a nerve fibre that functions by releasing a catecholamine, the identity of which is unknown, or one known to release adrenaline.

(b) *Some other adjectives describing nerve fibre function* NANC is an acceptable abbreviation of non-adrenergic, non-cholinergic for peripheral efferent nerve fibres when the identity of the transmitter(s) is unknown other than the fact that neither (nor) adrenaline nor acetylcholine is involved. It should be defined when introduced. NANCergic, e-NANC (or NANC-e) and i-NANC (or NANC-i) are not acceptable terms.

Glutamatergic, not glutaminergic, should be used to describe nerve fibres releasing glutamate. In referring to peptide-releasing nerve fibres, (e.g. those that may release substance P or vasoactive intestinal peptide) the nomenclature to be used is peptidergic (X), e.g. peptidergic (SP), peptidergic (VIP), not SPergic, VIPergic.

The terms 5-hydroxytryptamine (5-HT) and 5-hydroxytryptaminergic (i.e. nerves releasing 5-hydroxytryptamine) are preferred to those of serotonin and serotoninergic. The term 5-HTergic is not acceptable, except to avoid frequent repetition of 5-hydroxytryptaminergic.

Likewise, the terms purinergic (ATP) and purinergic (adenosine) are preferred.

5 *Enzymes*

The IUB Enzyme Commission (EC) number and full name (Enzyme Nomenclature 1984, Academic Press, New York and London) must be quoted when first mentioned in text. Subsequently the accepted trivial name is used. Trivial names may be used in the title.

6 *Other nomenclature requirements*

(a) *Racemates* Authors must state unambiguously in the Methods section of papers which isomers were used, e.g. (+)- or (−)-propranolol, and must bring to the attention of the reader the composite character of drugs that are mixtures of stereoisomers. Furthermore, the implications of the composite nature of such drugs studied for the interpretation of the data measured and the conclusions drawn must be made explicit. Note that the terms d- or l- for dextro- and laevo-rotatory are now obsolete, and the prefixes (+)- or (−)- respectively should be used. Capital D and L refer to the absolute configurations and of course remain acceptable when appropriate.

(b) *Platelet activating factor (acetyl-glyceryl-ether-phosphorylcholine)* The acronym to be used is PAF (not AGEPC, Paf, Paf-acether or other variant). The alkyl chain should be specified for synthetic PAF e.g. C_{16}-PAF.

(c) *Ligands for NMDA receptors* N-methyl-D-aspartate (NMDA) and N-methyl-DL-aspartate (NMDLA) are to be given in full when introduced in the text.

(d) *Purines* This term should not be used as a synonym for purine nucleotides or nucleosides.

(e) *Eicosanoids* The system of nomenclature to be used for eicosanoids is that published in Methods in Enzymology, (1990), **187**, 1–9. This scheme incorporates

recent changes in the style of abbreviation of hydroperoxy-, epoxy- and oxo-unsaturated fatty acids e.g. 12(S)-hydroperoxyeicosatetraenoic acid which was formerly abbreviated as 12(S)-HPETE now becomes 12(S)-HpETE. In manuscripts, the first use of the full chemical name of any eicosanoid should indicate double bond geometry when this is known.

(f) *Peptide nomenclature* The preferred style is capital letters to designate the first letter of the word. Otherwise, upper and lower case letters should be used (e.g. Enk-IR, enkephalin-like immunoreactivity). When numbers are used these should be placed after a hyphen on the same line as the abbreviation, e.g. ET-1.

(g) *Cell line* Cell type, sources and originating species need to be defined.

(h) *Molecular biology* Abbreviations pertaining to molecular biological techniques need to be defined or presented in such a way that they can be recognised by the non-specialist e.g. the oligonucleotide sequence, TAGC.

PREPARATION OF MANUSCRIPTS

Authors are strongly recommended to read the full *Instructions to Authors* and *Nomenclature Guidelines for Authors* (*Br. J. Pharmacol.* 1992, **105,** 245–254) before submitting a manuscript for publication in the *British Journal of Pharmacology.* The manuscript and cover letter should be checked against the following list before mailing.

The original and one copy of the manuscript must be supplied. Manuscripts must be typed in double-line spacing on one side of A4 paper, in type not smaller than 12 characters per inch or 10 point. Both copies to include Tables and a set of labelled Figures. One set of Figures without numbers or letters is also to be included. The text to be arranged in the following sub-sections:

1. **Title**—To have no more than 150 characters on a separate page, which should also include a Short Title (50 characters maximum) and the name and address of the author for correspondence.
2. **Summary**—To be arranged in numbered paragraphs (Full Papers) or a single paragraph (Special Reports).
   —to include aims, principal results and conclusions.
   —to include Key words (10 maximum) at end of summary.
3. **Introduction**—To contain concise statements of the problem and the aims of the investigation.
4. **Methods**—To have brief but adequate account of the procedures; *full names of drugs* (*including those referred to by manufacturer's code*), sources of drugs and statistical tests to be stated.
5. **Results**—To have no repetition of data in Figures, Tables and text.
6. **Discussion**—Findings and conclusions to be placed in context of other relevant work.
   NB Simple repetition of results and unwarranted speculation are not acceptable.
7. **Acknowledgments**—Sources of support. Sources of drugs not widely available commercially.
8. **References**—All references in the text to be included in the Reference List and *vice versa.* References in alphabetical order with complete citations; Journals publishing 'in press' papers identified.
   References to manuscripts submitted to other journals but not yet accepted are not allowed.
9. **Tables**—Each on a separate page and prepared in accordance with current requirements of the Journal.
10. **Figures**—Both labelled and non-labelled Figures to be prepared in accordance with current requirements of the Journal (see *Instructions to Authors,* 1992, **105,** 245–251) and provided with Figure Number and Authors' names on back (*in pencil*)
    —each legend to be typed on a separate page and carrying keys to symbols
11. **Manuscripts**—To be accompanied by a declaration signed by each author that
    (a) results are original
    (b) approval of all persons concerned has been given to submit manuscripts for consideration (see also 12b)
    (c) the same material is neither 'in press' (i.e. is in proof or has definitely been accepted for publication) nor under consideration elsewhere. Furthermore it will not be submitted or published elsewhere before a decision has been reached by the Editorial Board of the *British Journal of Pharmacology* and will not be submitted elsewhere if accepted by the *British Journal of Pharmacology.*
    (d) Copyright assignment is included
12. **Cover letter**—To state clearly
    (a) Corresponding author's full postal address, telephone, telex or Fax number
    (b) where appropriate, that *either* ethical approval has been given for investigation *or* Company or Institutional permission to publish work has been received.
13. **Reminder**—Packaging to be sufficiently robust to protect Figures and to withstand mailing.

Failure to comply with *Instructions to Authors* may lead to substantial delays in processing, review and publication and may even jeopardize acceptance of the manuscript.

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